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Standardization of Mass Production in Three Isolates of Nucleopolyhedrovirus of *Helicoverpa armigera* (Hübner)

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Abstract: The effect of four major parameters, viz., larval age and weight, inoculation dose, incubation temperature and time of harvesting the larvae, on the production of three isolates of nucleopolyhedrovirus of *Helicoverpa armigera* (*Ha*NPV), viz., Ooty (OTY), Coimbatore (CMB) and Negamum (NGM) were evaluated. Early 5th instar larvae recorded the maximum yield of virus per larva when inoculated with a dose of 5×10^5 POB $larva^{-1}$ and incubated at a temperature of 25°C. Also, highest POB yield was recorded when virosed larvae were harvested as cadavers. However, among the isolates tested in this study, CMB isolate collected from Tamil Nadu, India, showed the highest yield per larva in all of the conditions.

Key words: Nucleopolyhedrovirus, geographical isolates, *Helicoverpa armigera*, mass production

INTRODUCTION

Since viral pathogens are obligate in nature, they must be necessarily multiplied on their natural live hosts from which they have been collected (Narayana, 2003). The selection of a virus for pest management depends not only on its bioefficacy but also on the ease of mass production (Sherman, 1985). Nucleopolyhedroviruses find extensive use in assisted control programmes for early management of pests (Carner and Yearian, 1989; Rabindra *et al*., 2003; Dolinski and Lacey, 2007). While propagation of the virus in cell culture continues to receive increasing attention (Shuler *et al*., 1995), *in vivo* production has been found to be the only feasible method for large scale propagation of virus (Hunter *et al*., 1998). There has been continuous flow of information on the *in vivo* production of baculoviruses, notably the nuclear polyhedrosis viruses in many insects (Shapiro, 1986; Ebora *et al*., 1990). However, to increase the yield of three *Ha*NPV isolates collected from India and selection the highly promising viral isolate under *in vivo* conditions a series of laboratorial studies were conducted. The present investigations were therefore undertaken to evaluate the effect of stage of the host larvae, incubation temperature and dose of virus inoculum as well as the time of harvest on the *in vivo* yield of three *Ha*NPV isolates.

MATERIALS AND METHODS

The *H. armigera* culture used in the study was maintained on a semi-synthetic diet based on Shorey and Hale (1965) in Bio-Control Research Laboratories (BCRL), Bangalore, India. Three Indian isolates of *Ha*NPV collected from Coimbatore (CMB), Negamum (NGM) and Ooty (OTY) of Tamil Nadu state, India, were used in this study during the year 2005. These isolates were passaged through early fifth instar larvae of host insect at 25±1°C to get uniformity in their virulence since they had been stored in the refrigerator for prolonged period of time. All the experiments were performed in insect virology laboratory of Project Directorate of Biological Control (PDBC), Bangalore, India.

Effect of larval age and weight on virus yield: Mid 4th, late 4th, early 5th and mid 5th instar larvae of *H. armigera* were used to study the influence of insect age on virus yield. Semi-synthetic diet without formaldehyde was prepared and filled in 5 mL glass vials up to 1/3rd height of the vial. A quantity of 10 µL of virus suspension (5×10^5 POB $larva^{-1}$) was applied onto the diet surface using a micropipette providing a dose of 1965.78 POB mm^{-2}. The suspension was spread uniformly

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over the diet surface with the polished blunt end of a glass rod (6 mm). Larvae in the four age groups were weighed individually in an electronic balance and transferred to treated diet. The treatments were replicated three times and included a control for each age group and isolate. Each treatment had 40 insects. After inoculation, the larvae were incubated at 25±1°C in an incubator. The mortality in different treatments was recorded at 24 h interval. Upon death, the cadavers were collected, transferred to sterile vials and the number of harvestable cadavers in each treatment was recorded. The samples were frozen immediately.

