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Full Length Article

Bacillus thuringiensis and Nuclear Polyhedrosis Virus for the Enhanced Bio-control of *Helicoverpa armigera*

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Abstract

A number of entomopathogenic microbial agents infect a wide array of insects and cause epizootics from time to time. *Bacillus thuringiensis* (*Bt*) and Nuclearpolyhedrosis virus (NPV) are two important potential pathogens of *Helicoverpa armigera*. Investigations were carried out on integration of *B. thuringiensis* and Nuclearpolyhedrosis virus to determine its impact on survival of second and fourth instar larvae under laboratory conditions. Both *Bt* and NPV were applied using diet incorporation method. Larvae continued to feed for 48 h when a batch of fifteen insects was exposed to diet mixed with any of the treatment. Mortality was recorded every 48 h until larvae died or pupated. The susceptibility of *H. armigera* larvae decreased with older stage as greater mortality was recorded in second instar larvae (95.45%) in comparison to fourth instar larvae (82.48%) in combined application of *Bt* and NPV. Application rate of *Bt* was the key for type of interaction as only one synergistic interaction (CTF≥20) was recorded when lower dose of *Bt* was combined with NPV, rest of the combinations were additive and independent (CTF≤20). Reduction in the pupation, adult emergence and egg eclosion was found directly related to pathogenicity of the applied agents. This study would be helpful to extend the integrated use of pathogens under field condition to many important insect pests. © 2015 Friends Science Publishers

Keywords: *Bacillus thuringiensis*; *Helicoverpa armigera*; Integration; Nuclearpolyhedrosis virus; Synergism

Introduction

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) is among the most threatening plant pests, cosmopolitan in distribution and polyphagous in herbivorous nature (Wakil *et al*., 2009a, b; 2010). The voracious feeding habit of it has occasionally subjected shortage of food with its drastic quantitative and qualitative losses for the day by day increasing population of the globe (Liu *et al*., 2006). It is extremely mobile and polyphagous pest with worldwide distribution and significance with very wide range of hosts found in almost every season of the year both in cultivated and wild flora. *H. armigera* is devastating pest notorious for its diversified preference of hosts, feeding on foliage and fruiting structures, concealed feeding habit, great mobility, adaptable and multivoltine nature (Kambrekar *et al*., 2009).

Economically important cultivated crops especially cotton, maize, soyabean, sorghum, groundnuts, pigeon pea, chick pea, tobacco, tomato and other vegetables make them primary concern due to their dietary and economic value. It is recorded damaging at least 60 cultivated important crops and about 67 other plant species in 39 families. In Pakistan, Saleem and Younas (1982) found it feeding on about 14 plant families in Punjab. Rapid adaptability to environment is another liability with this insect and more than 200 types of cultivated and wild plant from 30 families are regarded as the host in China only (Liu *et al*., 2006).

Rapid adaptability and surprising rate of resistance development in *H. armigera* to many a number of insecticide chemistries have been verified globally (Martin *et al*., 2000; Nguyen *et al*., 2007). Almost all the group of insecticides in Pakistan suffered field failure owning to resistance particularly carbamates (Ahmad *et al*., 2001), organophosphates (OPs) (Ahmad *et al*., 1999) and pyrethroids (Ahmad *et al*., 1997; 1998). Consequently the array of resistance development in insects feels no limit covering almost all the conventional insecticides (McCaffery, 1998; Kranthi *et al.*, 2001; Rashid *et al*., 2013).

