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BIOASSAY OF NEW ISOLATE OF *HELICOVERPA* NUCLEAR POLYHEDROVIRUS AGAINST *HELICOVERPA ARMIGERA* (HUBNER)

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ABSTRACT
Two nuclear polyhedrosis virus isolates of *Helicoverpa armigera* (*HaNPV*) from Lucknow, Uttar Pradesh (North India) was isolated, purified and subjected to TEM and bio-efficacy experiments. Recommended artificial diet was inoculated with different viral concentrations and used to conduct bioassays on fifth instar larvae. The highest number of OBs produced per early fifth instar larvae was found 342 x 10^8 POBs/ mL and 164 x 10^7 POBs/mL in BHA and RAU isolates, respectively. The dose-inhibitory response of the third instar larvae of *H. armigera* to the virus isolates revealed that isolate BHA to be the best in biological activity with LC 50 value (2.74x10^4 POBs /mL) and LC 90 value (4.29x10^5 POBs /mL). The maximum third instar larval mortality of 100 % was achieved at inoculation dose of 2 x 10^7 POBs/ mL followed by 4x 10^6 POBs/ mL with mortality of 98.33%; whereas, minimum mortality was reported in control. BHA isolate resulted in higher mortality of test insects than isolates obtained from Centre of Integrated Pest Management, Lucknow (CIPMC) and Biocontrol Laboratory, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut in North India.

INTRODUCTION

Helicoverpa armigera Hubner (Lepidoptera: Noctuidae), is currently placed on Annex I, A II of Council Directive 2000/ 29/EC, which reflect the notorious nature of this pest prevailing in almost all the countries of the world including India (EPPO, 2006). In India, states like Andhra Pradesh, Madhya Pradesh, Karnataka, Assam, Punjab, Bihar, West Bengal, Haryana, Uttar Pradesh, Odisha and Gujarat is reported to have significant damage due to this pest wherein the loss estimates vary from year to year, place to place and from crop to crop. As a pest, *H. armigera* still are controlled with chemical insecticides and practically 30% of total chemical insecticides are used for managing this pest alone on different crops (Yaqoob *et al*, 2006, Meena & Raju, 2014, and Gadhiya *et al.,* 2014a), however this practice resulted in environmental pollution and impact in the long term leading to pest resistance and pest resurgence. This negative consumer perception of pesticides drives attention towards eco-friendly alternatives.

Now a day's integrated approach is the noble idea to manage crop pests as it involves minimum burden of chemical pesticides on the environment (Jat & Ameta, 2013, Ahmad and Chandel, 2004 and Patel *et al*., 2009). Therefore current practices applied towards appropriate management of *H. armigera* need to be evaluated in reference to bioagents and agro-climatic conditions of the niches. In the fields, natural mortality of *Helicoverpa* larvae can be seen due to infestation of nuclear polyhedrovirus. Completely compatible with the all biologically based IPM approaches, field trials on chickpea in India showed that *HaNPV* at economic applications could manage *H. armigera* more effectively than chemical insecticides or commercially formulated *B. thuringiensis* (Cherry *et al.*, 2000).

Considerable emphasis is being laid on the bio-efficacy of nuclear polyhedrovirus (NPV) as a biopesticide (Caballero *et al*., 1992, Herz *et al*., 2003, Shapiro-Ilan *et al*., 2012, Magholi *et al*., 2014 and Gadhiya *et al.,* 2014b) and product is registered under a range of trade names, viz, Bio-control-VHZ, Elcar, Gemstar LC and Viron-H in different countries. But factors like variation in susceptibility of baculovirus isolates to host insects, genotypic variants and differences in their pathogenicity and virulence against the natural populations of the pest limits the use of NPV as biopesticides (Parnell *et al.*, 2002; Cooper *et al.*, 2003 and Mehrvar *et al,*. 2008). Moreover, genotypic variants of *Helicoverpa* NPV have been reported by numerous researchers (Geetha and Rabindra, 2000; Lua *et al.*, 2002; Cory *et al.*, 2005). One of the best example for variation in the pathogenicity of different isolates is *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV). The virus was registered in the United States (SpodX), The Netherlands and other countries (Kolodny-Hirsch *et al.*, 1997; Smits and Vlak, 1994) but the laboratory studies specify that Spanish populations of *S. exigua* are significantly more susceptible to a native Spanish isolate (named SeMNPV-SP2) than they are to Spod-X (Belda *et al*., 2000). Different geographic isolates of virus reported to have immense differences in their pathogenicity and virulence all over the world (Rabindra, 1992 and Battu & Arora, 1996).