The cadavers were homogenized individually using a glass pestle and mortar with distilled water. The homogenate was transferred to a measuring cylinder and volume made up to 25 mL with distilled water. Enumeration of polyhedra was performed in this stage and the following parameters were calculated:

$$\text{Yield larva}^{-1}\text{(POB)} = \frac{\text{POB mL}^{-1} \times \text{Suspension volume (mL)}}{\text{Total No. of cadavers}}$$

Yield per 100 inoculated larvae (POB) = Yield larva^{-1} × Corrected larval mortality (%)

$$\text{Productivity Ratio (POB)} = \frac{\text{Yield larva}^{-1}}{\text{POB inoculated larva}^{-1}}$$

Effect of inoculation dose on virus yield: The effect of inoculation dose on the yield of the virus was studied against early 5th instar larvae. The larvae were exposed to different doses of 1965.87, 393.17 and 78.63 POB mm^{-2} of the isolates. Aliquot of 10 µL virus suspension was applied on the diet surface as mentioned earlier. Each treatment had 40 larvae in three replications for each isolate. After inoculation the larvae were incubated at 25±1°C in an incubator. Untreated controls for the respective isolates and doses were maintained similarly. The mortality was recorded at 24 h interval and the experiment was terminated on 10th day. The cadavers were collected daily, frozen and processed and yield was assessed as described earlier.

Effect of inoculation temperature on virus yield: Early 5th instar larvae were exposed to 1965.87 POB mm^{-2} of diet surface of each viral isolate. Similar procedure was followed as described above except the incubation temperatures were either room temperature (28-31°C), 25±1°C, or 30±1°C.

Effect of period of larval harvest on virus yield: Early 5th instar larvae of *H. armigera* were allowed to feed on diet surface treated with viral dose of 1965.87 POB mm^{-2} and incubated at 25±1°C for each NPV isolate. The infected larvae were harvested five, six, seven and eight days after inoculation. Also, a treatment of harvesting the cadavers was included for comparison. An untreated control was also maintained. Each treatment was replicated three times with 40 larvae per replication.

The larval cadavers were transferred to sterile vials, plugged with cotton and frozen immediately. The cadavers were then processed and POBs were enumerated as described earlier.

Statistical analyses: Analyses of variances (ANOVA) were carried out using SAS software version 6.12 and means were separated by Duncan's Multiple Range Test (DMRT). All data in percentage were transformed to arcsin $\sqrt{}$percentage and the data from POB yields were subjected to log transformation and then analyzed. The larval counts were also transformed to $\sqrt{x+0.5}$ values. The probit analyses in various experiments were carried out in a Statistical Package for Social Sciences (SPSS), version 10.0 for windows.

RESULTS

Virus yield in relation to larval age and weight: A significant influence of larval age and weight on the per cent larval mortality, yield larva^{-1}, yield per 100 inoculated larvae and productivity ratio of the isolates was observed in the assay (Table 1). Of the different larval ages, early 5th instar recorded significantly the highest yield and productivity with all the three isolates tested. Mid 4th, late 4th and mid 5th instars registered decreased production of POB.

The initial larval weight was critical for obtaining higher POB yield in all the isolates tested in this study. The maximum POB yield was related to the early 5th instar larvae with initial weight range of 65.46-68.13 mg (Fig. 1).

Virus yield in relation to inoculation dose: Optimization studies on inoculation dose revealed that larval mortality increased as the inoculum dose advanced. Highest mortality of 83.77, 82.90 and 82.05% was recorded at inoculum dose of 1965.87 POB mm^{-2} of NGM, OTY and CMB isolates, respectively (Table 2). Similarly, the virus yield per larva and for 100 inoculated larvae was significantly higher at this dose of all the three isolates. The productivity ratio of all the isolates decreased progressively as the dose of inoculation advanced.

