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Isolation, characterisation and bioassay study of *Spli*NPV associated with *Spodoptera litura* Fab

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Abstract
Dead larvae of *Spodoptera litura* were collected from different parts of Bengaluru. Some larvae were diseased and the cuticles were ruptured, discharging of white body fluid on plant parts. *Spodoptera litura* Nucleo polyhedro viruses were isolated from the diseased larvae of various populations of *Spodoptera litura*. Observation of discharged body fluid under light microscope revealed that the large number of spherical particles resembling as viral occlusion bodies (OBs). The virus was characterized as *Spodoptera litura* nucleo polyhedro virus (*Spli*NPV) based on morphological and biological characterisation. Scanning electron microscopy of POBs purified from diseased larvae revealed polyhedral particles of size approximately 0.860-2.171. µm. The transmission electron microscope (TEM) images of the POBs revealed the irregular and tetrahedral shape. The bioassay study showed highest LC_{50} values of NPV against 3rd instar larvae of *Spodoptera litura* indicated 15679.1 POBs/ml, against second instar larvae indicated 11231.5 POBs/ml and against first instar larvae LC_{50} was 8430.4 POBs/ml.

Keywords: Nucleo polyhedro viruses, *Spodoptera litura*, bioassay

Introduction
The growing awareness about the consequences of indiscriminate use of chemical pesticides on one hand and the environmental and health hazards on the other have compelled the scientists to device newer approaches to minimize pest populations on the crop plants. Disadvantages of unilateral approach of controlling crop pests with synthetic insecticides have dictated the need for developing cost effective, eco-friendly and safe pest management strategies.

Fortunately, the lepidopteran pests are susceptible to several entomopathogens, which play a major role in the natural regulation of their populations, and if properly utilized, can be useful augmentative biocontrol agents (Rabindra *et al.*, 1994) [4]. In this direction, biopesticides like viruses are increasingly being used as alternatives to chemicals in the management of noxious insect pests.

Insect viruses are potential biological control agents of agricultural pests. Most viral agents used to manage insect pests are either nuclear polyhedrosis viruses (NPV) or granulosis viruses (GV) both of which belong to the family Baculoviridae (Erayya *et al.*, 2013) [2]. The nucleo polyhedro virus (NPV) produces large occlusion bodies containing numerous rod-shaped enveloped virions in the nuclei of infected cells. NPV is mono specific and is extremely unlikely to present a risk to any non-target species, making it highly suitable for use as insect pest control agent, particularly in urban areas and nature reserves. The genus NPV is characterized by the presence of polyhedral-shaped viral occlusions containing randomly occluded viral particles (Martins *et al.*, 2005) [6].

Spodoptera litura Fabricius (Lepidoptera: Noctuidae) (tobacco cutworm) is polyphagous and has about 150 host species (Trang and Chaudhari, 2002) [10]. The tobacco cutworm is a strong flier, and disperses long distances annually during the summer months. It is one of the most economically important insect pests in many countries including India, Japan, China, and other countries of Southeast Asia. This pest may become serious during the seedling stage. Considering the reliability and suitability of *Spli*NPV in terms of economic and ecological reasons, its utilization in pest management has received a great deal of significance.

The main objective of this study is isolation and characterisation of *Spodoptera litura* NPV, Survey was conducted under natural epizootic conditions in and around Bengaluru rural for collection and isolation of NPV from major *Spodoptera litura* pests. Typical symptoms of NPV “tree top” were observed, infected and dead larvae were collected and observation was done under light microscope in laboratory.

Material and Methods

Collection and extraction of baculovirus

The NPV was isolated from the dead larvae of *Spodoptera litura* which was collected from cabbage farm, brought to NBAIR, Hebbal, Bengaluru. To release occlusion bodies (OBs), the diseased larvae were homogenized, using a sterile pestle and mortar for 4 min with 5 ml of distilled water. The suspension was filtered twice through a double-layered muslin cloth and then the filtrate was centrifuged (Remi, cC24 BL, India) at 500 rpm for 1min to remove the larger particles. The supernatant was suspended in (5ml) distilled water and centrifuged at 5000 rpm for 20 min to collect the pellet containing polyhedra. The pellet containing OBs was resuspended in (5 ml) distilled water and stored at 5°C. The polyhedral occlusion bodies were counted using a haemocytometer.

