Molecular and biological characteristics of some geographic isolates of nucleopolyhedrovirus of *Helicoverpa armigera* (Lep.: Noctuidae)

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Abstract

Seven geographic isolates of *Helicoverpa armigera* NPV were characterized by PCR-RFLP techniques and their comparative virulence to second instar larvae of *H. armigera* (Hübner) were compared before and after exposure to simulated sunlight as well as open weather conditions on cotton and tomato plants. The PCR products of *25K FP* region showed no difference among the isolates (780 bp). The PCR amplification products revealed genetic diversity among the 7 *Hear*NPV isolates when they were digested individually using four different restriction enzymes (*Hind*III, *Bam*HI, *Pst*I, and *Eco*RI). The bioassays of the *Hear*NPV isolates against the second instar larvae of *H. armigera*, before and after exposure to simulated sunlight, revealed a range of variation in their biological activity. However, the NGM isolate was the most virulent with the lowest LC_{50} and LT_{50} values. The dose-inhibitory response of the second instar larvae of *H. armigera* to the virus isolates revealed NGM isolate to be the least susceptible to simulated sunlight, having the highest ID_{50} value (462.9 W/m^2). Experiments on persistence of *Hear*NPV isolates on cotton and tomato plants under open weather conditions showed that the persistence of the viral isolates was relatively higher on tomato than on cotton.

Key words: Nucleopolyhedrovirus, *Helicoverpa armigera*, geographic isolates, *25K FP* gene, biological activity, simulated sunlight, host plant

چکیده

هفت جدایه از ویروس *Helicoverpa armigera* NPV با تکنیک PCR-RFLP توصیف گردید و بیماری‌زایی نسبی آنها نسبت به لاروهای سن دوم *H. armigera* (Hübner) قبل و بعد از پرتوتابی با شبه نور خورشید و شـرایط محـیط آزاد روی دو گیاه پنبه و گوجه‌فرنگی مورد مقایسه قرار گرفت. محصول PCR حاصل از ژن *25K FP* تفاوتی بین جدایه‌ها نشان نداد (۷۸۰ جفت باز). محصول پلیمراز به دست آمده از جدایه‌ها پس از آنالیز جداگانه با چهار نوع آنزیم (*Hind*III، *Bam*HI، *Pst*I و *Eco*RI) تنوع ژنتیکی بین جدایه‌های ویروس *Hear*NPV را نمایان ساخت. زیـست‌سـنجی جدایـه‌هـای *Hear*NPV علیـه لاوهای سن دوم *H. armigera* قبل و بعد از پرتوتابی با شبه نور خورشید، طیفی از تغییرات در فعالیت زیستی آنها را آشکار نمود. جدایه‌ی NGM با کمترین میزان LC_{50} و LT_{50} به عنوان بیماری‌زاترین جدایه شناخته شد. واکنش غلظت - بازدارندگی لاروهای سن دوم *H. armigera* نسبت به جدایه‌های ویروس نشان داد که جدایه‌ی NGM کمترین میزان حساسیت به شـبه نور خورشید را با داشتن بیشترین مقدار ID_{50} (٤٦٢/٩ W/m^2) دارا است. آزمایش‌های به عمـل آمـده روی میـزان پایـداری جدایه‌های *Hear*NPV روی گیاهان پنبه و گوجه‌فرنگی در شرایط محیط آزاد نشان داد که پایداری جدایه‌های ویروس به طور نسبی روی گیاه گوجه‌فرنگی بیشتر از پنبه می‌باشد.

واژگان کلیدی: ویروس چنـدوجهی هـسته‌ای، *Helicoverpa armigera*، جدایـه‌هـای جغرافیـایی، ژن *25K FP*، فعالیـت زیستی، نور خورشید مصنوعی، گیاه میزبان

Introduction

Members of the genus *Helicoverpa* Hardwick are pests of worldwide significance (Matthews, 1991). On a global scale, only a few insect pests continue to receive wide

attention of scientific community and *Helicoverpa armigera* (Hübner) is one of them. This pest attacks several crops like chickpea, pigeonpea, soybean, sunflower, cotton, tomato, sorghum, etc. (Rabindra & Jayaraj, 1995). The pest has reportedly developed resistance to all the major groups of synthetic organic insecticides including synthetic pyrethroids, organophosphates and cyclodienes (Ahmad & McCaffery, 1988; Armes *et al.*, 1992), while it is highly susceptible to its nuclear polyhedrosis virus (Rabindra & Subramaniam, 1974). However, inactivation of viruses on foliage of plants has been a major problem in the development of viral insecticides for use in insect management systems. Variation in susceptibility of baculovirus isolates to different host plants has also been evaluated by several authors (McLeod *et al.*, 1977; Young *et al.*, 1977; Olofsson, 1988; Tuan *et al.*, 1989; Killic & Warden, 1991; Raymond *et al.*, 2005). Basically, persistence of viruses on foliage is influenced by sunlight, pH, temperature and moisture (Young, 2000). The ultraviolet rays of the spectrum both in natural sunlight and artificial radiation are inactivating the insect pathogens (David, 1969; Bullock *et al.*, 1970; Morris *et al.*, 1995) and may greatly reduce their effectiveness (Shapiro & Bell, 1984). Solar inactivation has been a major impediment for large-scale use of entomopathogenic viruses (Entwistle & Evans, 1985), and is at least partially responsible for inconsistent field efficacy (Watanabe, 1987).