Table 1: Effect of larval stage on the yield of *Ha*NPV isolates at an inoculation dose of 5×10^5 POB $larva^{-1}$ and an incubation temperature of 25±1°C

*Ha*NPV isolates	Larval stage	Mean harvestable cadavers (out of 40)	Yield $larva^{-1}$ ($\times10^9$ POB)	Yield per 100 inoculated larvae ($\times10^{11}$ POB)	Productivity ratio ($\times10^6$ POB)
CMB	Mid 4th	39.67	2.69d	2.67c	0.54d
	Late 4th	39.67	4.12c	4.09b	0.83c
	Early 5th	34.33	6.73a	5.73a	1.35a
	Mid 5th	29.33	5.81b	4.22b	1.16b
NGM	Mid 4th	40.00	1.79d	1.79c	0.36d
	Late 4th	38.00	3.45c	3.27b	0.69c
	Early 5th	33.67	6.45a	5.38a	1.29a
	Mid 5th	30.33	4.55b	3.42b	0.91b
OTY	Mid 4th	40.00	1.95d	1.95c	0.39d
	Late 4th	35.33	3.56c	3.12b	0.71c
	Early 5th	33.33	5.83a	4.83a	1.17a
	Mid 5th	29.33	4.63b	3.33b	0.92b

In a column, for each isolate means followed by the same letter (a-d) are not significantly different (p = 0.05) by Duncan's Multiple Range Test (DMRT)

Table 2: Effect of dose of inoculum on the yield of *Ha*NPV isolates achieved from early 5th instar larvae at an incubation temperature of 25±1°C

*Ha*NPV isolates	Dose (POB mm^{-2})	Larval mortality (%)	Yield $larva^{-1}$ ($\times10^9$ POB)	Yield per 100 inoculated larvae ($\times10^{11}$ POB)	Productivity ratio ($\times10^6$ POB)
CMB	1965.87	82.05a	6.87a	5.64a	1.37c
	393.17	81.39a	6.13b	4.99b	6.13b
	78.63	74.56b	2.51c	1.87c	12.57a
NGM	1965.87	83.77a	6.49a	5.44a	1.30c
	393.17	80.39b	5.30b	4.26b	5.30b
	78.63	74.56c	2.32c	1.73c	11.58a
OTY	1965.87	82.90a	5.87a	4.86a	1.17c
	393.17	80.33b	4.99b	4.01b	4.99b
	78.63	74.56c	2.19c	1.63c	10.93a

In a column, for each isolate means followed by the same letter (a-c) are not significantly different (p = 0.05) by DMRT