The enumeration of the POB was done using a standard haemocytometer of depth factor 0.1 mm (Weber, England). The samples were diluted 100-1000 folds and counted (Evans and Shapiro, 1997) [3] by using a phase contrast microscope. Ten replicates of counts were made to arrive at the concentration of the virus. After assessment of the strength, the suspensions were stored in refrigerator at 4°C till use.

Counting, standardization and determination of NPV dosage

The NPV could be seen as bright refractive irregular shaped crystals, called as occlusion bodies (OBs) or polyhedral inclusion bodies (PIBs) under phase contrast microscope. The concentration of any sample of NPV could be explained in terms of number of occlusion bodies per ml of solution (OBs/ml).

The polyhedra could be easily counted by using an improved Neubauer haemocytometer. It is a thick glass slide having a central depression. The depression was divided into two halves. Each half has a fine ruled grid of squares. The dimensions of these grids are defined. A glass cover slip was placed on the depression. This makes a chamber above the depression. A sample of 8µl was poured into the chamber by using a micropipette and kept for 10 minutes to facilitate the settling of the polyhedral bodies at the bottom of the slide. The polyhedra were counted under phase contrast microscope at X 400. The polyhedral bodies which were present completely in the centre of the square counted. The polyhedral touching the top and left side of the square were counted, while the polyhedra touching the bottom and right side were excluded. The number of PIBs per ml of the sample was determined by using the following formula,

$$\text{Number of inclusion bodies (PIBs/ml)} = \frac{D \times X}{N \times K}$$

Where,
D = Dilution factor
X = Total number of polyhedra counted
N = Number of squares counted
K = Volume above one small square in cm^3 ($2.5 \times 10^{-7} cm^3$)

Area of each small square was 1/400 mm^2 = 0.0025 mm^2. Depth of the chamber was 0.1mm. Volume of liquid above a single small square was 0.0025 mm^2 x 0.1mm= 0.00025 mm^3. To convert it into cm^3 it was multiplied by 1/1000, to get a volume of 2.5×10^{-7} cm^3, above 1 small square. Hence, $K=2.5x10^{-7}cm^3$.

The dosage of each geographical isolate of NPV was indicated as LE (Larval Equivalents) and one LE was equal to the quantity of NPV obtained from one full grown, infected larva. In general, one LE was equal to 6×10^9 PIBs/ml. The recommended dose of NPV for field application is 250-500 LE/ha ($1.5\text{-}3.0 \times 10^{12}$ PIBs/ml) (Grzywacz *et al.*, 2004) [4].

Electron microscopy morphology

The morphological studies of the extracted OBs were carried out under a scanning electron microscope (SEM) and a transmission electron microscope (TEM). The purified OB suspensions were taken in vials, fixed in 2.5% (v/v) glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 2 h at 4 °C. The samples were then post-fixed in 2% (v/v) aqueous osmium tetroxide prepared in 0.1M phosphate buffer (pH 7.4) for 1 hour and dehydrated in graded ethanol series (Martins *et al*., 2005) [6]. The samples were finally dried to a critical drying point. Stubs with double-sided conductivity carbon adhesive tape and sputter coated with gold for 20 s, using an automated sputter coater (Model: EMITEC - SC 7620), were used for sample mounting. The coated samples were examined directly under a SEM (Quanta 250, FEI, Netherlands) at 10 kV with a spot size of 3.5 and a pressure of 60 Pa. The sample images were visualized and photographed at × 40,000 magnification. The amplified photographs developed by a scale were used to measure the sizes of the OBs.

For TEM studies, the pellets of OBs were initially fixed in 2.5% (v/v) glutaraldehyde in 0.05M phosphate buffer (pH 7.2) for 24h at 4°C and again fixed in 0.5% (v/v) aqueous osmium tetroxide in the same buffer for 2h. After the post-fixation, samples were dehydrated in a series of graded alcohol; the dehydrated sample was mounted on 300 mesh carbon-coated copper grid. The sample was stained by saturated aqueous uranyl acetate and counterstained with lead citrate and viewed under TECNAI 120 Kv TEM (FEI, Netherlands). The sizes of the OBs and nucleocapsids were measured directly from the amplified photographs using a precision ruler and compared to the magnification of the photograph.