Moreover, genotypic variants of *Helicoverpa* NPV have been reported by many researchers (Hughes *et al.*, 1983; McIntosh & Ignoffo, 1983; Monroe & McCarthy, 1984; Geetha & Rabindra, 2000; Lua *et al.*, 2002; Cory *et al.*, 2005). Different geographic isolates of NPV revealed vast differences in their pathogenicity and virulence against the natural populations of the pest all over the world (Rabindra, 1992; Battu & Arora, 1996). Therefore, the biological activity is an important element in the evaluation of the potential of the virus as a biocontrol agent (Geetha & Rabindra, 2000). Besides, nucleopolyhedroviruses isolated from the same host species in different geographical regions frequently show restriction fragment length polymorphisms (RFLP) (Getting & McCarthy, 1982; Kislev & Edelman, 1982; Crook *et al.*, 1985; Shapiro *et al.*, 1991; Vickers *et al.*, 1991; Geetha & Rabindra, 2000). More recent studies at geographically scales have shown that restriction endonuclease (REN) profiles of NPVs isolated from individual caterpillars also vary both within and between populations of the same host species (Laitinen *et al.*, 1996; Parnell *et al.*, 2002; Cooper *et al.*, 2003). However, the development of DNA technology has provided a number of methods to detect differences at the DNA sequence level. One such technique is the polymerase chain reaction (PCR), which allows precise recognition of nucleic acids in biological samples that could not be detected by other methods (Mullis *et al.*, 1986). The *25K FP* gene, which encodes a 25

kDa protein and is essential for virion occlusion and polyhedron formation (Lua *et al.*, 2002), was the region for amplification in this study in which seven geographic isolates of *H. armigera* NPV were characterized by PCR-RFLP techniques and their comparative virulence to the second instar larvae of *H. armigera* were compared before and after exposure to simulated sunlight, as well as open weather conditions on cotton and tomato plants.

Materials and methods

A laboratory culture of *H. armigera* was maintained on a semi-synthetic diet (Shorey & Hale, 1965) based on hydrated chickpea seeds. The *Hear*NPV isolates used in this study were obtained from Project Directorate of Biological Control (PDBC), Bangalore, India. The various isolates of the virus used in this study are listed in table 1. Since the samples of these isolates had been stored under refrigerated condition (3 ± 2°C) for various periods, initial serial passages of the viral isolates (1×10^7 OB/ml) were made in early fifth instar larvae of *H. armigera* incubated at 25 ± 1°C. The virus isolates were multiplied and bioassayed in a facility away from the host culture laboratory.

Table 1. *Hear*NPV isolates studied for strain selection.

Sl. No.	Origin	Abbreviation
1	Parbhani, Maharashtra	PRB
2	Mumbai, Maharashtra	MUM
3	Rahuri, Maharashtra	RHI
4	Ooty, Tamil Nadu	OTY
5	Coimbatore, Tamil Nadu	CMB
6	Negamum, Tamil Nadu	NGM
7	Hyderabad, Andhra Pradesh	HYD

Virus purification and DNA extraction

DNA was extracted from different viral isolates using a modification of the technique described by Smith & Summers (1979). The oligonucleotides 5′-ACG GAC TGG ATG AGC TTC-3′ (primer 1) and 5′-CGG TAC TCG GTA AAT CTG-3′ (primer 2) were used to amplify the entire *25K FP* gene including upstream and downstream regions of the gene. The reaction mixture (50 µl) contained 10x PCR buffer, 5.0 µl; dNTPs (10mM), 1.0 µl; primers (20 pmol) each 0.25 µl; taq polymerase, 0.2 µl and template DNA (50 ng), 3.0 µl. The final volume was made up to 50 µl with sterile distilled water.

The amplification for both primers was undertaken using PCR with thermal cycling protocols as follows: initial start at 94°C for 3 minutes, denaturation at 94°C for 1 minute,

annealing for 1 minute at 50°C, extension at 72°C for 1 minute and incubation for 10 seconds at 72°C. The number of amplification cycles was 30. PCR products were separated by electrophoresis on a 0.8% agarose gel and visualized by ethidium bromide staining.

RFLP analysis of PCR products

PCR products were digested with four different restriction enzymes, *viz.*, *Hind*III, *Bam*HI, *pst*I, and *Eco*RI. Reaction mixture consisted of enzyme (10 units), 1.0 µl; buffer, 2.5 µl; PCR product, 10.0 µl and sterile distilled water, 11.5 µl. The reaction mixtures were incubated at 37°C for 5 hours. The restriction products were separated by electrophoresis in 2.0% agarose gel and visualized by ethidium bromide staining. The molecular data was subjected to cluster analysis using the "Statistica" software. The dendrogram of relationships among the isolates was derived by cluster analysis of similarities between isolates, using the unweighted pair-group method by Euclidean analysis.

Biological activity

The virus isolates were serially diluted in distilled water to achieve the desired concentrations ranging from 1.966 to 0.0006 OB/mm^2 of the diet surface (with five times reduction in each treatment). Early second instar larvae of *H. armigera* were released individually in the treated glass vials and incubated at 25 ± 1°C. Each treatment had 50 larvae and replicated thrice. Ten µl aliquots of each viral isolate (of different concentrations) was spread on semi-synthetic diet using a blunt end glass rod. The virus induced mortality was recorded daily starting from the third day to the tenth day after inoculation. These bioassays were repeated three times for each isolate and the average was taken. Larval mortality in control was corrected using Abbott's correction formula (Abbott, 1925). Variation in virulence of different geographical isolates of NPV was measured by computing the LC_{50} and LT_{50} values. Also, relative activities were calculated for both LC_{50} and LT_{50} values (Shapiro & Argauer, 2001).

Simulated sunlight susceptibility

A sunlight simulator, Atlas Suntest CPS$^+$/XLS$^+$ (Atlas Material Testing Technology GmbH, Vogelsbergstra βe, 22), which mimics the natural sunlight both in intensity as well as spectrum, was used as a source of simulated sunlight. This device uses a xenon lamp with a filter to illuminate a chamber of 20 × 28 × 21 cm in area with light similar to the solar spectrum at the Earth's surface, from UV-B (280-320 nm) through the visible portion of the

spectrum (Farrar *et al.*, 2003; Lacey & Arthurs, 2005). It has been showed that the rate of degradation of *Anagrapha falcifera* NPV under simulated sunlight was similar to that under natural sunlight. Also the solar simulator provided light at a flux level higher than sunlight (*i.e.*, greater intensity of irradiance). This enables faster exposure in the simulator to equivalent amounts of accumulated radiant energy in the environment (Lacey & Arthurs, 2005).

