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DEMONSTRATION OF MASS PRODUCTION, FORMULATION AND APPLICATION OF A BACULOVIRUS FOR MANAGEMENT OF THE TEAK DEFOLIATOR, *Hyblaea puera*

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ABSTRACT

The study addressed mass production of HpNPV and developing and field-testing of an effective virus formulation. One of the preliminary actions in the project was the establishment of a laboratory for HpNPV mass production. The laboratory design incorporated the concept of spatial separation of experiment and HpNPV production space. Separate routes of entry for field collected and laboratory reared larvae were provided. In connection with refining of method of rearing the host insect (*H.puera*), a novel insect rearing tube with a detachable diet cup was designed and evaluated.

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In connection with standardization of mass production, virus productivity was quantified with reference to different larval instars, dosage, incubation period and temperature. Of the three larval stages *i.e.,* third, fourth and fifth instars studied, the maximum POB yield per unit diet (3.3 x 10^9 POBs) was obtained from fourth instar larvae dosed at 10^5 POBs per larva and incubated for a period of 72 h p.i. The harvestable larvae accounted as high as 98 %. The temperature turned out to be one of the major factors determining the productivity of the virus The maximum POB yield was registered at the dosage 1 x 10^5 POBs per larva and the temperature 25 ± 2 °C.

The method of inoculation plays an important role in virus mass production. The different inoculation methods evaluated for identifying the best method were: Application of virus suspension using micropipette on to (A) semi synthetic diet dispensed in rearing tubes; (B) leaf disc kept in rearing tubes; spraying virus suspension using atomizer on to (C) semi synthetic diet dispensed in the rearing tubes; or to tender teak leaves (D). The study revealed that the methods of inoculum presentation had a great influence on NPV productivity as well as percentage of harvestable larvae. The percentage of harvestable larvae under all methods varied significantly from 86 to 95 %. In the case of methods A, B and C, the mean percentage of retrievable larvae did not vary but was significantly higher than in D The productivity ratio was the highest in method C (15,400 POBs) and varied significantly from rest of the methods. Method A was the second most productive (10,560 POBs) followed by methods B (7,163 POBs) and D (7,200 POBs). When compared in terms of easiness, method C was found to be the easiest as it did not involve cumbersome activities such as micropipetting, leaf disc preparation, added larval transfer etc.

The economics of mass production of the HpNPV using the laboratory-reared and field-collected host larvae were compared. The data were based on 7,645 Laboratory-reared (LR) larvae and 8,925 Field-collected (FC) larvae during the period of 2000-2001. The virus production was carried out using fifth instar larva inoculated with 10^6 POBs/larva. The virus yield/larva in LR and FC larvae was $4.8x10^8$ and $3.6x10^8$ POBs respectively. The cost of production of HpNPV/larva was higher in the case of LR (Rs. 0.82) in comparison with FC (Rs.0.72). However, upon considering the yield/larva from both sources, the cost of HpNPV required for spraying in one hectare at the rate of 1.63 x 10^{11} POBs worked out to be Rs. 279/- and Rs. 317/- in the case of LR and FC respectively. A marginal difference in the virus yield was found affecting the cost of the virus produced

The following seven formulations were developed and tested of which six were with additives: 1. Wettable Powder formulation (WP (A-); 2. Wettable Powder with Additives (WP (A+); 3. Wettable Powder by Lactose Co-precipitation (WP-Co-prec. (A-); 4. Wettable Powder by Lactose Co-precipitation with Acetone (WP- Co-prec.

(A+); 5. Wettable Powder - Freeze dried (WP-FD); 6. Wettable Powder - Micro Encapsulation (WP-Encaps); 7. Flowable Concentrate (FC). The additives included talc as carrier, activated carbon as UV (ultra violet rays) protectant and polyvinyl alcohol as dispersing agent. The process-linked loss the virus during formulation ranged from 1.2% to 8.1% with regard to all powder/encapsulation formulations. The LD_{50} value of all the formulated products except WP- Co-prec. (A+) were comparable to that of unformulated HpNPV. The LD_{50} values obtained for WP- Co-prec. (A+) was 320 times less virulent than the unformulated HpNPV and 280 times less virulent than WP-FD formulation which retained 90% of the original activity.

Regarding the ultra violet stability, unformulated HpNPV, retained the original activity of 37.8 % after exposure to sunlight for a period of 9 hours. However, in the case of in WP-FD and WP-Encaps the original activity remaining (OAR) under the same exposure condition was 88.5% and 86% respectively. The study indicated that formulation increased the viability of HpNPV under natural sunlight by 2.34 folds. It was also able to deduce that the WP-FD formulation is more stable to different periods of exposure than the rest of the formulations. Out of the seven formulations developed, WP-FD was field-tested, the performance of which was as good as or even better than the unformulated HpNPV.

Under storage at room temperature (28°C–30°C), the unformulated crude suspension lost its infectivity almost completely by 18 months. WP and WP-L retained about 50 % of the original activity. The maximum OAR of 76 % was registered with WP-FD formulation. Storage of WP-FD at $4^{o}C$ was found to be detrimental with loss of about 70 % original activity by 18 months storage

The biosafety evaluation of the WP-FD formulation against silk worm and Indian mynah revealed that the formulation was totally safe. *In vitro* biosafety evaluation was carried out using three cell lines namely, Sf-9 (*Spodoptera frugiperda*), Hep-2 (Human larynx) and Vero (Africal green monkey kidney). The impact of the virus on the various cell lines tested was negative establishing the biosafety of the HpNPV formulation.

The study also gave an opportunity to understand genetic variation within HpNPV population. Eight HpNPV isolates could be characterized using Restriction Endonuclease analysis wherein Hind*III* was used as the restriction enzyme. The estimated molecular weights of the genome of HpNPV isolates ranged from 79.37 kbp to 112.14 kbp.

1. INTRODUCTION

The teak defoliator, *Hyblea puera* (Cramer) (Lepidoptera, Hyblaeidae) is one of the most serious pests of teak. Outbreaks occur every year in India, over extensive areas. The impact assessment of this insect pest carried out by Nair *et al*. (1985) showed a loss of about 44 % of the potential volume increment in young teak plantations. Several management options have been considered in the past and in the recent years (Beeson, 1941; Nair *et al*., 1995; Sudheendrakumar, 1986; 1997). In general the emphasis was on economically as well as ecologically acceptable biocontrol options.

Baculoviruses are considered to be potential biocontrol agents of many agricultural and forestry pests in many countries. Baculoviruses are double stranded DNA viruses and are known exclusively from arthropods. This large family of viruses comprises two genera (i) Nucleopolyhedrovirus (NPV) and (ii) Granulovirus (GV) (Murphy *et al*., 1995). At least 800 known isolates of Baculoviruses were reported from more than 400 host species, mostly in Lepidoptera and Hymenoptera (Vlak and Hu, 1997). Their efficacy, specificity, and production of secondary inoculum make them attractive compared to broad-spectrum insecticides as ideal components of IPM systems (Huber, 1986; Groner, 1990; Cunningham, 1995).

During the past decade, the Nucleopolyhedrovirus, HpNPV (Sudheendrakumar *et al*., 1988) has received considerable attention owing to its biocontrol potential. Preliminary bioassays were carried out by Mohamed-Ali *et al*. (1990) to evaluate the efficacy of this virus against third and fourth instar *H. puera* larvae. The median lethal dose values for these instars showed that the virus was highly pathogenic to the test larvae. The persistence studies indicated that UV light has a significant negative impact on viability of the virus. However, the influence of heat (heating at 40 °C for seven days) and pH (holding virus at pH 4 and 9.2 for 6 h) has negligible effect on this virus.

Nair *et al*. (1998) attempted mass production of HpNPV from field collected as well as laboratory reared host larvae. The high percentage of parasitism and bacterial contamination encountered became major drawbacks in the virus mass production programme using larvae from the field. To overcome these draw backs, attempts were made to produce NPV from laboratory maintained *H. puera* larvae. The laboratory-reared larvae were found to be high yielding than field collected ones (7.5×10^7 POBs per larva against 4×10^6 POBs per larva) and the risk of parasitism as well as bacterial contamination was very low but all those were at the cost of hiked expense. In the above study, information on disease transmission and epizootiology has been generated which indicated intra-host persistence of the virus and transovarian transmission from parental generation up to the second filial generation under controlled laboratory conditions. In the same study, the performance of the crude virus under field conditions was also evaluated. Nair *et al.* (1996; 1998) reported that one-time foliar application of a crude preparation of HpNPV at the rate of 10^5 POBs per ml of the spray fluid, at the earliest sign of each infestation, could give 70 to 76 % protection of foliage in teak plantations.

The preliminary field trials brought out the need for understanding the interaction of the host (*H. puera*), the virus (HpNPV), environmental factors, host tree (teak), and spray technology to provide a framework for determining optimal dosages for this biocontrol agent. Sudheendrakumar *et al*. (2001) studied the interaction of these five primary variables. Information on host biology including larval feeding habits and larval distribution patterns on host tree was generated. Following a series of ranging bioassays, dosage mortality relationships for larval stages were found out. Virus productivity relationships for larval stages were assessed for semi-purified

virus. Under artificial rainfall system (upto 200mm) the effect of rainfall on virus applied on teak plants was studied which indicated rapid loss of virus activity. No effect of either leaf surface or leaf volatiles on HpNPV was observed. Studies on virus application system indicated that ground based ULV sprayer Stihl SR 400 is effective for trees up to 14m height. A theoretical model for dosage estimation was predicted and the parameters predicted by the model were field-tested in 1997 and 1998 and the optimal dosage rates to achieve >95 % mortality of the target larvae were calculated.

One of the prime requirements in a biological control programme involving an entomopathogen is the large-scale availability of the pathogen for field application. At present the only viable option for large-scale production of baculovirus is *in vivo* replication of the virus in the homologous host (Cherry *et al*., 1997). In earlier studies attempts have been made to mass-produce HpNPV using both field collected as well as laboratory reared *H. puera* larvae (Nair *et al*., 2001). The virus yield reported using laboratory-reared larvae was 2×10^8 POBs per fifth instar larva. So far no systematic study has been made to select the optimum larval stage of the host insect, dosage, incubation period, microclimate or inoculation method favoring maximum virus production. Since yield as well as cost determines the viability of using NPV as a biocontrol agent, it becomes imperative to look into the possibilities of optimizing *in vivo* mass production techniques, which is known for its highly variable nature.

Lack of field stability of entomopathogenic viruses after application has long been considered a primary factor limiting commercialization of these important insect microbial control agents. High-energy short wave ultra violet rays in sunlight has a significant role in reducing the infectivity of baculoviruses exposed to field conditions (Jaques, 1977). Several studies have shown that baculoviruses lost most of their insecticidal activity with in a few days, and sometimes within a few hours, after application in the field (Watanabe, 1951; Aizawa, 1955). Studies on comparison of HpNPV persistence and activity in direct sunlight and in darkness indicated that when exposed to full sunlight, mortality of third instar larvae fed with known quantities of virus dropped from 97% to 70% over a six-hour period. This rate of decay continued up to about 24 hours, after which it was stabilised at 13 % mortality up to 12 days (Sudheendrakumar *et al*. 2001). This suggested the need for developing a product formulations which are stable from the time of production to the time of its use and which can be effectively applied using a variety of pesticide application equipments. The process of developing a formulation also involves adding a wide variety of substances, which will improve the performance of NPV in the field. Substances that function as UV protectants, wetting, sticking and thickening agents, anti evaporants and appropriate carriers can be built into the formulation so that the end user can use the product as a single entity without bothering about various additives.

The present study was undertaken to fill in the existing lacunae in our knowledge to develop an efficient management strategy against the teak defoliator using HpNPV. The aspects considered hereunder-included establishment of a pilot mass production unit for HpNPV, development and filed evaluation of product formulations and safety testing of the formulated products.

2. GENERAL METHODS

The methods followed in general are presented here. Methods pertaining to specific experiments are mentioned under respective chapters.

2. 1. Laboratory rearing of *H. puera*

For obtaining larvae required for various bioassays, a continuous culture of *H. puera* was maintained in the laboratory of the Kerala Forest Research Institute (KFRI), Subcenter, Nilambur throughout the study period. Various steps involved in establishing a culture of *H. puera* are depicted in Fig. 2.1.

For establishment of the host culture, *H. puera* pupae were collected from the teak plantations at Nilambur and brought to the laboratory. Pupae were surface sterilized by soaking in 5 % sodium hypochlorite solution for 5 min and washed under tap water for another 2 min. The pupae were then air dried and allowed to emerge. The newly emerged moths were fed with 10 % (v/v) honey solution provided on sponge pieces. Sponge pieces soaked in diluted honey will be provided anew every day. It was found that fecundity increased with the time allotted for free movement before mating and hence, equal number of male and female moths were released into mating cages (30 cm x 30 cm) for one day. The moths were fed with 10 % honey solution. On the second day, moths were sexed on the basis of the morphological features of their legs (Sudheendrakumar, 2003). After sexing pairs were set and transferred to wide mouthed bottles (20 x 10cm) covered with cotton cloth, which served as substratum for oviposition.

Egg laden cloth was removed daily and sterilized by soaking in 2 % sodium hypochlorite solution for five minutes and air-dried. When the eggs were about to hatch, the cloth was transferred to glass bottles provided with fresh tender teak leaf so that the newly emerged larvae could shift to the leaves and start feeding. Eggs hatch in about two days. The neonates were reared on tender teak leaves until third instar. From third instar onwards the larvae were reared on an artificial diet with slight modifications from what had standardized by Mathew *et al*. (1990). The ingredients of the diet required for preparing 1kg of diet are given in Table.2.1.

