Pakistan J. Zool., vol. 50(6), pp 2229-2237, 2018. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2018.50.6.2229.2237

Molecular Identification and Pathological Characteristics of NPV Isolated from *Spodoptera litura* (Fabricius) in Pakistan

Jam Nazeer Ahmad[1,2,*], Rashid Mushtaq[1], Samina Jam Nazeer Ahmad[1,2], Sumaira Maqsood[3], Ishita Ahuja[4] and Atle M. Bones[4]

[1]*Intergrated Genomics Cellular Developmental and Biotechnology Lab, Department of Entomology, University of Agriculture, Faisalabad*

[2]*Plant Stress Physiology and Molecular Biology Lab, Department of Botany, University of Agriculture, Faisalabad*

[3]*Institute of Agricultural Sciences, University of the Punjab, Lahore*

[4]*Department of Biology, Norwegian University of Science and Technology, NO-7491 Trondheim, Norway*

Article Information
Received 28 May 2018
Revised 21 June 2018
Accepted 10 July 2018
Available online 05 October 2018

Authors' Contribution
JNA, RM and SJNA presented the ideas and wrote the manuscript. RM, SJNA and SM reviewed the literature. JNA, IA, and AMB critically analyzed and edited the manuscript.

Key words
***Spodoptera litura*, PCR, NPV, Biopesticide, DNA barcoding, Nucleopolyhedro-viruses.**

ABSTRACT

The cotton army worm *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) is a destructive pest of various field crops and vegetables in Pakistan. Development of biopesticide is an attractive strategy to minimize the problems of pest resistance, environmental pollution and human health concerns. The isolates of *S. litura* nucleopolyhedroviruses (SltNPV) were collected from infected larvae fed on natural cotton crops. The NPV was isolated from the larvae and viral occlusion bodies (VOBs) were detected using a light microscope. The toxicity of native isolates against *S. litura* also studied by testing different concentrations (1 x 10^2 POB (Polyhedral occlusion bodies) mL^{-1}- 1 x 10^{10} POB mL^{-1}) from the occlusion bodies produced from NPV isolates against 2nd, 3rd, 4th and 5th instar larvae of *S. litura.* The rapid and sensitive polymerase chain reaction (PCR) technique was used for the molecular detection of NPV gene from native NPV diseased insect. Multiple sequence alignment and phylogenetic analysis were performed to compare *Sl*NPV- FSD15 based on *Lef-8* with other Lef-8 genes sequences clearly showed that our *Sl*NPV-FSD15 isolate belongs to *Spodoptera litura* associated NPVs. The biological activities of this NPV isolates were investigated under laboratory condition. The highest mortality of *S. litura* was observed at early instars. Against second instars of *S. litura,* LC_{50} values of NPV isolate ranged from 1.92×10^3 to 3.64×10^3OB/ml with LT_{50} values of 69.30 h to 72.80 h, respectively. This study showed highly effectiveness and provides an opportunity to cut down the use of synthetic approaches and develop safe biological/microbial insecticides from NPV isolates, which in future may effectively control *S. litura.*

INTRODUCTION

Spodoptera litura Fabricius (Lepidoptera: Noctuidae) is also known as pigweed caterpillar or tobacco cutworm is cosmopolitan and polyphagous pest which cause severe losses in southern and northern districts of Pakistan (Ahmed *et al*., 2016; Saleem *et al*., 2016) and it is widely distributed throughout Asia, Africa and Europe (Nathan *et al*., 2005; El-Helaly, 2013). It is a destructive pest of numerous economically important cash crops such as cotton, groundnut, soybean, tomato, sweet potato, onion, clover chili, cauliflower and cabbage (El-bendary and El-Helaly, 2013; Saleem *et al*., 2016). In vegetables, it attacks leaves, stem, fruiting points and occasionally, also seed pods resulting in considerable yield loss. Current methods to control this pest are based on the use of conventional insecticides which causes the development of resistance in the pest and further has a negative environmental and human impact. Extensive use of synthetic insecticides has led to outbreaks of insecticide resistance in *S. litura* (Bhatti *et al*., 2013; Ahmed *et al*., 2015). The development of resistance in *S. litura* against organochlorine, organophosphates and synthetic pyrethroids has been reported in the areas of cotton belt of south Punjab, Pakistan (Ahmed *et al*., 2015). Thus this situation demands to search out some ecological safe alternative like microbial pathogen to control this notorious pest. An attractive and alternate tool for *S. litura* control is the use of entomopathogens. Several species of insect viruses, bacteria, fungi and protozoa were isolated and investigated for control of *S. litura* (Çakici *et al*., 2014). In recent years, more than 600 insect species belonging to order Lepidoptera, Hymenoptera, Diptera, Orthoptera, Coleoptera, Neuroptera, Thysanura, and Trichoptera infected with baculovirus have been isolated

* Corresponding author: jam.ahmad@uaf.edu.pk
0030-9923/2018/0006-2229 $ 9.00/0

(Haase *et al*., 2015). Baculoviruses are insect-specific, diverse group of double-stranded DNA circular viruses with genome in the region of 80-180 kbp. Baculoviruses belong to the family baculoviridae which is further divided into two genera, the Nucleopolyhedroviruses (NPVs) and Granuloviruses (GVs) (Hu *et al*., 2003; Jehle *et al*., 2006; Barreto *et al*., 2005). The NPV s are cuboidal shaped have occlusion body of 0.4 to 2.5 µm in size visible under electron microscope (Moser *et al*., 2001; Shapiro *et al*., 2004). Lepidopteran pests, *Spodoptera exigua* (Murillo *et al*., 2001; Wu *et al*., 2012; Khattab, 2013). *S. litura* (Lavina *et al*., 2001; Martins *et al*., 2005; Laarif *et al*., 2011), *Helicoverpa armigera* (Lepidoptera: Noctuidae) (Mehrvar *et al*., 2007; Kumar *et al*., 2012; Noune and Hauxwell, 2015) and *Malacosoma americanum* (Demir *et al*., 2014) have shown susceptibility to several isolates of NPV. Horizontal transmission of baculoviruses from one host to another occurs when a susceptible insect ingests food contaminated with occlusion bodies (OBs) (Takahashi *et al*., 2015). Several studies have reported the isolation of NPVs from *S. litura* (Lavina *et al*., 2001; Martins *et al*., 2005; Lucien *et al*., 2009; Laarif *et al*., 2011; Kumar *et al*., 2011), *H. armigera* (Figueiredo *et al*., 1999; Ogembo *et al*., 2007; Mehrvar *et al*., 2008; Kumar *et al*., 2012; Noune and Hauxwell, 2015) *S. exigua* (Murillo *et al*., 2001; Wu *et al*., 2012; Khattab, 2013), *Trichoplusia ni* (Erlandson *et al*., 2007) *Diaphania pulverulentalis* (Pachippan *et al*., 2012), Turnip moth (Jakubowska *et al*., 2005) and *Chrysodeixis includens* (Alexandre *et al*., 2010).