Hence, intensive research is required to develop a virus isolate with more virulence than the existing ones from indigenous areas and optimization of virus yield is crucial for minimizing the cost of production and for its large scale-production using in vitro technology. This necessitates the exploration of indigenous virus strain to combat the natural population of host insects at particular agro-climatic conditions. For successful management of *Helicoverpa armigera,* the viable and virulent indigenous strain should be evaluated. The present study emphasizes on microscopic evaluation of indigenous strains of *HaNPV* from Uttar Pradesh, India along with bioassay for their efficacy as marketable and easily applicable bio-pesticide.

MATERIALS AND METHODS

Collection of infected *Helicoverpa armigera* larvae from Lucknow dsitrict

Different villages, viz, Sonikpur, Maankhera, Vijaipur, Naguamau, Roodahi, Singanamau, Indaura,Tikatganj, Vishrampurva, Choti Devraikala, Asti, Bhaulli, Itaunja, Kalyanpur, Prithvipur, Paikaramau, Bhikhapurva and Paharpur were randomly selected for collection of diseased larvae and data analysis. Typical disease symptoms of baculovirus were observed in larvae on chickpea and pigeonpea crops. The diseased larvae were collected into separate vials and brought to the Biocontrol Laboratory, Lucknow University for further studies.

Extraction of indigenous type isolates of *HaNPV* from field collected larvae

The viral occlusion bodies were extracted from individual diseased larvae by macerating them with 3 mL of 0.1% sodium dodecyl sulphate (SDS) and filtered through three-layered muslin cloth several times to remove the insect debris. The filtrate was centrifuged at 5000 rpm for 5min (Sridhar *et al.,* 2011).

Rearing of *Helicoverpa* larvae

Healthy third instar larvae collected from the field were transferred on to synthetic diet in sterilized plastic containers and reared according to techniques given by Krishnareddy and Hanur (2015). The culture had been reared for two generations before bioassay studies were initiated. Once they entered late third instar stage, they were used for the multiplication of virus.

In vivo production of *HaNPV*

The virus was propagated in laboratory stocks of healthy third instar *H. armigera* larvae. Virus inoculation was done by the surface contamination method as given by Evans and Shapiro (1997). 100 μL of *HaNPV* containing 1x 10^8 POBs/mL was dispensed over the surface of diet in the tray and spread uniformly over the surface using blunt end of glass rod and allowed it to dry for 10-20 min. Late third instar larvae were transferred to virus-infected diet and incubated at 25 $\pm$ 1°C.

Microscopic studies

Third instar larvae of *H. armigera* were fed on artificial diet preimpregnated with 1x 10^8 POBs/mL of virus suspension. The larvae were exposed singly on the impregnated diet and allowed to feed for 24 hr., thereafter they were transferred on fresh diet. Infected larvae were dissected at 96 h after exposure, in normal saline solution (Davenport and Wright, 1985). Dissected material was fixed overnight in a Bouin-Duboscq fixative. The fixed tissues were processed routinely for sectioning. Paraffin (M.P. - 60°C) was used to embed the tissue which was sectioned by microtome at 6µm and stained with Giemsa stain with acid hydrolysis. The stained section of tracheae and nervous tissue were examined under high power microscope.

Extraction of polyhedral occlusion bodies (POBs) from laboratory reared diseased larvae for TEM

The POBs were extracted from individual larvae according to Christian *et al.,* 2001 with slight modification. On mortality of fifth instar larvae, midgut was homogenized in deionized water and strained through 35-mm mesh nylon cloth to remove large debris. The virus particles were obtained by dissolving the ploy hedra with 0.05 M. Na2CO3 and 0.05 M NaCl in the proportion of 10:1 and left for 20-30 min. Then, this mixture was processed by differential centrifugation at 5000 and 16000 rpm for 10 to 15min, respectively (Remi C24BL: cooling centrifuge). The polyhedral thus extracted, were further ascertained by examination under light microscope and TEM and kept in a refrigerator for use.