The third instar larvae were transferred to individual sterilized plastic tubes (7 cm x 2.5 cm) containing one ml of the artificial diet. The tubes were closed with perforated cap and kept inverted in slanting position in aluminum trays to keep the diet free of faecal pellets. The faecal pellets were removed from the tubes daily. The larvae pupated inside the diet tubes are removed and washed in 2% sodium hypochlorite solution for sterilization. Sterilized pupae were washed in tap water, air-dried and are then kept in glass bottles for emergence. Diseased larvae, if any, found in the culture were removed immediately. At times, healthy larvae collected from the field were introduced to the culture for maintaining the vigor of the culture. A total of fifty-five generations of *H.puera* were successfully reared out in the laboratory during the study period.

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Deleted: As the eggs were pasted to the cloth, the cloth as such was surface sterilized with 0.5 per cent sodium hypochlorite solution for 10 minutes. The cloths was then air dried at room temperature (27-32°C) and was kept in sterile glass bottles along with fresh tender teak leaves. So that the newly emerged larvae could shift to the leaves and start feeding . ¶

Table 2.1. Ingredients of artificial diet for rearing *H.puera* larvae

Ingredients	Quantity
Binders	
Agar agar	20 g
Distilled water	1000 ml
Nutritional elements	
Teak leaf powder	20 g
Kabuligram flour (*Cicer arietnum*)	100 g
Yeast extract powder	10 g
Casein	30 g
Multivitamin and mineral mixture (Becadexamine)	800 mg
Vitamin E (Tocopheryl Acetate)	400 mg
Antimicrobial agents	
Streptomycin sulphate	0.125 mg
Tetracycline hydrochloride	0.125 mg
Sorbic acid	1 g
Ascorbic acid	3.5 g
Methyl-para- hydroxybenzoate	1.5 g
Formaldehyde 10 %	2 ml

2. 2. HpNPV inoculum preparation

The *Hyblaea puera* nucleopolyhedrovirus (HpNPV) used in the study was obtained from a stock culture maintained in the Entomology laboratory of KFRI at Nilambur. The virus was multiplied by infecting fifth instar *H. puera* larvae. The larvae were individually fed with HpNPV on leaf discs (0.5 cm diameter) at a dosage of 10^6 POBs per larva. The infected larvae were then maintained individually on virus free artificial diet at 28 $\pm$ 4 °C and 60 $\pm$ 10 % relative humidity (RH) in rearing tubes (5.5 cm x 2.3 cm) covered with a perforated lid. After 96 h post infection, fully infected and dead larvae were retrieved, frozen and processed for virus extraction. The viral POBs were isolated from the larval cadavers by cutting the abdominal epithelium releasing fluid loaded with POBs into a sterilized petri dish. The larval extract was then filtered to remove insect debris, if present. The cotton muslin cloth folded over to twice thickness was placed in a filter funnel and the extract was allowed to drip through into a conical flask. Double the amount of distilled water was added in to the filtrate and vortexed thoroughly. The well-mixed solution was then transferred to clean centrifuge tubes and centrifuged for 5 min at 5000 rpm and the supernatant was removed and discarded. The pellet obtained was resuspended in distilled water by vortexing thoroughly. This was again centrifuged for 5 min at 5000 rpm to

remove the supernatant. The pellet was made up to known volume based on the pellet size, and vortexed well enough to break NPV clumps.

The enumeration of POBs in purified suspension was carried out under a light microscope using a standard haemocytometer (improved Neubauer's haemocytometer (0.1mm depth)) (Hunter-Fujita *et al*., 1998a; Rabindra *et al*., 2001). The POBs of NPV are highly refractive protein crystals that show up as bright refractive bodies under illumination. They can be seen clearly under phase contrast illumination with a magnification of x 400. Prior to enumeration, viral suspension was vortexed to facilitate equal distribution of polyhedra and dissociation of clumps of polyhedra. Working standard was prepared by necessary dilutions with distilled water. Haemocytometer along with the cover slip was cleaned by rinsing in ethanol (70%) and wiped till dry with clean tissue. Haemocytometer was placed over a clean flat surface with cover slip on top of the slide exactly over the depression in the counting chamber. The virus suspension (10μl) was introduced in to the chamber directly so that the chamber will be filled completely. Pressing down the cover slip firmly, fixes it over the chamber by the capillary attraction of the drops. Only the specially thickened cover slip designed for use with hemocytometer was used. The virus suspension was kept undisturbed for 5min to settle down in the same focal plane. Ambient conditions to perform enumeration could be met with at air-conditioned room since it prevents fast drying up off the film. The haemocytometer was fixed over the stage of a phase contrast microscope and the counting area was fixed at low power with appropriate settings. The objectives were focused on to the polyhedra dispersed in the centrally located squares, and fine adjustments were made.

The central squares in the haemocytometer are divided into 5 x 5 squares equally. Each of these 1/25 squares is further subdivided into 16 smaller squares. The OB count was made systematically in the five larger squares seen diagonally (i.e., first large square from first row, second large square from second row like wise) so that polyhedra in 80 smaller squares will be known. The polyhedra within each smaller square and those touching the top and left-hand sides alone were counted. If there are more than 5 polyhedra per smaller square counting will be difficult and necessary dilutions were made. The counts were taken in three replicates and average was worked out.

The number of polyhedra (POBs) per ml was calculated by using the formula:

$$\text{Number of POBs per ml} = \frac{DxX}{NxV}$$

Where,

D = Dilution of the suspension

X = Number of polyhedra counted

N = Number of smaller squares counted

V = Volume in milliliters above a small square (0.00000025 ml)

After enumeration, virus was stored at ⁻20°C as stock for further use.

2. 3. Bioassays

The relationship between dosage of virus and mortality was quantified by probit analysis (Finney, 1971). Probit analysis uses dosages of virus as an independent variable, the total number of insects treated at each dosage, and number of insects

killed at each dosage as a dependent variable. The result obtained is the dosage needed to kill 50% (or different levels) of the insects, or median lethal dose, the LD_{50}. In addition to dosage-mortality data, time-mortality data are often developed from the same bioassays. When the insect is exposed to the pathogen for a short, restricted period, and then removed to untreated diet the estimate made is median survival time (ST_{50}) and is determined by probit analysis.

The test insects used for bioassay were from a laboratory culture maintained as described above. Required doses of viral inoculum were prepared by serial dilution of the semi purified viral stock prepared earlier using distilled water. Inoculation medium used for presenting virus preparation to test insects was either leaf disc made out of tender teak leaf or semi synthetic diet, to which required dosages of inoculum was applied individually using a micropipette or sprayed using a chromatographic sprayer. The larvae, which remained unfed after the specified inoculation period was discarded. The selected larvae were then kept in a controlled microclimatic condition. Observations were made on the response of the test insect at specific time points. According to the need of the objectives of the study, larval samples (dead/live) were collected and freezed. For estimating the viral production, larvae obtained for each time point were homogenized using tissue homogenizer and the homogenate was made up to known volumes with distilled water. Enumeration was carried out using improved Neubauer's hemocytometer.

3. INFRASTRUCTURE DEVELOPMENT FOR *IN VIVO* MASS PROUDCTION OF HPNPV

3. 1. Biocontrol Laboratory

One of the preliminary actions in the project was the establishment of a laboratory for HpNPV mass production. The laboratory design incorporated the concept of spatial separation of experiment and HpNPV production space. Separate routes of entry for field collected and laboratory reared larvae were provided.

The design of the biocontrol laboratory is shown in Fig. 3.1. The building had a total plinth area of 110.83m^2 and carpet area of 80.73m^2. HpNPV mass production unit in this building is of an area 31.52 m^2. For related research, a room is assigned for bioassay in this building. While this laboratory was designed for virus multiplication, the host insect was reared in another building about 100m away to avoid any contamination.

Fig. 3.1. Design of the NPV biocontrol laboratory

3. 2. Designing insect rearing tubes

For rearing of *H. puera* larvae on artificial diet, glass/polypropylene tubes were being used (Nair *et al*., 1998). During scaling up of insect culture for HpNPV production, the glass tubes were found difficult to handle. Inadequacies are also met with while rearing larvae in one piece cylindrical plastic tubes: spillage during diet dispensing, poor capture efficiency while inoculum spraying, difficulty in pupa collection and difficulty in cleaning of rearing tubes. To overcome the problems met with a two-piece rearing tube (referred to as A hereafter), a three-piece rearing tube (referred to as B hereafter) was designed and evaluated. The two-piece tube consists of two parts- main body measuring 55mm in length and 23mm in diameter and a perforated cap (Fig. 3.2-A). The three-piece tube consists of a main body with both ends open (55mm x 23mm), a diet cup (20mm x 22mm) and a perforated cap (Fig. 3.2-B). The uniqueness of this tube is the detachable diet cup, which can be fitted into the main body of the tube. The material used for the production of this tube is polypropylene of high grade and is autaclavable. The transparency of the tube ensures easy observation of the insect growing inside the tube.

Fig. 3.2. A - Two piece tube; B - Three piece tube

3. 3. Evaluation of rearing tubes

A comparative evaluation of the 2 types of tubes was made in terms of the quantity of virus received in the tubes (virus capture efficiency) during inoculum dispensing and also easiness in dispensing diet and easiness in handling. The virus capture efficiency of the two rearing tubes was estimated by carrying out a dummy experiment.

3. 3. 1. Materials and methods

The main bodies of Tube A and diet cups of Tube B were stacked vertically in two separate trays (140 containers per tray) and were filled with 2.5 ml distilled water each. A known quantity of the virus was then sprayed into tubes/diet cups stacked in trays. Spraying of HpNPV was carried out using a chromatographic sprayer. The sprayer was held at 10 to 15cm above and the whole virus was uniformly sprayed into the tubes. After spraying, the water from each set were pipetted out and vortexed well. POBs present in the water were enumerated using improved Neubauer's hemocytometer. In the experiment, water was used instead of semi synthetic diet, so as to get an accurate count of the POBs received in the tubes.

3. 3. 2. Results

The data on capture efficiency of the two types of containers are presented in Table 3.1.

Table 3.1. Capture efficiency of two types of rearing tubes

Treatment replicates	Capture efficiency	
	Tube A	Tube B
T1	2.38×10^5	9.2×10^5
T2	2.48×10^5	8.9×10^5
T3	2.86×10^5	7.6×10^5
Mean $\pm$ S.E.	$2.57 \times 10^5 \pm 14{,}621$	$8.56 \times 10^5 \pm 49{,}103$

The capture efficiency of the rearing containers varied significantly by LSD, P = 05. Out of 1.86 x 10^6 POBs sprayed on to each diet tube, the diet cup of the Tube B was able to capture 8.56 x 10^5 POBs as against 2.57 x 10^5 POBs captured in tube A. Here the virus capture efficiency was found to be 30 % more in the diet cup of tube B which is attributable to the short length of the diet cup compared to the long bodied tube A. The added advantage of tube B is that the used diet can be changed and diet cup containing fresh diet can be fixed to the main body without disturbing the larva. Similarly the main body can also be changed without disturbing the larva. Above all, being a three-piece tube it is quite easy to remove silken threads and faecal pellets while cleaning. The short height of the diet cup of Tube B also supports an efficient diet dispensing. Thus Tube B could be an excellent option for rearing *H. puera* larvae for NPV mass production as well as for laboratory culture maintenance. The same tube may also be useful for rearing of other lepidopteran larvae of similar behaviour and size.

4. HpNPV MASS PRODUCTION

4. 1. optimisation of *in vivo* mass production techniques

4. 1. 1. Introduction

During the early phase in the development of nucleopolyhedrovirus as insect control agents, the virus Polyhedral Occlusion Bodies (POBs) were extracted from field collected diseased larvae (Hofmaster and Ditman 1961). This was followed by a period when field-collected inocula were used to infect host colonies maintained in greenhouses (Hall, 1957; Elmore, 1961). The labour intensive and cumbersome procedures for producing host plants and advent of semisynthetic diet led to the culturing of insects in laboratory as sustained source of host material for virus multiplication. Now, the simplest method of nucleopolyhedrovirus production is *in vivo* multiplication where, the host larva is inoculated with a small dose of viral inoculum and then incubated until death. The virus occlusion bodies are isolated through maceration, filtration and purification.

In the case of *H.puera* larvae so far no systematic study has been made to select the optimum life stage of the host insect, dosage, incubation period, microclimate or inoculation method favoring maximum virus production. Inadequacies like, spillage during diet dispensing, poor capture efficiency while inoculum spraying, and difficulty in pupal collection and difficulty in cleaning of the tubes were also met with while rearing larvae in cylindrical plastic tubes. These inadequacies warranted improvement of the rearing tubes.

4. 1. 2. Optimization of larval stage, dosage, incubation period and temperature

4. 1. 2. 1. Materials and methods

H. puera larvae (third, fourth and fifth instar) for the study were selected from the insect culture maintained as described earlier. Larvae used for quantification of virus growth were within a narrow weight range (third instar (9 to 13mg), fourth instar

(26 to 37 mg), and fifth instar (80 to 110mg)). HpNPV inoculum prepared from the stock solution was presented to the test larvae (at a dose of 10^2, 10^3, 10^4, 10^5 and 10^6 POBs per larva) on individual teak leaf disc (0.5 cm^2 area) using a 0.5 to 10 µl micropipette.. Larvae fed on leaf discs treated with water served as control. A single larva was placed in each rearing tube containing treated leaf disc. A minimum of 130 larvae were used per dose. The larvae that consumed the whole leaf disc within 6 h period were selected for the study and transferred to rearing tubes containing virus free semi synthetic diet.