Alternatives to merely application of conventional synthetic insecticides are being investigated for their concerns related to environment and insect resistance problems (Carlton *et al*., 1986; Brousseau *et al*., 1998; Ullah *et al*., 2014). Microbial insecticides especially bacterial formulations made from *B. thuringiensis* are quite effective against number of economically important insect pests because they are not harmful to humans, other mammals, or non-target species. Insect resistance to *Bt*-formulations reported seem to be developed less quickly and less strongly as compared to resistance to chemical insecticides (Tabashnik *et al*., 1994) because *Bt*-toxins are quite

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complex in action mechanism against target insects (Tabashnik, 1992) and for insects to develop resistance against *Bt*-toxins require several important mutations (Carlton *et al.*, 1986). Viral insecticidal formulations are powerful agent in biological control of agricultural and forest pests because of their appropriateness and safety as they are simply formulated and deployed to target the particular host. Infection process by NPV is favored by alkaline pH (9.5–10) of the midgut with some enzyme mediated virus degradation activity that ultimately initiate polyhedral dissolution and virion release. Virions after release are directly exposed to the chemical conditions within the midgut lumen and ultimately result in insect death due to breakdown of midgut lining and cessation of feeding (Zhu *et al.*, 2014). Mainly the susceptible hosts for nuclear polyhedrosis viruses (NPVs) are arthropod and preferably lepidopterous insect pests (Liu *et al*., 2006).

The combined use of microbial formulations has attained greater repute among agricultural community as a successful tool in integrated pest management (IPM) strategies (Purwar and Sachan, 2006) as the value of individual application of any agent could suffer seriously if insect could be able to develop widespread resistance against it (Qayyum, 2010). Against *H. armigera*, both microbial products i.e. *B. thuringiensis* and NPV work either in synergistic or additive way, the action being dependent on several factors. This study aims to defeat the developmental success of *H. armigera* to augment it as susceptible host for pathogenicity of both microbial agents. Major intention was to determine the reliable combination between two entomopathogens that can enhance the biocontrol of *H. armigera* at lower doses by offering more lethal effects which on the other hand are achieved at higher doses when applied alone. This integration helps to reduce the quantity of microbial agents required to achieve sustainable insect management and hence to cut down the application cost. Moreover, the appraisal and integration of entomopathogens can help to identify their insecticidal prospective to supplement the synthetic insecticides which are threatening the natural ecosystem and human health.

Materials and Methods

Insect Culture

Larvae of *H. armigera* were obtained from stock culture reared in IPM vegetable laboratory, Department of Entomology, University of Agriculture, Faisalabad. Larvae were reared on modified artificial diet (Wakil *et al*., 2011) containing chickpea flour (125 g), red kidney beans (125 g), canned tomato paste (25 g), yeast (40 g), agar (17 g), vitamin mixture (10 mL) mixed thoroughly in distilled water (1300 mL). Adults of *H. armigera* were fed with 10% honey solution and provided with coarse surface of tissue as nappy liner to lay eggs (Fig. 1).

Bacillus thuringiensis toxin

B. thuringiensis var kurstaki (*Btk*) was obtained from isolate collection of IPM microbial control laboratory, Department of Entomology, University of Agriculture, Faisalabad originally obtained from National Center for Genetic Engineering and Biotechnology (BIOTEC). The isolate *Btk* is quite lethal to lepidopterans and is generally characterized by the production of both bipyramid and cuboidal parasporal crystal proteins (Obeidat *et al*., 2004) and carry *cry* 1 and *cry* 2 genes (Etinkaya, 2002). Toxin separation from the inert material was preceded by dissolving 1g *Bt* powder in 2 mL of sterile distilled water and streaking it gently on *Bt* nutrient agar media. Media for growth contained 15 g agar, 1.5 g beef extract, 5 g NaCl, 5 g peptone, 1.5 g yeast extract, and 1,000 mL distilled H_2O admixed with appropriate antibiotic. The crystal and spores formed after sporulation were centrifuged at 16,000 rpm for 15 min at 4°C temperature to extract required *Bt* toxin (Crecchio and Stotzky, 2001; Hernández *et al*., 2005). This resulted in pellet formation that was washed thrice in cold 1 M NaCl and re-suspended in 1 M NaCl. Concentration of spore-crystal mixture was estimated by the optical density at 600 nm (Hernández *et al*., 2005) in 1:100 dilutions. Toxin obtained was then stored in the refrigerator until used (Wakil *et al*., 2013).

