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Sub-lethal Dose Reponses of Native Polyhydroviruses and Spinosad for Economical and Sustainable Management of *Spodoptera litura* in Pakistan

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JNA, RM and SJNA designed and conducted the experiment. RM, MAM, SJNA and JNA wrote the manuscript. JNA, MAM, IA and AMB analyzed the results. SJNA, AMB, MM, MT, ZA SM critically reviewed the manuscript.

Key words
***Spodoptera litura*, Nucleopolyhedrovirus, Spinosad, mortality, Synergism, Pest management**

ABSTRACT

In the present investigation, laboratory trials were conducted to investigate the synergistic, additive or antagonistic effect of three sub-lethal dose rates (2 x 10^3, 4.5 x 10^3, and 6 x 10^3 PIB/Larva) of native isolated Nucleopolyhydrovirus (NPV) from *Spodoptera litura* and Spinosad (0.01 ppm) against 3rd and 4th instar larvae collected from three different geographical areas of Punjab (Pakistan). A difference in larval mortality, pupation, adult emergence and egg ecluson was observed. The higher but sub-lethal dose rate of NPV with Spinosad exhibited synergistic interaction, while the rest of the combinations were found additive in all the tested populations. The results confirmed the population of *S. litura* from Rahim Yar Khan Region least susceptible, and that of Faisalabad highly susceptible. It may be inferred that the mixtures of the correct sub-lethal doses of Spinoad and NPV in combination may be used against destructive pests such as *S. litura*. This strategy also has a great potential in insecticide resistance management (IRPM) against such pests in vegetable and major crop growing areas of Pakistan.

INTRODUCTION

Spodoptera litura (Lepidoptera, Noctuidae) also known as a tobacco caterpillar is a serious polyphagous and cosmopolitan insect pest of vegetable and ornamental crops (Nathan and Kalaivani, 2005; Shaurub *et al*., 2014; Trang and Chaudhari, 2002). This insect pest attacks leaves, buds and flowers resulting in a serious decline in terms of quality and quantity of the produce. Its management on *Brassica* crops has become a challenge due to its high reproductive rate as well as damage potential. It has been estimated that *S. litura* can cause 25-100% economic losses (Dhir *et al*., 1992; Prayogo *et al*., 2005). Farmers mostly rely on use of synthetic insecticides to curb this insect pest, which causes serious hazardous threats to environment, human health, and development of resistance in insect pests along with harmful residual effects on wild life (Aydin and Gürkan, 2006). An indiscriminate use of chemical insecticides has resulted in insecticide resistance in *S. litura* population in Pakistan (Ahmad *et al*., 2007; Ahmad *et al*., 2008; Shad *et al*., 2012). It is important to explore different eco-friendly alternatives as use of nucleopolyhedrosis virus successfully reduced *S. litura* population (Ahmad *et al*., 2018). Recently, various pests and diseases of different crops have been identified from Pakistan for their proper control (Ahmad *et al*., 2019a, b; Shareef *et al*., 2019; Ahmad *et al*., 2019; Ahmad *et al*., 2017; Manzoor *et al*., 2018)

The use of biocontrol agents like baculoviruses especially against the important agricultural and forest pests is a pesticide alternative control method, which is completely eco-friendly and environmentally benign (Popham *et al*., 2016; Rao *et al*., 2015; Tang *et al*., 2011;

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Ahmad *et al*., 2018). The Baculoviridae comprises of 600 viruses, including two genera, the NPVs and the granuloviruses (Hu *et al*., 2003). The cuboidal shaped NPV is host specific and used as a safe microbial pesticide. Under favorable conditions, it multiplies in the field and reduces the natural pest population (Kumari and Singh, 2009). NPVs have great potential against Lepidoptera insects (Kumari and Singh, 2009; Rios-Velasco *et al*., 2011; Tang *et al*., 2011; Zhang *et al*., 2015).

Spinosad (Sp) is a natural derived mixture of two *macrocylic* lactones spinosyns A and D produced during fermentation of bacterium *Saccharopolyspora spinosa* (Mertz and Yao, 1990; Aydin and Gürkan, 2006). The Environmental Protection Agency of U.S. has classified Sp. as a reduced-risk compound due to its environmentally benign characteristics. Spinosad in contrast to synthetic insecticides have low mammalian toxicity and no toxic effect on non-target organisms (Sparks *et al*., 1998). Due to its safer mode of action and compatibility with NPV, the mixture of Spinosad+NPVs have been evaluated successfully against lepidopteron insect pests (Jackson *et al*., 2014; Mendez *et al*., 2002; Figueroa *et al*., 2015; Wang *et al*., 2013).