Transmission electron microscopy

Morphology of the POBs extracted from individual larvae was studied under transmission electron microscope (TEM). Pellets of POBs were fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24 h at 4^0 C and post fixed in 0.5% v/v aqueous osmium tetroxide in the same buffer for 2 h. After the post fixation samples were dehydrated in a series of graded alcohol (Sridhar *et al.,* 2011). Prepared samples were stained with 4% lead citrate and observed under TEM (Jeol JEM 1400: Jeol Ltd., Tokyo, Japan).

Bioassay

The virus isolates were serially diluted in distilled water to achieve the desired concentrations ranging from 2x 10^7 to 2.48 x 10^2 POBs/mL on the diet surface (Evans *et al.,* 1997). Ten µL aliquots of BHA isolate of different concentrations was spread on semi-synthetic diet using a blunt end glass rod (Diet plug bioassay). Early third instar larvae of *H. armigera* were released individually in the treated glass vials containing diet and incubated at 25 $\pm$ 1°C. Each treatment had 38-40 larvae and replicated thrice. Larval mortality in control and in treatment was recorded. Bioassay was conducted at 25 $\pm$ 1°C and 65 $\pm$ 5% humidity under 16 h light : 8 h darkness photoperiod and larval mortality was recorded every 24 h until the larvae either died or pupated.

Comparison of different *HaNPV* isolates

The treatment include virus suspensions of both the isolates (BHA and RAU), formulation of BHA isolate (crude virus suspension 1x 10^9 POBs/mL + diet enhancer + Glycerol 10%) and viral suspensions obtained from different Institutions (Centre of Integrated pest management, Lucknow and Biocontrol laboratory, Meerut) including water solution without virus as a control with three replications. Diet plug bioassay was performed on third instar larvae. Experimental parameters measured were larval mortality of *H. armigera* for 20 days of

observation. Research results then analyzed using Analysis of Variance (ANOVA), and if there are significant differences in outcome data, followed by Duncan's multiple range test on standard 5 %.

Statistical analysis

For bioassay analysis, the larvae that were unable to move and feed were proclaimed dead. Percentage mortality was corrected by the following equation (Abbott 1925; Duddield & Jordan 2000):

$M\ [\%] = [(t - c)/(100 - c)] \times 100,$

Where, M – corrected mortality;

c – Percentage mortality in controls;

t – Percentage mortality in treatments

From the corrected mortality data, the probability integral of the chi-square distribution, regression equation, slope and lethal concentrations (LC50 and LC90) were calculated in order to find out the efficacy of *HaNPV* against test insects (Finney, 1971).

RESULTS AND DISCUSSION

Collection of indigenous isolates of *HaNPV*

Two indigenous isolates of *Helicoverpa* nuclear polyhedrovirus were collected directly from *Helicoverpa armigera* third and fifth instar larvae feeding on chickpea crop from village Bhaulli (BHA isolate) and Raudahi (RAU isolate) at Lucknow, Uttar Pradesh.

Examination of discharged body fluid of diseased larvae under phase contrast microscope revealed large number of round and crystalline particles resembling as POBs. Microscopic studies revealed the presence of polyhedral occlusion bodies in the lumen of the midgut at 24 hour post infection. After 72 hours post infection, morphologically different areas of infected region having hypertrophied cells with disintegrated nucleus and vacuolated cytoplasm along with lysis of cell wall were seen. Tracheal epidermal cells filled with virus particles were seen and it was observed that subsequent dissemination of virus infection in the host central nervous system is brought out by tracheal system after 96 hours of post infection (Figure 1). Also showed marked histopathological changes, but not up to 72 hpi preparations; it appears probably that these cells are secondary site of infection.

The tracheal cells develop lyses after viral infection, thus resulting in release of virus particles in central nervous system through neural lamella which is in conformity with previous reports (Brancalhao, 2002). During process of internal infection in CNS, lyses of nerve, glia, and perineurium cells in ganglia and connectives were not reported. Similar finding was also reported by Torquato *et al*., 2006 in *B. mori*.