Preparation of NPV

H. armigera nuclear polyhedrosis virus (NPV) was provided by Agri Life (Hyderabad) India. NPV for *H. armigera* is a member of the occluded virus group of baculoviruses which have unusually complex structure. It contains double-stranded deoxyribonucleic acid genome enclosed within a nucleocapsid in the polyhedral inclusion body. The viral DNA is heterogeneous in size with variable contour lengths (15–45 mμ), rod or ellipsoids in shape and exhibit molecular weight of about 30×10^{-6} daltons. The polyhedral occlusion bodies (POB) of NPV generally vary in both shape and size (0.65–1.93 μ in dia) and can be observed easily under the light microscope. The genome of *H. armigera* NPV is 131 kb and contains 135 open reading frames with protein encoding potential (Zhu *et al*., 2014). Second instar larvae of *H. armigera* were infected with a NPV suspension (1×10^{7} POB mL^{-1}) by spraying on artificial diet. Propagation of nuclear polyhedrosis virus was continued in insect midgut and 7 days after infection, midguts were homogenized, strained through fine mesh to remove debris. Pure virus was isolated from cadavers of *H. armigera* by sucrose gradient centrifuging (Hostetter and Puttler, 1991). The concentration of virus in suspension (POB mL^{-1}) was determined after several counts under microscope using a Neubauer haemocytometer (Cory and Myer, 2004).

Toxicity Assays

Insecticidal efficacy of *Bt* and NPV was evaluated against newly hatched 2nd and 4th instar larvae. Both *Bt* and NPV

were applied using diet incorporation method and diet piece of 0.5 g were offered to insects placed in glass vials (7 cm height, 3 cm in diameter). The artificial diets were mixed with two concentrations of *Bt* (0.5 and 1.0 µg g^{-1}) and NPV (2.3×10^6 and 4.6×10^6 POB mL^{-1}) or a combined solution of them in 1:1 ratio. Fifteen larvae were considered as a treatment which was repeated thrice. Larvae were fed on the treated artificial diets for 48 h. After 48 h, surviving *H. armigera* larvae were transferred into new vials containing non treated fresh diet. Bioassay were conducted at 25°C in 60–70% relative humidity, with a 14:10 h photoperiod. Percent pupation, adult emergence and egg eclosion was recorded hereafter.

Statistical Analysis

Mortality for both NPV and *Bt* was corrected for control mortality using Abbott's (1925) formula and subjected to analysis with Minitab software (Minitab, 2002) while significance of means were separated by Tukey's HSD test (Sokal and Rohlf, 1995). Type of interaction was determined by equation CTF = $(O_c\text{-}O_e)/O_e \times100$, where CTF is the co-toxicity factor, O_c is the observed percentage mortality resulted from the combined application, and O_e the expected percentage mortality, that is, the total percentage produced by each of the treatments used in the combination. The interactions were categorized into three groups: a positive factor of 20 or more meaning synergism, a negative factor of 20 or more meaning antagonism, and any intermediate value (i.e., between −20 and +20) was considered additive (Mansour *et al*., 1966; Wakil *et al*., 2012).