Several studies have been reported in which the synthetic insecticides in combination with virus occlusion bodies enhance the effect of baculoviruses, especially against *Lymantria dispar* (Cook *et al*., 1996), *S. litura* (Nathan and Kalaivani, 2005; Nathan and Kalaivani, 2006; Shaurub *et al*. 2014; Trang and Chaudhari, 2002), *H. armigera* (Arrizubieta *et al*., 2016; Wakil *et al*., 2012), and *Pieris brassicae* (Lepidoptera: Noctuidae) (Bhandari *et al*., 2009). Therefore, keeping in view the importance of low input based crop production and reduction of pesticide load on the vegetable crops, the present study was undertaken to isolate native NPV and to assess their efficacy individually or in combination with commercially available Spinosad against larvae of *S. litura* from selected districts of Punjab province, Pakistan.

MATERIAL AND METHODS

Insect culture

The *S. litura* larvae used in bioassays were collected from crop fields from, Faisalabad, Rahim Yar Khan and Layyah districts of Punjab, Pakistan. The larvae were identified, and mass reared in the Integrated Genomics Cellular Developmental and Biotechnology (IGCDB) laboratory, Department of Entomology, University of Agriculture (UAF), Faisalabad, Pakistan (Fig. 1) at 25 ± 2ºC, 75 % RH and a photoperiod of 14:10 h (L: D) (Fig. 1A) following the method of Saljoqi *et al*. (2015) and Ahmad *et al*. (2018) with slight modification. The artificial diets consisted of chickpea flour 150g, sorbic acid 0.75 g, yeast powder 24g, agar 8.4 g, vitamin mixture 5ml, ascorbic acid 2.35 g, methl-4-hydroxy benzoate 1.5 g, d H2O 550 ml and streptomycin 0.75 g. The diet was stored at 4ºC until use.

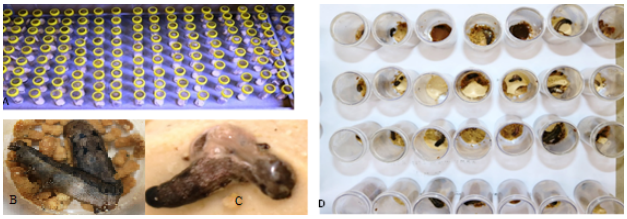

Fig. 1. **A,** Artificial Rearing of *Spodoptera litura* on artificial diet under laboratory conditions; **B,** NPV infected larvae of *S. litura* collected from cotton field (RY Khan) and **C,** propagated in laboratory; **D,** Mass culturing of NPV infected larvae through feeding on diet mixed with NPV suspension.

Insecticide

The commercial liquid formulation of Spinosad (Dow AgroSciences, Pakistan), containing a spinosyn A to spinosad D ratio of approximately 85:15 was prepared.

Viral isolation, suspension preparation and treatments

The 3rd and 4th instar larvae of *S. litura* from different districts of Punjab were collected and stored in IGCDB laboratory, Department of Entomology, UAF, Faisalabad. The NPVs from *S. litura* infected insects (Fig. 1B) from Rahim Yar Khan, Multan, Faisalabad and DG Khan Districts were identified on molecular level (Ahmad *et al*., 2018). In this study, we used isolate RY7 of NPVs collected from Rahim Yar Khan District. For mass culturing, the infected larvae suspension from Rahim Yar Khan was mixed in artificial diet (Fig. 1D). The infected cadavers were homogenized in distilled water and filtered through 3 layers of muslin cloth to remove large debris, then; suspension was centrifuged at 16000g for 45 min (Shapiro *et al*., 2005; Green *et al*., 2006). For purification of virus, the pellets were washed three times and kept at 5ºC. The concentration of polyhedral occlusion bodies (POBs) as stock solution (2 x 10^8 POB L^{-1}), (3 x 10^8 POB L^{-1}), and (4 x 10^8 POB L^{-1}) were prepared from indigenous NPV using Neubauer haemocytometer. From stock solution, 1 ml suspension from each concentration were prepared as NPV1 (2 x 10^5 POB mL^{-1}), NPV2 (3 x 10^5 POB mL^{-1}), and NPV3 (4 x 10^5 POB mL^{-1}). For bioassay, 10 µl NPV-1 (2 x 10^3 POB/larva), 15 µl NPV-2 (4.5 x 10^3 POB/larva) and 20 µl NPV-3 (6 x 10^3 POB/larva) were used to study synergistic, additive or antagonism effect against *S. litura* test population belonging to distinct geographical area of Punjab, Pakistan.

