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Isolation and characterization of baculoviruses from major lepidopteran insect pests infesting jute, *Corchorus olitorius* Linn

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Abstract
Jute hairy caterpillar, *Spilosoma obliqua* Walker (Arctiidae: Lepidoptera) and jute semilooper, *Anomis sabulifera* Guenee (Noctuidae: Lepidoptera) duo are the important lepidopteran insect pests infesting jute and causes damage by defoliation. Epizootic caused by a nucleopolyhedrovirus (NPV) was observed in the jute field infested with *S. obliqua* and *A. sabulifera* during a routine survey in farmer's fields. The NPV was isolated and characterized based on *polyhedrin* gene of NPV which resulted in an amplicon size of 700bp each for the both *Spob*NPV and *As*NPV. Bioassay studies with second instar larvae of *A. sabulifera* and *S. obliqua* revealed the median lethal concentration (LC_{50}) of *As*NPV as 5.37 × 10^4 OBs/ml and 2.44 x 10^4 OBs/ml at 72 HAT (Hours After Treatment). The *Spob*NPV at the highest POB count @3.2 x10^6 OBs/ml caused cent per cent larval mortality, similarly *As*NPV with POB (Poly Occlusion Bodies) concentration @2.8 x10^6 OBs/ml resulted in 83 per cent larval mortality. Thus spray application of both *Spob*NPV and *As*NPV can be used as biocontrol agents in Integrated Pest Mangement of lepidopteran insect pests infesting jute.

Keywords: Jute, Bihar hairy caterpillar, *Spilosoma obliqua*, Jute semilooper, *Anomis sabulifera*, nucleopolyhedrovirus, polyhedrin gene

Introduction
Jute (*Corchorus olitorius*; Family: Malvaceae) is an important commercial bast fibre crop. The cultivation of this crop is mostly confined to Indo-Gangetic plain [1] constituting the states of West Bengal, Bihar, Odisha and Assam, all of which contribute to more than 80% of total jute production in India [2]. Jute cultivation owes significance in providing livelihood and support to small and marginal farmers and industrial workers of Eastern India. Currently, the average productivity of jute in our country is 2.5 t/ha [3]. Biotic and abiotic factors in duo are responsible for paving the way for declining productivity *vis-a-vis* quality of the fibre. The pest infestation results in approximately 20-25% yield loss and also deteriorating the quality of fiber across the different jute growing regions of India. In worst case scenario these pests occur in gigantic numbers manifesting the complete failure of the crop causing huge loss to jute cultivators. Of the many insect pests encompassing different insect orders, lepidopteran pests are of utmost significance.

Spilosoma obliqua Walker (Arctiidae: Lepidoptera), once considered as a sporadic pest has now attained a regular and polyphagous pest status [4]. More than 126 plant species belonging to 25 families, including pulses, oilseeds, cereals, vegetables, mulberry, turmeric, bast fibre crops viz., jute, mesta and ramie, medicinal and aromatic plants, are been reported to be infested by this pest and causing tremendous economic loss [5, 6]. The pest is accredited as a major pest of natural bast fibre crop jute (*C. olitorius*, *C. capsularis*). Recently the outbreak of this pest has been documented both in jute and sunnhemp during 2011 and 2012 [7]. Female moth lays about 1000 eggs in a cluster which upon hatching the caterpillars feed gregariously and scrapes chlorophyll content of the leaves during the early larval stages while the late larval stages feed solitarily resulting in severe defoliation.

Jute semilooper, *Anomis sabulifera* Guenee (Noctuidae: Lepidoptera) is another potential and major pest of jute, with larvae being the most destructive stage of the pest that causes heavy damage to the crop by defoliation [8]. The repeated infestation by this pest compromises crop growth and induces profuse branching [9] there by resulting in ultimate reduction of fiber yield upto the tune of 30.50 - 37.50% on important ruling varieties of *C. olitorius* [10, 11]. The pest as a pod borer is known to feed on both pods and unripe seeds in jute crop cultivated for seed production [12, 13].