The *Hear*NPV isolates were irradiated using a Suntest machine for evaluation of their persistence under simulated sunlight condition. Five hundred µl of each viral isolate (1×10^7 OB/ml) prepared in 0.1% Teepol was applied onto the surface of plastic sheets (6 × 12 cm) using a micropipette. The suspension was spread uniformly over the sheets with the blunt end of a sterile 6 mm polished glass rod and air-dried before exposing to simulated sunlight. These virus-smeared sheets were irradiated in a suntest machine (Atlas CPS^+/XLS^+) at 500 W/m^2 for 90 minutes.

After exposure, each irradiated sheet was eluted with distilled water and collected in microfuge tubes, labelled, and re-enumerated. For evaluation of the effect of simulated sunlight on virulence of the viruses, two sets of experiments were conducted. In one study, each viral isolate was exposed to different exposure doses (275-750 W/m^2) for 90 minutes to compute their ID_{50} values (median inhibitory dose) using a concentration of 3.93 OB/mm^2. In another experiment the LC_{50} values were estimated for each virus isolate. Here the exposure dose was constant ca 500 W/m^2 for the period of 90 minutes. The effective concentrations of polyhedra used in the second experiment were 3.9317 to 0.0006 OB/mm^2 with five times dilution in each treatment. Bioassays were performed with the recovered viral isolates as described earlier. Non-irradiated virus for each isolate served as a control in addition to general check and larval mortality was recorded from third to tenth day after inoculation.

Evaluation of persistence of *Hear*NPV isolates on different host plants

A set of experiments were conducted to identify the extent of persistence of different *Hear*NPV isolates on different host plants. The host plants, *viz.*, tomato and cotton were grown in pots under open weather conditions.

Tomato seedlings were sown in pots and allowed to grow for 60 days, whereas, cotton plants were 45 days old when the experiment was conducted. An area of 20 mm diameter was marked with a marker pen on the upper surface of leaves of the plants. The suspensions of different virus isolates were prepared in 0.1% Teepol and a quantity of 10 µl of each viral isolate containing 1×10^5 OB/ml was applied using a micropipette onto the leaf in the labelled

area. Viral suspensions were distributed uniformly by smearing with a polished blunt end of a glass rod (6 mm). Five leaves were inoculated per treatment per day and the pots were exposed to the open weather conditions. Inoculation of separate sets of plants was done for seven consecutive days, so that on the seventh day, plants containing virus treated leaves exposed to weathering for 0, 1, 2, 3, 4, 5 and 6 days would be available. On the seventh day, leaves from the treated plants were removed and placed individually, treated surface upward. Ten newly moulted second instar larvae of uniform age and size were released on each leaf. Then a sterilized glass vial (50 × 20 mm) was inverted on the treated area of the leaf, so that the larvae are forced to feed only on the upper surface of the leaf. This was replicated thrice. After 24 hours of feeding, the larvae were individually transferred to 5 ml glass vials carrying semi-synthetic diet lacking formaldehyde and plugged with sterile cotton. Mortality was recorded daily till the tenth day.

Results

Molecular characterization of *Hear*NPV isolates

Genomic DNA from seven isolates of *Hear*NPV was isolated and the region of *25K FP* gene was amplified using the primers 1 and 2. The region amplified from all seven isolates showed no differences between the isolates. The fragment length for all isolates was 780 bp (fig. 1). The PCR amplification products from seven isolates were individually digested with four different restriction enzymes, *viz.*, *Hind*III, *Bam*HI, *Pst*I and *Eco*RI. Each unique fragment pattern (haplotype) produced by an endonuclease was designated by a letter (A-J) (table 2). *Hind*III had 6 distinct patterns (fig. 2.A), *Eco*RI 3 distinct patterns (fig. 2.B), *Bam*HI 3 distinct patterns (fig. 2.C) and *Pst*I 5 distinct patterns (fig. 2.D). With the enzyme *Hind*III, only RHI and PRB isolates showed the same haplotypes. *Bam*HI revealed the haplotype similarities between NGM and RHI; OTY, CMB and PRB; and also between HYD and MUM isolates. With the enzyme *Pst*I, the only similarity determined was between OTY and CMB isolates. *Eco*RI showed a series of haplotype similarities among NGM, MUM and PRB, and also between RHI, HYD and CMB isolates.

The cladogram constructed after cluster analysis revealed two major clusters formed at 92.77 per cent (3.18 genetic distance) dissimilarity (fig. 3). The first major cluster consists of NGM, RHI, PRB, and MUM isolates with 86.13 per cent dissimilarity. The RHI and PRB isolates made up the sub group with 66.89 per cent similarity or 33.11 per cent (2.24) genetic distance. The second major cluster consisted of OTY, CMB, and HYD isolates with 71.78 per cent dissimilarity, whereas, OTY and CMB showed a similarity up to 82.64 per cent or 17.36

per cent (2.0) genetic distance. Matrix of genetic distances among the seven *Hear*NPV isolates showed that NGM and HYD were the most diverse isolates (3.61 genetic distance) and OTY and CMB were the closest isolates with the genetic distance of 2.0 (table 3).

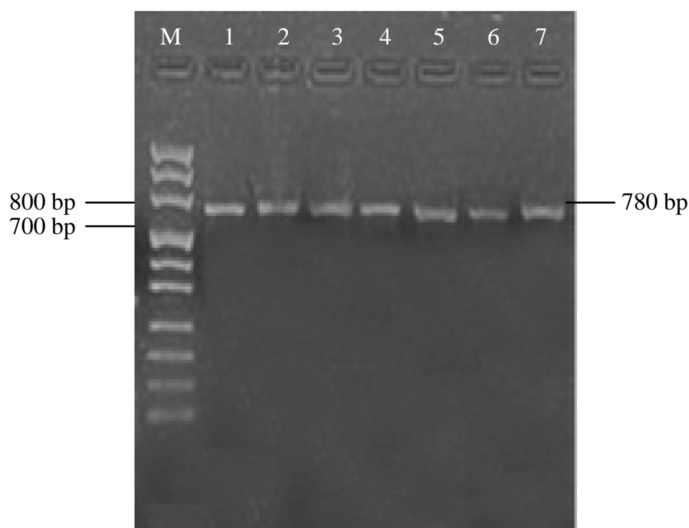

Figure 1. Agarose gel electrophoresis of PCR products of *25K FP* gene region amplified from *Hear*NPV isolates. Lanes: M, molecular marker; 1, NGM; 2, RHI; 3, OTY; 4, HYD; 5, BARC; 6, CMB and 7, PRB isolate.