For quantification of virus growth, 10 larvae of each dose were sampled at every 12 h interval till 120 h p.i. and freezed to death. Weight of the larvae sampled at each time point was recorded. The larvae for each lot were triturated separately using tissue homogeniser and the homogenate was made up to known volumes with distilled water. Enumeration was carried out using improved Neubauer's haemocytometer. Three replicates were maintained per dosage per larval instar. The test larvae were maintained at 26 ± 2°C and RH of 60 ± 10%.

For identification of optimum temperature regime for HpNPV mass production, fourth instar larvae within a narrow weight range (80 to 110 mg) were used. Cohorts of 10 larvae were fed with 104, 105 and 106 POBs per larva, presented on leaf discs. After 6 h of inoculation, the treated larval cohorts were incubated at 15, 25 and 35 ± 2 °C temperature regimes under a 12L: 12D photoperiod. Control larvae were placed at each of the above temperature regimes. POB yield per larva was estimated at every 12 h interval by enumerating the larval homogenate using improved Neubauer's haemocytometer.

The selection of the better combination of the larval stage, dosage, incubation period and temperature regime is based on the POB yield per unit diet. POB yield per unit diet is calculated as follows:

$$\text{POB yield per unit diet} = \frac{\text{POB yield per larva x percentage of harvested larvae}}{100}$$

4. 1. 2. 2. Results

Optimum larval stage, dosage and incubation period

The harvest percentage of third, fourth and fifth instar larvae fed with different doses of virus inoculum through time course of infection is presented in Fig.4.1.

Fig. 4.1. Proportion of larvae harvested for 3 larval stages against different doses

With the increase in the incubation period, the harvest percentage was found decreasing in all larval stages. In the case of third instar larvae, cent percent harvest was registered at the lower doses (10^2 to 10^4 POBs per larva) during the early periods of incubation (till 60h p.i.). At higher doses (10^5 and 10^6 POBs per larva) the percentage harvest showed a decreasing trend initially (from 12 h p.i. onwards) and it deepened towards the end of the incubation period. In the case of fourth and fifth instar larvae, the percentage harvest showed a decreasing trend towards the end of the incubation period in all the doses tested. But the percentage harvest was found to increase in successive doses for the same incubation period in contrary with that of third instar larvae.

It has been observed (Biji, 2004) that during the initial phase of infection there was an increase in the number of POBs produced in all the doses tested (10^2 to 10^6 per larva) for a particular larval stage. However, towards the end of the infection cycle, maximum POB production was obtained only from lower doses (10^2 to 10^4 per larva). The mean maximum POB yield per larva obtained for the three larval stages (third to fifth instar) is presented in Table 4.1. Of the three larval stages, the maximum POB yield per larva (3.6×10^9 POBs) was obtained from fourth instar larvae dosed at 10^6 POBs per larva and incubated for a period of 72 h p.i. It may be noted that only 90 % of the larvae could be harvested in this case. Achieving maximum virus production per larva, associated with inoculum loss due to over kill or pupation at lower doses does not make a production system efficient. It would be advantageous to go for a higher recovery rate at the expense of a lower average yield. Keeping this in mind for practical mass production, an alternative value, "mean POB yield per unit diet" was arrived at, by considering the POB yield per larva and harvest percentage together. The maximum mean POB yield per unit diet against different doses of HpNPV tested against three larval instars is given in Table 1. It may be seen that mean maximum POB yield per unit diet (3.3×10^9 POBs), was obtained from the fourth instar larva fed with 10^5 POBs, where 98% of the larvae were harvested. Eventhough the 90 % harvested treatment group yielded maximum POBs per larva, it could yield only 3.2×10^9 POBs per unit diet, which was at an expense of 10^6 POBs. Here, the lower mean yield per larva was compensated by 98 % harvest rate.

Table 4. 1. HpNPV yield per larva and yield per unit diet against different doses for different stages

Larval stage	Mean maximum POB yield per larva	Percentage larval harvest	Mean maximum POB yield per unit diet	Log. dose	Mean larval wt. at dosing	Age at dosing	Incubation period (days)	Mean larval wt. at harvest
III	$1.67x10^8$	90	$1.4x10^8$	2	11	4	72	36
IV	$3.6x10^9$	90	$3.2x10^9$	6	31.5	6	72	72
	$3.4x10^9$	98	$3.3x10^9$	5	31.5	6	72	76
V	$3.4x10^9$	100	$2.3x10^9$	6	95	8	84	176

Statistical analysis of the data of three instars of larvae indicated that the POB yield per unit diet varied significantly between larval stages at $p < 0.5$ by LSD. In all the stages, doses 10^4, 10^5 and 10^6 POBs per larva were found to be mutually replaceable since their variation was not found to be significant. Significant variation was observed in the POB yield per unit diet at different incubation periods within a dose for all the stages tested. The maximum POB yield per unit diet (3.3×10^9 POBs per unit diet) obtained from fourth instar larva was found to be significantly varying from the rest of the treatment cases.

Optimum temperature regime

The maximum POB yield obtained per unit diet for fourth instar larvae through different doses and different temperatures are presented in Table 4. 2. In the three doses tested the maximum POB yield per unit diet was obtained from the larvae incubated at 25 °C (3×10^9 POBs against 10^5 POBs per larva) followed by 15 °C (9.25×10^8 against 10^6 POBs per larva) and 35 °C (7.82×10^8 against 10^4 POBs per larva). From the results it is evident that the temperature turns out to be one of the factors determining the productivity of the virus. The maximum POB yield per unit diet was registered at the dosage 10^5 POBs per larva and the temperature 25 ± 2 °C. The virus yield at this temperature-dosage combination was significantly higher than that in the rest of the combinations. In the case of the other dosages tested, maximum virus yield was obtained at 25 ± 2°C followed by 15 ± 2°C and 35 ± 2 °C.

Table 4. 2. Mean maximum POB yield per cell obtained at different temperatures

Dosage POBs per larva	Temp (°C)	No. of larvae tested	Mean max. POB yield per unit diet
10^4	15	43	$3.68x10^8{}_b$
	25	38	$7.82x10^8{}_a$
	35	39	$1.83x10^8{}_c$
10^5	15	36	$1.41x10^9{}_e$
	25	38	$3x10^9{}_d$
	35	42	$7.05x10^8{}_f$
10^6	15	34	$4.35x10^8{}_h$

	25	46	$9.25x10^8{}_g$
	35	45	$2.17x10^8{}_i$

Note: In a column, means followed by the same subscript(s) do not differ significantly by LSD (P=0.05)

4. 1. 3. Evaluation of inoculation methods

4. 1. 3. 1. Materials and Methods

Different virus inoculation methods evaluated :

Application of virus suspension using micropipette on to

A. semi synthetic diet dispensed in rearing tubes
B. leaf disc kept in rearing tubes

Spraying virus suspension using atomizer on to

C. semisynthetic diet dispensed in the rearing tubes
D. tender teak leaves

Concentration of virus stock prepared for inoculation was 10^7 POBs per ml and presentation of inoculum was carried out in such a way that, every treatment lot get equal amount of inoculum irrespective of the method of inoculum presentation. In the case of method A, 10 µl of the prepared inoculum was applied onto artificial diet in rearing tubes, and for method B inoculum was applied on to a leaf disc of 1.5 cm^2 area placed in a rearing tube, both using a 5 to 50 µl micropipette. Under method C, the virus suspension was sprayed on to the diet filled rearing tubes and under method D, the virus suspension was sprayed on to the whole leaf.

In methods, where the inoculation medium used was the artificial diet, the virus suspension was allowed to spread on the diet and then the test larvae were introduced into the diet filled rearing tube. This was the case for methods A and B, and the inoculation period is continuous. Methods where leaf discs were employed as inoculation medium the virus suspension was allowed to dry on the surface and then the test larvae were introduced into the rearing tube. Here the inoculation period was 6 h and the larvae, which completely fed the leaf disc, were transferred to virus free diet for the rest of the experimental time. When the whole leaf was selected as an inoculation medium the test larvae were transferred on to the virus applied leaf kept in a glass container of 20 x 10cm size. After 12h of inoculation period the larvae were transferred into virus free diet for the rest of the experimental time. Each treatment was replicated thrice with 10 larvae per replicate and a control was set where distilled water was used instead of virus suspension. The whole experiment was conducted at a temperature of 26 ± 2 °C, humidity of 60 ± 3 % RH and a photoperiod of 12: 12 (L: D).

Observations were made on easiness in inoculation and percentage of harvestable larvae. Larvae obtained for each time point were homogenized using tissue homogenizer and the homogenate was made up to known volumes with distilled water. Enumeration was carried out and POB yield per larva was calculated. The difference between the size of the infecting inoculum and the per-larval POB yield is expressed as the productivity ratio (PR) (Hunter-Fujita *et al.*, 1998b):

$$PR = \frac{\text{No. of POBs yielded per larva}}{\text{No. of POBs ingested per larva}}$$

4. 1. 3. 2. Results

The yield parameters (harvestable larvae and Productivity ratio (PR) under different inoculation methods with respect is presented in Table 4. 3.

Table 4. 3. Comparison of yield parameters with respect of inoculation methods

Methods compared	No. of larvae tested	Larvae harvested (%)	Productivity ratio (PR) per larva	Easiness
A	45	95_a	$10,560_a$	+++
B	45	91_{ab}	$7,163_b$	+
C	45	94_a	15,400	++++
D	52	86_b	$7,200_b$	++

Note: In a column, means followed by the same subscript(s) do not differ significantly by LSD (P=0.05). Easiness is assessed on an increasing scale from + (difficult) to ++++ (easiest).

The percentage of harvestable larvae under all methods varied significantly from 86 to 95 %. In the case of methods A, B and C, the mean percentage of retrievable larvae did not vary but was significantly higher than in D (for details on methods compared see Section (4. 1. 3. 1.). The productivity ratio was the highest in method C (15,400 POBs) and varied significantly from rest of the methods. Method A was the second most productive (10,560 POBs) followed by methods B (7,163 POBs) and D (7,200 POBs). When compared in terms of easiness, method C was found to be the easiest as it did not involve cumbersome activities such as micropipetting, leaf disc preparation, added larval transfe, etc.

4. 1. 4. Discussion

A central problem associated with *in vivo* mass production is that of obtaining maximum virus production at the expense of minimal requirements. Based on this study, it was evident that the larval stage, inoculum dose, incubation period, temperature and inoculation methods exerted a great control over NPV yield as well as percentage of harvestable larvae.

When the data on the virus production for different larval stages were analysed, it was found that the mean maximum POB yield per larva for both the fourth and fifth instar larvae were registered against a dose of 10^6 POBs per larva. However, in the case of third instar larvae the same was against a dose of 10^3 POBs per larva. For third instar larvae, it was also found that manipulating the inoculum dose had only a limited effect on productivity. By increasing the dose from 10^2 to 10^3 per larva raised NPV yields significantly, but increase in the dose beyond the 10^3 threshold could not increase the yield per larva further. The rise in mean yield found between 10^2 and 10^3 could be due to the increased proportion of the inoculated larvae getting successfully infected rather than increase in the POB per infected larvae. At the lower doses, less number of larvae became infected successfully while at the optimum dose all were infected and showed elevated POB counts. For fourth and fifth instar larvae this optimum dose might be 10^6 POBs per larva. Even though it may be possible to increase the yield by increasing the dose further, it would be at the expense of larvae inept to harvest. This appears to be the reason for obtaining mean max. POB yield per unit diet for fourth instar larvae against a dose of 10^5 rather than against a dose of 10^6.

The time of harvesting is found to play a major role in determining virus productivity. Maximum productivity is obtained when the infected larvae were harvested between 60 and 96 h p.i. which is found to be dose dependent. In *Spodoptera littoralis*, the peak virus productivity was found in insects harvested between 6 and 8 days post inoculation and before that very few POBs were found (Grzywacz *et al*., 1998). In HpNPV infected *H. puera* larvae, the inoculum build up during the initial 12 h p.i. was at faster rate. In the case of *S. littoralis* the low POB production period corresponds to the primary infection in the midgut epithelial cells and most of the POB production occurs only later during the second phase of infection when the fat body, hemocoel and tracheal cells gets infected. However, in the case of *H.puera* the fat body, hemocytes *etc*., gets infected at first resulting in faster build up of virus inoculum during the initial phase (Biji, 2004).

The better performance of the temperature regime 25 ± 2°C over different inoculum doses tested revealed that, this regime is optimal for the multiplication of HpNPV in *H. puera* larva. At 35 ± 2°C the POB yield per unit diet registered was the least. This might be due to the combined effect of reduced larval growth and the larval death at a faster rate without rendering enough time for the virus to get multiplied. At very low temperature (15 ± 2°C) the elongated life span appears to counteract the reduction in larval growth resulting in improved POB yield per unit diet than in larvae kept at 35 ± 2°C. Partial starvation was also met with in the larvae maintained at lower and higher temperatures due to desiccation of semi synthetic diet. Synchronization of larval growth during incubation of viral diseases can be very sensitive to temperature fluctuations (Hunter-Fujita *et al*., 1998b). For *H. zea*, Shieh (1989) found that a deviation of just over 1.0°C could result in as much as 50% (larval size) deviation in a seven-day incubation period. Boucias *et al*. (1980) reported variation in susceptibility of *Anticarsia gemmatalis* to NPV maintained at different temperature regimes. In the case of the larvae maintained at 26.7 °C, the NPV replicated efficiently but the replication was slower at 15.6 °C.