Table I. Larval mortality (% ± SE) of third-instar larvae of *S. litura* from three different field populations of Punjab province, Pakistan after treatment with NPV and Sp, individually, and in combination (NPV+Sp).

Treatments	Actual mortality			Expected mortality			CTF and type of interaction		
	Faisalabad	Layyah	RYK	Faisalabad	Layyah	RYK	Faisalabad	Layyah	RYK
Control	4.8 ± 1.19G	3.7 ± 1.25G	2.4 ± 1.19E						
NPV1	21.6 ± 2.28F	13.6 ± 2.47F	9.57 ± 3.41E						
NPV2	27.8 ± 2.38H	23.5 ± 2.09E	20.31 ± 3.1D						
NPV3	32.4 ± 2.37E	29.7 ± 1.76E	31.0 ± 1.45C						
Sp	47.9 ± 1.87D	42.6 ± 1.88D	40.05 ± 2.1C						
NPV1+Sp	65.0 ± 2.2C	57.2 ± 1.16C	39.3 ± 2.24C	69.6	56.3	49.7	-6.5 (Additive)	1.7 (Additive)	-21.5 (Antagonistic)
NPV2+Sp	86.0 ± 2.38B	68.7 ± 1.23B	68.9 ± 1.30B	75.8	66.1	60	13.5 (Additive)	3.8 (Additive)	13.8 (Additive)
NPV3+Sp	97.7 ± 1.13A	91.5 ± 2.29A	86.3 ± 1.57A	80.4	72.3	70.8	21.1 (Synergistic)	26.6 (Synergistic)	22.9 (Synergistic)
F	256	264	168						
DF	7,71	7,71	7,71						
P	< 0.01	< 0.01	< 0.01						

CTF, Co-toxicity factor; Sp, 0.01ppm; NPV-1, 2×10^3 POB/larva and NPV-2, 4.5×10^3 PIB/larva; NPV-3, 6×10^3 PIB/larva. Within columns, means (±SE) sharing the same letter within each population do not differ significantly (Tukey,s test, $P \leq 0.05$). Upper case letters show significance across the columns.

Table II. Larval mortality (% ± SE) of fourth-instar larvae of *S. litura* from three different field populations of Punjab province, Pakistan after treatment with NPV and Sp, individually, and in combination (NPV+Sp).

Treatments	Actual mortality			Expected mortality			CTF		
	Faisalabad	Layyah	RYK	Faisalabad	Layyah	RYK	Faisalabad	Layyah	RYK
Control	3.2 ± 1.25H	2.4 ± 1.19H	1.6 ± 1.04E						
NPV-1	17.5 ± 2.89G	12.8 ± 2.56G	8.9 ± 1.55E						
NPV-2	25.9 ± 1.93F	23.4 ± 4.54F	17.9 ± 3.10D						
NPV-3	29.0 ± 1.92E	32.4 ± 1.93E	25.5 ± 1.80D						
Sp	42.2 ± 2.42D	38.6 ± 3.53D	37.5 ± 2.01C						
NPV-1+Sp	59.2 ± 1.46C	57.5 ± 3.29C	35.3 ± 0.94C	59.7	51.4	46.5	-0.85 (Additive)	11.8 (Additive)	-23.9 (Antagonistic)
NPV-2+Sp	76.9 ± 2.04B	73.5 ± 2.84B	63.9 ± 1.68B	68.2	62.0	55.5	13.5 (Additive)	18.5 (Additive)	15.2 (Additive)
NPV-3+Sp	92.2 ± 1.88A	87.1 ± 1.36	80.3 ± 2.26	71.2	71.1	62.9	21.1 (Synergist)	22.6 (Synergistic)	27.6 (Synergistic)
F	225	107	197						
DF	7,71	7,71	7,71						
P	< 0.01	< 0.01	< 0.01						

For abbreviations and statistical details, see Table I.