Table 2. Haplotypes of *25K FP* gene region of *Hear*NPV isolates.

***Hear*NPV isolates**	***25K FP* region (bp)**	***Hind*III (720-400 bp)**	***Bam*HI (720-150 bp)**	***Pst*I (600-250 bp)**	***Eco*RI (760-400 bp)**
NGM	780	AD*	ABDE	DE	A
RHI	780	BD	ABDE	---	BC
OTY	780	CEFJ	BDE	B	B
HYD	780	CEGI	CDE	E	BC
MUM	780	BEH	CDE	A	A
CMB	780	E	BDE	B	BC
PRB	780	BD	BDE	C	A
No. of distinct patterns	—	6	3	5	3

* Letters indicate each unique fragment pattern produced by the specific enzyme.

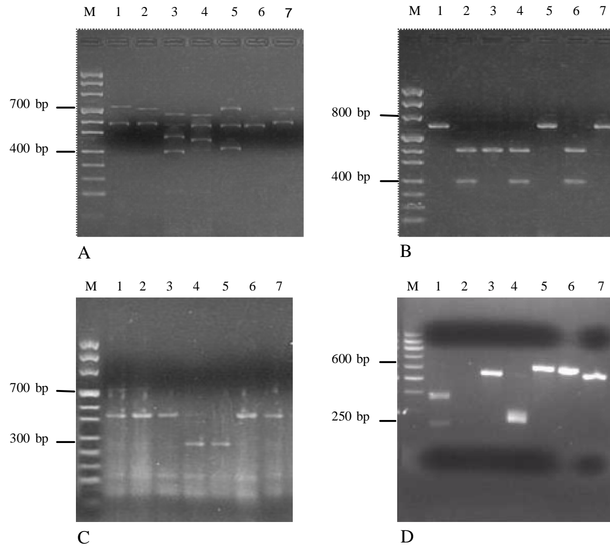

Figure 2. RFLP profiles of *Hear*NPV isolates generated from restriction analysis of *25K FP* gene region digested with different enzymes: A, *Hind*III; B, *Eco*RI; C, *Bam*HI and D, *Pst*I. Lanes: M, molecular marker; 1, NGM; 2, RHI; 3, OTY; 4, HYD; 5, BARC; 6, CMB and 7, PRB isolate.

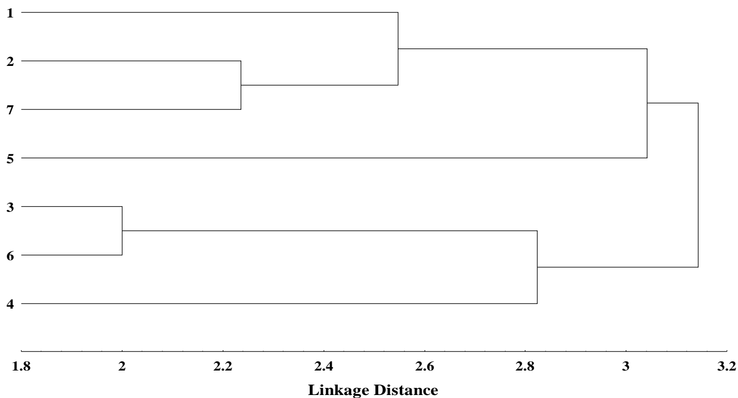

Figure 3. The dendrogram of similarities among restriction fragment length polymorphism of *Hear*NPV isolates derived by cluster analysis, using the unweighted pair-group method by Euclidean analysis. 1, NGM; 2, RHI; 3, OTY; 4, HYD; 5, MUM; 6, CMB and 7, PRB isolate.

Table 3. Matrix of genetic distances among the seven *Hear*NPV isolates.

*Hear*NPV isolates	NGM	RHI	OTY	HYD	MUM	CMB	PRB
NGM	0.00						
RHI	2.65	0.00					
OTY	3.46	3.00	0.00				
HYD	3.61	3.16	3.00	0.00			
MUM	3.32	3.16	3.32	3.16	0.00		
CMB	3.16	2.24	2.00	2.65	3.00	0.00	
PRB	2.45	2.24	3.16	3.51	2.65	2.83	0.00

Biological activity of *Hear*NPV isolates

Before exposure: bioassays of seven isolates of *Hear*NPV against the second instar larvae of *H. armigera* under laboratory conditions revealed a range of variation in their biological activity. The NGM isolate was the most virulent with the lowest LC_{50} value (0.02859 OB/mm^2), followed by the isolates of OTY, PRB, CMB, RHI, MUM and HYD (table 4). The order of LC_{50} values in the case of seven isolates of *Hear*NPV against second instar larvae was NGM < OTY < PRB < CMB < RHI < MUM < HYD. Similarly, LT_{50} value of NGM isolate was the lowest (97.8 hours) followed by CMB, OTY, PRB, MUM, HYD and RHI isolates (table 5).

Table 4. Probit analysis of concentration-mortality response of the second instar larvae of *H. armigera* to *Hear*NPV isolates.

*Hear*NPV isolates	LC_{50} (OB/mm^2)	Fiducial limits		Slope "b" ± SE	χ^2* (n-2)	Relative activity†
		Lower	Upper			
NGM	0.02859	0.02349	0.03789	0.58 ± 0.09	1.50	2.75
OTY	0.03139	0.02404	0.04652	0.49 ± 0.08	1.80	2.50
PRB	0.03590	0.02514	0.06116	0.43 ± 0.08	0.65	2.19
CMB	0.04415	0.02978	0.07686	0.49 ± 0.08	2.04	1.78
RHI	0.04455	0.03165	0.07007	0.60 ± 0.08	0.68	1.76
MUM	0.07154	0.04106	0.13721	0.46 ± 0.08	2.20	1.10
HYD	0.07851	0.04180	0.18003	0.42 ± 0.08	2.12	1.00

* All lines are insignificant at $p < 0.05$.
† All lines were compared with the highest LC_{50} (HYD).