Mass rearing of *Spodoptera litura*

The initial culture of *S. litura* was obtained by collecting the larval stages from the infested crop like castor and cabbage grown in the university farm. The larvae where then reared in cylindrical transparent glass jars (42 cm height; 30 cm diameter) having a piece of blotting paper at their bottom. Fresh tender leaves of castor were provided as food for larvae. This food was changed twice a day. The top of the glass jar was covered with muslin cloth secured firmly with rubber band. Fully developed last instar larvae were handpicked and transferred to glass jars having 7.5 cm thick layer of fine soil at the bottom. Moths which emerged from the pupae were used for building up of subsequent culture. Newly emerged male and female were confined into large glass jars along with folded pieces of black blotting paper for mating and egg laying.

The freshly emerged male and female moths were confined to single glass jar in the ratio of 2:3, respectively. Five such sets were maintained for egg laying. The inner side of each glass jar was lined with blotting paper to create favourable conditions for egg laying. A piece of circular blotting paper

was placed at the bottom of each glass jar. Cotton swab soaked in 10 per cent sugar solution was kept suspended in each glass jar as adult food. The cotton swab was changed periodically. The eggs laid on blotting paper were collected by cutting away the piece of paper along with the egg mass. The egg masses were then transferred to glass jars containing fresh castor leaves and reared further as described earlier. The mass culture of the host was thus prepared and maintained in the laboratory during the entire period of investigation. Second instar larvae of *S. litura* were randomly selected from this culture and used further for evaluation of effectiveness and shelf-life of *Spli*NPV.

Bioassay study of *Spli*NPV on different instars of *Spodoptera litura*

Median lethal concentration (LC_{50}) of *Spli*NPV to first, second and third instar larvae was estimated by using leaf disc bioassay method. Viral suspensions of 1x 10^{11}, 1x 10^{10}, 1x 10^{9}, 1x 10^{8}, 1x 10^{7}, 1x 10^{6} and 1x 10^{5} PIBs/ml, were prepared in aqueous (0.05%) Tween 20 (v/v) along with untreated control was tested in the study. Ten microliters of viral suspensions was spread on castor leaf, air dried and individually placed inside the multicavity trays. A blunt end polished glass rod was used to distribute the suspension containing the virus uniformly over the diet surface. Each well constituted an independent replication and three wells were used per viral suspension, constituting 50 insects per treatment. Different instar *Spodoptera litura* larvae starved for about 6 h were released individually into the wells which were then covered with a lid. The larvae consumed the diet within 12 hrs. It is maintained at 26 ± 2 °C and 60–70% relative humidity. In control, larvae were allowed to feed on castor leaves treated with aqueous (0.05%) Tween20. Larval mortality rates were recorded at daily intervals. Mortality due to viral infection was recorded up to 9 days post inoculation.

The mortality data recorded was corrected depending upon the mortality in the control, by adopting Abbott's formula as follow

T: Percentage mortality in treatment

C: Percentage mortality in control

The probit analysis for determination of median lethal concentration by using the statistical package for social sciences (SPSS) software.

Result and Discussion

Collection of baculovirus

Severe infestation of pod borer, *Spodoptera litura* was observed during the regular survey in the Bengaluru rural region. Few diseased larvae of *Spodoptera litura* were found to harbour the virus. The body fluid of the dead diseased larvae was found on cabbage plant parts. Observation of discharged body fluid under a light microscope revealed numerous spherical particles resembling baculoviral OBs.