All NPVs have molecularly been characterized by restriction endonuclease mapping of viral DNA, showing that they can be distinguished from each other by one or more DNA restriction enzyme fragments. At biological level, some of these strains have presented better insecticidal activities, which make them more suitable to *S. litura* (Martins *et al*., 2005; Laarif *et al*., 2011) *H. armigera* (Ogembo *et al*., 2007; Mehrvar *et al*., 2008) and *S. exigua* control (Khattab, 2013). The present study was planned to identify NPVs as a biological control agent of *S. litura* from local Spodoptera NPV infected population. Therefore, a simple molecular procedure suitable for diagnosis of viruses in their natural hosts was adopted and the evaluation of its biological activity against different instars is being reported.

MATERIALS AND METHODS

NPV diseased insect collection

The original virus isolates were obtained from diseased *S. litura* larvae collected from cotton field of PARS-UAF. The larvae that showed baculovirus infection symptoms were brought to laboratory (Fig. 1A) and examined to confirm the presence of virus by light microscope with Giemsa staining according to Mustafa *et al*. (2001), in which a thin smear of infected worm tissue was mounted and air dried. The smear was immersed for 1-2 min in Giemsa, rinsed under running tap water for 5-10 sec then the smear was stained for two hours in 10% Giemsa stain (10g of Giemsa dissolved in 100ml distilled water). The dye was rinsed off in running tap water for 5-10 sec and allowed to air dry, then examined under a light microscope to detect the Occlusion Bodies (OBs). After the examination, the diseased larvae were kept at -20°C until the purification of OBs (polyhedra).

Light microscope examination

Moribund larvae showing specific disease symptoms were individually examined for the presence of polyhedral inclusion bodies using light microscope. A wet smear of the homogenized liquid using a drop of haemolymph or a small part of larval tissue was spread on a glass slide. The slide was then dipped in 10% Giemsa's stain for 10 min. The excess stain was then washed with running water for 5-10 sec (Wigley, 1976). The prepared smear was examined using the oil immersion of phase contrast microscope. The smear test would allow recognition of the occlusion bodies of nuclear polyhedrosis viruses NPV.

NPV production and purification

The diseased larvae of 3rd to 5th instar of *S. litura* were collected from cotton fields in Faisalabad, Pakistan, in 2013. The infected larvae showing baculovirus presence symptoms were brought to IGCDB laboratory and stored at -20 °C until the isolation of OBs (polyhedra). Isolation and purification of the NPV was performed in 4th instar of *S. litura*. Using haemocytometer (Hausser Scientific), NPV viral concentrations were quantified under a light microscope with six counts per hemocytometer. A stock suspension of NPV was prepared. A dilution of various concentrations 1 x 10^2- 1 x 10^9 OBs/ml concentration was prepared from the stock suspension. Twenty-five larvae were placed in 50 ml tube and homogenized. But, for NPV isolation from *S. litura* infected samples, larvae were treated with 0.1% SDS (1 ml/larva) for 1 night at 4°C and filtered through 5 layers of cheese cloth. POBs were settled at bottom of tube by centrifugation at 3600g for 10 min at room temperature in 50 ml centrifuge tubes. Before final isolation, the pellets were re-suspended in 0.5% SDS and centrifugation and re-suspension were repeated with 0.3 M NaCl. Then the OBs were finally re-suspended in distilled water. Then, following the protocol of Cheng *et al*. (1990), POBs were further purified according to sucrose gradient centrifugation method.

