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# Establishment of Cell Line from *Helicoverpa armigera* (Hübner) Embryo: Effect of Temperature on its Susceptibility to Baculoviruses

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**Attempts were made to establish new cell lines from the embryos of *Helicoverpa armigera* (Hübner) to study its susceptibility to baculoviruses. Three primary cell cultures were initiated from the embryo of *H. armigera*. Among the three cultures, *Ha*E9 alone produced sufficient cell growth to allow subculturing and led to establishment. Population doubling time (PDT) of the cell line *Ha*E9 ranged from 3.63 days to 4.75 days for five consecutive generations. Susceptibility studies of different baculoviruses revealed that *Ha*E9 was permissive for *Hear*NPV alone. Studies on the influence of incubation temperature regimes (26$^{o}$, 27$^{o}$ and 28$^{o}$C) on cell infection and polyhedral occlusion body (POB) yield of *Hear*NPV in *Ha*E9 cell line revealed that the temperature regime of 27$^{o}$C could yield a maximum of 5.63 and 5.75x10$^{7}$ POB/ ml in 20$^{th}$ and 21$^{st}$ passage respectively. Bioassays with cell cultured viruses in comparison with *in vivo* produced virus had similar effects against 2$^{nd}$ instar *H. armigera* larvae with $LC_{50}$ values of 0.029 and 0.017 POB/mm$^{2}$, respectively.**

**Keywords**: *Helicoverpa armigera*, Cell line, Population doubling time, Temperature

The American bollworm, *H. armigera* is a serious pest of cotton, legumes and vegetables in India and South East Asia (Singh *et al*., 2002). Development of resistance to chemical pesticides made this insect as a prime target for biological control, including insect viruses. Insect cell lines offer an attractive alternative for *in vivo* production of nucleopolyhedrovirus. Cell cultured viruses are free from undesirable viral or microbial contamination, secondary biotypes and insect debris. Though several cell lines from larval and pupal reproductive tissues of the genera *Helicoverpa* had been established (Lynn *et al*., 1988), the yield of polyhedral occlusion bodies (POB) in these cell lines were lower than the cell lines established from embryos of other lepidopteran pests (Lynn *et al*., 1989). *In vitro* produced virus must also be comparable to the *in vivo* produced one so as to be a successful control agent. Hence, in the present study, attempts were made to establish new cell lines from the embryos of *H. armigera,* test the cell lines thus established for their susceptibility to baculovirus infection at different temperature regimes and compare *in vitro* produced virus with *in vivo* produced virus for its efficacy.

## Materials and Methods

### *Establishment of cell lines from embryos*

Twenty four hours old *H. armigera* eggs were collected from the laboratory reared healthy colonies maintained at the Biocontrol Laboratory, Tamil Nadu Agricultural University, Coimbatore. The embryos were isolated from eggs by the method described by Lynn (1998) and were transferred to tissue culture flasks (25 cm$^{2}$) containing five ml of TNM-FH medium. The flasks were incubated at 27.5$^{0}$C in a cooled incubator.

In primary cultures, once in 5-7 days, four ml of the spent medium was removed and replaced with fresh medium. For the established cell lines, sub culturing was done at the confluence stage.

### *Population Doubling Time (PDT)*

At log phase, a viable cell count was made using the trypan blue exclusion method.The cell suspension was diluted with fresh TNM-FH medium so as to get an initial concentration of 5x10$^{5}$ viable cells/ml. Suspensions of five ml from this stock cells were dispensed into sterile disposable 25 cm$^{2}$ tissue culture flask and were incubated at 27.5$^{o}$C in a cooled incubator. Four flasks were used.Out of four, two were used for daily counts and other two for subculturing. Two samples of 200 µl cell suspension were drawn daily after repeated gentle flushing with the medium and counts on the viable cells were made in quadruplicate. The counts were continued for ten days. Studies were continued for five generations following the same procedure.

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The population doubling time (PDT) was calculated according to the formula of Hayflick (1973).

$$PDT = \frac{(t_2-t_1)\log^2}{\log N_2 - \log N_1}$$

Where,

$N_1$ - Number of cells initially ($t_1$) seeded

$N_2$ - Number of cells at any time ($t_2$) of the log growth phase

***Susceptibility of cell lines to NPV and GV***

Early fifth instar, *H. armigera* larvae were inoculated with *Hear*NPV, *Sl*NPV and *Px*GV @ $5x10^7$ POB/ml and $5x10^7$ OB/ml for NPV and GV respectively by diet surface treatment method. Three days after inoculation, the haemolymph from twenty host larvae was collected by cutting prolegs and introduced into TNM-FH medium containing 0.5 per cent cysteine to prevent melanization. The medium with the haemolymph was passed through 0.45 mm membrane filter (Millipore$^R$) and the filtrate containing free virions were used to infect the cell lines.