Chemical control of this pest is difficult and uneconomical because the pest infests on several weed plants [6]. Spray control is no longer considered a sustainable strategy ever since indiscriminate usage of pesticides has led to resistance development, resurgence and residue issues *vis-a-vis* causing serious environmental hazards. New research strategies are rifting suggesting that biocontrol technology especially entomopathogenic insect viruses *viz.*, baculoviruses are opined to be an effective, alternative, environment friendly, excellent and low cost management options in jute ecosystem combating the pest menace caused by both jute hairy caterpillar, *S. obliqua* and jute semilooper, *A. sabulifera*. Hence, the present study was undertaken to isolate, characterize and evaluate the efficacy of nucleopolyhedro viruses infecting lepidopteran pests of jute.

Materials and Methods

Isolation and extraction of nucleopolyhedrosis virus

The nucleopolyhedrosis virus was isolated from dead *S. obliqua* and *A. sabulifera* larvae showing typical "*Wipel Krankheit* or *Tree Top Disease*" with characteristic viral infection symptoms (Figure 1; Figure 2) and found hanging from jute plants at farmer's field located in Haringhatta, North 24 Paragnas while surveying during an epizootic in October 2019. The dead larvae of both *S. obliqua* and *A. sabulifera* were individually transferred to small sterile eppendorf tubes (3 ml) and crude homogenate of the virus was extracted by grinding a single larval cadaver in sterile distilled water. To remove the larval debris, the extract was filtered through four layers of cheese cloth the resulted filtrate was centrifuged for 1 min at 500 rpm to remove large particles. The supernatant was again resuspended for 20 minutes and centrifuged at 5000 rpm and pellet was collected. The pellet was resuspended in 100 ml of sterile distilled water. The number of occlusion bodies (OBs) in the filtrate were adjusted to 3.2×10^6 OBs/ml and 2.8 x 10^6 OBs/ml for *Spob*NPV and *As*NPV respectively using a Neubauer haemocytometer in aqueous solution of 0.05% Tween 20 (v/v).

Multilication of nucleopolyhedrosis virus

First instar larvae of *S. obliqua* and late instar larvae of *A. sabulifera* were collected from the jute fields of Beraberia and Ratanpur of North 24 Paragnas and were reared separately in Biocontrol Laboratory, Crop Protection Division, ICAR-CRIJAF, Barrackpore till pupation at 25 °C and 80-85% relative humidity (RH) on natural diet *viz.*, jute leaves, *C. olitorius* (JRO-204) in plastic containers. After adult emergence from pupae of both the insect pests, male and female moths were fed with 10% honey solution and allowed to mate. The eggs laid by female moths were used to maintain the healthy populations of the insect in the laboratory for further experimental studies.

Third instar larvae of both *S. obliqua* and *A. sabulifera* were used for multiplication of the respective nucleopolyhedrosis virus. Multiplication of the nucleopolyhedrosis virus was done by smearing 1 ml of the corresponding viral suspension uniformly on a fresh disinfected jute leaf on both sides and air dried for 1 min. A total of thirty 4th instar larvae of both *S. obliqua* and *A. sabulifera* in three replicate (10 larvae/replication), pre-starved for 2 h were allowed to feed on the virus inoculated leaf for 24 h kept in pertiplates (15 cm diameter). The larvae were later on transferred on to fresh natural diet kept in petriplates (15 cm diameter) and were maintained at 25 °C till death.

Fig 1: *S. obliqua* larvae infected with NPV

Fig 2: *A. sabulifera* larvae infected with NPV

Molecular characterization of *Spob*NPV and *As*NPV

The two nucleopolyhedrosis viruses initially isolated from diseased larvae collected from the fields were used for characterization of the *polh* gene. The Occlusion Bodies (polyhedra) from the infected larvae of both the insect pests were purified by centrifugation at 2,500 rpm once for 2 min. The debris was pelleted while that of the OB remained intact within the supernatant. Nucloepolyhedrosis virus was isolated by suspension was again subjected to centrifugation by which OB's settled at the sides of the walls of the centrifuge vial, which were lateron suspended in sterile distilled water and stored at -20 °C for further use. DNA extraction was performed using the traditional 0.1M sodium carbonate solution [14]. The viral suspension was treated with 1% SDS and 20 mg/ml of proteinase K followed by phenol:chloroform:isoamylalcohol (1:1:1) extraction with the final ethanol precipitation. DNA was resuspended in TE buffer and extracted DNA was visualized in 0.8% agarose gel. For the amplification of *polh* gene, primer set was designed as species-specific primers according to the *Spob*NPV sequences in the Genbank database of National Centre for Biotechnology Information. The primer sequence consisted of forward primer 5'-ATGCCAGACTTCTCGTACCG-3' while the reverse primer constituted the sequence 5'-TAATACGCGGGACCGGTGAAT-3'. For polh gene amplification, each PCR reaction comprised of 50 ng template DNA, 0.25 U of Taq DNA polymerase, 10x Taq buffer, 2.5mM $MgCl_2$, 2.5mM of each of four dNTPs and 1µl each forward and reverse primer with final volume of PCR mixture made upto 50 µl by adding nucleus free water. The PCR consisted of an initial denaturation step of 95 °C for 3 min and 35 cycles of 95 °C for 30 sec, 70 °C for 1min, 55 °C for 1