Plate 1: NPV infected *Spodoptera litura* larvae in cabbage crop

Table 1: Collection and isolation of Nucleo polyhedrosis viruses (NPVs) from major insect pests

Name of the virus	Host	Crop	Location	Isolate name
Spodoptera litura NPV	*Spodoptera litura*	Cabbage	Bengaluru Rural	*Spli*NPV NBAIR1

Table 2: Electron microscopic characterisation of *Spli*NPVs isolated from Spodoptera litura insects pests

NPV	Scanning electron microscopy		Transmission electron microscopy	
	Shape of OBs	Size of OBs (µm)	Shape of OBs	Size of Obs (µm)
*Spli*NPV	Irregular and tetrahedral	0.860 - 2.171	Irregular and tetrahedral	0.500-0.660

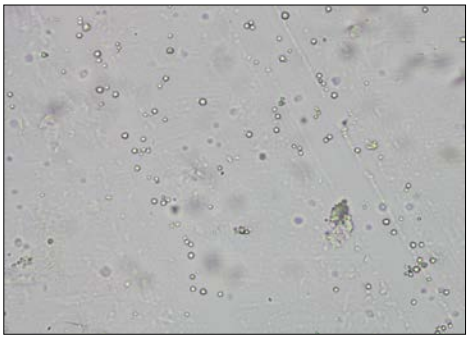

Plate 2: POBs View under light microscope (40X)

Electron microscopic studies revealed the typical baculovirus OBs of type nucleopolyhedrovirus (NPV) with polyhedral structures. Under SEM, the OBs of *Spli*NPV appeared as irregular tetrahedral shapes and sizes ranging from 0.860-2.171 µm (Plate 3). TEM studies of the OBs also revealed the tetrahedral shape (Plate. 4). Sridhar Kumar *et al.* (2011) [9] reported OBs of three major Lepidopteran pests revealed multiple nucleocapsids in each envelop, which were bacilliform shape. Senthil Kumar *et al.* (2015) [8] also recorded that the OBs are tetrahedral in shape in *Spilarctia oblique*.