The study also revealed that the methods of inoculum presentation had a great influence on NPV productivity. The major variation between the inoculation methods was the difference in the length of time the larvae were allowed to feed on the virus medium. In the case of methods A and C, feeding of inoculum was continuous and the productivity ratio per larva registered maximum in these methods when compared to methods B and D where the virus feeding time was 6h (virus on leaf disc) and 12h (virus on whole leaf) respectively. Hedlund and Yendol (1974) reported that in the case of *Porthetria dispar* (L) the length of virus feeding time significantly affected the mean number of POB yielded per larva. In *P. dispar*, virus productivity in the continuously fed larvae was significantly greater than that of the larvae fed for 96 h. In *H. puera*, there was a significant difference in productivity ratio for methods A and C inspite of the virus feeding time being the same. This can be explained partially by the presence of a highly significant interaction between the time fed on the inoculum and the distribution of the virus in the medium. In method A, the chances of accumulation of inoculum in the medium was high as the inoculum was dispensed using a micropipette which would result in fast feeding of the accumulated inoculum by the larvae. In method B, the inoculum was dispensed using an atomizer allowing uniform spreading of the virus on the medium, which permitted uniform feeding, by the larvae. Thus the variation in virus productivity is also attributable to the method of inoculum presentation as suggested by Hunter-Fujita *et al*. (1998b).

Even though reports citing various methods of virus inoculum presentation were available (Shieh, 1989; Jones, 1994; Hunter-Fujita *et al*., 1998b) a critical evaluation of these methods for selecting an optimum method for mass-producing

NPV is not available as such. Although the productivity ratio discussed here was achieved when larvae fed with 10^7 POBs per ml on a continuous basis, it is not known whether any other combinations of viral concentrations and controlled feeding period would give a better result. Based on percentage of harvestable larvae, POB productivity ratio per larva and ease of inoculation, the method C (spraying virus suspension on to semi synthetic diet dispensed in rearing tubes) is found to be the most efficient method of inoculation for *in vivo* mass production of HpNPV

4. 2. Production statistics for the project period

4. 2. 1. *H.puera* insects processed in the rearing center

For the *invivo* production of NPV on a regular basis it is important to have a stabilized culture of the host insects in the laboratory. A laboratory culture was started in August 2000 using fifth instar larvae collected from an outbreak population at Valluvasserry, Nilambur. The larvae collected have a high level of parasitism but the survivors of the first generation could initiate a series of generations in the laboratory. Further additions of insects (third and fourth instar) from the field were made during the second week of September 2000. Beyond which no further additions was needed during the reporting period. The larvae from the continuous culture were used for bioassay experiments using HpNPV and for the mass production of the baculovirus. Statistics of the larvae processed during the project period is given in Table 4. 4. Figure 4. 1. represents percentage split up of processed larvae to no. of eggs for the periods 2000-2001 and 2001-2002.

Table 4.4. Larvae processed in the culture room

Period	No. of eggs	No. of larvae used for production	Larvae used for Experiment	Total no. of larvae processed
2000-2001	1,14,032	25,187	1,030	26,217
2001-2002	8,51,553	90,000	67,500	1,57,500
Grand total	**9,65,585**	**1,15,187**	**68,530**	**1,83,717**

Fig. 4. 2. Split up of insects processed in the culture room

4. 2. 2. HpNPV production in the mass production unit during the project period

Availability of sufficient stock of NPV was a prerequisite for the proposed studies. There fore attempts were made to build up a large stock of the material based on the mass production methods opted earlier (2000-2001) and based on the optimized *in vivo* mass production techniques (2001-2002). As the laboratory level we have, for instance, produced enough NPV to spray around 452 ha at around $2x10^{11}$POBs/ha using high volume sprayer. Total HpNPV production during the project period is given in Table 4.5.

Table 4. 5. HpNPV production in the mass production unit

Period	Larvae inoculated	Larvae harvested	POB yield per inoculated larva	Total HpNPV production (POBs)
2000-2001	24,810	20,377	$3.53x10^{8}$	$8.75x10^{12}$
2001-2002*	90,000	72,900	$1.01x10^{9}$	$9.16x10^{13}$
Grand total	**1,14,810**	**93,277**	**$6.3x10^{8}$**	**$9.04x10^{13}$**

**HpNPV production following optimized mass production protocol*

4. 2. 3. Economics of *in vivo* mass production from laboratory reared larvae

In a mass production approach, even the slightest increase in the POB yield could make the pest management strategy more economically feasible. The economics of mass production of the HpNPV using the laboratory-reared and field-collected host larvae was compared. The data were based on 7,645 laboratory-reared (LR) larvae and 8,925 field-collected (FC) larvae during the period of 2000-2001. The virus production was carried out using fifth instar larva inoculated with 10^{6} POBs/larva. The virus yield/larva in LR and FC larvae was $4.8x10^{8}$ and $3.6x10^{8}$ POBs respectively. The cost of production of HpNPV/larva was higher in the case of LR (Rs. 0.82) in comparison with FC (Rs.0.72). However, upon considering the yield/larva from both sources, the cost of HpNPV required for spraying in one hectare at the rate of $1.63x10^{11}$ POBs worked out to be Rs. 279 and Rs. 317 in the case of LR and FC respectively. A marginal difference in the virus yield was found affecting the cost of the virus produced. HpNPV mass production parameters and cost estimates of from laboratory reared and field collected larvae were given in Table 4. 6. and 4. 7.

Table 4. 6. HpNPV mass production parameters of LR and FC larvae

Sl. no	HpNPV mass production parameters	Source of larvae	
		Laboratory-reared	Field-collected
1	Larvae inoculated	6,950	7,319
2	Larvae retrieved (%)	90.7 ± 5.03 (68-97)	84.2 ± 11.17 (86-96)
3	Virus yield (POBs)/larva	$4.8x10^{8}$ ± $1X10^{8}$ ($3.8x10^{8}$- $6x10^{8}$)	$3.6x10^{8}$ ± $2X10^{8}$ ($1 x10^{8}$ - $5.8x10^{8}$)
4	Productivity ratio (virus yield/ virus dosage)	241 ± 55 (190-300)	178 ± 100.85 (50-250)
5	Estimated virus production cost/larva (Rs.)	0.82	0.70

6	Virus production (OBs/Rupee)	5.85×10^8	5.14×10^8
7	Production cost of inoculum for spraying* 1 ha teak plantation (Rs)	279	317

*When ULV spraying done @1.63×10^{11}/ha. Dosage estimated based on LD_{50} value for third instar larvae. Figure in parentheses shows the range values.

Table 4.7. Break-up of expenditures involved in mass-producing HpNPV

Task/materials		Expenditure (Rs.)	
		Laboratory	Wild
Larval collection	Labour	0.00	0.10
	Transport	0.00	0.15
Diet	For rearing third instar upto fifth instar and through post infection	0.26	0.26
	For rearing fifth instar and through post infection	0.10	0.00
Inoculation		0.09	0.00
Retrieval of infected larvae (labour cost)		0.09	0.09
Washing of rearing tubes etc. (labour cost)		0.10	0.10
Larval transfer to the diet tubes	Fifth instar	0.09	0.00
	third instar	0.09	0.00
TOTAL		**0.82**	**0.70**

In industrialized countries the high cost of labor for *in vivo* mass production of viruses made it difficult in the market place. However, in developing countries, with their much lower labor costs, mass producing viruses *in vivo* is a realistic option.

4. 3. modified protocol for *in vivo* mass production of hpnpv

Based on the result of the study, the protocol for mass production of HpNPV has been modified as described below (Plate 4.1)

Rearing Container: The new improved three-piece tube.

Larvae: Fourth instar larva coming under the weight range of 0.27 – 0.36 mg is the ideal stage for *in vivo* mass production of HpNPV.

Inoculation: In order to infect the host larvae, the diet dispensed in the diet cup of new improved rearing tubes is sprayed with HpNPV solution at a concentration of 10^5 OBs per larvae using a chromatographic sprayer. Selected larvae are then transferred into the diet cup and closed using hollow cylinder with the perforated cap.

Incubation and larval harvest: Larvae, were harvested after an incubation period of 72h at a temperature of 25 ± 2°C and frozen on harvesting at -20°C for extraction of the virus.

Extraction and purification of HpNPV: The frozen larvae are macerated in the homogenizer (OMNI 5000 International) with sterile 0.1% Sodium Dodecyl Sulphate (SDS) to prevent the clumping of Polyhedral Occlusion Bodies (POBs). The homogenate is filtered using a muslin cloth to remove coarse insect debris. The filtrate is again filtered, using muslin cloth of three layers. The virus suspension is centrifuged at 100xg for 10 minutes for removing the large contaminant particles

Deleted: the larvae were allowed to feed on artificial diet dispensed with HpNPV in each rearing tube, four hours before inoculation. The diet tubes were arranged in an aluminum tray and the inoculum (HpNPV) at a concentration of $1x10^5$ PIBs/larvae was sprayed on the diet surface using a chromatographic sprayer.

Deleted: which

Deleted: lauryl

and the supernatant is collected and again centrifuged at 6000xg for 25 minutes in a REMI R-24 centrifuge (with angle rotor) to produce a pellet, comprising mainly of virus POBs. For removing the SDS the pellet is resuspended in distilled water. Once the POBs settled at the bottom, the supernatant is removed. This process is repeated thrice and the retrieved POBs are enumerated using improved Neubauer's haemocytometer, and stored at 4°C. A realistic model for small-scale production of HpNPV is described in Fig. 4. 2 which may be scaled-up when large quantity of the virus is required.

5. HPNPV FORMULATIONS

5. 1. Introduction

One of the limitations of baculovirus applications is that the virus could be rapidly deactivated by environmental factors. The key elements leading to the degradation of NPVs in the field are UV light, rainfall and possible effects from the host plant (Evans and Harrap, 1982). In a field trial carried out in teak plantations in Nilambur it was observed that the killing potential of HpNPV dropped from 97% to 70% over a six hour exposure to sunlight. Further the rate of decay continued up to about 24 hours and by 12 days it was about 13%. When subjected to various levels of artificial rainfall (up to 200 mm per hour) there was rapid loss of virus activity and the mortality of the test larvae dropped from 90 % to 15% (Sudheendrakumar et al., 2001). Hence the need for developing a stable formulation of HpNPV was felt.

5. 2. Materials and Methods

5. 2. 1. Preparation of HpNPV formulations

The formulations were developed foloowing the general methods summarized by Hunter-Fugita *et al.* (1998) with suitable modifications wherever required.

5. 2. 1. 1. Wettable Powder formulation (WP (A-))

Since the active ingredient (HpNPV) is insoluble in water, a wettable powder formulation was tried. A stock suspension of HpNPV (380 ml) containing $6.7x10^{11}$ OBs was taken and centrifuged. The sediment was poured into glass petridishes and dried in an incubator maintained at a temperature of 30 °C and 30 % RH for three days. The dried virus was powdered using pestle and mortor and stored in dark bottles at room temperature (31ºC to 39 ºC).

5. 2. 2. 2. Wettable Powder with Additives (WP (A+))

The stock suspension of HpNPV containing $2.93x10^{12}$ OBs was taken and centrifuged. With the sedimented OBs 98 g of talc, 4 ml of Plantowet (a surfactant) and 4 % activated carbon were added and mixed thoroughly and the resultant slurry was dried in the incubator at 30 °C and 30 % RH for a day. The dried virus was powdered using pestle and mortar and stored in dark bottles at room temperature (31 ºC to 39 ºC).

5. 2. 1. 3. Wettable Powder by Lactose Co-precipitation (WP-Co-prec. (A-))

Fifty ml of acetone was added to 500 ml aqueous suspension of virus containing $9x10^{11}$ POBs in the presence of 4% dissolved lactose. This resulted in the precipitation of both POBs and lactose. The precipitate was recovered by filtration and air-dried. The dried powder was ground and stored at room temperature.

5. 2. 1. 4. Wettable Powder by Lactose Co-precipitation with Acetone (WP- Co-prec. (A+))

HpNPV polyhedra were sedimented from virus suspension (500ml) containing $2.93x10^{12}$ POBs by co-precipitation with lactose. The pellet containing the OBs was resuspended in 50ml of acetone and mixed thoroughly with 98g of talc to make

slurry. The slurry was kept overnight under room temperature for evaporation of acetone. To this preparation, 4ml of plantowet and 4% activated carbon were added and mixed thoroughly in a pestle and mortar. The powder obtained was stored at room temperature.

5. 2. 1. 5. Wettable Powder - Freeze dried (WP-FD)

Freeze drying is a process of removing the water from a frozen product by sublimation. As the ice sublimates, it leaves voids in the dry residual material making it easy to dehydrate, thus preserving chemical structure and biological activity of the product.

This formulation was prepared by mixing 100ml of HpNPV suspension containing $3.1x10^9$ POBs per ml with a talc (4 %), 4g of activated carbon and 4ml of Plantowet and 1% Polyvinyl Alcohol. The slurry was mixed well and dried in a freeze drier (CHRIST ALPHA 1-2). The dried powder was stored in airtight container and stored at room temperature.

5. 2. 1. 6. Wettable Powder - Micro Encapsulation (WP-Encaps)

Encapsulated formulations are relatively new. The active ingredient is encapsulated using a polymer to allow prolonged release of the pesticide over a period of time. Encapsulation provides a slow release mechanism and usually is more expensive than other formulations. However, the long-term benefit often makes this formulation feasible. Here each viral particle is encapsulated by starch which is a UV protectant to maximize field persistence and thereby increasing effectiveness.

HpNPV was purified by sucrose gradient centrifugation. Purified HpNPV (30ml) containing $6.1x10^{10}$ OBs was added to distilled water (10 ml) and 1g of refined coconut oil, which was previously mixed with 750 mg of activated carbon. This preparation was then mixed vigorously with10g of pregelatinized starch. The mixture was refrigerated at 5ºC for three days (to promote retro gradation of starch) and dried in the incubator at 30ºC. The powder was blended with 5g of pearl cornstarch, and then pulverized using pestle and mortar. The product was stored at room temperature.