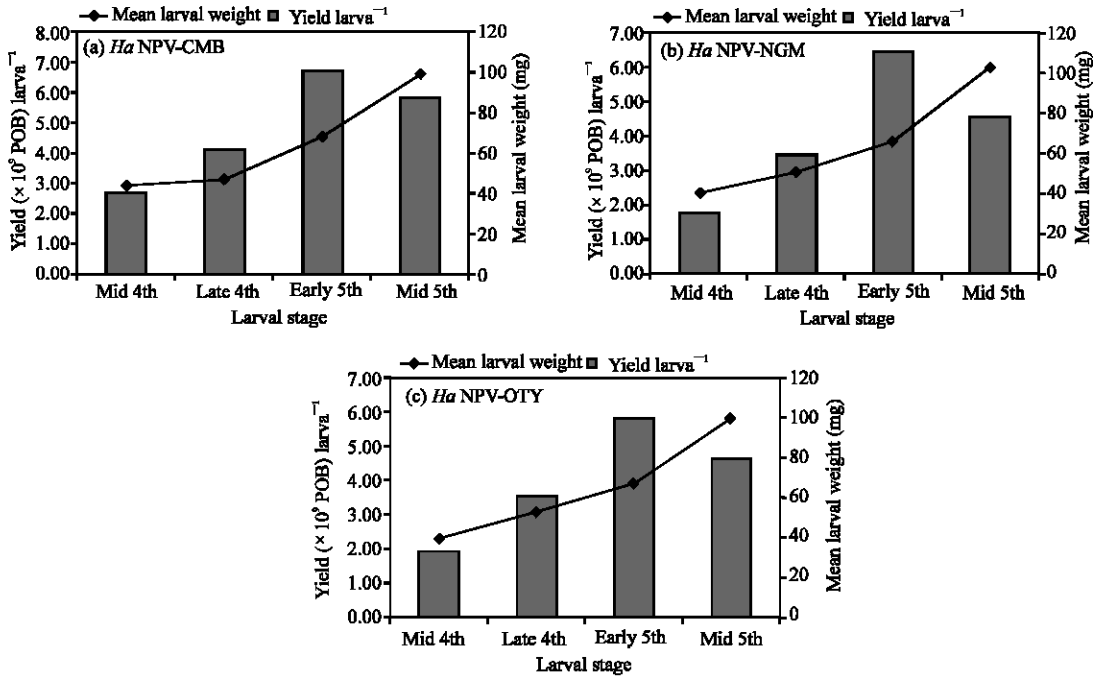

Fig. 1: Effect of larval stage and weight on the yield of *Ha* NPV isolates at an inoculation dose of 5×10^5 POB $larva^{-1}$ and an incubation temperature of 25±1°C

Virus yield in relation to incubation temperature: The maximum yield of virus per larva and yield per 100 inoculated larvae were obtained for all the viral isolates when larvae were incubated at 25°C after virus inoculation as compared with that of room temperature and 30°C (Table 3). The productivity was

Table 3: Effect of incubation temperature on the yield of *Ha*NPV isolates achieved from early 5th instar larvae inoculated with 5×10^5 POB $larva^{-1}$

*Ha*NPV isolates	Temperature (°C)	Mean harvestable cadavers (out of 40)	Yield $larva^{-1}$ ($\times10^9$ POB)	Yield per 100 inoculated larvae ($\times10^{11}$ POB)	Productivity ratio ($\times10^6$ POB)
CMB	Room*	34.33	5.65b	4.80b	1.13b
	25°C	33.67	6.87a	5.66a	1.37a
	30°C	34.00	5.10b	4.25b	1.02b
NGM	Room*	35.33	5.39b	4.73b	1.08b
	25°C	35.67	6.37a	5.54a	1.27a
	30°C	33.33	4.91b	4.29c	0.98b
OTY	Room*	35.67	4.89b	4.34b	0.98b
	25°C	34.33	5.98a	5.04a	1.20a
	30°C	33.00	4.55b	3.90c	0.91b

*: The temperature ranged 28-31°C during the period of study. In a column, for each isolate means followed by the same letter (a-c) are not significantly different (p = 0.05) by DMRT

Table 4: Effect of harvest period on the yield of *Ha*NPV isolates achieved from early 5th instar larvae inoculated with 5×10^5 POB $larva^{-1}$ at an incubation temperature of 25±1°C

*Ha*NPV isolates	Harvest period (DAI)†	Mean harvestable cadavers (out of 40)	Yield $larva^{-1}$ ($\times10^9$ POB)	Yield per 100 inoculated larvae ($\times10^{11}$ POB)	Productivity ratio ($\times10^6$ POB)
CMB	Five	5.33	1.48d	0.17e	0.30d
	Six	11.33	3.44c	0.91d	0.69c
	Seven	20.00	4.36bc	2.06c	0.87bc
	Eight	33.33	4.96b	4.09b	0.99b
	Cadaver	36.67	6.34a	5.80a	1.27a
NGM	Five	6.00	1.25d	0.16e	0.25d
	Six	14.00	3.11c	0.98d	0.62c
	Seven	23.00	3.87bc	2.18c	0.77bc
	Eight	34.33	4.39b	3.75b	0.88b
	Cadaver	38.67	5.83a	5.60a	1.17a
OTY	Five	5.00	1.02d	0.13e	0.20d
	Six	12.33	2.82c	0.77d	0.56c
	Seven	21.67	3.29bc	1.75c	0.66bc
	Eight	33.00	4.01b	3.27b	0.80b
	Cadaver	37.33	5.50a	5.07a	1.10a