At log growth phase of the cell line, the spent medium was removed and 5ml of medium containing the virus inoculum was added and allowed for adsorption time of two hours with periodical rocking of flasks. The medium was then removed and the cells washed twice gently with sterile Hank's balanced salt solution (HBSS). After removing the HBSS completely, five ml of fresh TNM-FH medium was added. The cells were incubated at different temperature regimes *viz*., 26, 27 and 28°C to find out the influence of incubation temperature on the cell susceptibility at 20$^{th}$ and 21$^{st}$ passages of subculturing.

***Rate of infection of cells***

The rate of invasion of cells by NPV and GV was calculated by counting the total number of cells and the number of cells with polyhedron and GV in a single microscopic field (magnification 200 x) before harvest in four replicates.

***Harvesting of* in vitro *cultured virus***

The virus-infected cells were harvested 10 days post inoculation. The cells were sonicated (Branson 450$^R$) in ice bath to release the polyhedra and the polyhedra were separated from the cell debris by differential centrifugation at 200 *g* for one min and at 780 *g* for five min. The polyhedra in the pellet were collected and the POB strength was assessed using a haemocytometer (Weber, England) (Evans and Shapiro, 1997).

***Biological activity of virus multiplied* in vitro *in comparison with* in vivo *produced virus***

The biological activity of *in vitro* produced virus was compared with *in vivo* produced virus against Standard diet surface treatment method. Concentrations of virus ranging from 5 x $10^3$ to 1.6 POB/ml were prepared by serial dilution. Aliquots of 10 ml suspensions of the virus were dispensed on the diet surface of area of 254.34 mm$^2$ which resulted in doses *viz.*, 19.659, 3.932, 0.786, 0.157, 0.031 and 0.006 POB/mm$^2$. Thirty numbers of second instar larvae of uniform size were released individually at the rate of 10 in three replicates for each dose. A set of 30 larvae was maintained as control. Observations on mortality were recorded at 12 h interval from third to tenth day after treatment (Jeyarani *et al*., 2006).

***Data analysis***

The data on population doubling time of the cells and its susceptibility to viruses was subjected to analysis of variance (ANOVA) using IRRISTAT software and means were separated by LSD (Gomez and Gomez, 1984). The data on concentration mortality responses of larvae to *Hear*NPV produced by both *in vivo* and *in vitro* was subjected to probit analysis (Finney, 1962). The $LC_{50}$ values and slopes of concentration mortality lines were determined by probit analysis using SPSS advanced version 10.00 for Windows (SPSS, Chicago, IL).

## Results and Discussion

***Establishment of cell lines from embryos***

Several attempts were made to establish new cell lines from *H. armigera* embryos. However, three

**Table 1. Responsiveness of *H. armigera* embryos in cell line establishment**

| Cell line | Response of embryos | | |
|---|---|---|---|
| | Attachment | Cell migration | Cell proliferation |
| *Ha*E1 | + | + | - |
| *Ha*E2 | + | - | - |
| *Ha*E3 | - | - | - |
| *Ha*E4 | - | - | - |
| *Ha*E5 | + | - | - |
| *Ha*E6 | + | + | + |
| *Ha*E7 | + | + | + |
| *Ha*E8 | - | - | - |
| *Ha*E9 | + | + | + |
| *Ha*E10 | + | + | - |

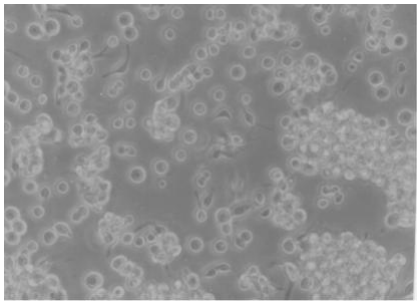

**Plate 1. *Ha*E9 Cell line** second instar larvae of *H. armigera* by following

embryonic cultures *viz*., *Ha*E6, *Ha*E7 and *Ha*E9 alone had active cell proliferation and were established as primary cultures (Table 1). When subculture was attempted in these primary cultures,

**Table 2. Population doubling time of *Ha*E9 at different passages**

| Treatment | PDT (in days)* ± SE |
|---|---|
| I | 3.63 ± 0.25 |
| II | 4.50 ± 0.29 |
| III | 4.25 ± 0.50 |
| IV | 4.75 ± 0.50 |
| V | 4.50 ± 0.29 |
| SEd = 0.13; CD (0.05)= 0.27; CV % = 8.58 | |

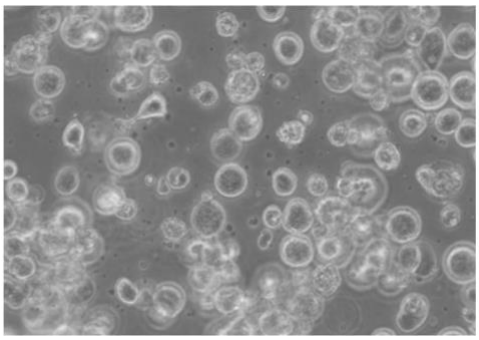

**Plate 2. *Ha*E9 infected with HearNPV**

only one culture (*Ha*E9) (Plate 1) was established. Hink (1972) reported that the establishment of cell lines may not be possible in all attempts. According to him, cell multiplication and migration from explants may begin from one to several days after initiation of cultures and continues upto 4-6 weeks, then the proliferation may ceases but the cells remain viable for weeks, months or almost a year.