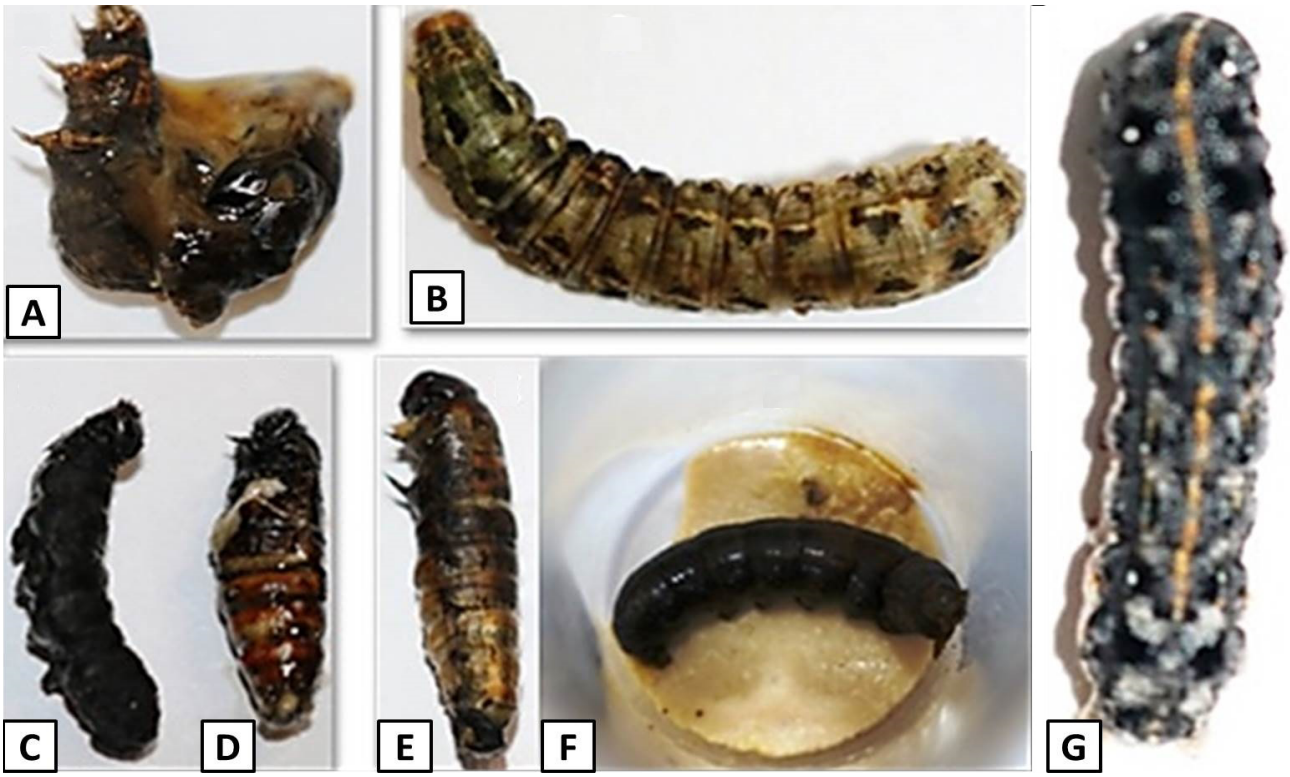

Fig. 1. NPV infected *S. litura:* **A,** typical NPV symptom of NPV ruptured infected larvae, usually die 4–9 days after infection, disintegrate and release a virus-laden fluid; **B,** slow move NPV infected swollen malformed pale brown larva; **C**, slow move NPV infected swollen malformed dark larva from 5th instar; **D and E**, malformed pre-pupae; **F**, laboratory propagative NPV infected larva; **G**, healthy larva.

DNA extraction and PCR

DNA was extracted from NPV infected larvae of *S. litura* with DNeasy Tissue kit (Qiagen, Hilden, Germany) according to manufacturer protocols. Quantity and quality of extracted virus DNA was estimated using UV spectrophotometer (Pico200) and by the ethidium bromide 1.5% agarose gel electrophoresis, respectively. The DNAs obtained were stored at -20°C until used. The primer nucleotide sequences based on NPV conserved gene late expression factor (Lef-8) was carried out for PCR in a reaction volume of 48 µl. The reaction volume contains ddH2O (33 µl), 10Xreaction buffer (5 µl), 1 mM dNTPs (5 µl), 200 µM of each forward and reverse primers (1 µl), and 1 µl of purified DNA (~ 50 ng). The lef-8 specific degenerate primers (prL8-1 and prL8-2) developed by Lange *et al*. (2004) was used in PCR. The amplification of DNA was accomplished with a PCR thermal cycler (PeqStar, Germany) under following PCR cycling conditions. DNA samples were preheated to 95°C for 4 min, followed by 35 cycles with denaturation temperature of 95°C for 2 min, annealing temperature of 46°C for 2 min, and extension temperature at 72°C for 1 min. Then, final extension at 72°C for 2 min was included for final amplification. In the first step of PCR, 0.2 µl of Taq polymerase and 1.8µl its dilution buffer was added in each tube making final reaction volume up to 50 µl. The PCR product was analyzed by 1% agarose gel electrophoresis at 80 V for 1 h. After the PCR, the gel was stained with ethidium bromide and photographed under a UV light. Gels were stained in solution of ethidium bromide (2µg/mL) and were visualized under UV light using fisher scientific gel documentation system (Syngene™ IG3) for the confirmation of virus presence.

Nucleotide sequencing and phylogenetic analysis

PCR product (745 bp) was purified using commercial kit and sequencing was performed and analyzed on AbiPrism 3100 Genetic Analyzer apparatus (Applied Biosystems, USA). The resulting Lef-8 sequence has been deposited in international database (NCBI). NPV Lef-8 sequences identified by Blast sequence homology were downloaded from Genbank database (http://www.ncbi.nlm.nih.gov). All Lef-8 sequences were aligned with ClustalW and phylogenetic analysis was performed under

MEGA6 software (Tamura *et al*., 2013).

Insect rearing and bioassay study

The *S. litura* larvae used in this experiment derived from cotton fields of Punjab and reared on artificial diet consisting of chickpea flour 150g, sorbic acid 0.75 g, yeast powder 24g, linseed oil 6 ml, agar 8.4 g, vitamin mixture 0.02 g, ascorbic acid 2.35 g, methl-4-hydroxy benzoate 1.5 g, d H2O 550 ml and streptomycin 0.75 g. The rearing was done under controlled laboratory condition (25± 2°C, 70± 5 RH, 14:10 h light: dark photoperiod). The diet was stored at 4°C until use. About fifty 2nd and 3rd instar *S. litura* larvae were transferred in individual plastic vials containing 0.5-1.5 g thin layer of artificial diet until they reached at fourth larval stage. Then, they were transferred to boxes containing 3 cm thick layer of vermiculite (<0.5 mm grain size) for pupation. Additional artificial diet was provided shortly before pupation. Pupae were collected and incubated at 25°C. All the larvae were maintained at 25±2°C, 75% r.h and 16:10 (D:L) photoperiod (Jehle *et al*., 2013).