Transmission electron microscopy

Electron transmission studies of BHA isolate revealed typical baculovirus polyocclusion bodies (POBs). The polyhedral structure of *HaNPV* is formed basically by virions, matrix of polyhedron protein and viral envelope or polyhedral envelop. The POBs of *HaNPV* appeared as crystalline structures of

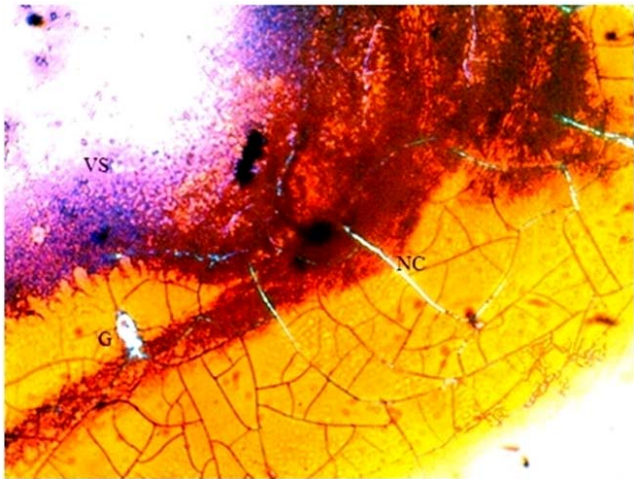


Figure 1: Photomicrography of *HaNPV* infected larvae: infected nerve cells (NC) and ganglion (G) in fifth instar larvae of virogenic stroma (400X magnification)

Table 1: Yield of *HaNPVs* from fifth instar larvae of *Helicoverpa armigera*

HaNPV isolate	Age of the larvae used	Larvae used	Concentration of virus for inoculation	Virus yield POBs/ mL
BHA	Third instar	Field collected	-	232×10^8
BHA	Fifth instar	Lab reared	10^8	342×10^8
RAU	Fifth instar	Field collected	-	129×10^7
RAU	Fifth instar	Lab reared	10^8	164×10^7

Table 2: Larval mortality and percentage pupation of 3rd instar larvae treated with various concentration of NPV isolate BHA

Number of POBs / mL	Number of larvae treated	% mortality	Incubation period(days)	% pupation	Adult emergence Healthy adult	Malformed adult
$2x\ 10^7$	40	100	2.87	0	0	0
$4x\ 10^6$	40	98.33	5.79	0	0	0
$8x\ 10^5$	40	90.59	6.67	1.66	0	100
$1.6x\ 10^5$	38	80.00	7.12	3.66	0	100
$3.2x\ 10^4$	40	75.83	8.60	16.33	10.20	89.79
$6.4x\ 10^3$	40	26.66	10.73	70.2	78.57	21.53
$2.48\ x10^2$	38	0.25	13.95	97.7	93.7	6.3
Control	38	00	16	100	98	2

Table 3: Probit analysis of concentration-mortality response of the third instars larvae of *H. armigera* to *Ha*NPV isolate BHA

LC_{50}	Fiducial limit Lower	Upper	LC_{90}	Fiducial limit Lower	Upper	Regression equation
$2.74x10^4$	$1.71x10^4$	$4.26x10^4$	$4.29x10^5$	$2.36x10^5$	$9.90x10^5$	$y = -4.76 + 1.073 \pm 0.67$

Line is insignificant at $p < 0.05$.

Table 4: Results of analysis of variance for *Helicoverpa* larvae mortality in each treatment

Source of variance	Sum of Squared	Degree of freedom	Mean square	F count	F table 5%
Between treatment	15607.19	5	3121.44	551.49	3.1
Residual treatment	67.87	12	5.66		
Total	15675.04	17	922.06		