Bioassay

The 3rd and 4th instar larvae of *S. litura* were infected with three concentrations of NPV: (2×10^8; 3×10^8 and 4×10^8 POB L^{-1} as stock solution, designated here as NPV-1, NPV-2 and NPV-3. A desired quantity of 10, 15 and 20 µl obtained from each 1 ml stock NPV suspension was incorporated in an artificial diet. Mortality, pupation, adult emergence and egg eclosion of *S. litura* were determined on single concentration of Spinosad (0.01 ppm) and three concentrations of NPV individually, and

in respective combinations. The NPVs and Spinosad was applied by incorporating it with artificial diets and mixing in shaker for even distribution. Cubes of 2mm^2 from prepared diet were cut and soaked in respective NPV and spinosad concentrations. In this way, different batches of artificial diets were arranged in sequence to provide the test chemicals to the target insects. The pre-starved (12 h) larvae (n=215/treatment)) of 3rd and 4th instar from each district were put separately in the plastic vials (base radius 2.4 cm × height 6 cm). A piece of the artificial diet (2mm^2) from each treatment was given to 3rd and 4th instar larvae until complete consumption. The larvae served with untreated diet were designated as control. After being fed, the larvae were removed and then released in plastic vials containing an artificial diet until the larvae died or pupated. All the bioassays were carried out at 25 ± 2°C, 70± 5 % R.H. and L16: D8 h photoperiod. Each treatment was replicated three times, and each bioassay was repeated thrice independently to avoid the phenomenon of pseudo-replication. The data regarding mortality was recorded regularly until pupation. The larvae were poked with a blunt needle and those which were unable to move in a coordinated manner were considered dead (Ma *et al.*, 2008). The data regarding pupation and adult emergence was also recorded thereafter. The emerged adults were allowed to mate freely for each treatment and egg-hatching percentage was also calculated to observe further NPV effect.

Data analysis

The mortality means were corrected using Abbott's (1925) formula and the data was processed through one-way ANOVA using Minitab 13.2. The synergistic, additive and antagonistic interaction between the treatments were determined by the equation CTF = (Oc-Oe)/Oe ×100. CTF represents the cytotoxicity triggering factor where observed mortality (Oc) is calculated by combination of insect derived NPV isolates, and the expected mortality (Oe) is the sum produced by each concentration of NPV isolate used in experiment (Mansour *et al.*, 1966). If the cytotoxicity factor has a positive value > 20 the interaction is considered synergistic, negative value of 20 or above means antagonistic, while any value between 20 and -20 is considered additive. Where the treatment effects were observed to be significant, the means were compared using Tukey-Kramer (HSD) test at P = 0.05 (Sokal and Rohif, 1995).

RESULTS

Larval mortality of S. litura

In single treatments, the highest larval mortality was observed with application of Sp in populations from Layyah ($F_{7,71}$ =264; P < 0.01), followed by populations from Faisalabad ($F_{7,71}$ =256, P < 0.01), and RYK ($F_{7,71}$ =168; P < 0.01) districts. The dose rate of NPV3+Sp caused maximum larval mortality in populations from Faisalabad, followed by the populations from Layyah and RYK (Table I) (Fig. 2).

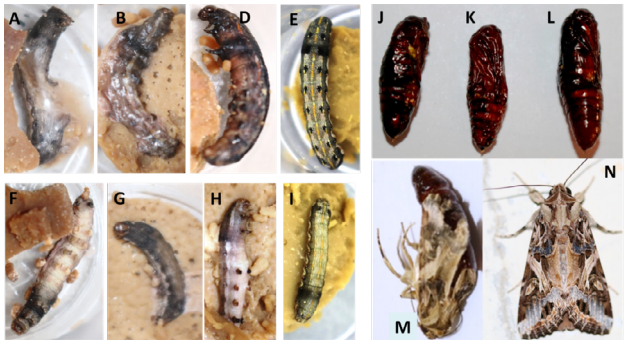

Fig. 2. Different stages of Healthy and treated *S. litura,* **A-D** The larvae from 4th Instars showing NPV symptoms treated with NPV1-3+Sp, **F-H** NPV and Spinosad treated dead larvae of 3rd Instars, **EI** Healthy (Control) larvae of 4th and 3rd Instars, **JKM** Severe malformed pupae and Adult after treatment, **LN** Healthy (Control) Pupa and Adults.