Results

Greater susceptibility was shown by second and fourth instar *H. armigera* larvae when challenged by artificial diets containing the combination of NPV and *Bt* than any of them alone. The effectiveness of the both agents was marked against both the larval instars and significant differences were recorded for treatment and instars but their interaction in combined ANOVA were found non-significant (Table 1). For the larval mortality, both additive and synergistic interactions were recorded as the determining factor was the concentration of both agents. Mortalities were higher in all combination of *Bt* and NPV than the individual application of these in both larval stages. Second instar larvae were found to be more susceptible to pathogenic action of both agents than the fourth instar. Second instar larvae infected with *Bt* (0.5 and 1.0 µg g^{-1}) showed mortality of 31.21±2.72 and 34.23±4.75% while NPV (2.3×10^6 and 4.6×10^6 POB mL^{-1}) treated larvae harbored 39.57±2.71 and 45.07±3.78% mortality. Integration of *Bt* at lower concentration (0.5 µg g^{-1}) with higher concentration of NPV (4.6×10^6 and 4.6×10^6 POB mL^{-1}) resulted in synergistic action (CTF≥20) with mortality of 95.45±1.59 while rest of the interactions were independent of each other showing additive effect (CTF≤20). Similar trend of increased mortality with increased concentration of *Bt* and NPV was recorded in fourth instar *H. armigera*, however, mortality was higher in case of individual NPV application than the *Bt* at both concentrations. In simultaneous application of *Bt* and NPV, three of the four interactions were additive whereas combining *Bt* at 0.5 µg g^{-1} with NPV at 4.6×10^6 POB mL^{-1} enhanced the effect of each other resulting mortality 82.48±5.18% more than the expected (Table 2).

With regards to proportion of insects succeeded in pupating, the interaction of *Bt* and NPV varied greatly and significantly (second instar $F_{8,80}$= 140, P≤0.01, fourth instar $F_{8,80}$= 106, P≤0.01). Combined action of both agents was proved to be more fatal than the individual application and lower proportion of pupation (0.74±0.74 and 8.89±2.72%) was observed in second and fourth instar respectively in larvae treated with combined application of *Bt* and NPV in concentration where synergistic interaction was recorded in mortality. Apart from untreated larvae, highest level of pupation (62.96±1.96 and 68.14±3.09%) was observed in lower concentration of *Bt* (Table 3).

Adult emergence and egg eclosion were found inversely related to pathogenicity of *Bt* and NPV. Significant differences were observed for treatments and instars among individual and combined treatments (Table 1). Lowest adult emergence (0.00±0.00 and 5.92±2.34%) and egg hatchability (0.00±0.00 and 3.59±2.06%) was recorded in larvae treated with *Bt*-1 + NPV-2 in second and fourth instar, respectively whereas highest level was recorded in larvae treated with *Bt*-1. Combined application exerted more hazardous effect on adult emergence and egg eclosion than the individual applications (Table 3).

Discussion

While exploiting the role of microbial agent in IPM, a number of constraints exist. Microbial agents are slow acting, persistently low in nature and need repeated applications to ensure an ample quantity of microbes thriving to pathogenize the target host. Combined application of microbes may help to meet some of the challenges faced in their individual use as these agents in combination are proved more pathogenic, more persistent and rapid in action. Many of the insecticide resistance related issues can be solved by the intelligent use of the entmopathogens as they make use of different tactics than the synthetic insecticides. The decrease in survival pressure from the target sites that previously resulted in resistance development against insecticides may render the resistant insects as susceptible to microbial action. *H. armigera* is one of the important insect that plagues a number of economically important crops. Resistance development in *H. armigera* against a wide array of insecticides has left this insect as unmanaged in field failure cases more often. Entomopathogens had always a priority in tackling insecticide related resistance (Cloyd, 2014).

Table 1: Factorial analysis of mortality, pupation, adult emergence and egg eclosion of *H. armigera* exposed to NPV and *B. thuringiensis*

Source of variance	df	Mortality		Pupation		Adult emergence		Egg eclosion	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Instar	1	39.7	0.000	22.7	0.000	19.07	0.000	6.32	0.013
Treatment	8	129.7	0.000	243.1	0.000	209.27	0.000	95.03	0.000
Treatment × instar	8	1.33	0.232	0.50	0.85	0.25	0.98	0.13	0.998
Error	144								
Total	161								

Table 2: Mean mortality (%±SE) of second and forth instar *H. armigera* larvae treated with NPV and *B. thuringiensis*