†: Days after inoculation. In a column, for each isolate means followed by the same letter (a-e) are not significantly different (p = 0.05) by DMRT

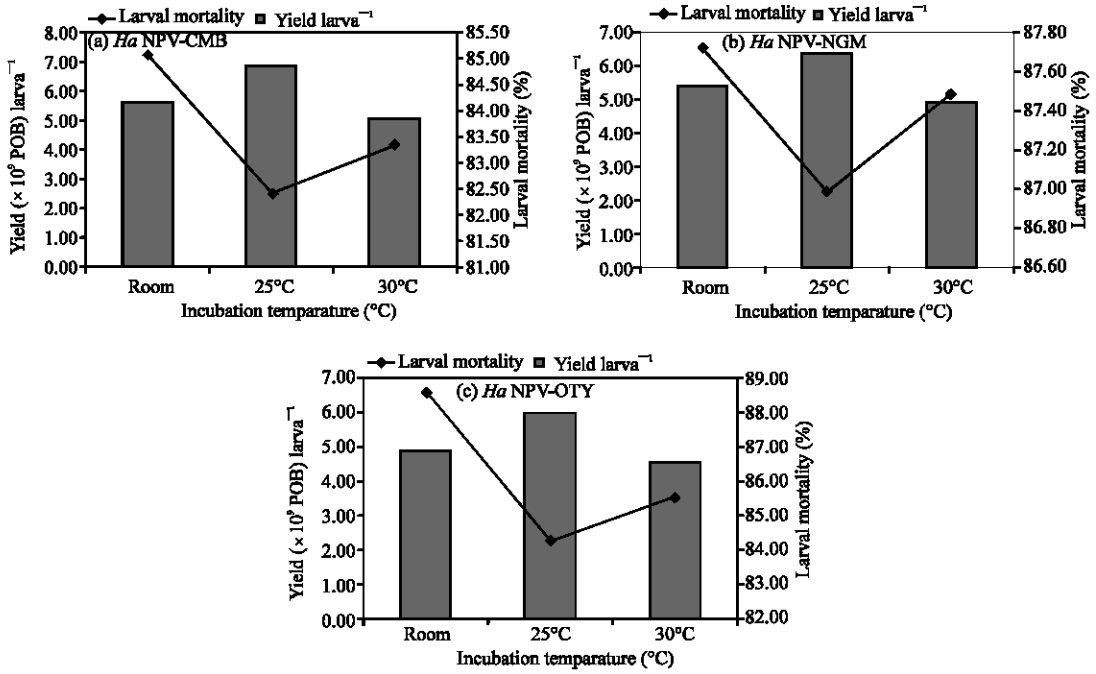

Fig. 2: Effect of incubation temperature on the yield of *Ha*NPV isolates and larval mortality achieved from early 5th instar larvae inoculated with 5×10^5 POB $larva^{-1}$