All the bioassays were performed according to Lucien *et al*. (2009) with slight modification. Briefly, Virulence of NPV was tested against 2nd, 3rd 4th and 5th instar larvae of *S. litura*. Molted larvae were distinguished by their head capsule slippage and transferred to individual vials for 12 h. Different NPV suspensions (1 x 10^2; to 1x 10^9 POB mL^{-1}) were prepared and 5-10 µl of each viral concentration was socked with fresh piece of artificial (2mm^2) in vials. Molted Larvae were allowed to feed on the treated artificial diets until complete consumption. After consuming artificial diet, 25 larvae were individually shifted on diet without virus suspension. Larvae served with virus free diet constituted the healthy controls. LC_{50} and LT_{50} values were determined from data observed every day. Concentrations of OBs were determined with haemoctometer under phase contrast microscopy at 400X magnification. All the treatments were replicated thrice and maintained at 25±2°C, 75% r.h. and L16: D8 h photoperiod.

Statistical analysis

The data were analyzed using Probit analysis software to arrive at lethal concentration of virus required to cause 50% mortality (LC_{50}) and lethal time required to cause 50% mortality (LT_{50})

RESULTS

NPV isolates and VOBs

The native *S. litura* NPV isolates were obtained from infected larvae collected from cotton field of Post graduate Agriculture Research Station (PRAS) of University of Agriculture Faisalabad. The symptoms of viral infection on *S. litura* collected from the cotton field were liquefied ruptured larval body (Fig. 1A), and slow motion swollen larvae with red color cuticle (Fig. 1B). The field collected virus isolate was cultured in a *S. litura* laboratory colony (Fig. 1C-F). Then infected propagated colony was then purified and kept at -80°C for further studies.

The viral occlusion bodies (VOBs) from diseased larvae were observed under a light as well as inverted microscope. VOBs appeared as polyhedral and negatively stained particles after Giemsa staining. Under light and inverted microscope, NPV associated occlusion bodies were observed (Fig. 2).

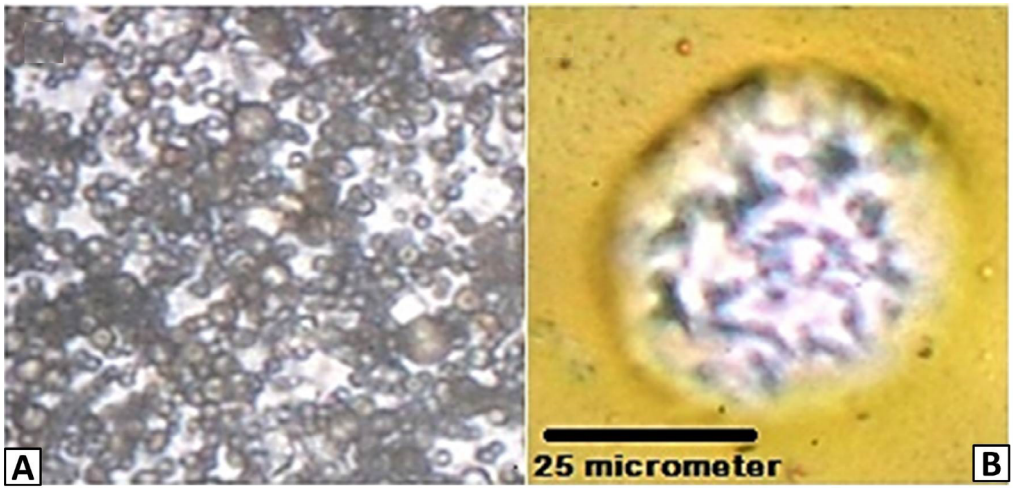

Fig. 2. **A,** NPV infected *S. litura* viral occlusion body's polyhedra under light microscopy; **B**, degenerating (ruptured) infected *S. litura* cell.

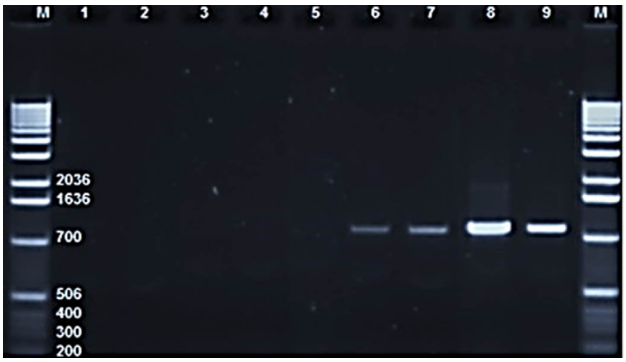

Fig. 3. PCR detection of NPV from *S. litura* infected samples from Pakistan by using NPV LEF8- specific primers: Lane 1-3, non-infected *S. litura* larva; Lane 4-8, NPV infected *S. litura* larva (Pak-15 Faisalabad strain); Lane M, 1 kb DNA marker (Invitrogen).

Amplification of NPV specific gene

The infected samples collected from Faisalabad district were analyzed for PCR detection. The partial codons sequence of the late expression factor-8 (Lef-8) gene was amplified using specific PCR primers as a single fragment at correct length (~745 bp) (Fig. 3).

DNA sequencing and phylogenetic analysis

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Phylogenetic analyses were conducted in MEGA6 (Tamura *et al*., 2013) for the comparison of the SlNPV-FSD15-lef-8 gene sequences with the sequences of other lef-8 genes of various nucleopolyhydroviruses (NPVs) available in GenBank. The nucleotide sequence of the native NPV isolate (Slt NPV-FSD15) is composed of 745 bp, submitted at Gen Bank NCBI. This sequence was aligned using ClustalW with the NCBI available nucleotide sequences of NPVs associated with different insects. The molecular phylogeny of nucleotide sequence of the SlNPV-FSD15-lef-8 showed maximum homology (99-100%) with nucleotide sequence of SpliNPV from *S. litura* of Germany, Japan and India origin (AY706581.1, AB326103.1, JF2760358.1, AB583682.1, AB581187.1). The *Helicoverpa armigera* associated NPV formed separate clade clearly distinguished from *S. litura* NPV isolates with lowest similarity percentage index (Fig. 4).