Simulated sunlight inactivation of *Hear*NPV isolates: in this experiment each *Hear*NPV isolate was exposed to seven exposure doses (ranging from 275 to 750 W/m^2) for 90 minutes and their activity was evaluated against the second instar larvae of *H. armigera*. Results showed that the activity of each viral isolate decreased as the exposure doses increased (table 6).

Table 5. Probit analysis of time-mortality response of the second instar larvae of *H. armigera* to *Hear*NPV isolates at a dose of 1.966 OB/mm^2.

*Hear*NPV isolates	LT_{50} (h)	Fiducial limits		Slope "b" ± SE	χ^2* (n-2)	Relative activity†
		Lower	Upper			
NGM	97.8	94.4	100.2	8.28 ± 0.50	4.25	1.24
CMB	104.6	101.6	106.3	5.97 ± 0.35	3.26	1.16
OTY	105.3	101.8	108.8	7.60 ± 0.43	5.63	1.15
PRB	109.9	105.4	110.2	5.77 ± 0.34	5.15	1.10
MUM	113.6	109.6	115.6	10.0 ± 0.60	2.43	1.06
HYD	118.7	116.3	122.0	9.93 ± 0.61	9.12	1.05
RHI	120.8	117.3	124.3	10.85 ± 0.70	4.71	1.00

* All lines are insignificant at $p < 0.05$.
† All lines were compared with the highest LT_{50} (RHI).

Table 6. Experiments on *Hear*NPV isolates with different simulated sunlight exposure doses for 90 minutes on the second instar larvae of *H. armigera*.

*Hear*NPV isolates	Per cent larval mortality (± SE) at different simulated sunlight exposure doses (W/m^2)							
	0	275	500	550	600	650	700	750
NGM	99.5 ± 0.9^{a}	60.0 ± 0.9^{a} (39.7)	53.7 ± 0.3^{a} (46.1)	47.9 ± 0.3^{a} (51.9)	43.5 ± 0.5^{a} (56.3)	39.6 ± 0.4^{a} (60.2)	36.7 ± 0.7^{a} (63.1)	32.0 ± 0.2^{a} (67.8)
OTY	99.3 ± 0.3^{a}	59.9 ± 0.6^{a} (39.7)	53.5 ± 0.4^{a} (46.1)	47.8 ± 1.3^{a} (51.9)	42.67 ± 0.3^{a} (57.0)	37.0 ± 0.2^{b} (62.8)	35.3 ± 1.3^{a} (64.5)	32.0 ± 0.1^{a} (67.8)
CMB	98.9 ± 0.2^{a}	58.9 ± 0.7^{ab} (40.4)	53.3 ± 0.3^{a} (46.1)	46.7 ± 0.4^{a} (52.8)	41.1 ± 0.9^{ab} (58.4)	36.7 ± 1.1^{b} (62.9)	32.2 ± 0.4^{b} (67.4)	31.1 ± 0.4^{a} (68.6)
MUM	98.9 ± 0.5^{a}	56.7 ± 0.3^{bc} (42.7)	52.2 ± 0.4^{ab} (47.2)	44.4 ± 0.7^{b} (55.1)	38.9 ± 0.5^{b} (60.7)	36.7 ± 0.7^{b} (62.9)	32.2 ± 0.2^{b} (67.4)	31.1 ± 0.3^{a} (68.6)
PRB	99.5 ± 0.3^{a}	60.0 ± 0.4^{a} (39.67)	53.3 ± 0.4^{a} (46.4)	42.2 ± 1.1^{b} (57.6)	34.4 ± 0.3^{c} (65.4)	34.4 ± 0.7^{bc} (65.4)	28.9 ± 0.8^{cd} (70.9)	26.7 ± 0.7^{b} (73.2)
HYD	97.8 ± 0.1^{a}	56.7 ± 0.3^{bc} (42.0)	48.9 ± 0.7^{c} (50.0)	42.2 ± 1.1^{b} (56.8)	34.4 ± 0.1^{c} (64.8)	33.3 ± 0.9^{dc} (65.9)	27.8 ± 0.3^{d} (71.6)	25.6 ± 0.3^{b} (73.9)
RHI	98.9 ± 0.1^{a}	55.6 ± 1.0^{c} (43.8)	51.1 ± 0.5^{cb} (48.3)	43.3 ± 0.3^{b} (56.2)	36.7 ± 0.1^{c} (62.9)	$32.2 \pm 0.^{7d}$ (67.4)	$31.1 \pm 0.1b^{c}$ (68.6)	26.7 ± 0.6^{b} (73.0)

Figures in parenthesis represent per cent inhibition of viral activity.
Means followed by the same letter in a column are not significantly different ($p = 0.05$) by DMRT.

The maximum larval mortality was achieved from NGM isolate ranging 60-32 per cent at dose 275 to 750 W/m^2; whereas, this range was the lowest in the case of RHI isolate (55.6-26.7 per cent) (table 6). This result was reinforced by ID_{50} values. Accordingly, NGM isolate showed the highest ID_{50} value (462.9 W/m^2) which was 1.16 times more than that of RHI isolate (as the lowest) indicating a higher tolerance to UV radiation (table 7).

Another set of experiments were conducted to compare the biological activity of *Hear*NPV isolates after exposure to 500 W/m^2 for 90 minutes. Results of the bioassay against second instar larvae of *H. armigera* revealed that NGM isolate was the most active with a LC_{50} value of 0.04146 OB/mm^2 (table 8). The order of LC_{50} values was NGM < OTY < CMB

< MUM < PRB < HYD < RHI. The order of LT_{50} after artificial irradiation was NGM < OTY < CMB < PRB < MUM < HYD < RHI (table 9).