Fig. 3. Graphical Sketch, different stages of healthy and NPV infected larvae of *S. litura.* **A,** typical NPV infected symptom as attached with walls of vials downward (4th Instars); **B**, Different NPV infected dead larvae instars; **C,** NPV infected pupae; **D,** Malformed NPV infected Adults; **E,** Healthy larvae; **F,** Healthy pupae; **G,** Healthy adults.

For fourth-instar larvae of *S. litura*, among single treatments, the Spinosad alone caused maximum larval mortality, which was observed in the following order;

Faisalabad ($F_{7,71}$=225, P < 0.01), Rahim Yar Khan ($F_{7,71}$ =197, P < 0.01), and Layyah ($F_{7,71}$ =107; P < 0.01), as compared to the untreated control (Table II). Similarly, as it was observed for second-instar larvae, NPV3+Sp treatment showed significantly highest larval mortality for populations from Faisalabad and Layyah districts. The treated larvae showed infection symptoms with swollen and ruptured bodies releasing fluids (Figs. 2 and 3)

Table III. Pupation (%±SE) of third-instar and fourth-instar larvae of *S. litura* from three different field populations of Punjab province, Pakistan after treatment with NPV and Sp, individually, and in combination (NPV+Sp).

Treatments	Faisalabad	Layyah	RYK
Third-instar *S. litura*			
Control	94.8 ± 0.98A	96.3 ± 1.18A	97.0 ± 1.17A
NPV1	73.3 ± 2.22B	82.3 ± 2.93B	87.4 ± 1.73B
NPV2	66.7 ±1.57B	70.1 ± 2.94C	71.1 ± 1.92C
NPV3	55.6 ± 1.57C	59.3 ± 2.59D	60.0 ± 1.12D
Sp	41.5 ± 1.85D	50.4 ± 1.95D	48.9 ± 2.23E
NPV1+ SP	27.4 ± 3.41E	33.4 ± 0.00E	47.4 ± 2.06E
NPV2+ SP	8.9 ± 2.48F	21.5 ± 1.85F	23.7 ± 2.74F
NPV3+ SP	0.0 ± 0.00	2.9 ± 1.17G	7.4 ± 1.73G
F	267	235	253
Df	7,71	7,71	7,71
P	< 0.01	< 0.01	< 0.01
Fourth-instar *S. litura*			
Control	95.6 ± 1.57A	97.0 ± 1.61A	98.5 ± 0.97A
NPV1	79.3 ± 1.73B	85.2 ± 1.48B	89.6 ± 1.61B
NPV2	67.4 ± 1.73C	74.1 ± 0.74C	74.8 ± 2.42C
NPV3	57.8 ± 2.48D	67.5 ± 1.34C	68.1 ± 1.48C
Sp	47.4 ± 0.74E	55.6 ± 2.23D	56.3 ± 1.61D
NPV1+ Sp	29.6 ± 1.17F	42.2 ± 2.24E	26.7 ± 1.57E
NPV2+ SP	15.6 ± 1.11G	28.1 ± 1.48F	32.6 ± 1.73E
NPV3+ Sp	3.7 ± 1.17.H	8.9 ± 3.1G	13.3 ± 1.57F
F	419	242	342
Df	7,71	7,71	7,71
P	< 0.01	< 0.01	< 0.01

For abbreviations and statistical details, see Table I. n= 215/treatment where n is number of each 3rd and 4th instar larvae individually used in experiment)

Pupation and adult emergence

The combined treatments of NPV+Sp for 3rd larval instars population of RYK region showed lowest pupation and adult emergence as compared to single treatments (Tables III, IV). The morphological and physiological alteration was observed from larval-adult *S. litura* population treated with different concentrations of NPVs+Sp (Fig. 2). Likewise, during bioassay of fourth-instar *S. litura*, Sp with high dose rate of NPV application against various populations showed decreasing pupation trend (RYK: $F_{7,71}$ =342, P < 0.01; Layyah: $F_{7,71}$ =242, P < 0.01; Faisalabad: $F_{7,71}$ =419, P < 0.01) (Table IV), however, the adult emergence was in ascending order (Faisalabad: $F_{7,71}$ = 411, P< 0.01; Layyah. $F_{7,71}$ =229, P < 0.01; RYK: $F_{7,71}$ =166, P < 0.01) (Tables III, IV).