5. 2. 1. 7. Flowable Concentrate (FC)

Flowables are formulated by impregnating the active ingredient on a diluent or carrier. These particles then are suspended in a small amount of liquid as a thick, paste-like material.

Virus suspension (200 ml) containing $2.8\ x10^{11}$ POBs was mixed with 5% Carboxy Methyl Cellulose, 4% of carbon and Plantowet and the resultant suspension was stored at 4ºC.

5. 2. 2. Evaluation of HpNPV formulations

5. 2. 2. 1. Laboratory evaluation of HpNPV formulations

HpNPV formulations were compared on the basis of physical loss of POBs during processing, variations in biological activity after processing and persistence against natural sunlight.

Estimation of process linked physical loss of OBs

The physical loss of HpNPV while processing regarding each formulated product was estimated by quantifying the viral POBs before and after formulation.

Estimation of process linked loss of biological activity of formulated products

Effect of formulation on Virulence of the virus

To evaluate the efficacy of various formulations of HpNPV, bioassays were carried out using early fifth instar *H.puera* larvae. The test larvae were fed with three concentrations (10^2, 10^4 & 10^6 POBs) of each of the seven formulations and of an unformulated crude suspension (as standard) through leaf disc method. Bioassay was replicated thrice with an untreated control. For each treatment 90 larvae were used.

Effect of sunlight on viability of formulated products

The stability of each formulation to sunlight was estimated by exposing them to sunlight. Twenty-four teak plants of comparable height (in the range 100-150cm) and exposed to sunlight were selected at Nedumgayam teak plantation in Nilambur in May 2002. Ten microlitre of the prepared virus suspension containing 10^8 OBs per ml was applied on precisely defined areas (marked using a ballpoint pen) on the upper leaf surface. Separate plants were used for different formulations. The leaves were removed from the plants at intervals of 0, 3, 6 and 9 h after virus application and the marked leaf area containing the known dose of virus was cut out and fed to individual *H. puera* larvae. After 5h, the larvae were transferred individually to tubes containing artificial diet. For each formulation, the bioassay was replicated twice with 30 larvae per replicate. Observations on larval death were recorded daily from 48 h of ingestion until death or pupation of the test insect.

Cumulative larval mortality for 96h was used to compare differences between the various formulations and sunlight exposure periods. The percentage original virus activity remaining was computed for each formulation at all time points of exposure to sunlight as per the formula given below (Ignoffo *et al*., 1977).

$$\text{Percentage of OAR} = \frac{\text{Percentage of larval mortality after exposure}}{\text{Percentage of larval mortality before exposure}} \text{ X100}$$

5. 2. 2. 2. Field evaluation of freeze dried formulation

Based on the performance of the formulated products in laboratory evaluation, the freeze-dried formulation was found to be the best and was selected for field-testing. As a standard for comparison unformulated crude virus sample was also included in the study. The field trial was carried out during a major outbreak of the teak defoliator in Panayangodu teak plantations (Nilambur Forest Range) in April 2002.

Using RCBD (Randomized Complete Block Design) technique, selected area under teak plantation was divided into three blocks of equal size. Each block was again divided into three treatment plots containing ten trees each. Between each plot, a strip of 20m width was left untreated as buffer. The treatments included freeze dried formulation, crude HpNPV and a plain water control. The experiment was carried out

in such a way that each block received all the three treatments. Within a block, the plots receiving a particular treatment were randomly selected.

Since the trees in the outbreak area was in the range of 15 to 17m in height, a high volume sprayer has to be used for virus application instead of an ultra low volume sprayer, which was used during earlier field trials. The HpNPV application parameters like dosage and quantity of inoculum was recalculated for the high volume sprayer. Field dosage was estimated for third instar larvae based on LD_{90} for that instar. The quantity of spray fluid per tree was flexible *i.e.,* required for uniform coverage depending on the foliage. The tank mix had a concentration of 2 $x10^7$ OBs per litre. Each tree within the treatment plot was individually sprayed using a motorized Birla Yamaha high volume motorised sprayer, which was set to a spray tank pressure of 40 kg/cm^2. The quantity of spray solution applied for each tree was one litre. The assessment of treatment effect was recorded as follows:

The field larval sampling method suggested for *H. puera* by Nair *et al*. (1998) was adopted for the study. Larval sampling was done on three trees per plot before the treatment and at 48, 72 and 96h post spraying. From each of the tree sampled, three shoots each from top, middle and bottom levels were taken from which dead and live larval samples were collected randomly to identify the cause of mortality. The total number of larvae present in each shoot was also tabulated. Larvae sampled were stored individually in sterile polythene covers and smears were prepared for each larva and checked for HpNPV infection under a light.

5. 2. 3. Estimation of shelf life of HpNPV

The shelf life of the different formulations of HpNPV was quantified at two different storage conditions *viz*. at room temperature and at 4 ºC.

Samples of the four Wettable Powder formulations along with unformulated HpNPV were stored at temperature (28ºC – 30ºC). A sample of WP-FD was also stored at 4 ºC. he WP-FD formulation was stored in a glass container and the other wettable powder formulations were stored in black polypropylene bottles for a period of 18 months. The infectivity of the HpNPV formulations was determined before and after storage by challenging early fifth instar teak defoliator larvae.

The infectivity loss during storage was computed as:

Activity ratio = LD_{50} value obtained before stoage/LD_{50} value obtained after storage

The infectivity before and after storage was compared with that of crude suspension of HpNPV stored in similar conditions in each case.

5. 2. 4. Data analysis

From the data obtained from the laboratory and field trials, mean percentage mortality was calculated, which was transformed into probit values and the significant difference was analyzed using Duncan's multiple range tests at 95 % probability level. The percentage Original Activity Remaining (OAR) was calculated as per Ignoffo *et al*. (1977) and the data was subjected to analysis of variance. Mean and Standard Error was calculated using the statistical package SPSS 10.0 and compared using ANOVA and the LSD for test for significance ($p<0.05$). The dosage-mortality data were subjected to probit analysis (Finney, 1971) to calculate the respective LD_{50} value of each formulation.

5. 3. Results

5. 3. 1. Laboratory Evaluation of HpNPV formulations

5. 3. 1. 1. Process linked physical loss of OBs

The process-linked loss of the virus for each of the formulations is given in Table 5. 1. The loss of the virus ranged from 1.2% to 8.1% with regard to all formulations except the FC, which had absolutely no loss. This is because of the fact that the FC has not undergone any physical transformation and retrieval processes as in the case of the other formulations.

Table 5. 1. Virus lost during formulation

Formulation	Quantity of NPV Used	Quantity of NPV in final product	Virus lost (%)
FC	$2.8x10^{11}$	$2.8x10^{11}$	0
WP-FD	$3.1x10^{11}$	$3.06x10^{11}$	1.2
WP-Encaps	$6.15x10^{10}$	$6x10^{10}$	2.4
WP- Co-prec. (A+)	$2.9x10^{12}$	$2.74x10^{12}$	5.5
WP- Co-prec. (A-)	$1.3x10^{12}$	$1.2x10^{12}$	7.6
WP (A+)	$2.93x10^{12}$	$2.7x10^{12}$	7.8
WP (A-)	$6.75x10^{11}$	$6.2x10^{11}$	8.1

5. 3. 1. 2. Process linked loss in biological activity of formulated products

Virulence

The virulence of different formulated products of HpNPV on *H. puera* is given in Table 5.2. The LD_{50} value of all the formulated products except WP- Co-prec. (A+) were comparable to that of unformulated crude virus suspension. The LD_{50} values obtained for the Wettable Powder formulation produced by Lactose Co-precipitation with Acetone was 320 times less virulent than the unformulated HpNPV and 280 times less virulent than the highly efficient formulated product *i.e.*, WP-FD formulation which retained 90 per cent of the original activity.

Sunlight persistence

The OAR after exposure to sunlight for different time intervals varied between the formulated products (Table 5. 3.). After 3 h exposure to sunlight the maximum OAR was observed in WP-Encaps formulation (100 %) and FC formulation (100 %) against a minimum OAR in WP (A-) formulation (86.4 %). When the exposure period increased to 9 h the OAR decreased to 37.8 % in the case of unformulated crude HpNPV against a maximum OAR in WP-FD (88.5 %) and WP-Encaps (86 %) formulations that were significantly varying from the rest of the formulations. This result clearly indicated that formulation increases the viability of HpNPV under natural sunlight by 2.34 folds. It was also able to deduce that the WP-FD formulation is more stable to different periods of exposure than the rest of the formulations.

Table 5. 2. Laboratory evaluation of biological activity of HpNPV formulations

Formulations	No. of insects	LD_{50} values	95 % confidence limits lower - upper	Slope (SE)	Original activity remaining (% OAR)
Crude virus	187	0.052^{a}	0.029 – 0.095	1.302(0.22)	100.0
WP-FD	147	0.058^{a}	0.024 – 0.128	1.0(0.16)	90.0
WP- Co-prec. (A-)	195	0.079^{a}	0.046 – 0.141	1.271(0.17)	65.0
WP-Encaps	209	0.089^{a}	0.035 – 0.199	0.673(0.09)	57.0
FC	200	0.105^{a}	0.049 – 0.203	0.872(0.12)	49.0
WP (A-)	201	0.165^{a}	0.089 – 0.298	1.387(0.18)	31.0
WP (A+)	241	8.148^{ab}	3.629-18.772	0.579(0.07)	0.6
WP- Co-prec. (A+)	215	16.454^{b}	3.581- 50.82	0.97(0.14)	0.3

LD_{50} in a column followed by the same subscripts are not significantly different, LSD, P= 0.05; SE - Standard Error

Table 5. 3. Infectivity of HpNPV formulations on exposure to natural sunlight

Formulations	Mortality (%)	OAR after different exposure periods (%) (Time in hours)			
	'0'hr	6 Am* (0 h)	9 Am (3 h)	12 Noon (6 h)	3 Pm (9 h)
WP-FD	100_{b}^{a}	100	96.5_{ab}^{bc}	94_{ab}^{b}	88.5_{ab}^{b}
WP-Encaps	100_{a}^{a}	100	100_{a}^{c}	91_{b}^{ab}	86_{b}^{b}
WP- Co-prec. (A+)	84.5_{a}^{b}	100	95.3_{a}^{a}	84.02_{a}^{ab}	67.5_{bc}^{a}
WP (A+)	79.5_{a}^{b}	100	97.4_{a}^{a}	86.5_{a}^{ab}	62.9_{bc}^{a}
FC	100_{a}^{a}	100	100_{a}^{c}	82_{b}^{ab}	55.5_{c}^{a}
WP (A-)	100_{a}^{a}	100	86.4_{b}^{a}	49_{c}^{a}	40_{c}^{a}
WP- Co-prec. (A-)	100_{a}^{a}	100	90.5_{b}^{ab}	47_{c}^{a}	39_{c}^{a}
Crude HpNPV	100_{a}^{a}	100	89_{b}^{ab}	56.5_{c}^{a}	37.8_{d}^{a}

Note: Means followed by the same superscripts in a column and means followed by the same subscripts in a row are not significantly different (P<0).
*For comparison, the OAR of each formulated product at 0 h is taken as cent percent irrespective of their killing efficiency.

5. 3. 2. Field evaluation of WP-FD formulation against *H. puera* population

The number of *H. puera* larvae found infected by WP-FD formulation and crude HpNPV in the experimental teak plots prior to and after treatment is given in Table 5.4. The larval samples collected from all the plots immediately before HpNPV application (0 h) were free from NPV infection. In the case of the larval samples collected two days post spraying, 3% and 6.4% of the larvae in the plots sprayed with crude suspension and formulated product respectively were found infected with HpNPV. By fourth day of HpNPV application, the infected larvae increased to 47.6 %

and 42.9 % in the plots treated with crude NPV and formulated NPV respectively. However, in the control plot during the second and third days of sampling, a very low level infection was recorded (1.2 - 7.0 %). But this incidence of infection did not increase further. Even though the disease incidence in plots treated with crude suspension and formulated product were significantly varying with respect to control plots the variation between themselves was not found to be significant. However, it was observed that the freeze-dried formulated product is as good as or even better than the crude HpNPV suspension under field conditions.

Table 5. 4. Mortality of *H. puera* larvae in the experimental teak plots treated with HpNPV

Treatment	Larvae infected (%) *			
	Hours post spraying			
	0	48	72	96
Untreated control	0(108)	$1.2(75)_a$	$7.0(30)_a$	$0(34)_a$
Formulated product (WP-FD)	0(121)	$6.4(108)_b$	$31.6(79)_b$	$47.6(21)_b$
Crude suspension	0(93)	$3.0(69)_b$	$22.0(37)_b$	$42.9(19)_b$

** Figures in parenthesis indicate total number of larvae sampled per treatment. In columns, the difference between values followed by the same subscript is not statistically significant*

5. 3. 3. shelf life of HpNPV formulations

5. 3. 3. 1. Shelf life at room temperature

The result of the bioassay to determine the infectivity loss of various formulations of HpNPV during stored at room temperature for eighteen months is presented in Table 5. 5.

It can be seen that the unformulated crude suspension lost its infectivity almost completely by 18 months storage at room temperature. Ewhile WP-Encaps lost about 79% of the original activity by the same period, WP (A+) and WP (A-) retained about 50% of the original activity. The maximum remaining activity of 76 % was registered with WP-FD formulation after a shelf life of 18 months.