Table 7. Probit analysis of irradiation dose-inhibitory responses of the second instar larvae of *H. armigera* to *Hear*NPV isolates with simulated sunlight.

***Hear*NPV isolates**	**ID_{50} values (W/m^2)**	**Fiducial limits**		**Slope "b" ± SE**	**χ^2* (n-2)**	**Relative activity†**
		Lower	**Upper**			
NGM	462.9	370.5	531.2	1.58 ± 0.35	3.55	1.16
OTY	456.6	367.8	521.8	1.65 ± 0.35	3.93	1.15
CMB	447.0	359.4	510.1	1.69 ± 0.35	4.76	1.12
MUM	421.1	317.9	488.5	1.56 ± 0.35	4.17	1.06
PRB	418.8	345.9	471.7	2.05 ± 0.36	6.21	1.05
HYD	409.5	326.4	467.1	1.87 ± 0.36	4.62	1.03
RHI	398.9	304.3	461.1	1.72 ± 0.35	5.76	1.00

* All lines are insignificant at $p < 0.05$.
† All lines were compared with RHI as the lowest ID_{50} value.

Table 8. Probit analysis of concentration-mortality response of the second instar larvae of *H. armigera* to *Hear*NPV isolates after exposure to simulated sunlight at 500 W/m^2 for 90 minutes.

***Hear*NPV isolates**	**LC_{50} OB/mm^2**	**Fiducial limits**		**Slope "b" ± SE**	**χ^2* (n-2)**
		Lower	**Upper**		
NGM	0.04146	0.02691	0.05534	1.81 ± 0.56	1.99
OTY	0.05117	0.04884	0.07635	0.50 ± 0.04	1.83
CMB	0.05421	0.04425	0.07213	1.70 ± 0.65	2.10
MUM	0.07941	0.05229	0.10128	4.16 ± 0.37	5.15
PRB	0.08751	0.05625	0.12529	0.94 ± 0.35	3.56
HYD	0.12801	0.09780	0.16492	1.03 ± 0.64	7.43
RHI	0.13863	0.10143	0.18178	2.63 ± 0.97	4.16

* All lines are insignificant at $p < 0.05$.

Table 9. Probit analysis of time-mortality response of the second instar larvae of *H. armigera* to *Hear*NPV isolates after exposure to simulated sunlight at 500 W/m^2 for 90 minutes.

***Hear*NPV isolates**	**LT_{50} values (h)**	**Fiducial limits**		**Slope "b" ± SE**	**χ^2* (n-2)**
		Lower	**Upper**		
NGM	124.9	121.9	126.7	7.95 ± 0.42	3.81
OTY	128.2	124.5	131.1	8.61 ± 0.43	3.14
CMB	129.2	125.0	133.3	5.18 ± 0.25	4.52
MUM	136.8	134.0	140.6	6.97 ± 0.29	3.86
PRB	144.0	139.9	147.7	8.34 ± 0.50	5.07
HYD	152.8	149.1	155.3	5.59 ± 0.30	4.93
RHI	156.7	152.4	160.9	7.45 ± 0.41	6.55

* All lines are insignificant at $p < 0.05$.

Relative susceptibility of *Hear*NPV to irradiation: the biological activity of the *Hear*NPV isolates was altered when they were irradiated by simulated sunlight under

laboratory conditions. However, the different isolates varied in their susceptibility. Accordingly, NGM was most tolerant with a LC_{50} value of 0.04146 OB/mm^2. After irradiation by simulated sunlight this isolate was 1.45 times less virulent than non-irradiated samples (table 10). The order of inactivation of the isolates under simulated sunlight was MUM < CMB < NGM < OTY = HYD < PRB < RHI. The relative inactivation in terms of LT_{50} was in the order of OTY < CMB < PRB < MUM < NGM < HYD < RHI (table 10).

Table 10. Comparison of biological activity and relative simulated sunlight susceptibility of different viral isolates.

***Hear*NPV isolates**	**LC_{50} values**			**LT_{50} values**		
	Before exposure (OB/mm^2)	**After exposure (OB/mm^2)**	**Relative inactivation***	**Before exposure (h)**	**After exposure (h)**	**Relative inactivation***
NGM	0.02859	0.04146	1.45	97.8	124.9	1.28
OTY	0.03139	0.05117	1.63	105.3	128.2	1.22
CMB	0.04415	0.05421	1.23	104.6	129.2	1.24
MUM	0.07154	0.07941	1.11	113.6	144.0	1.27
PRB	0.03590	0.08751	2.44	109.9	136.8	1.25
HYD	0.07851	0.12801	1.63	118.2	152.8	1.29
RHI	0.04455	0.13863	3.11	120.8	156.7	1.30

* Calculated by dividing LC_{50} and LT_{50} values after exposure by LC_{50} and LT_{50} values before exposure for each isolate.

Persistence of *Hear*NPV isolates on cotton and tomato plants

A pot culture experiment was conducted to study the relative persistence of *Hear*NPV isolates on cotton and tomato plants. The results of the experiments showed that the virus lost its activity rapidly as the days of exposure to the weather condition on the host plants advanced (table 11). By the second day, more than 50 per cent of the viral activity was lost on both plants. By the fourth day the original activity remaining (OAR) was reduced to about 20 per cent on cotton and by another two days it fell to about 10 per cent. Up to the fourth day, the NGM recorded significantly higher OAR compared to the other isolates on cotton. On tomato also, the viruses lost their activity steadily as the days advanced. However, it was interesting to see that from the third day onwards the persistence of the virus isolates was significantly higher on tomato than on cotton. For example, the NGM recorded an OAR of 34.2 % on the third day on cotton while it was 45.2 % on tomato. Similarly, on the sixth day it was 10.5 % on cotton and 25.8 % on tomato and the difference was statistically significant (table 11).

Table 11. Relative persistence (OAR %) of *Hear*NPV isolates on cotton and tomato plants.