Table IV. Adult emergence (%±SE) from third-instar and fourth-instar larvae of *S. litura* from three different field populations after treatment with NPV and Sp, individually, and in combination (NPV+Sp).

Treatments	Faisalabad	Layyah	R Y Khan
Third-instar *S. litura*			
Control	94.1 ± 1.34A	95.6 ± 1.12A	96.3 ± 1.17A
NPV1	65.9 ± 1.33B	74.8 ± 1.85B	80.0 ± 1.92B
NPV2	55.6 ± 1.11C	62.9 ± 3.35C	71.1 ± 1.56C
NPV3	46.7 ± 1.57D	59.2 ± 4.44Cd	62.2 ± 1.11D
Sp	40.7 ± 1.73D	49.6 ± 1.17D	56.3 ± 1.61D
NPV1+ Sp	22.9 ± 1.17E	30.4 ± 3.16E	35.5 ± 1.57E
NPV2 + Sp	4.5 ± 2.23F	14.1 ± 2.06F	17.0 ± 1.61F
NPV3 + Sp	0.0 ± 0.00F	0.74 ± 0.74G	3.7 ± 1.59G
F	484	166	423
Df	7,71	7,70	7,71
P	< 0.01	< 0.01	< 0.01
Fourth instar *S. litura*			
Control	94.8 ± 1.85A	96.3 ± 1.61A	97.8 ± 1.11A
NPV1	72.6 ± 1.73B	86.6 ± 2.48B	88.1 ± 1.48A
NPV2	60.7 ± 0.74C	72.6 ± 2.82C	76.29 ± 3.86B
NPV3	57.8 ± 1.11C	64.5 ± 1.57C	68.1 ± 2.89B
Sp	49.6 ± 1.61D	51.9 ± 1.48D	63.7 ± 2.74C
NPV1+ Sp	34.1 ± 1.74E	37.0 ± 2.51E	45.9 ± 2.06D
NPV2 + Sp	14.8 ± 2.15F	24.5 ± 1.92F	28.9 ± 1.11E
NPV3 + sp	0.0 ± 0.00G	7.4 ± 1.34G	11.1 ± 1.57F
F	411	229	166
df	7,71	7,71	7,71
P	< 0.01	< 0.01	< 0.01

For abbreviations and statistical details, see Table I.

Egg eclosion

Maximum egg eclosion in second-instar larvae of *S.*

litura was observed in larval populations from RYK (F $F_{7,71}$ = 28.1; P < 0.01) and Layyah ($F_{7,71}$ = 32.4; P < 0.01) in both individual and combined treatments. However, minimum egg eclosion was observed in population from Faisalabad ($F_{7,71}$ = 41.6; P < 0.01) at the highest sub-lethal dose rate of NPV (6 x 10^3 PIB/larva) with sub-lethal dose rate of Sp (0.01ppm) (Tables V). Similarly, in 4th instar, the same trend (RYK: $F_{7,71}$ = 16.4, P < 0.01; Layyah: $F_{7,71}$ =21.1, P < 0.01; and Faisalabad: $F_{7,71}$ = 57.2; P < 0.01) was observed, where NPV and Spinosad were applied in combination at the dose rates of (6 x 10^3 PIB/larva) and 0.01 ppm (Tables V). Overall, eclosion rates decreased significantly in the treatments where higher concentration of NPV and sub-lethal dose of Sp were applied simultaneously.

Table V. Egg eclosion (%±SE) of third-instar and fourth instar larvae of *S. litura* from three different field populations treated with NPV and Sp, individually, and in combination (NPV+Sp).