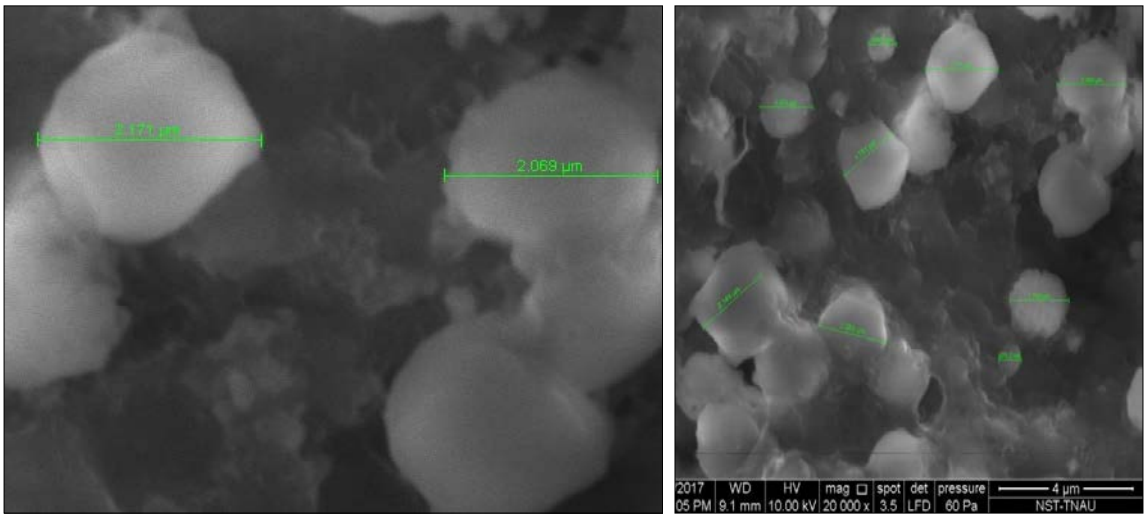

Plate 3: SEM micrograph of *Spli*NPV

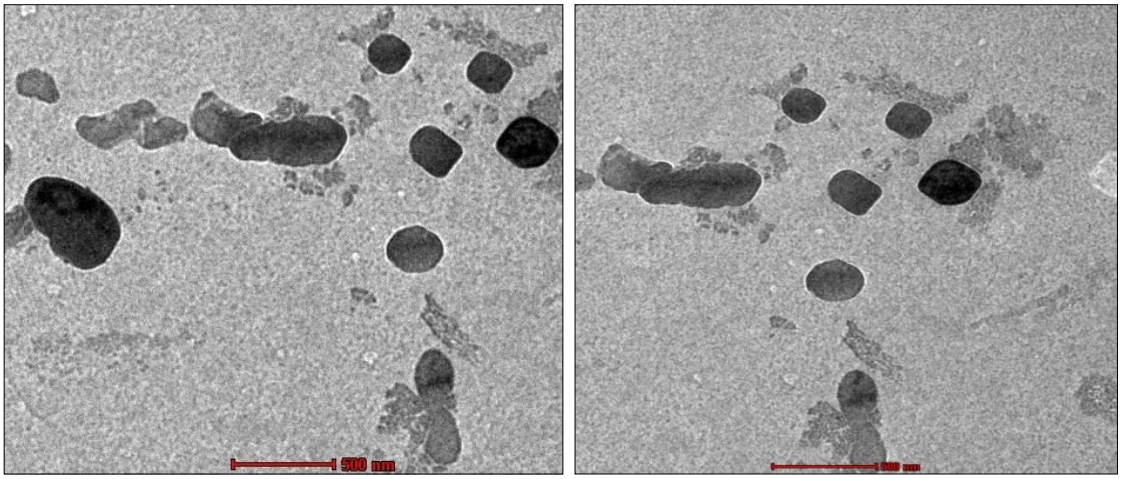

Plate 4: TEM micrograph of *Spli*NPV

Bioassay study with different larval instar of *Spodoptera litura* at different concentrations of *Spli*NPV

The *Spli*NPV solution was prepared and LC_{50} values for first, second and third instar larvae were assessed using independent bioassay method. The mortality of larvae was recorded on alternate days for six days post-treatment. The assays were carried out thrice and the pooled larval mortality data were subjected to probit analysis using the software POLO[22] to calculate the LC_{50} values for larval instars. The Chi-square (χ^2) values were statistically significant for the concentration, which implied heterogeneous response of the test population of *Spodoptera litura* to all the dosage is detailed in Table 3 and Fig 1.

In first instar larvae, highest mortality percentage at concentration of $1x10^6$ POBs/ml was 84 per cent (number of larvae dead were 42) followed by concentration of $2x10^5$ POBs/ml in first instar larvae 84 per cent (42 number of larvae dead), at concentration of $4x10^4$ POBs/ml in first instar larvae 74 per cent mortality (37 number of larvae dead) followed by $8x10^3$ POBs/ml showing 56 per cent (28 number of larvae dead) followed by $1.6x10^3$ POBs/ml showing 38 per cent mortality (larvae dead were 19) the lowest per cent was obtained in $3.2x10^2$ POBs/ml concentration with 6 per cent mortality (number of larvae dead was 3), whereas control showed zero.

In second instar larvae, highest mortality percentage at concentration of $1x10^6$ POBs/ml was 80 per cent (number of larvae dead were 40) followed by concentration of $2x10^5$ POBs/ml in second instar larvae 76 per cent (38 number of larvae dead) at concentration of $4x10^4$ POBs/ml in second instar larvae 72 per cent mortality (37 of larvae dead) followed by $8x10^3$ POBs/ml showing 52 per cent mortality (26 number of larvae dead) followed by $1.6x10^3$ POBs/ml showing 32 per cent mortality (larvae dead were 16), the lowest per cent was obtained in $3.2x10^2$ POBs/ml concentration with 10 per cent mortality (number of larvae dead were 5), whereas control showed zero number of larvae dead and mortality.

In third instar larvae, highest mortality percentage at concentration of $1x10^6$ POBs/ml was 76 per cent (number of larvae dead were 38) followed by concentration of $2x10^5$ POBs/ml in third instar larvae 74 per cent (37 number of larvae dead) at concentration of $4x10^4$ POBs/ml in third instar larvae 68 per cent mortality (34 of larvae dead) followed by $8x10^3$ POBs/ml showing 50 per cent (25 number of larvae dead) followed by $1.6x10^3$ POBs/ml showing 30 per cent mortality (larvae dead were 15), the lowest per cent was obtained in $3.2x10^2$ POBs/ml concentration *i.e.*, 14 per cent mortality (number of larvae dead was 7), whereas control showed zero mortality.