Table 5. 5. Shelf life of HpNPV formulations at room temperature (28°C – 30°C)

Formulation	Storage period (month)	No. of insects	LD_{50} ($x10^4$)	Fiducial limits	Slope	% Activity loss
Crude virus	0	187	0.052	0.029 – 0.095	1.302(0.22)	0
	18	71	1.301	0.298 – 5.327	0.6(0.12)	99.96
WP (A-)	0	201	0.165	0.089 – 0.298	1.387(0.18)	0
	18	157	0.316	0.119 – 0.792	0.58(0.08)	48.00
WP (A+)	0	195	0.079	0.046 – 0.141	1.271(0.17)	0
	18	194	0.166	0.008 – 0.367	0.62(0.08)	52.00
WP-FD	0	147	0.058	0.024 – 0.128	1.0(0.16)	0
	18	80	0.076	0.033 – 0.191	1.3(0.23)	24.00
WP-Encaps	0	209	0.089	0.035 – 0.199	0.673(0.09)	0
	18	131	0.42	0.190 – 0.93	0.89(0.129)	79.00

5. 3. 3. 2. Shelf life of WP-FD formulation at 4°C

The storage stability of HpNPV in freeze-dried formulation as compared to crude virus when stored at 4 °C is given in Table 5. 6. The loss of original activity in the case of WP-FD formulation was 59 % and 69 % after a storage period of 6 months and 12 months respectively. However, in the case of crude virus, the loss of activity for the same storage period was 15 % and 29 % respectively. It can be seen that the crude virus was able to retain infectivity better than the freeze-dried formulation when stored at 4 °C. Thus it can be confirmed that storage at 4 °C is not advisable for the freeze-dried formulation. By the end of 18 months, the loss of infectivity in the case of crude HpNPV was about 44 %. Storage of WP-FD at 4 °C thus appeared to be not a requirement as it can be well be stored at room temperature by retaining about 80 % original activity for a period of about 18 months.

Table 5. 6. Effect of storage at 4ºC on the pathogenicity of the crude and formulated HpNPV tested on fifth instar larvae of *H. puera*

Formulation	Storage period (month)	No. of insects	LD_{50} ($x10^4$)	Fiducial limits	Slope	% Activity loss
Crude virus	0	187	0.052	0.029 – 0.095	1.302(0.22)	0.00
	6	155	0.061	0.03 – 0.121	0.93(0.13)	15.00
	12	82	0.073	-	0.77(0.15)	29.00
	18	77	0.093	0.02 – 0.341	0.65(0.13)	44.00
WP-FD	0	147	0.058	0.024 – 0.128	1.0(0.16)	0.00
	6	83	0.141	0.026 – 0.516	0.57(0.11)	59.00
	12	80	0.188	0.063 – 0.574	0.81(0.2)	69.00

5. 4. Discussion

Field trials using HpNPV had already shown that the virus is very much unstable to the environmental influences (Sudheendrakumar *et al*., 2001). However, in the present study various formulations tested have proved to be more stable than unformulated virus.

The process of developing product formulations confronts various impediments of which the process linked physical loss and loss of biological activity of the virus are the major aspects. In the present study, the wettable powder formulations registered a loss of virus ranging from 1.2 % to 8.1 %. Comparatively the lowest loss was observed in the case of the freeze-dried formulation (WP-FD) and the microencapsulated formulation (WP-Encaps) (1.2% and 2.4% respectively). Not much similar information is available on the formulations of other NPVs for a comparison. With regard to loss of biological activity, WP formulations, WP (A+) registered the lowest original activity of 0.6 % compared to WP (A-) (31 %). Both WP formulations were prepared by long duration, low temperature air-drying of the HpNPV suspension. The only difference between these formulations was the presence of additives (talc, carbon, and plantowet) in the former and the absence of the additives in the latter. It is possible that in the case of WP (A+) the process was not able to develop a stable formulation with protective additives without compromising the biological activity of HpNPV. The interaction of the additives with the virus is likely to have a deleterious effect on HpNPV.

The WP-Co-prec. (A+) and WP- Co-prec. (A-) were prepared by co-precipitation of HpNPV with lactose and acetone. The difference between these formulations was the presence of the additives in the former and absence of the additives in the latter. While WP-Co-prec. (A+) lost almost all the original activity, WP- Co-prec. (A-) could retain 65 % of its original activity. The adverse effect of acetone on the active agent might be the reason for this variation in performance. Ignoffo *et al*. (1976) found that a commercial formulation of acetone-dried preparation of virus diseased larvae mixed with lactose was less effective than water based formulation against *H. armigera*. Ethiraju *et al*. (1988) also reported that the efficiency of *Helicoverpa armigera* NPV was affected in the case of dust formulation prepared involving acetone. It may be seen that both WP (A+) and WP-Co-prec. (A+) containing additives irrespective of the process of preparation registered more than 99 % loss in biological activity as compared to the crude virus. The formulations, WP-Encaps and FC retained 57% and 49% of original activity respectively. It appears that the interaction of the additives with the virus had some adverse effect on the biological activity of the NPV.

Compared to all other formulations, the freeze-dried (WP-FD) formulation performed superior by retaining 90% of the original activity. This indicated that freeze-drying process is able to develop a stable formulation with protective additives without compromising the biological activity of HpNPV. Freeze drying involves freezing aqueous suspension of baculovirus occlusion bodies (OBs) and volatilization under vacuum for complete removal of moisture. Retention of stability after freeze-drying has been confirmed in the case of many NPV formulations (Kennedy and Sathiah, 2001). Eventhough additives had an adverse effect on HpNPV in the case of most of the other WP formulations (WP (A+), WP-Co-prec. (A+), WP-Encaps), such adverse effect was not found in the case of WP-FD. It could be possibly because that a true interaction of the additives with the virus did not take place as the process of formulation was carried out at a very low temperature.

The crude virus registered the lowest original activity after 9 h exposure to sunlight (38 %). As in the case of crude virus, WP (A-) and WP-Co-prec. (A-) both without any additives, also lost their infectivity very drastically during the period of exposure of 9 h (6 AM to 3 PM). Various workers reported inactivation of occluded insect viruses due to UV radiation in the past (Cantwell, 1967; Bullock, 1967; Singh and Battu, 2001). According to Ignoffo *et al*. (1976), the rapid inactivation of NPV in the environment is primarily due to the UV irradiation associated with sunlight and it is mostly assumed that the damage is caused to the viral DNA. Hence, protection of NPV against UV inactivation deserves much attention while developing formulations.

In the case of the HpNPV formulations with additives, carbon (activated charcoal) was used as UV protectant. In general, the formulations with additives and carbon as UV protectant were found retaining high level of original activity on exposure to sunlight. The highest loss of infectivity was recorded during the hours 9 AM to 3 PM. Carbon is known to absorb infrared rays through the visible UV wavelengths. Carbon is usually employed in a very finely divided form such as activated charcoal/carbon. The UV protection ability of carbon in virus formulations have been studied by various workers (Jaques *et al*., 1971; Ignoffo *et al*., 1972; Bull *et al*., 1976). Ignoffo *et al.* (1991) reported that *Hz*SNPV formulations containing carbon with 15 % corn oil achieved 95% original activity remaining compared to 20 % original activity remaining for those formulations without carbon. Activated charcoal/carbon is a less expensive ingredient and can be easily and locally produced by most developing nations (Hunter Fujita *et al*., 1998a).

Among the formulations developed, the starch encapsulated formulation, WP-Encaps and the freeze-dried formulation, WP-FD retained a comparable original activity of 86% and 88.5% respectively. Dunkle and Shasha (1988; 1989) demonstrated that starch encapsulation technology (Shasha, 1980) could be successfully used to produce UV stable NPV formulations. Starch, a low-cost and abundant natural polymer is composed of linear chains (amylose) and highly branched chains (amylopectin). When starch is gelatinized in water and cooled, the amylose retrogrades (*i.e.* the chain in dispersion associate, become insoluble and precipitate). Ignoffo *et al.* (1991) prepared granular formulation of *H.armigera* NPV based on corn starch and solar protectants, which are readily degradable in insect gut and proved that all sunlight exposed starch encapsulated formulations of *Hz*SNPV containing a UV protectant were significantly more active against tobacco budworm larvae than controls without UV protectant.

Eventhough the performance of WP-FD and WP-Encaps were comparable, the process of preparation of WP-Encaps appeared to be cumbersome as it required gradient purified NPV for better quality of the encapsulated product. Apart from this WP-Encaps requires more ingredients than WP-FD which include starch, pearl corn starch, coconut oil in addition to the usual ingredients like carbon, talc and plantowet. While WP-FD can be prepared within a day, preparation of WP-Encaps requires about three days as the virus with the ingredients has to be refrigerated at 5^{o}C for three days to promote retrogradation of starch. Compared to WP-Encaps, WP-FD requires only semi purified virus for preparing the formulation. It is known that compared to pure virus, unpurified NPV with haemolymph content of the host larvae containing proteins, fats and other substances can act as stickers and UV protectants (Cherry *et al*., 2000). A crude preparation and a formulation of *Ag*MNPV containing an UV protectant presented a half life of approximately seven and eight days, respectively, compared to a half life of about three days for the partially purified *Ag*MNPV (Moscardi, 1983; 1986). Hence, it appears that WP-FD containing semipurified HpNPV could have an additional advantage over WP-Encaps in resisting UV degradation.

Based on the efficacy and the UV stability, WP-FD was selected for the field evaluation. The infection percentage recorded in the experimental plots treated with WP-FD formulation and the crude HpNPV was significantly different from that in untreated plots. However, there was no significant difference regarding the infection percentage between the plots treated with WPFD and the crude virus. Under UV stability experiment (section. 5. 3. 2.), It was found that the crude virus may lose its virulence when exposed to sunlight. However, a loss of infectivity was not reflected in the result obtained from the field experiment, as the performance of both crude virus and WP-FD was comparable. This could have happened only if the virus was consumed by the host larvae immediately after its application on the teak leaf before it was subjected to UV inactivation. However, in the case of WP-FD which is highly UV stable there could be only a minimum loss of virus activity even if it is kept exposed to sunlight for about 9h. Hence a delay in consuming WP-FD formulation by the host larvae need not have any adverse effect on the percentage mortality. However, while fixing the field dosage the virus activity loss if anyt may be needs to be taken into account.

In the case of storage of HpNPV, the crude virus lost its potency during prolonged storage of 18 months at room temperature. Jenkins and Grzywacz (2000) reported that in the case of unpurified NPV suspension, the loss of virulence was substantial during longterm storage. However, the mechanisms of inactivation have not been well understood. Some factors such as UV light are well known to cause the inactivation of NPVs even when it stored is as suspension (Ignoffo and Garcia, 1992). It was suggested that an appropriate UV-opaque packaging could be used to

prevent this. The NPV occlusion bodies are also pH-sensitive and while very stable between pH 4.0-9.0, activity is reported to deteriorate outside this range (Ignoffo and Garcia, 1966). It appears that the storage life of crude HpNPV suspension can be increased by storing it in an opaque container and by maintenance of appropriate pH.

Loss of activity of virus formulations stored at ambient temperature has been reported by various workers (Kaupp and Ebling, 1993; Lewis and Rollinson, 1978; Moscardi *et al.,* 1989; Gessler *et al.,* 1991). In this study, the HpNPV formulation, WP-FD stored at room temperature performed better than all the formulations by retaining 76% original activity at the end of the 18-month storage period. All other formulations including the microencapsulated formulation lost most of their infectivity. Rabindra *et al*. (1989a) reported that the wettable powder formulation of NPV of *S. litura* lost 22.6% of its original activity when stored for a period of six months. According to them the loss of virulence in the formulations could be due to either the alkaline condition of the formulation or due to either storage at room temperature.

Crude HpNPV lost 44% of its initial virulence when stored at 4 ^{o}C for a period of 18 months. Based on earlier studies Stuermer and Bullock (1968) and Pawar and Ramakrishnan (1971) observed that continuous exposure to low temperature could reduce the infectivity of NPVs. The freeze-dried formulation stored at 4 ^{o}C when taken out of shelf 12 months after storage had lost 69 % of its activity. It also showed change in its physical characteristics by becoming clumpy and undissolvable in water. This could probably be because of retention of any moisture in the product at the time of packing. It is concluded that storage at 4^{o}C is not advisable for the freeze-dried formulation.

Short shelf life of many of the virus formulations is a major challenge. The moisture content and contaminant microbial load will be crucial factors affecting the storage stability of many water-based formulations. In India, most of the NPV formulations sold for *H. armigera* and *S. litura* are simple aqueous suspensions with no stabilizers or UV protectants and hence do not have adequate efficacy, shelf life and field persistence (Rabindra, 2002).

The freeze-dried wettable formulation, WP-FD which has been developed with due consideration of the above problems has been selected as the potential HpNPV formulation. The process of development of WP-FD ensures retention of biological activity as high as 90%. The product also ensures high-level protection against UV degradation. Apart from this WP-FD can be stored at room temperature up to 18 month with retention of original activity as high as 76 %. Being a dry powder formulation, the product can be kept without any microbial contamination. Field evaluation has also established the efficacy of this product.

6. BIOSAFETY OF HpNPV

6. 1. Introduction

Baculoviruses are naturally occurring pathogens exclusively known from arthropods and mostly from insects. They are not infective to plants or vertebrates and have been used for biological insect control for more than 100 years. However, as in the case of any novel insecticide, the potential impact of a viral pesticide on the environment has also to be strictly evaluated before it is used for field application. Unlike chemical insecticides, biological agents are capable of replicating and persisting in the environment for many years. The use of pesticides (insecticides, fungicides, weedicides, plant growth regulators and biopesticides including microbial pesticides) are regulated in India under the Insecticides Act, 1968, and rules framed there under. For this purpose the pesticides should be included in the "Schedule" to the Insecticides Act. One of the requirements for registration of nucleopolyhedrovirus under section 9 (3) of the Insecticides Act, 1968 is the data on safety testing (Pawar, 2002).