Treatments	Faisalabad	Layyah	RYK
Third-instar *S. litura*			
NPV1	61.5 ± 4.70B	69.0 ± 6.40Ab	74.4 ± 4.98A
NPV2	49.5 ± 6.74Bc	58.8 ± 6.50Bc	67.7 ± 4.95Ab
NPV3	38.9 ± 4.39Cd	45.3 ± 4.77Cd	49.4 ± 4.84Bc
Sp	25.3 ± 4.43De	36.5 ± 5.06Cde	40.5 ± 5.56Cd
NPV-1+ Sp	16.6 ± 2.18Ef	25.3 ± 6.67De	34.1 ± 7.04Cd
NPV-2 + Sp	9.9 ± 3.02Ef	16.6 ± 6.00Ef	19.7 ± 9.60De
NPV-3 + Sp	0.00 ± 0.00F	0.00 ± 0.00F	1.70 ± 1.15E
Control	83.4 ± 5.63A	90.6 ± 1.61A	92.4 ± 2.62A
F	41.6	32.4	28.1
df	7,71	7,71	7,71
P	< 0.01	< 0.01	< 0.01
Fourth-instar *S. litura*			
NPV1	72.2 ± 4.92B	85.6 ± 6.35A	92.4 ± 1.92A
NPV2	62.8 ± 5.00Bc	72.4 ± 5.15Ab	78.3 ± 2.39Ab
NPV3	46.3 ± 6.01Cd	55.6 ± 10.09Bc	65.2 ± 3.88Abc
Sp	34.8 ± 3.51De	45.7 ± 4.77Bcd	54.7 ± 7.45Bcd
NPV-1+ Sp	23.1 ± 3.76Ef	34.5 ± 10.27Cde	40.0 ± 6.45Cde
NPV-2 + Sp	17.0 ± 2.76Fg	23.5 ± 4.18De	29.9 ± 4.74De
NPV-3 + Sp	4.9 ± 2.00G	11.8 ± 3.20E	10.2 ± 2.87E
Control	92.7 ± 1.59A	93.9 ± 1.92A	87.9 ± 16.19A
F	57.2	21.1	16.4
Df	7,71	7,71	7,71
P	< 0.01	< 0.01	< 0.01

For abbreviations and statistical details, see Table I.

DISCUSSION

Lepidoptera comprises of many agricultural and forest pests, which can be controlled by theuse of biological insecticides (Nathan and Kalaivani, 2006). Excessive use of synthetic chemical insecticide has created insecticide resistance, pest resurgence along with environmental and health complications (Cherry *et al*., 1997), which urges the researchers to find out some environmentally safer control agents to control the agriculturally important insect pests. Combining microbes with bio- rational insecticides is an ideal tool to overcome resistance in insect pests (Ahmad *et al*., 2018). *S. litura* is one of the most destructive pests that cause serious economic losses to many cash crops. It has been reported that entomopathogens have the ability to tackle insecticide resistance related issues. The rotation of control materials also helps to lessen the onset of insecticide resistance (Zahn and Morse, 2013). In current study *S. litura* showed retarded growth, decreased life span of male and female and increased larval and pupal duration after viral infection. The enhanced pathogenicity of NPVs has been observed against different vegetable and ornamental crops (Arrizubieta *et al*., 2014; Rios-Velasco *et al*., 2011; Ahmad *et al*., 2018). The pathogenicity of viral infection varies in different larval instars because of attraction of neonate larvae towards light, being very active and consumes large areas of leaf during feedings (Gothama *et al*., 1995; Smits and Vlak, 1988). A similar kind of response was also reported by other researchers (Nathan and Kalaivani, 2005, 2006) for Azadirachtin (AZA) and NPVs against the *S. litura*. Moreover, increase in pupal duration from third to fourth-instar larvae of *S. frugiperda* has been observed by Zamora-Avilés *et al*. (2013). The cutworm larvae died more rapidly at the higher and combined dose rate of AZA and *S. litura derived* NPV (SpltNPV). In contrast, the contradictory result was also obtained by Wakil *et al*. (2012), who found that fourth and fifth-instar larvae of *H. armigera* were more susceptible to viral infection than the second-instar larvae. The present study showed that Sp is more effective against forth instar larvae in all tested populations. Wang and co-workers (Wang *et al*., 2009, 2013) obtained similar results at low concentration of Spinosad against *H. armigera* and *S. exigua*. It has been reported that Sp has a novel mode of action primarily acting on nicotinic acetylcholine receptor and further on butyric acid receptors resulting in paralysis of insects (Capinera and Froeba, 2014). Spinosad delays egg hatching ability, fecundity, extend larval developmental time, reduce- weight gain, pupation ratio, pupal weight, adult emergence and adult longevity, which makes the insect pest more vulnerable against entomopathogenic insect (Wang *et al*., 2013; Malik *et al*.,