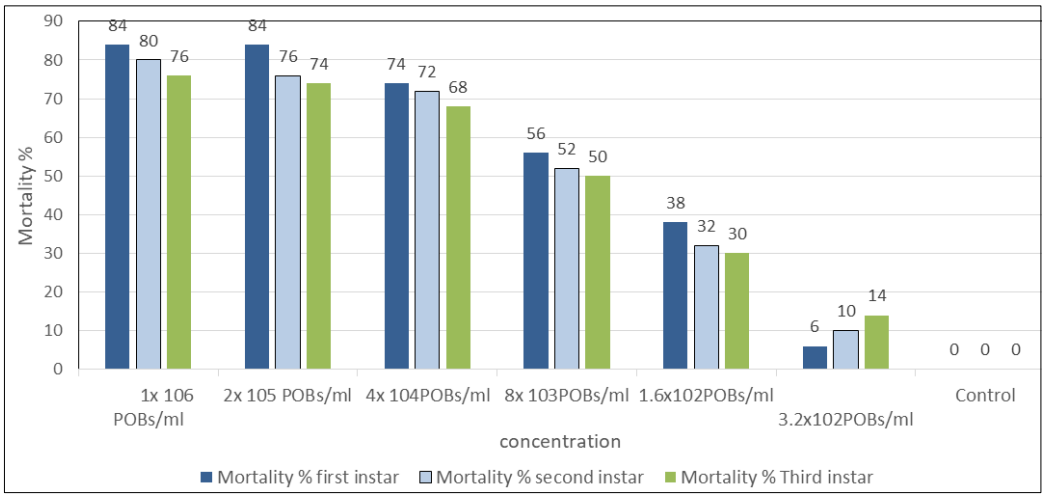

Fig 1: Larval mortality in different instars of *Spodoptera litura* by *Spli*NPV isolate to different concentration

Table 3: Bioassay study with different larval instars of *Spodoptera litura* by *Spli*NPV isolate at different concentrations

*Spli*NPV Concentration POBs/ml	Number of larvae used	No. of larvae dead		
		Instars		
		first	second	third
1x 10^6 POBs/ml	50	42^a	40^a	38^a
2x 10^5 POBs/ml	50	42^a	38^b	37^b
4x 10^4 POBs/ml	50	37^b	36^c	34^c
8x 10^3 POBs/ml	50	28^c	26^d	25^d
1.6x10^3 POBs/ml	50	19^d	16^e	15^e
3.2x10^2 POBs/ml	50	3^e	5^f	7^f
Control	50	0^f	0^g	0^g

Note: Figures in parenthesis are the square root transformed values for number of larvae per plant
**Pooled mean of three observations
In each column, means followed by same alphabets are statistically on par by DMRT (p=0.05)

In probit regression analysis of mortality, number of larvae used were 50 in number, Slope value obtained in case of first instar larvae was 0.66±0.07, 0.58±0.07 at second instar and 0.052± 0.06 slope value at third instar larvae (Table 4). Fiducial limits (95%) of first instar larval range was 4613.1 to 14586.0, second instar 6181.15 to 21822 and third instar larvae was 6947.69 to 27898.8, the LC_{50} value of first instar larvae was 11.67 POBs/ml, second instar larvae was 7.30 POBs/ml and third instar larvae was 4.99 POBs/ml. This results revealed that the LC50 values higher in third instar larvae as compared to first instar larvae.

Table 4: Probit regression analysis of mortality data of *Spli*NPV against *Spodoptera litura*

Instars	No. of larvae used	LC50 (POBs/ml)	Slope+SE	95% Fiducial limits			
				Lower	Upper	$\chi 2$	DF
First	50	8430.4	0.66±0.07	4613.1	14586.0	11.67	4
Second	50	11231.5	0.58±0.07	6181.15	21822	7.30	4
Third	50	15679.1	0.52±0.06	6947.69	27898.8	4.99	4

The *Spodoptera litura* larvae infected with *spli*NPV showed typical baculovirus OBs that have crystalline structures of variable shapes and sizes ranging from 0.860 - 2.171 µm. Most of the POBs were tetrahedral in shape. The bioassay study showed the highest LC_{50} values in 1x10^6 OBs/ml against the different instars of *Spodoptera litura* larvae. NPV is safe and friendly to the environment, and it could be an ideal component for the biological pest management approach to control the pod borer, which is a major insect pest of pulses and other vegetable crops.

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