min, and a final extension step 72 °C for 10 mins. The nucleotide sequence was submitted to GenBank and accession number were obtained thereof.

Bioassay Studies

Median lethal concentration response (LC_{50}) of *Spob*NPV and *As*NPV against second instar larvae of both *S. obliqua* and *A. sabulifera* were carried out by leaf disc bioassay method. For the bioassay studies, viral suspensions of 10^4, 10^5 and 10^6 OBs/ml were prepared in aqueous 0.05% Tween 20 (v/v). The 100µl of viral suspension was smeared on unsprayed fresh jute leaf, air dried and individually placed inside petri plate (19 cm diameter). Each plate was considered as individual replication and therefore three replicates were maintained per viral suspension. The bioassay study for both *Spob*NPV and *As*NPV, thus comprised of three replications per viral suspension treatment and thirty 2nd instar larvae per replication along with a control treatment. Second instar larvae of *S. obliqua* and *A. sabulifera* were pre-starved for about 2h and then were released individually into the petriplates. The control treatment comprised of leaves treated with aqueous 0.05% Tween 20. Larval mortality data was recorded at every 12 h interval up to sixdays post inoculation to deduce log- dose probit assay and median lethal concentration for both *Spob*NPV and *As*NPV against 2nd instar larvae of *S. obliqua* and *A. sabulifera*. Larval mortality data observed were corrected by using Abbott's formula [15] and further subjected to probit analysis [16] using SPSS software version 16 for calculating LC_{50}. Control treatments were in mortality exceeded 20 per cent were discarded and bioassay was repeated.

$$\text{Abbott's Corrected Mortality \%} = \frac{(\text{\% mortality in treatment} - \text{\% mortality in control})}{(100 - \text{\% mortality in control})} \times 100$$

Results and Discussion

During the natural baculovirus epizootics both the lepidopterous pest diseased larvae; *S. obliqua* and *A. sabulifera* appeared swollen, glossy, flaccid and moribund. The larvae crawled to the top of the twigs where fed and hanged downwards in inverted 'Λ' shape, a typical "*Wipfel krankheit*" or "Tree top disease" symptom. The diseased larvae were found dead, body tissues liquefied with cuticle ruptured and discharging of white body fluid on to leaves.

The nucleopolyhedrosis virus *Spob*NPV and *As*NPV isolated from the larvae of *S. obliqua* and *A. sabulifera* was characterized by the amplification of polyhedrin gene (Figure 3). The PCR product was of 700 bp (Fig 3; Fig 4). The sequence data generated for the *polyhedrin* gene were deposited in GenBank, National Centre for Biotechnology Information (NCBI) and accession number was obtained (MN648213). The deposition of the sequence data generated for the *polyhedrin* gene from *As*NPV is under process.

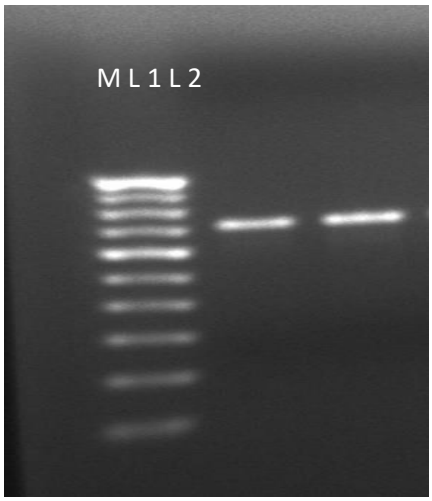

Fig 3: PCR detection based on polh gene at 700 bp (M = Marker (M):1000 bp ladder,
Lane 1 - 2 = *S. obliqua polh* NPV gene (Replication 1-2);