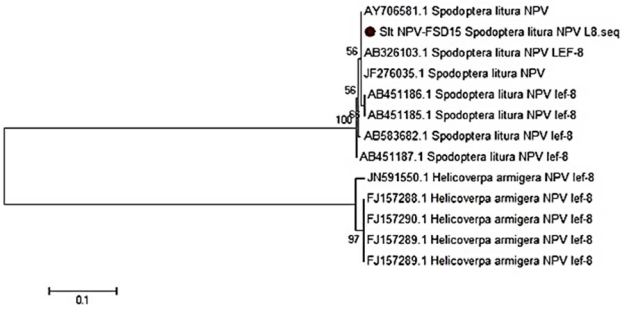

Fig. 4. Molecular phylogenetic analysis by maximum likelihood method of Pakistani isolate SltNPV-FSD15 strain based on nucleotide sequences with the corresponding partial late expression factor-8 (Lef-8) gene of 12 nucleopolyhydroviruses associated with *S. litura* (NPVs). *S. litura* associated NPV isolates origin and GenBank accession numbers used in the sequence analysis and phylogenetic tree construction; AY706581.1 S37 (Germany), JF276035.1 Banglore (India), AB451187.1 (AB326103.1, AB451187.1, AB583682.1 (Japan). The numbers represent bootstrap percentage values based on 1,000 replicates.

Biological activity of native SplNPV-FSD15 isolate

Bioassay of isolated strain of SpltNPV against 2nd, 3rd, 4th and 5th instars larvae of *S. litura* under laboratory condition show a wide range of variation in its biological activity. The LC_{50} values in three experiments of SpltNPV were inversely correlated with the age of the larvae, LC_{50} values being the highest for fifth instars. Similarly, LT_{50} was as low as 69-72 h for second instar larvae and regularly increased to reach at 144 to 146 h for fifth larvae instars. LT_{50} values were not significantly different between the different independent experiments. Bioassay result revealed that the SpltNPV strain isolated from *S. litura* was highly effective especially against 2nd, 3rd instar larvae. In first experiment, LC50 values for 2nd , 3rd , 4th and 5th instar larvae were 2.64×10^3, 2.92×10^4, 2.94×10^5, and 2.15×10^6 OBs/ml, respectively. Briefly, in the third experiment,

Table I.- LC_{50} (95% CL) values of SPLtNPV-FSD15 isolate against 2nd, 3rd and 4th instar of *Spodoptera litura.*

Instars	SPLtNPV-1					SPLtNPV-2					SPLtNPV-3				
	LC_{50}	Slope	X^2	df	P	LC_{50}	Slope	X^2	df	P	LC_{50}	Slope	X^2	df	P
Second	2.64×10^3	10298210	3.02009	1	0.082	1.94×10^3	10672645	3.07360	1	0.080	1.92×10^3	10672645	3.05460	1	0.081
Third	2.92×10^4	9727687	3.87286	1	0.049	1.66×10^4	10301158	4.45550	1	0.874	1.91×10^4	9898068	5.46667	1	0.019
Fourth	2.94×10^5	248157064	0.173039	1	0.677	2.68×10^5	69371413	0.0973090	1	0.755	3.9×10^5	55947328	0.09-3141	1	0.764
Fifth	2.15×10^6	65593222	0.449124	1	0.503	2.34×10^6	56888296	0.294854	1	0.587	3.96×10^6	54577160	0.295128	1	0.587

Table II.- LT_{50} (95% CL) value of SPLtNPV-FSD15 isolate against 2nd, 3rd, 4th and 5th instar of *Spodoptera litura.*

Instars	SPLtNPV-1					SPLtNPV-2					SPLtNPV-3				
	LT_{50} (h)	Slope	X^2	df	P	LT_{50} (h)	Slope	X^2	df	P	LT_{50} (h)	Slope	X^2	df	P
Second	72.50	0.917231	0.304227	1	0.581	70.4	0.948467	0.587796	1	0.443	69.30	0.953284	0.657373	1	0.417
Third	95.76	0.802226	0.117478	1	0.732	98.4	0.786250	1.49456	1	0.222	93.6	0.839378	1.10078	1	0.294
Fourth	121.6	0.713918	0.0030889	1	0.956	125.2	0.700997	0.0038591	1	0.950	123.5	0.693892	0.118227	1	0.731
Fifth	144.64	0.654180	0.0038249	1	0.951	146.8	0.660854	0.0632983	1	0.801	145.8	0.676337	0.0005908	1	0.981

for 2nd instars, the most virulent LC_{50} value (1.92 x 10^3 POB mL^{-1}) was observed, followed by 2nd and 1st experiment (Table I). The order of LC_{50} value in the case of three experiments of SpltNPV was second<third <forth <fifth instar. Similarly, LT_{50} value of 3rd spltNPV experiment was the lowest (59 h) followed by 2nd and 1st experiment (Table II).

DISCUSSION

The *S. litura* (Lepidoptera: Noctuidae) also called armyworm is one of the most destructive and notorious phytophagous insect pests on cotton, vegetables and other field crops. Recently various entomopathogens such as nematodes are being evaluated and utilized to control to *S. litura* (Safdar *et al.*, 2018). Microbial bio-pesticides based on native baculoviruses have great potential in agriculture with better insecticidal characteristics and higher safety' for environment. The NPV virus isolates was obtained from diseased *S. litura* collected cotton. The diseased larvae exhibited viral-like symptoms such as swollen bodies, moribund appearance when field collected and also when laboratory NPV treated. Similarly, because of deposition of occluded viruses (OVs), infected larval bodies become pale and their cuticle ruptured discharging body fluid and ultimately killing the insect. Similar results were reported by Toprak *et al*. (2005). The viral occlusion bodies (VOBs) of the Pakistani nucleopolyhedrovirus isolates (*Sl*NPV-FSD15) were detected using a light microscope by staining thin smear of infected larvae and drop of VOBs with Giemsa stain which appeared polyhedral and negatively stained particles. The developed bio informative tools and application of latest genomic data facilitated the identification of viruses because of good primer designing. Here, we also used molecular techniques to characterize native *S. litura* associated NPVs that were found identical to SpltNPVs from other countries and clearly different from *Helicoverpa armigera* and *Mythimna separata* NPVs (Kouassai *et al*., 2009). Further, LC_{50} values against 2nd -5th instars with LT_{50} values of this NPV isolate (SltNPV-FSD15) indicated a significant effective control against *S. litura*. Result obtained by Laarif *et al*. (2011) indicated that amino acid and nucleotide sequences of *S. littoralis* (Tun-SlNPV) nucleopolyhedrovirus strain was almost identical with different GenBank deposited sequences of NPVs (Clem *et al*., 2013; Ikeda *et al*., 2013).