The safety of HpNPV to higher organisms has so far not been worked out. It is known that the cultured mammalian cells are ideal for assessing the relative toxicity of a series of compounds (Perchermeier *et al*., 1994) and the rationale behind the use of Vero (Africal green monkey kidney cell line) cells is that these cells can be banked and well characterized. In addition, the continued use of animals is problematic from ethical and economic viewpoints. Sf-9 cells derived from *Spodoptera frugiperda* (ovary) cells are used for virus amplification and plaque assays. Hep-2 (Human larynx cell) cells are mostly used in virus and tumourigenity studies. Hence the present investigation was carried out to determine the in vivo and *in vitro* safety of HpNPV. Silkworm (*Bombyx mori*) was used for the *in vivo* study and three cell lines such as Hep2, Vero and Sf -9 cell lines were used for the *in vitro* study.

6. 2. Materials and Methods

6. 2. 1. *In vivo* safety assessment against silkworm (*Bombyx mori*)

The infectivity of HpNPV to the mulberry silkworm *Bombyx mori* was studied. Semipurified and formulated HpNPV produced in the laboratory were used for the present study.

A culture of *B. mori* (L.) was raised in the laboratory from the eggs obtained from the Sericulture Department, G.D. Naidu Agriculture University, Coimbatore, Tamil Nadu. Eggs were allowed to hatch and emerged larvae were reared upto second instar on mulberry leaves. Separate sets of mulberry leaves were sprayed with semipurified and formulated HpNPV solution of concentration of 10^8 OBs per ml. The leaves were air dried for 10-15 minutes. Thirty larvae (second instar) were allowed to feed on each set of virus treated leaves. A control set was maintained on untreated leaves. Control and treatments replicated thrice with 30 larvae per replication. Data on mortality if any, larval weight, pupation percentage, fresh weight of cocoons, shell weight and fecundity were recorded.

6. 2. 2. *In vivo* safety assessment against Indian mynah

As part of a study on the dispersal of HpNPV through birds, HpNPV was fed to an Indian mynah maintained in the laboratory. The bird was kept under observation for a period of three months.

6. 2. 3. *In vitro* safety assessment using cell lines

Cultured mammalian and insect cells are ideal for assessing the relative toxicity of a series of compounds. In this study, three cell lines namely, Sf-9, Hep-2 and Vero obtained from National Center for Cell Science, University of Pune Campus, Ganesh Khind, Pune were used. The rationale behind the use of Vero (Africal green monkey kidney) cell line rather than monkey kidney cells as such these cells can be banked and well characterized. In addition, the continued use of animals is not justified from ethical and economic viewpoints. Vero cells are sensitive to infection with SV-40, SV-5, measles, arbovirus, reovirus, rubella, simian adenoviruses, polioviruses, influenza viruses, parainfluenza viruses, respiratory syncytial viruses, vaccinia, and others. Sf 9 cell lines derived from *Spodoptera frugiperda* ovary were used for virus amplification and plaque assays. Hep-2 (Human larynx) cell lines are mostly used in virus and tumourigenity studies.

The cells were first checked under phase contrast microscope for contamination if any, and excess medium was removed and cells were incubated at appropriate temperature depending on the cell line. All the cells were grown as monolayers in plastic disposable tissue culture flasks. To maintain the cells within the log phase and to prevent them from entering their stationary phase, they were subcultured.

6. 2. 3. 1. Maintenance of Sf9 cell lines

Healthy Sf-9 cells were grown in TNFM Medium (pH- 6.2) containing 10 % of Foetal Bovine Serum. The cell culture medium was equilibrated at room temperature before using it. Subculturing of cells was carried out when the cells attained confluence growth on plates, which occurred thrice a week at a passaging ratio of 1:3. For subculturing, the medium was removed and kept undisturbed for 5 minutes. Then the sides of the flask were gently taped from all sides so as to dislodge the cells from the monolayers. The cells were then transferred to fresh tissue culture flask containing medium and serum and were maintained at 28 ºC.

6. 2. 3. 2. Maintenance of Hep-2 and Vero cell lines

Hep-2 and Vero cells were grown in Minimum Essential Medium (Eagle) (MEM (E) with Earle's BSS (pH - 7.2) containing 10 % of Fetal Bovine Serum. Metabolism of growing cells in a closed tube results in the production of CO_2 and acidification of the growth liquid. To counteract the pH decrease, a bicarbonate buffering system was employed in the culture medium. To keep the cells at physiologic pH (pH - 7.2), phenol red, a pH indicator, which is red at physiologic pH, purple at alkaline and yellow at acidic pH was added. The medium was sterilized by pressure filtration with 0.22 µm filters. The media were periodically checked for bacterial and fungal contamination using Nutrient Broth, Sabourauds and Thioglycolate media (HIMEDIA). The old acidic medium was removed and fresh medium was added 2-3 times weekly.

For sub culturing the cells, the medium was removed (old acidic medium), fresh TPVG (Phosphate buffered saline (PBS) with 0.1 % trypsin, 0.2 % versene and 0.5 % glucose) was added. After 1-2 minutes TPVG was removed. The culture flasks were kept at 37 ºC for 10 min. In between the flasks were taken out and the sides of the flasks were tapped. After 10 min, fresh medium was added and agitated to dissociate the cells from the surface. Homogenous suspension was made and seeded into new culture flasks. The cell density was estimated and subcultures with a density of 106 cells per flask were made.

6. 2. 3. 3. Estimation of cell density

The cell density of the master culture was estimated so as to seed the correct cell density while subculturing. The cell density was determined using an improved Neubauer's haemocytometer. After passaging, the cells were taken into a sterile centrifuge tube and centrifuged at 1000g for 3 min. The supernatant was carefully removed without disrupting the cell pellet. The pellet was resuspended by adding 2 ml of fresh medium through the sides of the tubes and mixed well. For enumeration, 0.2 ml of the cells was diluted in 0.7 ml of medium and 0.1 ml of trypan blue (2 % w/v, in Phosphate Buffer saline (PBS)) was added to it and mixed well. Trypan blue stain was used to distinguish viable cells from non-viable cells; live healthy cells appear bright, round and refractile and exclude the blue coloured dye; non-viable cells absorb the dye and appear blue in colour. From this, one drop was taken using Pasteur pipette and poured on to the sterile haemocytometer. The cells were counted under phase contrast microscope (x10 objective). All the cells within the 5 x 5 square grid of the haemocytometer, and touching the middle line of the triple line on the top and left of the squares were enumerated. This count gives the number of cells present in 0.1 µl of stock. Counting was repeated thrice to estimate the correct cell densities. Number of cells present in one ml was calculated by the following formula:

Cells per ml = Mean cell number x 10^4 x dilution factor

6. 2. 3. 4. Freezing and thawing cells

For freezing, the cell density was kept at 4 x 10^6 per ml. Then the cells were centrifuged at 400 rpm for 5 min. Supernatant was removed and freezing mixture *i.e.*, 4 ml of medium containing 0.5 ml of serum was added to the cells. The test tube was kept in a beaker containing ice and then 0.5 ml of DMSO (Dimethyl Sulphoxide) was added drop by drop. Quickly one ml of the aliquot was added in to freezing vials, the cells were frozen slowly, and initially it was placed in –20 ºC for 1 h, and then over night at –80 ºC and transferred in to liquid nitrogen.

The frozen cells were thawed after removing from the liquid nitrogen by gentle agitation in a 37ºC water bath at 30 seconds. The outside of the vial was rinsed with 70% ethanol and aseptically the cells were transferred into centrifuge tube, and 10ml of medium was added and centrifuged at 400 rpm for 5min, supernatant was removed and the pellets were resuspended in 10ml of fresh medium. This was transferred into a fresh tissue culture flask, and incubated at 27ºC for Sf-9 cells, for Hep 2 and Vero maintained at 37ºC.

6. 2. 3. 5. Preparation of virus inoculum

The crude and formulated HpNPV isolated from the laboratory reared *H.puera* larvae were used in these experiments. After gradient centrifugation, 5 ml virus suspension containing 1.3 x 10^9 POBs/ml was mixed with equal volume of 0.1M Sodium Carbonate with the suspension of polyhedra and stirred at 28ºC for 30 min. The pH of the suspension was adjusted to less than 8.0 by adding 100mM Tris-HCl, at pH 7.5 and passed through 0.45 µm disposable filter. Five dilutions of the filtrate (10^4, 10^5, 10^6, 10^7 and 10^8 POBs per ml) were used as inocula. The formulated HpNPV was also treated the same way and dilutions were prepared using tissue culture medium. One untreated control was kept. The experiment was replicated thrice.

6. 2. 3. 6. Inoculation of cells

To screen for HpNPV susceptibility, healthy cells of each cell line (Hep-2, Vero and Sf-9) containing 10^6 cells in one ml of growth medium were transferred to 25 cm^2

tissue culture flask (Nunclon) and incubated for three days (Sf-9 at 28ºC and Hep 2 and Vero cells at 37ºC). Before infecting the cells with virus, the cell lines were tested under the microscope to confirm all cells are viable. The cell lines, which were attached well and formed an even monolayer that is not too sparse, overcrowded or clumped (if they are too crowded the virus will not replicate efficiently) were selected. From the flasks containing cell lines the medium was removed and 1ml of each virus inoculum was added dropwise, very gently over the cells. In the case Sf-9, the flasks were maintained at 27ºC ± 0.5ºC for I h. Vero and Hep2 were incubated at 37ºC for 1 h. The flasks were rocked gently every 5 to 10 min to ensure an even coverage of virus over the cells. After 1 h incubation, the remaining inoculum and culture medium was removed. Using Pasteur pipette the medium was poured on to the other side of the flask so as to not dislodge the cells. The inoculated flasks of Sf-9 cells were closed and maintained at 28 ºC while the Hep 2 and Vero cells were kept at 37ºC. After inoculation the cells were examined with photographic ocular inverted phase contrast microscope every 4h interval for cytopathic effect (CPE) and photographed every 24h upto 5 days.

6. 3. Results

6. 3. 1. Safety of HpNPV to silkworm (*Bombyx mori*)

The results are presented in Table 6.1. It can be seen that the silkworm infected with HpNPV did not show any mortality. Larvae fed with crude and formulated HpNPV did not show variation with regard to larval weight, fresh weight of cocoons, shell weight and fecundity. Similarly there was no significant difference in the foresaid aspects between the treated larvae and the untreated larvae.

Table 6.1. Safety of HpNPV to *Bombyx mori* larvae

Treatment	Mean Larval weight (g) ± SD	Mean Pupation %	Mean Weight of cocoon	Mean Weight of cocoon shells	Mean Fecundity
Silkworm fed with untreated leaf	3.19 ± 0.15	87.90 ± 8.8	1.18 ± 0.16	0.17 ± 0.11	599.7 ± 15.6
Silkworm fed with crude HpNPV treated leaf	3.20 ± 0.19	84.14 ± 8.1	1.14 ± 0.22	0.14 ± 0.02	574.4 ± 31.1
Silkworm fed with formulated HpNPV treated leaf	3.19 ± 0.21	74.49 ± 5.2	1.15 ± 0.16	0.14 ± 0.03	561.0 ± 3.6
'F' Statistic	0.98 NS	0.16 NS	0.75 NS	0.27 NS	0.14 NS

NS-Not significant; SD – Standard deviation

6. 3. 2. Safety of HpNPV to the bird, Indian mynah

In the case of the mynah fed with NPV infected *H. puera* larvae, presence of the viral occlusion bodies was noted in the faecal droppings within 1 hr post feeding. Presence of viral particles in the faeces was observed until 24h post feeding. This suggested that the viral particle did not remain in the body of the bird for long time. After 24h post feeding, no viral presence was observed in the faeces. The test bird

behaved normally and remained healthy throughout the observation period of three months suggesting no deleterious effect of HpNPV.

6. 3. 3. *In-vitro* safety testing of HpNPV

The HpNPV treated Sf-9 cell lines were periodically observed under the microscope for the presence of occlusion bodies up to five days. But none of the Sf-9 cell lines were found infected (Plate 6.1: Fig 1, a-b) and the number of healthy cells were found increased. The treated cells were not showing any difference compared to the untreated cell lines. The same was the case with the HpNPV treated Hep 2 ((Plate 6.1: Fig 2, a-b) and Vero cell lines ((Plate 6.1: Fig 3, a-b) where no death of cells, giant cell formation or clumping of cells were found.

6. 4. Discussion

Nucleopolyhedroviruses are exclusively known from arthropods and considered to be host specific and safe. Safety test of more than 51 entomopathogenic viruses including more than 30 baculoviruses resulted in a long and complete safety record (Ignoffo, 1973; Burges *et al*., 1980a, 1980b; Groner, 1986). No adverse effect on human health has been observed in any of these investigations indicating that the use of baculovirus is safe and does not cause any health hazards. Eventhough NPVs are generally considered to be safe to non-target organisms, safety testing is mandatory for their registration as commercial pesticides. Hence, in the present investigation the safety of HpNPV was looked into in detail.

Non infectivity of crude HpNPV to lepidopteran forest pests like the caster semi looper, *Achaea janata*, ailanthus webber, *Atteva fabriciella*, Cassia semi looper *Catopsilia crocale*, ailanthus defoliator, *Eligma narcissus*, teak skeletonizer, *Eutectona machaeralis* (Ahamad, 1995), *Helicoverpa armigera*, *Spodoptera litura*, *Amsacta albistriga*, *Bombyx mori* (Rabindra *et al*., 1997) have been reported in the past. The present study has reconfirmed the host specificity of HpNPV and its safety to silkwom. The present study also revealed that HpNPV is safe to the Indian mynah, which may be applicable to all other birds in general. This information is valuable, as several birds have been reported to be present in teak plantations when teak defoliator outbreaks occur. It may be assumed that feeding on HpNPV infected teak defoliator larvae would no way be harmful to the birds.