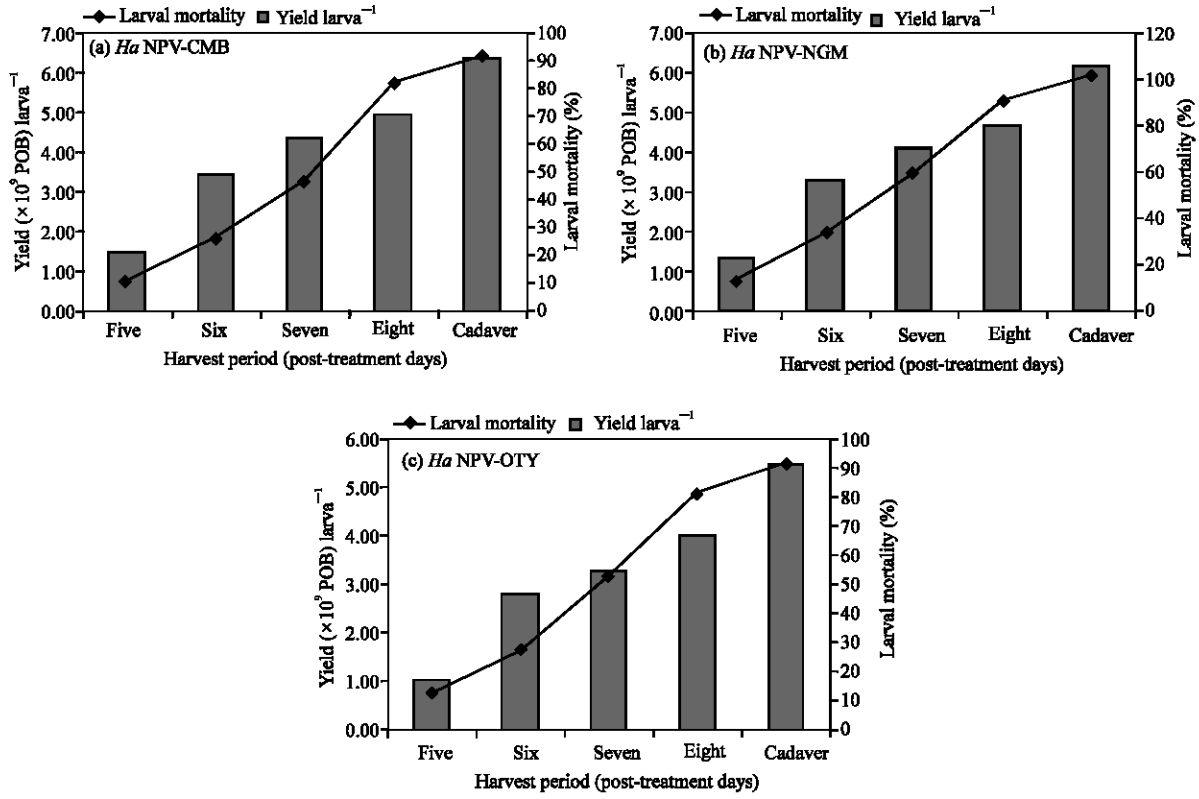

Fig. 3: Effect of harvest period on the yield of *Ha*NPV isolates and larval mortality achieved from early 5th instar larvae inoculated with 5×10^5 POB larva^{-1} at 25°C

also the highest at 25°C. The yield larva^{-1}, yield per 100 inoculated larvae and productivity ratio were minimum at 30°C.

Data on the larval mortality showed a rise in incubation temperature resulted in a significant increase in the per cent kill in all the isolates (Fig. 2).

Effect of periods of larval harvest on virus yield: There was a significant increase in larval mortality with increasing time of larval harvest in all the three viral isolates (Table 4). The yield larva^{-1}, yield per 100 inoculated larvae and productivity ratio were the highest when virosed larvae were harvested as cadavers. As the time of harvest decreased, POB yield larva^{-1}, yield per 100 inoculated larvae and productivity ratio were reduced (Fig. 3).