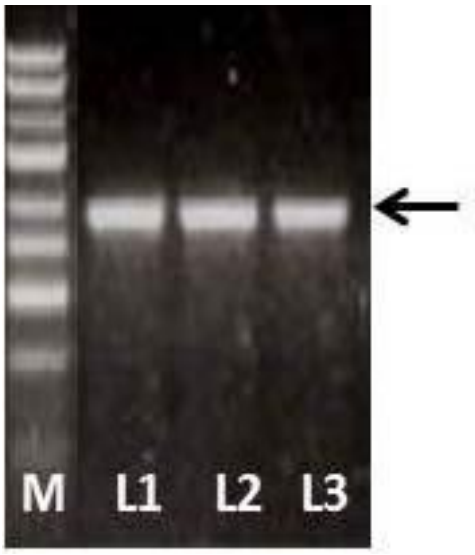

Fig 4: PCR detection based on polh gene at 700 bp (M = Marker (M):1000 bp ladder,
Lane 1-3 = *A. sabulifera polh* NPV gene (Replication 1-3)

Preliminary mortality studies using leaf dip bioassay with serial dilution of the OBs against second instar larvae of *A. sabulifera* and *S. obliqua* revealed the median lethal concentration (LC_{50}) of *As*NPV as 5.37 × 10^4 OBs/ml (F.L. 2.06 x 10^4 – 1.3 x 10^5) and 2.44 x 10^4 OBs/ml (F.L. 1.20 x 10^4 – 4.97 x 10^4) 72 HAT (Table 1). The *Spob*NPV at the POB counts (3.2 x10^6 OBs/ml and 6.8 x 10^5 OBs/ml) was successful enough to cause cent per cent larval mortality in the bioassay study with the 2nd instar larvae of *S. obliqua* (Fig 5), similarly *As*NPV with POB concentration (2.8 x10^6 OBs/ml) resulted in 83.33 per cent larval mortality in the bioassay studies with the 2nd instar larvae of *A. sabulifera* (Fig 6).

Table 1: Bioassay with NPV against 2nd instar larval stages

NPV	LC_{50} (POB's/ml)	95% Fudicial Limits		Heterogeneity	Slope	df
		LL	UL			
*Spob*NPV	2.44 x 10^4	1.20 x 10^4	4.97 x 10^4	13.23	0.89	6
*As*NPV	5.37 × 10^4	2.06 x 10^4	1.3 x 10^5	1.76	0.59	6

Owing to the innate ability of causing high epizootics, to occur naturally, ability to self-perpetuate, eco-friendly nature being host specific and non-hazard to beneficial arthropods, baculoviruses especially nucleopolyhedrovirus have enormous potential to be used as entomotoxic viral biopesticides. Though commendable research work pertaining to nucleopolyhedroviruses causing mortality to various lepidopteran insect pests has been studied *Spob*NPV and *As*NPV causing mortality to jute hairy caterpillar *S. obliqua* and jute semilooper, *A. sabulifera* are scanty. It's no astonishing fact to reveal that jute cultivation has hindered due to the menace created by both lepidopteran pests, *S. obliqua* and *A. sabulifera* which causes a total foliage loss up to 20–30% in jute [17] fields of West Bengal, India. Intermittent infection epizootics [18] in *S. obliqua* and *A. sabulifera* caused by *Spob*NPV and *As*NPV have be observed in the jute fields, isolation and characterisation of these both NPV's remained unleashed. The present study made an attempt for PCR amplification of the highly conserved nucelopolyhedrosis virus gene polh, which happens to be a powerful tool in identification of lepidopteran-specific baculoviruses [19, 18]. The LC_{50} value in the present study with *Spob*NPV against second instar *S. obliqua* was 2.44×10^4 POBs/ ml post 72 HAT. The median lethal concentration of *Spob*NPV against second and third instar larvae of *S. obliqua* studied elsewhere are 3.2×10^4 POBs/ ml; 4.46×10^5; 3.7×10^4 POBs/ ml; 4.7×10^4 POBs/ ml [20, 21, 22, 18]. In addition to field evaluation of *Spob*NPV in reducing the population of *S. obliqua* infesting several other crops have documented viz, 1.5×10^{12} POBs/ ml and 250LE [20, 23, 24, 25] studies revealed the possibility of *Spob*NPV in causing death to the 5th instar larvae of *S. obliqua* 6-7 DAT [18]. Based on median lethal concentration value obtained in the present study reveals the fact that this particular strain isolated from West Bengal happens to be the most virulent strain till date evaluated amongst the other strains assessed elsewhere in India. Geographical variation in virulence by various strains of *Spob*NPV causing mortality to larvae of *S. obliqua* is also been documented [26].