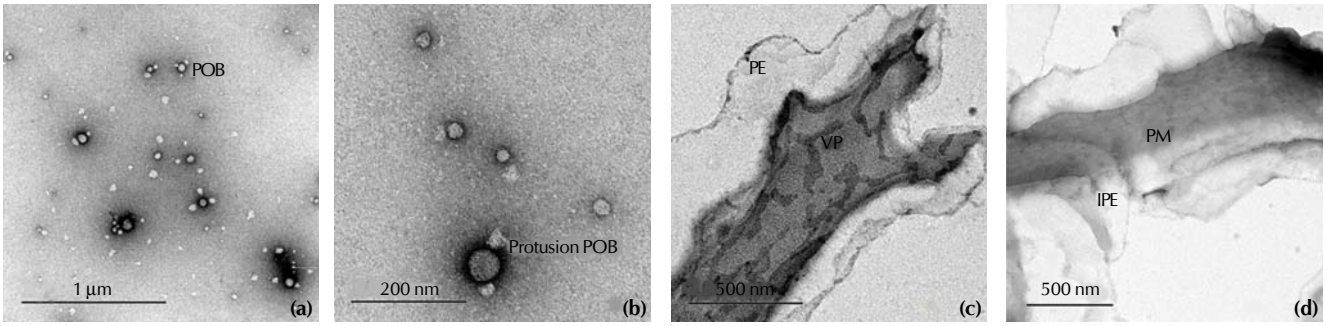
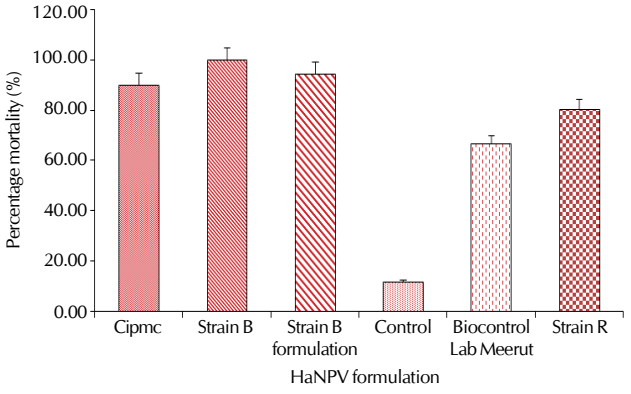


Figure 2: Transmission electron micrographs of POBs obtained from baculovirus infected larvae of *Helicoverpa armigera* a. POBs of different sizes, b. POB with protrusion c. polyhedral envelop (PE) and virus particles d. polyhedron protein matrix (PM) and irregular polyhedral envelop (IPE) Virus yield in fifth instar larvae



Graph 1: Percent mortality due to different strain of *HaNPV*

spherical shapes of size 0.9 to 2.15 ìm averaging 1.23ìm ± 2.45 in diameter. Majority of the POBs of *H. armigera* (present isolate BHA) are spherical while some of them are irregular in shape (Fig. 2a). The infectious virus particles were found randomly embedded in proteinaceous occlusion bodies. The virus particles, being rod shaped and inconstant in number, were asymmetrically arranged in the virus bundles. After the addition of 0·01 M Na_2CO_3 solution, the polyhedral body lost its shape instantaneously and at high magnification the negatively stained POBs samples precisely exposed the polyhedral envelop (PE) (Fig. 2c). It is loosely bound around the polyhedron and the distance between the PE and polyhedral crystalline matrix is not uniform around the polyhedron (Fig 2d). Jacob and Subramanian (1972) also reported that the size of POBs of *HaSNPV* (North India isolate) ranged from 0.5 to 2.5 ìm which is in conformity of present results. The present research also confirmed the discovery of a kind of cap like hemispherical shape (protrusion) at each end of virus bundle also discovered by Chang Li-ren and GawZan-yin in 1965 (Fig. 2b).

The number of OBs produced per fifth instar larva reared in laboratory was found 3.42×10^{10} POBs/ mL for *HaNPV* collected from village Bhaulli (BHA isolate) and 1.64×10^9 POBs/mL for *HaNPV* collected from village Raudahi (RAU) (Table 1).

Among the larval ages studied, Mehrvar, 2013 also recorded significantly high yield of 5.16×10^9, 4.56×10^9 and 3.33×10^9 OBs/ larva in the case of fifth instar larvae of NBN, MRG and MRD isolates of *HaNPV*. Considerable researches had earlier reported the same trends (Monobrullah and Nagata, 2000; Subramanian *et al.*, 2001; Subramanian and Mohankumar, 2006 and Mehrvar, 2012). Histopathological studies by Sathiah (2001) clearly stated that the growth of virus in fifth instar inoculated larvae progressed normally because of presence of adequate fat body which provide sufficient nutrition for the growth and development of POBs.

Bioassay

Bioassays of *Ha*NPV (BHA isolate) against the third instar larvae of *H. armigera* under laboratory conditions revealed deviation in their biological activity. For 3rd larval instar, mortality in-creased with *HaNPV* concentration rate (Table 2). The results specified a positive relationship between larval mortality and concentration values which varied statistically, based on the non-overlap of fiducial limits. Adult emergence in all

concentrations of *HaNPV* was significantly decreased compared with control. When 3rd instars larvae fed on artificial diet treated with *HaNPV*, mortality, pupation rate, larval period, and adult emergence showed significant variation in different concen-trations of *HaNPV*. The maximum third instar larval mortality of 100 % was achieved at a dose of 2 x 10^7 POBs/ mL followed by inoculation dose 4x 10^6 POBs/ mL with mortality of 98.33%; whereas, minimum mortality was reported in control (Table 2).