Treatments[£]	Days of exposure											
	1		2		3		4		5		6	
	T	C	T	C	T	C	T	C	T	C	T	C
PRB	71.2^{**ab}	75.9^{a}	$44.1^{ns\ de}$	43.8^{bc}	38.5^{**c}	30.4^{b}	29.7^{**b}	20.5^{bc}	27.3^{**b}	15.5^{ab}	24.9^{**ab}	9.3^{a}
CMB	$70.7^{ns\ b}$	71.6^{c}	$47.9^{ns\ bc}$	46.0^{a}	42.1^{**b}	30.7^{b}	32.5^{**a}	20.7^{bc}	28.9^{**a}	16.1^{a}	25.7^{**a}	9.9^{a}
MUM	69.8^{**bc}	74.8^{ab}	$45.8^{ns\ cd}$	46.0^{ab}	40.7^{**b}	30.5^{b}	30.0^{**b}	21.2^{bc}	28.3^{**ab}	16.2^{a}	25.6^{**a}	9.9^{a}
NGM	$72.7^{ns\ a}$	72.3^{bc}	50.5^{*a}	47.7^{a}	45.2^{**a}	34.2^{a}	33.1^{**a}	23.1^{a}	30.0^{**a}	17.1^{a}	25.8^{**a}	10.5^{a}
OTY	$72.3^{ns\ a}$	73.6^{bc}	$48.4^{ns\ b}$	47.3^{a}	43.4^{**b}	33.1^{a}	32.9^{**a}	21.4^{b}	28.5^{**ab}	15.4^{b}	25.8^{**a}	10.4^{a}
HYD	68.7^{**c}	74.6^{ab}	$42.7^{ns\ e}$	44.7^{b}	37.3^{**cd}	30.7^{b}	28.1^{**b}	19.8^{c}	24.9^{**c}	13.7^{c}	23.4^{**b}	9.3^{a}
RHI	69.7^{*bc}	74.0^{ab}	$43.5^{ns\ e}$	42.5^{c}	36.1^{**d}	27.5^{c}	29.2^{**b}	19.1^{c}	27.1^{**b}	13.8^{bc}	23.7^{**b}	9.9^{a}

£ All the treatments contained NPV at 10^5 OB/ml.
C, cotton and T, tomato.
Means followed by the same letter in a column are not significantly different (p = 0.05) by DMRT.
* Significant between the crops by Student "T" test.
** Highly significant between the crops by Student "T" test.
ns: non-significant between the crops at different intervals by Student "T" test.

Discussion

Molecular characterization of *Hear*NPV isolates

Baculoviruses are known to be highly variable, with isolates collected from the same species in different geographical locations frequently showing genetic variation and differences in their biology (Cory *et al.*, 2005). The present studies showed that the PCR products of amplified *25K FP* gene region were similar for all seven *Hear*NPV isolates tested in this experiment. With regards to *25K FP* gene, Lua *et al.* (2002) determined the same region from the wild-type *Ha*SNPV (in vivo produced) and *Hear*NPV serially passaged in cell cultures. No nucleotide differences within the *25K FP* gene were detected in any of these samples, resulting in a similar PCR fragment of 780 bp (fig. 1).

Monroe & McCarthy (1984) characterized the structural polypeptides of 12 baculovirus isolates (NPVs and GVs) obtained from different geographical regions of the world on four different species of the genus *Heliothis.* The results showed three major polypeptide profiles of isolates within each NPV phenotype, which differed in only one polypeptide whereas the two GV isolates differed by as many as five polypeptides. Somasekar *et al.* (1993) described two groups of *Hear*NPV isolates as genomically different, with *Hind*III digestion. Isolates from Ooty and Coimbatore were similar and formed a group among the isolates tested. The present findings along with others have clearly demonstrated that baculoviruses are very genotypically divers, and that this diversity occurs at a range of scales from between isolates collected from the same host species but different regions.

An earlier study of the whole viral genome using the same four restriction enzymes brought out the differences in the DNA profiles of some *Hear*NPV isolates (Geetha & Rabindra, 2000). Coimbatore, Negamum and Ooty isolates collected from Tamil Nadu showed almost similar polymorphism in their DNA profiles with all the cited restriction enzymes. Among the eleven isolates tested, Negamum isolate showed the lowest (64.43×10^6 Dalton) DNA molecular weight while that from Hyderabad was the highest (81.53×10^6 Dalton) (Geetha & Rabindra, 2000). Similarly, Sathiah (2001) characterized eight *Hear*NPV isolates at the genomic level using restriction endonucleases enzymes. Cladogram of the isolates with profile generated by the four cutter enzymes showed a closely genomic relatedness of Parbhani and Rahuri; and Fortune and Negamum isolates and some heterogeneity in others. Also, the cladogram showed that isolates Coimbatore I and Coimbatore II were out grouped.

Biological activity of HearNPV isolates

Bioassays against the second instar larvae of *H. armigera* with seven *Hear*NPV isolates revealed the existence of differential virulence as reflected by the LC_{50} and LT_{50} values (tables 4 and 5). Among the isolates tested, NGM was the most virulent against the second instar larvae of *H. armigera*. Similar differences in virulence among NPV isolates have been established in previous studies. Shapiro & Ignoffo (1970) found 56-fold difference in the activity of 35 *Helicoverpa* NPV isolates. Hughes *et al.* (1983) fractionated the time-mortality response of 14 *H. zea* NPV isolates into six activity classes. Rabindra (1992) reported that an isolate of *Hear*NPV obtained from the Nilgiris recorded a lower LC_{50} than those collected from Aruppukottai and Gujarat. Arora *et al.* (1997) and Odak & Rawat (1982) reported wide variations in the virulence and genetic make up of *Hear*NPV collected from different agroclimatic regions of Madhya Pradesh and Punjab, respectively. Somasekar *et al.* (1993) reported *Hear*NPV-OOT isolate was more potent than a few other isolates tested in their study. The concentration-mortality response of the second instar larvae of *H. armigera* to *Hear*NPV isolates showed that the isolate from Negamum was the most virulent while that from Rajasthan was the least virulent (Geetha & Rabindra, 2000). Similarly, Gopali & Lingappa (2001) showed that the local isolate (Gulbarga) exhibited the highest virulence among the *Hear*NPV isolates evaluated. Chandel *et al.* (2004) also evaluated five isolates of *Hear*NPV under laboratory conditions and stated that Kanpur and PCI-Bangalore isolates were the most virulent resulting in the highest mortality in *H. armigera* larvae. For the effective utilization of NPV against *H. armigera*, it is crucial to develop a highly virulent

isolate as a viral biopesticide. It would be desirable to bring all isolates reported earlier to be highly virulent, in a single experiment to identity the most virulent strain for further development and use in pest management. It would also be of interest to study the susceptibility of different population of *H. armigera* to the *Hear*NPV isolates.