***Population Doubling Time (PDT)***

The PDT of the embryonic cell line *Ha*E9 ranged from 3.63 days to 4.75 days in first to fifth passages of subculturing. The PDT did not differ significantly between passages (Table 2). Reuveny *et al*. (1993) reported that the cell growth was the maximum at 27$^{o}$C with 100 per cent viability after 120 h which is in confirmation with the present study.

**Susceptibility of cell lines to NPV and GV**

***Rate of infection of cells***

Results of infectivity tests *in vitro* with *Hear*NPV, *Sl*NPV and *Px*GV in *Ha*E9 cell line showed that the cell line was susceptible to *Hear*NPV alone (Plate 2). Lynn and Shapiro (1998) reported that the same cell line may vary in its ability to replicate viruses from different sources. This may be related to the actual cell type represented by the cultured cells, since it is clear that different tissues in the insect become infected at different times and produce different qualities of virus. The per cent cells infected by *Hear*NPV varied from 18.25 to 23.50 per cent at 26, 27 and 28$^{o}$C in 20$^{th}$ passage and 19.75 to 25.75 per cent in 21$^{st}$ passage of subculturing (Table 3). Lenz *et al*. (1991) reported greater variation in the percentage of infected cells of *H. zea* to HearNPV in clonal lines.

**Table 3. Susceptibility of *Ha*E9 cell line to *Hear*NPV**

| Temperature regimes | Per cent cell infected* ± SE in passage | | Yield* ± SE(x $10^7$ POB/ml) in passage | |
|---|---|---|---|---|
| | 20$^{th}$ | 21$^{st}$ | 20$^{th}$ | 21$^{st}$ |
| 26$^{o}$C | 18.25$^{b}$ ± 0.25 | 19.75$^{b}$± 0.75 | 4.06$^{b}$ ± 0.26 | 3.88$^{b}$ ± 0.43 |
| 27$^{o}$C | 23.25$^{a}$ ± 0.75 | 25.75$^{a}$ ± 0.75 | 5.63$^{a}$ ± 0.55 | 5.75$^{a}$ ± 0.60 |
| 28$^{o}$C | 23.50$^{a}$ ± 0.65 | 24.50$^{a}$ ± 0.65 | 3.00$^{c}$ ± 0.35 | 3.06$^{c}$ ± 0.21 |

| Treatment | SED | CD(0.05) | CD(0.01) | SED | CD(0.05) | CD(0.01) |
|---|---|---|---|---|---|---|
| Temperature (t) | 0.37 | 0.79 | 1.10 | 0.07 | 0.14 | 0.20 |
| Passage (p) | 0.46 | 0.97 | 1.34 | 0.08 NS | 0.18 NS | 0.24 NS |
| pt | 0.64 | 1.37 | 1.90 | 0.12 NS | 0.25 NS | 0.35 NS |

* In a column, means followed by similar letters are not significantly different (p£0.05) by LSD

With respect to virus yield, significantly the highest yield of 5.60 and 5.79x $10^7$ POB/ml was obtained in passage 20$^{th}$ and passage 21$^{st}$ at 27$^{o}$C compared to other temperature regimes (Table 3). The quantity and quality of baculovirus production in cell culture may be influenced by the strain of the

**Table 4. Concentration-mortality response of *H. armigera* to *in vitro* and *in vivo* produced *Hear*NPV**

| Particulars | λ(n-2) | Slope 'b'± SE | $LC_{50}$ (POB/mm$^2$) | Fiducial Limit |
|---|---|---|---|---|
| *In vivo* | 0.612 | 0.5438 ± 0.0994 | 0.017 | 0.003 - 0.045 |
| *In vitro* | 0.421 | 0.5109 ± 0.0933 | 0.029 | 0.006 - 0.077 |

$ Number of insects used per treatment was 180

* All lines are significantly a good fit (p£0.05)

virus, quality of the cells and the type of infection process it undergoes (Goodman and McIntosh, 1994).

***Biological activity of virus multiplied* in vitro *in comparison with* in vivo *produced virus***

Results of the bioassays of *in vitro* and *in vivo* produced *Hear*NPV against second instar showed $LC_{50}$ values comparable to that of *in vivo* produced *Hear*NPV. The $LC_{50}$ values had overlapping fiducial limits ranged from 0.003 – 0.077 POB/$mm^2$ (Table 4). Jeyarani *et al*. (2006) reported that the CBE-I strain of HearNPV after passage through *sf*21 was as virulent as *in vivo* produced virus to the larvae of *H. armigera*, which is in confirmation with the results obtained in the present study.

This study clearly indicated the ability of *Ha*E9 in the *in vitro* production of *Hear*NPV. However, further experiments need to be performed to examine the potential of the cell line in enhancing the productivity by continuous infection after several passages.

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