DISCUSSION

Virus yield in relation to larval age and weight: The yield and productivity parameters of the viral isolates were determined by larval age and weight at the time of inoculation. Of the different age groups studied, early 5th instar with a weight range of 65.46-68.13 mg recorded significantly the highest yield of 6.73×10^9, 6.45×10^9 and 5.83×10^9 POB larva^{-1} in the case of CMB, NGM and OTY isolates, respectively. Mid 4th, late 4th and mid 5th instar larvae registered lower POB production (Table 1). Teakle and Byrne (1989) found an exponential increase in the yield of the virus with age of the larvae of *H. armigera*. A 100-fold increase in the yield of the virus was noted in larvae of age 6 days compared to one day old larva. Ignoffo (1966) estimated that at least 6×10^9 POB were produced per larva in later instars of *H. zea*. Dhandapani (1990) reported in a study on *H. armigera* that the yield of POB larva^{-1} inoculated at the 5th instar stage was 2.40×10^9. Early 5th *S. litura* larvae was found to be an optimum stage for inoculation of virus to maximize the virus productivity (Tuan *et al.*, 1998; Subramanian *et al.*, 2001). Monobrullah and Nagata (2000) reported that 9-day-old *S. litura* larvae weighing 125-155 mg treated with 4.8×10^6POB larva^{-1} through diet resulted in maximum productivity of the NPV. Shieh (1989) reported that from *H. zea* larvae with the initial larval weight of 50-120 mg, the highest POB yields could be obtained. At this weight range, the larvae could continue their normal growth as

that of healthy insects until a day before death resulting in a higher amount of POB yields. These observations are in agreement with the present findings. When the larvae of *H. armigera* were allowed to feed on a constant dose, Whitlock (1977) found an inverse relationship between larval age at the time of inoculation and the mortality. In the present studies, mortality was significantly highest in the 4th and mid 4th instar larvae followed by early 5th and mid 5th instars. However, in all the *Ha*NPV isolates tested, yield per 100 inoculated larvae was the highest in the early 5th instar (Table 1).

Virus yield in relation to inoculation dose: A significant influence of inoculation dose on the per cent larval mortality and yield larva^{-1} was observed with all the isolates tested. The highest mortality occurred with the highest inoculation dose of 1965.87 POB mm^{-2}, recording 82.05, 83.77 and 82.90% for the isolates CMB, NGM, OTY, respectively. These results are in agreement with the findings of Narayanan and Jayaraj (2002) who observed a marked difference between doses as well as a significant interaction between dosages and larval instars. A mean number of 2.5×10^{10} POB was harvested from late 4th instars when inoculated at low dose of 1.1×10^{4} POB cup^{-1} compared to 1.2×10^{10} POB when inoculated at a higher dose of 1.1×10^{6}. A virus concentration of 3×10^{6} POB mL^{-1} by diet incorporation technique (Tuan *et al*., 1998) and 1×10^{8} POB mL^{-1} by diet surface contamination method (Subramaninan *et al*., 2001; Kumar and Rabindra, 2003) were found to be optimum for *in vivo* production of the virus. However, the productivity ratio progressively decreased as the dose of inoculation increased. This is in partial agreement with the findings by Bell (1991) who reported that a lower dose of virus can be used for achieving higher yield.

Virus yield in relation to incubation temperature: Data on evaluation of different incubation temperature on the yield of three *Ha*NPV isolates indicated that, maximum yield larva^{-1} was obtained at 25°C followed by room temperature and 30°C (Table 3). Similarly, the yield per 100 inoculated larvae and productivity was also maximum at 25°C. However, the maximum larval mortality occurred at room temperature followed by 30 and 25°C (Fig. 2).

Insect systems function optimally within a limited range of temperature (Chapman, 1998). Synchrony in larval development during virus multiplication is considered important, as the larvae are sensitive to temperature fluctuation. Any variation in optimal temperature will directly affect the larval growth as well as viral multiplication. So, handling this synchronization to get the maximum benefit in terms of yield and productivity of the virus was one of the major objectives in the present studies. Optimization of larval growth during the virus incubation will allow the virus to attain rate of its multiplication. Studies conducted by O'Reilly and Miller (1989) indicated that prolongation in larval growth, even beyond the period of a normal larval stage, would benefit the viral reproduction. Present findings are in agreement with that of previous studies.