In bioassay, increasing value of LC_{50} and LT_{50} with the age of the host showed the susceptibility of the different larval stages; decline in *S. litura* susceptibility to NPV is due to dilution effect because larval weight increased as the insect grows (Briese, 1986). Lucein *et al*. (2009) and Bhutia *et al*. (2012) also found that LC_{50} and LT_{50} values were increased as larval age increased showing - against SpltNPVN. However, Trang *et al*. (2002) reported that older larvae of *S .litura* were not affected through ingestion, but intra haemocoelic infection of NPV in mature insect resulted in insect death (Rao *et al*., 2015). SpltNPV isolate from Biocontrol Research Laboratory (BCRL) was found to be virulent against 3rd instar larvae of *S litura* larvae with LT_{50} value (122.16 d) at 1×10^6 OB's/ ml (Bhutia *et al*., 2012). Subramanien *et al*. (2005) described that LT_{50} values for larvae of *S. litura* was dose-dependent. The LC_{50} value for the larvae of *S. litura* increases 15,000 times in 2 day-old larvae as compared to 8th day larvae (Trang *et al*., 2002). Similar trend for LC50 of SpltNPV isolate against 2nd and 3rd were (3.5 9 $\times10^4$ and 2.4 9$\times$ 10^5 OBs/ ml) observed (Kumar *et al*., 2011). In summary, increasing dose of NPV caused significant reduction in the survival times of Lepidoptera larvae. This pattern was also observed in instar of *Mythima separata* and third instars of *S. litura* larvae (Koussoi *et al*., 2009). Minimum lethal time (LT_{50}) (96-216 h) of different instars of *S. litura* suggests that application of SpltNPV isolate as bio insecticide would be economic and attractive strategy for the control of *S. litura* (Lucein *et al*., 2009). Contradictory results were found by Koussoi *et al*. (2009) who observed that high LC_{50} of MSNPV (180 h) and Indian NPV isolates (240 h) against *M. separata* made clear that their sole application would not be an efficient strategy for insect control, but should become effective bio insecticides when combined with enhancers. In our case, the very good results of LC_{50} and LT_{50} showed highly effectiveness of Slt NPV-FSD15 isolate against all tested instars of *S. litura* as compared to other reported SltNPV isolates. The biological activity

of this isolate is under progress against other important lepidopterous pest of major agriculture crops.

ACKNOWLEDGEMENTS

AMB, JNA, SJNA and IA acknowledge the financial support from PAK 3004 Framework for Pak-Norway Institutional Co-operation Programme (FICP). The authors acknowledge the efforts of Abdullah and late Hafeez ul Rehman for insect collection and rearing of insect culture for experiments.

Statement of conflict of interest

The authors have no conflict of interest.

REFERENCES

Ahmed, Y.E., Shimaa, M.D., Marwa, M.E.S. and Ahmed, R.S., 2016. Molecular and biological characterization of a nucleopolyhedrovirus isolate (Egy-SlNPV) from *Spodoptera littoralis* in Egypt. *Int. J. Virol. mol. Biol.,* **5**: 34-45.

Ahmed, M.A.I., Sobhy, A.S.T., Farouk, A.A. and Samir, H.M.M., 2015. The effects of selected host plants on the efficacy of spinosad pesticide on cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) under laboratory conditions. *Adv. environ. Biol.,* **9**: 372-375.

Alexandre, T.M., Zilda, M.A.R., Saluana, R.C. and Maria, E.B.C., 2010. Evaluation of seven viral isolates as potential biocontrol agents against *Pseudoplusia includens* (Lepidoptera: Noctuidae) caterpillars. *J. Inverteb. Pathol*., **105**: 98-104. https://doi.org/10.1016/j.jip.2010.05.015

Barreto, M.R., Teixeira, F.F., Paiva, E. and Valicente, F.H., 2005. Effect of *Baculovirus spodoptera* isolates in *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) larvae and their characterization by RAPD. *Neotrop. Ent.,* **34**: 67-75. https://doi.org/10.1590/S1519-566X2005000100010

Briese, D.T., 1986. Insect resistance to baculoviruses. In: *The biology of baculoviruses* (eds. R.R. Granados and B.A. Federici), CRC Press, Coba Raton, Florida, pp. 237-263.

Bhatti, S.S., Ahmad, M., Yousaf, K. and Naeem, M., 2013. Pyrethroids and new chemistry insecticides mixtures against *Spodoptera litura* (Noctuidae: Lepidoptera) under laboratory conditions. *Asian J. Agric. Biol*., **1**: 45-50.

Bhutia, K.C., Chakravarthy, A.K., Doddabasappa, B., Narabenchi, G.B. and Lingaraj, V.K., 2012. Evaluation and production of improved formulation of nucleopolyhedrosis virus of *Spodoptera litura*. *Bull. Insectol*., **65**: 247-256.

Çakıcı, F.Ö., Sevim, A., Demirbağ, Z. and Demir, İ., 2014. Investigating internal bacteria of *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) larvae and some bacillus strains as biocontrol agents. *Turk. J. Agric. For*., **38**: 99-110. https://doi.org/10.3906/tar-1302-65

Cheng, X.W., Aguda, R.M. and Shepard, B.M., 1990. A nuclear polyhedrosis virus from the rice skipper. *Int. Rice Res. Inst. Newsl.,* **15**: 33-34.