Simulated sunlight inactivation

Though the most commonly used technique to increase the duration of virulence for viral insecticides involves the combination of virus with sunscreen substances (Witt & Hink, 1979), screening the most tolerant and highly virulent NPV isolate(s) within and between different geographical populations will also be crucial to improve their efficacy. Effect of simulated sunlight on the activity of *Hear*NPV isolates was studied under laboratory conditions through bioassay method. Results with the CMB isolate of *Hear*NPV showed that the virus was progressively inactivated as the exposure time to UV-source increased. The ID_{50} values decreased progressively as the time of exposure increased. There was significant difference in the virus-caused larval mortality between the NPV isolates after exposure to the different doses of simulated sunlight. The NGM isolate produced the highest larval mortality in all of the exposure doses ranging from 60.0 to 32.0 per cent indicating its relative tolerance to simulated sunlight radiation (table 6). The virus-caused larval mortality by OTY isolate was on par with NGM isolate except at 700 W/m^2. CMB isolate was the second nearest isolate to NGM.

Dose-inhibitory response of the second instar larvae of *H. armigera* to the virus isolates revealed NGM isolate to be least susceptible to simulated sunlight, having the highest ID_{50} value (462.9 W/m^2). The order of ID_{50} values for the isolates was NGM > OTY > CMB > MUM > PRB > HYD > RHI.

The other part dealt within the present work concerns the comparison of the biological activity of *Hear*NPV isolates after irradiation to simulated sunlight (at 500 W/m^2 for 90 minutes). Exposure to UV resulted in reduced larval mortality in all the *Hear*NPV isolates which varied between viral isolates. In the present study, NGM isolate collected from Tamil Nadu was found to be the most virulent (0.02859 OB/mm^2) among all isolates of *Hear*NPV tested in this study both before (table 4) and after (table 8) exposure to simulated sunlight. As already mentioned, the order of inactivation of the isolates under simulated sunlight was MUM < CMB < NGM < OTY = HYD < PRB < RHI. Even though, the rate of simulated sunlight-susceptibility of NGM isolate was more than MUM, the LC_{50} value of NGM was 1.92 times less than that of MUM after exposure to simulated sunlight (table 10).

Nucleopolyhedroviruses are known to be inactivated by exposure to UV rays (Morris, 1971). McLeod *et al.* (1977) reported that exposure of *H. zea* NPV to UV irradiation resulted in a substantial inactivation of the virus. Shapiro *et al.* (2002) found a progressive decrease in the larval mortality with increasing UV-exposure time. Further, they also determined the relationship between virus concentration and radiation-caused inactivation of *H. zea* NPV and *Spodoptera exigua* NPV. A 1000-fold difference in susceptibility to UV light has been identified in the virion population of *Galleria mellonella* NPV (Witt & Stairs, 1975). This heterogeneity in response to UV light is due to the genetic variability within the virus population.

Further studies should focus on identifying viral isolates more tolerant to UV radiation as well as the plant surface inactivating factors. The NGM isolate of *Hear*NPV itself may be subjected to repeated radiation on the host plant itself and a virus which can persist on the plants longer might be selected. A highly persistent virus will provide control for a longer period of time and lead to reduction in the number of sprays.

Persistence of *Hear*NPV isolates on cotton and tomato plants

A set of experiments was conducted to test the relative persistence of *Hear*NPV isolates on the foliage of cotton and tomato plants under open weather condition. Results showed that the virulence of different isolates progressively decreased as post-treatment days increased. Mortality data of the first three days on cotton and that of the first four days on tomato indicated that the NGM isolate was the most active on both cotton and tomato recording higher OAR values. Comparison of the data of the two crops revealed that the persistence of the virus was higher on tomato than cotton. Per cent original activity remaining after six days on cotton (10.5-9.3 per cent) was significantly less than that on tomato (25.8-23.4 %). Similar results were also obtained by several workers. Young & Yearian (1974) compared the activity of *Hz*NPV on the upper surface of host plant leaves for 96 hours and found the highest persistence on tomato followed by soybean and cotton. The least per cent of original activity remaining after 24-96 hours of exposure of *Heliothis* NPV to cotton leaves showed the rapid viral inactivation on the upper surface of leaves compared to other parts of the plant (Yearian & Young, 1974). Similar results were reported by Bullock (1967) and Young *et al.* (1977). Hugar *et al.* (1996) showed that the NPV of *Mythimna separata* could remain active only for 24 hours on foliage of sorghum plants.

Rabindra *et al.* (1994) studied the influence of host plants on the pathogenecity of NPV against *H. armigera* and reported that NPV exposed to the foliage of cotton registered higher

LC_{50} value compared to the other host plants. This is due to the alkaline nature of the cotton leaf surface. It is known that the leaf extracts from glandular hairs of the cotton plants inactivate the NPV of *Heliothis* spp. (Falcon, 1971).

These results indicate that the virus may be used more efficiently for the management of *H. armigera* on tomato than cotton. However, the problem of *H. armigera* is of greater concern on cotton since huge quantities of chemical pesticides are used on cotton. The problem of lower activity of the virus on cotton is overcome by the addition of adjuvants (Rabindra & Jayaraj, 1995) as well as by doubling the dose of virus. Fairly good control of *H. armigera* using the virus at the rate of 3×10^{12} OB/ha has been demonstrated on cotton (Muthuswami *et al.*, 1994). It may also be possible to identity a viral isolate having a relatively higher tolerance on cotton by screening a large number of geographic isolates of the virus.

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