2016). Organophosphates, carbamates, pyrethroids, and *Bacillus thuringiensis* (Bt) resistance in diamondback moth has been managed by using Spinosad (Zhao *et al*., 2006). The combined application of insecticides with myco-insecticide is an interesting strategy to control the important insect pests owing to the low concentration of insecticide which reduces the mechanism of resistance (Malik *et al*., 2016). The western corn rootworm (WCR), *Diabrotica virgifera* was controlled by the combined application of entomopathogen and pesticides under field condition (Rauch *et al*., 2017).

Previously, several researchers have reported higher mortalities of lepidopteron pest with combined application of NPV and Spinosad as compared to single application in both field and laboratory trials (Jackson *et al*., 2014; Mendez *et al*., 2002; Figueroa *et al*., 2015). In current bioassay, similar results were observed. Synergistic effects were found when high concentration of NPV with spinosad was applied against third and fourth-instar larvae of *S. litura* which is in accordance with the findings of (Figueroa *et al*., 2015), who observed synergistic interaction when NPV and Sp were applied against *S. frugiperda* (Lepidoptera: Noctuidae). However, the synergistic effect of NPV and Spinosad from our findings is in contrast with the observation of Mendez *et al*. (2002), who reported independent or antagonistic effect of NPV and Spinosad against *S. frugiperda.* The same effect was also reported by many scientists for NPV alone or with different bio rational insecticide against insect pest (Ahmad *et al*., 2018; Qayyum *et al*., 2015; Wakil *et al*., 2012; Pineda *et al*., 2014). The possible reason of NPV-Spinosad synergism is because of the physiological changes or chemical pressures caused by bio-rational insecticides in insects making them more susceptible to occlusion bodies. After infection, NPV spores easily penetrate in insect cuticle and subsequently OB productions increases causing larvae to become pale in color. The insects slightly swell and often move towards higher point of host plants and then ultimately die (Nathan *et al*., 2005; Kumar *et al*., 2008). It has been reported that virus infection prolonged larval molting duration because insect showed susceptibility against viral insecticides (Kumar *et al*., 2008). On the other hand, contrary to our results, the effect of antagonism at higher dose rate of NPV with imidacloprid has been observed (Trang and Chaudhari, 2002). In the present study, the antagonistic effect was found with low dose rate of Spinosad in combination with NPV against *S. litura* larvae. The independent or antagonistic interaction in this study could be due to decreased feeding or a change of gut pH (El-Helaly and El-bendary, 2013). The three different geographical populations of *S. litura* showed different mortality response to NPV and Spinosad, therefore, it is also important to observe the resistance development against NPVs and spinosad in Spodoptera population. This is the first study to elucidate the dose dependent synergistic and antagonistic effect of spinosad and native distinct NPV isolates against three different geographical population of *S. litura*. The high mortality and dose dependent distinct response in Faisalabad population contrary to Rahim Yar Khan Population of *S. litura* could be due to different geographical NPV isolate and insecticide resistance development in *S. litura* populatuon. Now, there is a need to fully characterize these locally isolated *S. litura* based NPVs on molecular level and test its effectiveness combined with some other chemical insecticides. The efficacy testing of various other NPVs against laboratory developed susceptible, resistance and field population of *S. litura* is under progress.

CONCLUSIONS

The present investigation is a novel study in which the indigenous isolates of NPV were applied alone and in combination with an insecticide for the first time against *S. litura* at various life stages. Our results indicate that among several single and combined treatments, NPV and Sp showed higher mortalities against all the larval populations of *S. litura*. Furthermore, the combination of sub-lethal doses of NPV+Sp could be a new and cost effective strategy against *S. litura* in IPM regimes. But it is also very important to study the difference in mortalities against different geographical population for specific NPVs. The decrease in use of synthetic insecticides mitigates the insecticide resistance problem and proves less hazardous for non-target organisms. However, laboratory trials on the efficiency of Sp+NPV mixtures requires validation in field studies as a potential IPM strategy against *S. litura* in vegetable and main crop growing areas of Pakistan.

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Statement of conflict of interest

Authors declare no conflict of interest.

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