Probit analysis of mortality data enabled calculation of the dose–response relationships for larval instar, obtaining the following regression equations and LC 50: $y = 1.073 \pm 0.67 - 4.76$ and LC 50 of 2.74×10^4 POBs/mL (with 95% fiducial limits $1.71 \times 10^4 - 4.26 \times 10^4$). The isolate was the most virulent with the LC 90 value of 4.29 x 10^5 POBs/mL. The fit of the transformed data was acceptable using the chi-squared test (Table 3).

Wide variations in the virulence and genetic makeup of *Ha*NPV collected from different agroclimatic regions of Madhya Pradesh, Gujarat and Punjab were also reported by numerous researches (Arora *et al.,* 1997, Geetha & Rabindra (1999), Gopali & Lingappa (2001), Rabindra, 1992, Somasekar *et al.,* 1993 and Odak & Rawat, 1982). Magholi (2014) reported 2.2 $\times 10^3$, 3.8 $\times 10^4$ and 6.6 $\times 10^5$ PIB/mL lethal concentrations values (LC 25, LC 50, and LC 75) of *HaNPV* for 2nd larval instars of *Plutella xylostella*, respectively. Chandel *et al*. (2004) reported that Kanpur and PCI-Bangalore isolates were the most virulent resulting in the highest mortality in *H. armigera* larvae.

Commercial formulations of the *HaNPV* were evaluated at PDBC, Banglore, India against third instar larvae of *H. armigera* under laboratory conditions (Srinivasa, 2008). Among the different formulation tested, the median lethal concentration of *HaNPV* isolate from PCI was found highest with 9.5 x 10^5 POBs per mL followed by 9.4 X 10^5 POBs per mL with MBP isolate and 15.6 x 10^5 POBs per mL with that of *HaNPV* of ZARS-G. The lowest LC50 was for isolate BPMS (3.12 x 10^4 POBs per mL) proving it as most virulent formulation among the different formulation tested.

Comparison of different *HaNPV* isolates against IIIrd Instar larvae of *H. armigera*

Results of statistical analysis indicates that diet plug bioassay in liquid dosage form on the larvae of *H. armigera* was very significant on larval mortality. Highest mortality of 100% was obtained in larvae treated with crude preparation of BHA isolate against the NPV formulation obtained from Centre of Integrated pest management, Lucknow and Biocontrol Laboratory, Meerut which reported 90.26 and 68.50 per cent mortality, respectively. RAU isolate also reported 80.26% mortality in third instar larvae (Graph 1).

The efficacy of BHA isolate and its formulation in liquid (with feeding stimulant mannitol) did not differ significantly from each other. Duncan's Multiple Range Test results indicate that both BHA and RAU isolate significantly manage the population of *H. armigera* exposed in the laboratory to cause mortality at range 90.26% - 100%. Several researchers also reported that the use of feeding stimulants added to carriers contaminated with OBs can result in superior pest control than without phagostimulant (Lasa *et al.*, 2009).

Results of analysis of variance indicate that all the treatments were very influential on the mortality (Table 4) of larvae of *H. armigera*.

The nuclear polyhedrovirus BHA isolate recorded significantly high yield of 3.42 x 10^{10} POBs/ mL for *HaNPV* as compared to different isolates reported from earlier workers. The efficacy of BHA isolate (LC 50: 2.74 x 10^4 POBs/mL) is also lower than the BPMS isolate (3.12 X 10^4 POBs per mL) which is also lower than the commercial nuclear polyhedrovirus (NPV) "Elcar" (1.4 x 10^6 POBs/ml) (Teakle *et al*., 1989). 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. The mean mortality of larvae exposed to virus mixed with 1% mannitol was not significantly different from that of larvae inoculated with virus alone suggesting that diet enhancer has the ability in protecting the stability of the virulence of the virus it carries under laboratory condition. Further research is needed to exploit the commercial formulation of BHA isolate.

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