Clem, R.J. and Passarelli, A.L., 2013. Baculoviruses: Sophisticated pathogens of insects. *PLoS Pathog*., **9**: e1003729. https://doi.org/10.1371/journal.ppat.1003729

Demir, I., Remziye, N., Lida, M.G. and Zihni, D., 2014. A highly effective nucleopolyhedrovirus against *Malacosoma* spp. (Lepidoptera: Lasiocampidae) from Turkey: Isolation, characterization, phylogeny, and virulence. *Turk. J. Agric. For.,* **38**: 462-470. https://doi.org/10.3906/tar-1307-32

El-bendary, H.M. and El-Helaly, A.A., 2013. First record nanotechnology in agricultural: Silica nano-particles a potential new insecticide for pest control. *Appl. Sci. Rep*., **4**: 241-246.

El-Helaly, M., Khattab, A.S., El-Salamouny, M., El-Sheikh, S. and Elnagar, M., 2013. Promising additives to protect the activity of *Baculovirus* biocontrol agent under field – sunlight conditions in Egypt. *J. Life Sci*., **7**: 495-500.

Erlandson, M., Newhouse, S., Moore, K. and Janmaat, A., 2007. Characterization of baculovirus isolates from Trichoplusia ni populations from vegetable greenhouses. *Biol. Contr.,* **41**: 256-263. https://doi.org/10.1016/j.biocontrol.2007.01.011

Figueiredo, E., Muñoz, D., Escribano, A., Mexia, A., Vlak, J.M. and Caballero, P., 1999. Biochemical identification and comparative insecticidal activity of nucleopolyhedrovirus pathogenic for *Heliothis armigera* (Lep., Noctuidae) larvae. *J. appl. Ent.,* **123**: 165-169.

Haase, S., Alicia, S.C. and Víctor, R., 2015. Baculovirus insecticides in Latin America: Historical overview, current status and future perspectives. *Viruses*, **7**: 2230-2267. https://doi.org/10.3390/v7052230

Hu, Y.C., Tsai, C.T., Chang, Y.J. and Huang, J.H., 2003. Enhancement and prolongation of baculovirus-mediated expression in mammalian cells: Focuses on strategic infection and feeding. *Biotechnol. Prog.,* **19**: 373-379. https://doi.org/10.1021/bp025609d

Ikeda, M., Yamada, H., Hamajima, R. and Kobayashi, M.,

2013. Baculovirus genes modulating intracellular innate antiviral immunity of lepidopteran insect cells. *Virology*, **435**: 1-13. https://doi.org/10.1016/j.virol.2012.10.016

Jakubowska, A., van Oers, M.M., Ziemnicka, J., Lipa, J.J. and Vlak, J.M., 2005. Molecular characterization of *Agrotis segetum* nucleopolyhedrovirus from Poland. *J. Inverteb. Pathol.,* **90**: 64-68. https://doi.org/10.1016/j.jip.2005.06.010

Jehle, J.A., Blissard, G.W., Bonning, B.C., Cory, J.S., Herniou, E.A., Rohrmann, G.F., Theilmann, D.A., Thiem, S.M. and Vlak, J.M., 2006. On the classification and nomenclature of baculoviruses: A proposal for revision. *Arch. Virol.,* **151**: 1257-1266. https://doi.org/10.1007/s00705-006-0763-6

Khattab, M., 2013. Isolation of nucleopolyhedrovirus (NPV) from the beet armyworm *Spodoptera exigua* (Hübner) (SpexNPV). *Int. J. Engin. Sci*., **4**: 75-83.

Kouassi, L.N.G., Tsuda, K., Goto, C., Mukawa, S., Sakamaki, Y. and Nakamura, M., 2009. Further studies on biological activity and identification of nucelopolyhedroviruses isolated from *S. litura* in Japan. *Biol. Contr.,* **54**: 537-548.

Kumar, C.S., Ranga, R.G.V., Sireesha, K. and Lava, K.P., 2011. Isolation and characterization of baculoviruses from three major lepidopteran pests in the semi-arid tropics of India. *Indian J. Virol.,* **22**: 29-36. https://doi.org/10.1007/s13337-011-0029-0

Kumar, M., Pradip, K.S. and Anirudh, K.S., 2012. Studies on pheromone catches of *Helicoverpa armigera* hubner and relation of moth activity with larval infestation on tomato in Baghpat Uttar Pradesh. *Int. J. Microb. Res. Technol.,* **2**: 1-3.

Lange, M., Wang, H. and Zhihong, H., 2004. Towards a molecular identification and classification system of lepidopteran-specific baculoviruses. *Virology*, **325**: 36-47. https://doi.org/10.1016/j.virol.2004.04.023

Laarif, A., Salhi, E., Fattouch, S. and Hammouda, M.H.B., 2011. Molecular detection and biological characterization of a nucleopolyhedrovirus isolate (Tun-SlNPV) from *Spodoptera littoralis* in Tunisian tomato greenhouses. *Annls. Biol. Res.,* **2**: 180-191.

Lavina, B.A., Padua, L.E., Wu, F.O., Shirata, N., Ikeda, M. and Kobayashi, M., 2001. Biological characterization of a nucleopolyhedrovirus of *Spodoptera litura* (Lepidoptera: Noctuidae) isolated from the Philippines. *Biol. Contr*., **20**: 39-47. https://doi.org/10.1006/bcon.2000.0877

Lucein, K.N.G., Katuso, T., Yositiskaand, S. and Masayuki, N., 2009. Further studies on biological activity and identification of nucleopolyhedroviruses isolated from *Spodoptera Litura* in Japan. *S. Pacific Stud.,* **30**: 1.