Cherry *et al.* (1997) indicated that the productivity was maximum at an incubation temperature of 25°C for *S. litura* and *S. exempta*. Huang (1995) reported that a temperature range of 24-27°C was optimum for 3rd and 4th instar *S. litura* larvae inoculated with 3.85×10^{5} and 3.85×10^{7} POB mL^{-1}, respectively, to maximize the *Sl*NPV yield. Incubation of early 5th instar *S. litura* larvae dosed with 3932.4 POB mm^{-2} at 25°C enhanced the NPV productivity to 6.623×10^{11} POB yield per 100 inoculated larvae, while it was only 1.779×10^{11} at 35°C (Subramanian *et al*., 2006).

Many laboratory studies have demonstrated that nucleopolyhedrovirus is inactivated by exposure to high temperature. McLeod *et al.* (1977) stated that increase in temperature from 15 to 45°C increased the LD_{50} values of *H. zea* NPV (29.8-349.2 POB mm^{-2} of diet surface). Stairs (1978) indicated that high temperatures caused direct inactivation of the virus and adversely affected the viral replication. Johnson *et al.* (1982) demonstrated the inhibition of virus activity against the velvet bean caterpillar, *Anticarsia gemmatalis*, at the extremes of temperature of 10 and 40°C. Kelly and Entwistle (1988) found an approximate linear relationship between the *Mamestra brassicae* NPV and the incubation temperature. Histopathological studies by Sathiah (2001) revealed that at 25°C, the growth of fat body in virus-inoculated larvae progressed normally during the early stages of infestation providing adequate substrate for the growth and multiplication of the virus. In *H. armigera* larvae, the virus multiplied at a slow pace at 25°C allowing the fat bodies to proliferate simultaneously. At higher temperatures the virus multiplied faster destroying the fat body before it could grow to provide greater substrate volume. Therefore, a good mass production facility should possess a temperature-controlled incubation chamber to provide a constant temperature of 25±1°C.

Virus yield in relation to period of harvest: Harvesting of virosed larvae is usually considered as a laborious and time consuming job in all the virus production units. Therefore attempts were made to optimize the period of harvest as it has influence on biological activity of the virus and growth of secondary contaminants like bacteria. The POB yield was higher when virosed larvae were

harvested as cadavers than when harvested at different days after inoculation (Table 4). The POB yield per 100 inoculated larvae and productivity ratio were also higher in the case of all isolates when harvested from cadavers. The finding of this study is in contrast with that of Smith and Vlak (1988) who reported that production of *S. exigua* NPV did not increase after seven days of inoculation. However, Sathiah (2001) and Narabenchi (2004) state that harvesting the virosed larvae as cadavers will enhance the yield production of *Ha*NPV.

Ignoffo and Shapiro (1978) found that the activity of *H. zea* NPV processed from dead hosts was 7-9 times higher than those from live virosed larvae. Similarly, Shapiro and Bell (1981) evaluated the yield and biological activity of *Lymantria dispar* NPV at different times after infection. They showed that POB harvested from dead larvae were up to 7 times more active than from living infected larvae. Also, the virus yield increased up to about 2×10^9 POB larva^{-1} during the first 11 days and then remained constant.

CONCLUSIONS

Virus mass production *in vivo* in host larvae is the pragmatic method as of today. A variety of parameters have been addressed for increasing productivity of the *Ha*NPV. Present study revealed that factors like larval age and weight, dose of the inoculum, the incubation temperature and the period of harvest could enhance the *in vivo* yield of the virus. Of the larval age evaluated early 5th instar larvae recorded the maximum yield. The inoculation dose of 1965.87 POB mm^{-2} and the incubation temperature of 25°C registered the highest *in vivo* virus yield. Also, the POB yield was recorded maximum range when virosed larvae were harvested as cadavers. However, among the isolates tested in this study, CMB isolate collected from Tamil Nadu, India, showed the highest yield in all of the conditions tested compared to the other *Ha*NPV isolates.

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