Being a monophagous pest and jute being the only host plant much appreciable research work regarding to *A. sabulifera* haven't been carried out except a single report [27] were the authors mentioned the POB's count between 0.775×10^9 to 3.729×10^9 with an average of 2.106×10^9 per larva and with cross-infectivity five other species of Lepidoptera. In the present study the LC_{50} determined against 2nd instar larvae of *A. sabulifera* by *As*NPV was 5.3×10^4 POB's / ml post 72HAT.

In the present day scenario control management strategies for curbing jute insect pest menace is relied mainly on conventional chemical control methods. Integrated Pest Management mediated through biological control agents like *Protapanteles obliquae*, *Meterous spilosomae*, *Blepherella lateralis*, entomopathogenic fungi like *Beauveria bassiana* to delimit hairy caterpillar menace and *Sisyropa formosa* to culminate semilooper are given importance for contemplating and foregoing the ill effects of insecticide usage, impetus on microbial control agents encompassing baculoviruses *viz.*, NPV's are to be given prime importance virtue of their numerous advantages by encouraging mass multiplication and proper formulation giving a sheer importance to quality control parameters..

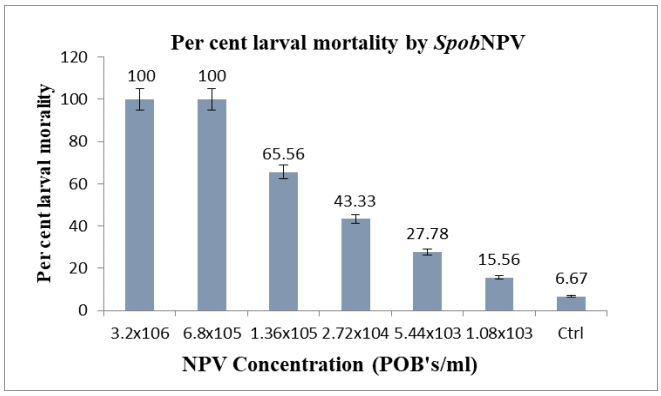

Fig 5: Larval mortality by *Spob*NPV against 2nd instar larvae of *S. obliqua*

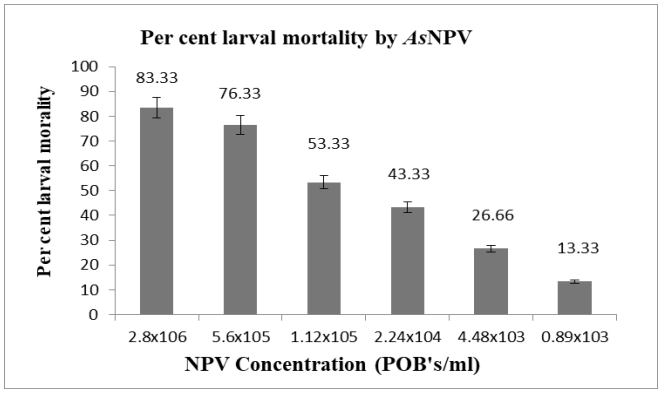

Fig 6: Larval mortality by *As*NPV against 2nd instar larvae of *A. sabulifera*

Conclusion

The present study discloses the entomopathogenic effects of *Spob*NPV and *As*NPV in controlling the two major lepidopteran defoliators' hairy caterpillar and semilooper infesting jute. The findings confirms the pathogenicity of the baculoviruses on these two important jute pests which can be incorporated as a microbial biocontrol component in Integrated Pest Management of jute insect pests.

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