Martins, T., Montiel, R., Medeiros, J., Oliveira, L. and Simoes, N., 2005. Occurrence and characterization of a nucleopolyhedrovirus from *Spodoptera littoralis* (Lepidoptera: Noctuidae) isolated in the azores. *J. Inverteb. Pathol*., **89**: 185-192. https://doi.org/10.1016/j.jip.2005.06.012

Mehrvar, A., Rabindra, R.J., Veenakumari, K. and Narabenchi, G.B., 2007. Standardization of mass production in three isolates of nucleopolyhedrovirus of *Helicoverpa armigera* (Hübner). *Pak. J. biol. Sci.,* **10**: 3992-2999. https://doi.org/10.3923/pjbs.2007.3992.3999

Mehrvar, A., Rabindra, R.J., Veenakumari, K. and Narabenchi, G.B., 2008. Evaluation of adjuvants for increased efficacy of HearNPV against *Helicoverpa armigera* (Hubner) using suntest machine. *J. biol. Sci*., **8**: 534-541. https://doi.org/10.3923/jbs.2008.534.541

Moser, B.A., Becnel, J.J., White, S.E., Alfonso, C., Kutish, G., Shanker, S. and Almira, E., 2001. Morphological and molecular evidence that *Culex nigripalpus* baculovirus is an unusual member of the family Baculoviridae. *J. Gen. Virol*., **82**: 283-297. https://doi.org/10.1099/0022-1317-82-2-283

Murillo, R., Muńoz, D., Lipa, J.J. and Caballero, P., 2001. Biochemical characterization of three nucleopolyhedrovirus isolates of *Spodoptera exigua* and *Mamestra brassicae*. *J. appl. Ent.,* **125**: 267-270.

Mustafa, Y., Remziye, N. and Zihni, D., 2001. Viral control of the European Pine Sawfly, Neodiprionsertifer (Geoffroy) in Turkey. *Turk. J. Biol*., **25**: 419-425.

Nathan, S.S., Chung, P.G. and Murugan, K., 2005. Effect of biopesticides applied separately or together on nutritional indices of the rice leaf folder, *Cnaphalocrocis medinalis*. *Phytoparasitica,* **33**: 187-195. https://doi.org/10.1007/BF03029978

Noune, C. and Hauxwell, C., 2015. Complete genome sequences of seven *Helicoverpa armigera* SNPV-AC53-derived strains. *Genome Announc*., **4**: e00260-16.

Ogembo, J.G., Chaeychomsri, S., Kamiya, K., Ishikawa, H., Katou, Y., Ikeda, M. and Kobayashi, M., 2007. Cloning and comparative characterization of nucleopolyhedroviruses isolated from African bollworm, *Helicoverpa armigera*, (Lepidoptera: Noctuidae) in different geographic regions. *J. Insect Biotechnol. Sericol*., **76**: 39-49.

Pachiappan, P., Narayanaswamy, K.C. and Aruchamy, M.C., 2012. Molecular characterization of

polyhedrin gene of nuclear polyhedrosis virus of mulberry leaf-roller, *Diaphania pulverulentalis* (Hampson). *Euro. J. biol. Sci*. **5**: 11-14.

Rao, G.V.R., Kumar, C.S., Sireesha, K. and Kumar, P.L., 2015. Role of nucleopolyhedroviruses (NPVs) in the management of lepidopteran pests in Asia. In: *Biocontrol of lepidopteran pests* (eds. K.S. Sree and A. Varma). Springers, pp. 11-52.

Safdar, H., Javed, N., Aleem, S.K. and Arshad. M., 2018. Reproduction potential of entomopathogenic nematodes on armyworm (*Spodoptera litura*). *Pakistan J. Zool.,* **50**: 771-774. http://dx.doi.org/10.17582/journal.pjz/2018.50.2.sc3

Saleem, M., Husain, D., Ghouse, G. and Susan, W.F., 2016. Monitoring of insecticide resistance in *Spodoptera litura* (Lepidoptera: Noctuidae) from four districts of Punjab, Pakistan to conventional and new chemistry insecticides. *Crop Prot.,* **79**: 177-184. https://doi.org/10.1016/j.cropro.2015.08.024

Shapiro, A.M., Becnel, J.J. and White, S.E., 2004. A nucleopolyhedrovirus from *Uranotaenia sapphirina* (Diptera: Culicidae). *J. Inverteb. Pathol*., **86**: 96-103. https://doi.org/10.1016/j.jip.2004.04.005

Subramanian, S., Rabindra, R.J., Palaniswami, S., Sathiah, N. and Rajasekaran, B., 2005. Impact of granulovirus infection on susceptibility of *Spodoptera litura* to insecticides. *Biol. Contr.,* **33**: 165-172. https://doi.org/10.1016/j.biocontrol.2005.01.007

Takahashi, M., Madoka, N., Yasumasa, S., Yasushi. S., Chikara, I. and Yasuhisa, K., 2015. Field efficacy and transmission of fast- and slow-killing nucleopolyhedroviruses that are infectious to *Adoxophyes honmai* (Lepidoptera: Tortricidae). *Viruses,* **7**: 1271-1283. https://doi.org/10.3390/v7031271

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S., 2013. MEGA6: Molecular evolutionary genetics analysis, version 6.0. *Mol. Biol. Evol*., **30**: 2725-2729. https://doi.org/10.1093/molbev/mst197

Tamura, K. and Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol*., **10**: 512-526.

Toprak, U., Bayram, Ş. and Gürkan, M.O., 2005. Gross pathology of SpliNPVs and alterations in *Spodoptera littoralis* Boisd. (Lepidoptera:Noctuidae) morphology due to baculoviral infection. *Tarim Bilimleri Derg*., **11**: 65-71.

Trang, T.T.K. and Chaudhari, S., 2002. Bioassay of nuclear polyhedrosis virus (NPV) and in ombination with insecticide on *Spodoptera litura* (Fab). *Omonrica,* **10**: 45-53.

Wigley, P.J., 1976. *The epizootiology of a nuclear polyhedrosis virus disease of the winter moth,* Operophtera burmata *L. at Wistman's Wood, Dartmoor*. D. Phil thesis, University of Oxford.

Wu, C.Y., Chen, Y.W., Lin, C.C., Hsu, C.L., Wang, C.H. and Lo, C.F., 2012. A new cell line (NTU-SE) from pupal tissues of the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae), is highly susceptible to *S. exigua* multiple nucleopolyhedrovirus (SeMNPV) and *Autographa californica* MNPV (AcMNPV). *J. Inverteb. Pathol.,* **111**: 143-151. https://doi.org/10.1016/j.jip.2012.07.022