No cytopathic effects were observed in any of the cell lines inoculated with crude as well as formulated HpNPV. The possibility of replication of baculovirus in vertebrates and mammals were investigated by challenging many vertebrate and human cell lines with OB of many baculoviruses by various workers. Although virus uptake of these cells was frequently reported, no evidences of virus replication or cytopathological effects were observed. The few early reports, which stated baculovirus replication in vertebrate cell lines (Himeno *et al*., 1967; McIntosh and Shamy, 1980) could never be demonstrated or confirmed in other laboratories.

Using a recombinant *Ac*MNPV containing the cat gene under the control of the Rous sarcoma virus terminal repeat promoter and the β-galactosidase gene under the control of the very late polyhedrin promoter reporter gene, expression was analysed in different invertebrate and vertebrate cell lines (Carbonell *et al*., 1985; Carbonell and Miller, 1987). No cat or β-galactosidase activity was detected in transfected mouse or human carcinoma cells. On the other hand, recent reports showed that recombinant *Ac*MNPV virus is efficiently taken up by human hepatocytes via an endosomal pathway. Recombinant *Ac*MNPV carrying the *Escherichia coli lacZ* reporter gene under control of the Rous sarcoma virus promoter and mammalian RNA processing signals showed considerable expression levels in the human liver cell

line HepG2, but at very low levels, or not at all, in cell lines from other tissues (Hofmann *et al*., 1995; Boyce and Bucher, 1996). Based on these findings it was suggested that baculovirus might be exploited for liver-directed gene therapy. From the view of baculovirus safety this results also showed that careful attention has to be paid to the promoters used to control heterologous gene expression in recombinant baculoviruses.

Safety of baculovirus is a topic, which needs to be reviewed very often. Adverse effects of baculovirus formulation on non-target organisms have been reported at least in few instances. In a recent study lethal effect of NPV of *Lymantria dispar* (LdMNPV) to larvae of coot calm (a molluscan species) *Mulinia lateralis* was reported (Gormly *et al*., 1996). Recently Elizabeth *et al*., (2001) reported the adverse effect of wettable powder formulations of *Anticarsia gemmatalis* NPV to a general pentatomid predator, *Podisus nigrispinus*, through generations. However, the unformulated, *A. gemmatalis* NPV was specific and safe to the predator. Further it was suggested that the observed effect could be due to some inert components present in the commercial formulation Narayanan (2002).

7. GENETIC VARIATION IN HpNPV POPULATIONS

7. 1. Introduction

Among insect viruses, members of the family Baculoviridae, especially NPVs have been considered as the most potential biocontrol agents compared to GV and CPV (Rabindra, 2001). The usefulness of NPVs in control of economically important pest species focused interest in selecting isolates with increased virulence and genetic stability. In the case of HpNPV, strain variation is not yet known and this chapter communicates the attempt made to collect HpNPV samples from the wild populations of *H. puera* species in the state of Kerala and compare them genetically.

7. 2. Materials and Methods

H.puera larvae showing typical symptoms of nucleopolyhedrosis were collected from the teak plantations at Nilambur (latitudes 11°10'N and 11°25'N and longitudes 76°10'E and 76°25'E) and Konni (latitudes 9°3'N and 9°19'N and longitudes 76°51'E and 77°13'E) regions in Kerala State of India during 2001-2002. Six samples: five from Nilambur and one from Konni were obtained. Another sample obtained in the year 1999 from *H. puera* insectary, at Kerala Forest Research Institute (KFRI) Subcenter, Nilambur was also included in the study. In order to compare the samples of HpNPV to a standard, a sample currently in use at HpNPV production unit at KFRI Subcenter, Nilambur was used.

H. puera larvae required for the study were obtained from the Entomology Laboratory at KFRI Subcenter Nilambur, Kerala, India, which had been reared over 50 cycles. The larvae were maintained on semi synthetic diet as per the methods described in Section 2.1. Viral isolates were propagated in newly molt fifth instar *H. puera* larvae (mean weight of 95.00 mg) by feeding larvae with POB contaminated semi synthetic diet (10^6 POBs per larva) and maintained at 26 ± 2 °C and 60 ± 10 % RH.

Larvae infected with individual NPV isolates were pooled but each isolate was handled separately to avoid any cross-contamination. The dead larvae were surface sterilized by immersing in 5 % Sodium Hypochlorite for 3 to 5 min. Abdominal epithelium of the selected larvae was cut using fine sterilized scissors releasing fluid loaded with POBs into a sterilized petri-dish. This was then vortexed for 10 sec with twice the amount of distilled water and filtered through 4 layers of muslin cloth. The filtrate was then centrifuged for 2 to 3 sec to pellet out insect debris. The supernatant was transferred carefully to a clean centrifuge tube and centrifugation was carried out for 2 min at 10,000 rpm. The pellet of NPV seen as a light coloured area was made up to one ml with distilled water and vortexed to resuspend the pellet. The concentration of the POBs was estimated and the viral suspension was stored at 4 °C as stock solution for experimental purpose.

To reveal the genomic variability in HpNPV samples, DNA characterization was carried out using the restriction endonuclease enzyme - *Hind*III by following the methods of Smith and Summers (1979). To perform REN analysis, an aliquot of purified POBs were incubated with 0.5M EDTA and proteinase K at 37 ºC for 1h 30 min in a Techne Dri-Block DB-2A heater. To this, 1M Sodium Carbonate was added and incubated at 37 ºC for 15 min to release the virions from POB. After adding 10 % SDS, the released virions were incubated at 37 ºC for 30 min and centrifuged at 10,000 rpm for 3 min to remove undissolved occlusion bodies. DNA from the pellet was extracted using phenol-chloroform DNA extraction method. The extracted DNA

was dialysed in 1x tris acetate EDTA buffer at 4 ºC for 36 h changing the buffer thrice at 12h interval.

Restriction enzyme digestions were performed according to the manufacturer's recommendations at 37°C for 4h. Restriction endonuclease enzyme used in this study was *Hind*III obtained from Gibco-BRL®. Electrophoresis was carried out using a BIORAD DNA subcell electrophoresis system using 0.6% agarose gel at 35V (22 mA current) over night. Lambda (λ) DNA cut with *Eco*RI and a 1 kb molecular weight marker were used as standards. The gels were stained with ethidium bromide solution and photographed under a UV-transilluminator (UVP) using Polaroid direct screen instant camera (Copal, DS34). The molecular weight of the fragments were calculated using 1D Ultra-Lum Total Lab Analysis Software-PC Version 2 (Total lab software, UK) based on the molecular weight of the co-migrating λ DNA and 1 kb ladder fragments and their migration distances.

In order to ascertain which samples of HpNPV generated different DNA profiles, banding patterns of all the 8 samples were compared to each other. Each band holding a unique position in the gel was numbered to form a list and every profile was visually checked to ascertain which bands in the lists it possessed. Presence or absence of a particular band was scored as "1" or "0", respectively and the scores were tabulated to produce rows of "ones and zeros". Once in a tabulated form, the row of ones and zeros for each sample was usually compared to all the others visually to identify those containing unique patterns of bands. The tables were then combined to form a single set of data, which was analysed in the multivariate statistical analysis programme, NTSYS-PC Version 2 (Exeter Software, New York State). Cluster analysis using the unweighted pair-group method with arithmetic means (UPGMA) was performed on the data set and a dendrogram produced showing levels of homology between isolates.

7. 3. Results and Discussion

Restriction endonuclease analysis of 8 HpNPV isolates using *Hind*III restriction endonuclease showed that despite being the same virus species, they are genetically distinct (Fig. 7. 1).

Fig. 7. 1. *Hind*III restriction endonuclease digest profile of DNA of HpNPV isolates. λ (marker), 1 (LST), 2 (NDM), 3 (SGD), 4 (KON), 5 (PGD), 6 (CUL), 7 (KKT), 8 (KKD), 1 kb (marker).

From the restriction profile, it is also evident that this genomic heterogeneity could be brought out using a single enzyme, *i.e. Hind*III. REN profiles of *Hind*III digested isolate genome were characterized by varied number of restriction fragments with obviously varying mobility. Following digestion by *Hind*III, the DNA of the isolates LST and KON produced 6 restriction fragments each; KKT and PGD isolates produced 9 restriction fragments each while the rest of the isolates produced 8 fragments. The DNA of LST and KON isolates was unique as they missed one of the three high molecular weight fragments found in rest of the isolates. The SGD isolate was found to be distinctive in having a 5.97 kb fragment instead of an 11.03 kb fragment. The estimated molecular weights of the genome of HpNPV isolates ranged from 79.37 kbp to 112.14 kbp.

Pooled analysis of similarity in isolates was carried out and based on the similarity coefficients, a dendrogram was constructed (Fig. 7. 2). Isolates LST, KON and CUL forms an individual group 'A' within a similarity coefficient of 0.91. Isolates PGD and KKT with similarity coefficient of 1.00 and forms group B that joins group A at a similarity coefficient of 0.86. Isolates NDM and KKD with a similarity coefficient of 0. 88 and forms group C that joins the above group at a similarity coefficient of 0.803. The isolate SGD stands alone and forms an out-group and joins other isolates at a similarity coefficient of 0.80.

Fig. 7. 2. Dendrogram showing homology between HpNPV isolates

This study on genetic variation in naturally occurring HpNPV population is of great practical value from an ecological point of view. The results of REN analysis confirmed existence of the genetic heterogeneity in HpNPV isolates. The genetic variation observed in the isolates collected from the Nilambur region needs emphasis, since variation to such extent might be reported for the first time in the case of regional isolates i.e., 5 natural variants from an area of 8,500 ha. The insertion of host DNA could be a major source of variation in the case of HpNPV isolates obtained from Nilambur region since a long association between HpNPV and H. puera existed in the region. Studies on population dynamics of *H. puera* in Nilambur using RAGEP technique were able to deduce that the out break populations and endemic populations are genetically distant. Their finding suggests the

immigration of moths from distant teak plantations as the causative for outbreaks (Chandrasekhar *et al*., 2005). This continuous intermixing of genetically distant host populations and occurrence of several generations in a year could add up to the co evolution aspect, but clear cut evidences are still to be obtained.

According to Possee and Rohrmann (1997), minor genetic differences can have biologically significant consequences in terms of the phenotypic characteristics of a virus. Biological studies with respect to the virulence and persistence of the variants will help to improve the quality of virus products by selecting the most competitive isolates. Hence, the prospects of developing highly effective and stable HpNPV biocide against teak defoliator using the most potential isolate or making use of the synergism offered by isolate combinations is worth attempting.

8. SUMMARY AND CONCLUSIONS

Baculoviruses have been used for biological control of a vide variety of insect pests of agricultural as well as forestry crops. However, a well understanding of both the pest and the pathogen is a prerequisite for the success of the biocontrol programme.

The important facets of an efficient biocontrol scheme involving a nucleopolyhedrovirus are (i) optimization of production techniques for greater efficiency in their production; (ii) formulation (to enable ease of application & increased environmental persistence); (iii) safety of the product; (iv) longer shelf life; and (v) field application strategies (dosage estimation and spraying techniques) (vi) screening for virus strain with increased pathogen virulence and speed of kill. Hence, the main purpose of this study was optimization of HpNPV mass production techniques, formulation of the effective agent and safety testing of HpNPV. Since it is known that variation in genetic makeup is an indicative of variation in biological activities an attempt to understand natural variation in HpNPV in terms of genetic makeup was also carried out.

As part of the virus mass production, method was standardized to maintain a continuous culture of the host insect in a specifically designed laboratory system using larvae reared on synthetic diet. A novel three piece insect rearing tube was designed in order to enhance the efficiency of insect rearing. The easiness in filling the diet, handling the insects and cleaning the tubes after use etc. were found to be the major advantages of the new tube design compared with the conventional rearing tubes. The study identified the best insect stage for optimizing the virus production in combination with optimum dose, incubation period and temperature. Accordingly fourth instar larva dosed with 10^5 POBs, incubated at 25 ± 2 °C temperature and harvested at 72 hour post infection yielded 3.6 x 10^9 POBs. Different inoculation methods were evaluated and the application of the virus using an atomizer on the diet surface within the rearing tubes was found to be the best and easiest to handle several tubes at a time.

Another major achievement of the study is the development of a product formulation of HpNPV. Out of the six wettable powder formulations and a flowable concentrate developed and evaluated, the Freeze Dried Wettable Powder formulation with activated charcol as UV protectant proved to be the most efficient from the point of view of virulence, UV stability, field efficacy and storage life of about 18 months with retention of a high percentage of original activity.

Biosafety tests of Freeze dried HpNPV formulation and crude HpNPV revealed their safety against insect, bird and insect, monkey and human cell lines.

The study brought out evidences of genetic varaiation within HpNPV population. It was found that the eight isolates discovered showed variation in the molecular weight ranging from 79.37 kbp to 112.14 kbp. This knowledge is valuable from the point view of selecting the best isolate in the biocontrol programme. However, a detailed study is warranted to understand the virulence and stability of the isolates and the efficacy *per se* and in combination.

It is expected that the HpNPV technology including the mass production of the virus, development of formulations and field application would soon find a place in forest working plan for management of the teak defoliator for enhancing productivity in teak plantations. The technology is also expected to transfer to the private teak growers soon.

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