Entomopathogen	Second instar			Fourth instar			
	Actual mortality	Expected Mortality	CTF	Actual mortality	Expected Mortality	CTF	Type of interaction
Bt-1+ NPV-1	82.43±3.03ab	70.79	16.44	64.60±3.36b	56.24	14.86	Additive
Bt-1+NPV-2	95.45±1.59a	76.30	25.10	82.48±5.18a	62.28	32.46	Synergistic
Bt-2+ NPV-1	74.71±4.08b	73.81	1.22	59.63±3.65b	59.95	-0.53	Additive
Bt-2+ NPV-2	78.57±4.90b	79.31	-0.93	61.64±4.16b	65.98	6.58	Additive
Bt-1	31.21±2.72c			24.07±2.26c			
Bt-2	34.23±4.75c			27.77±2.53c			
NPV-1	39.57±2.71c			32.17±3.18c			
NPV-2	45.07±3.78c			38.20±4.11c			
Control	2.964±1.17d			1.482±0.98d			
df	8, 80			8, 80			
F	78.5			52.9			
P	≤0.01			≤0.01			

CTF: Co-toxicity factor

Bt-1:0.5 and *Bt*-2: 1.0 µg g^{-1}; NPV-1: 2.3×10^6 and NPV-2: 4.6×10^6 POB mL^{-1}

Mean sharing the same letters within columns are not significantly different

Table 3: Pupation, adult emergence and eclosion (%±SE) of second and forth instar *H. armigera* larvae treated with *B. thuringiensis* and NPV

Entomopathogen	Second instar			Fourth instar		
	Pupation (%)	Adult emergence (%)	Egg eclosion (%)	Pupation (%)	Adult emergence (%)	Egg eclosion (%)
Bt-1+ NPV-1	9.63±3.16de	7.41±2.82de	3.81±1.54c	18.51±2.67de	14.07±3.75de	9.58±2.14c
Bt-1+NPV-2	0.74±0.74e	0.00±0.00e	0.00±0.00c	8.89±2.72e	5.92±2.34e	3.59±2.06c
Bt-2+ NPV-1	20.74±2.82d	17.78±4.01d	13.42±2.72c	30.37±4.02d	25.92±3.03d	21.28±3.42c
Bt-2+ NPV-2	14.07±3.22d	10.37±1.95de	7.70±2.21c	21.48±2.89d	17.78±3.33de	12.82±3.34c
Bt-1	62.96±1.96b	56.29±2.74b	50.34±8.61b	68.14±3.09b	63.70±3.53b	58.06±5.49b
Bt-2	59.25±3.75bc	52.59±3.03bc	47.93±4.35b	63.70±2.74bc	58.51±2.67bc	50.48±5.27b
NPV-1	51.85±2.42bc	46.67±2.93bc	40.67±5.27b	57.03±1.61bc	51.85±2.89bc	47.40±5.63b
NPV-2	48.88±2.22c	42.96±3.16c	37.24±5.27b	52.59±2.34c	47.41±2.59c	42.91±7.37b
Control	94.07±1.73a	91.85±1.85a	95.51±1.73a	95.56±1.92a	93.33±1.92a	97.13±1.21a
df	8, 80	8, 80	8, 80	8, 80	8, 80	8, 80
F	140	119	50.5	106	92.4	44.9
P	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01

Bt-1:0.5 and *Bt*-2: 1.0 µg g^{-1}; NPV-1: 2.3×10^6 and NPV-2: 4.6×10^6 POB mL^{-1}

Mean sharing the same letters within columns are not significantly different

The rotation of control materials also help to lessen the onset of insecticide resistance (Zahn and Morse, 2013).

Two or more entomopathogens can work in collaboration, thus broaden the spectrum of action, range of targeted hosts (Kalantari *et al.,* 2014), and lower the time to kill. It may be hypothesized that joint action of microbials may magnify the virulence expected in alone. In present study, *Bt* and NPV were found integrating in additive and synergistic way making these combinations as more virulent than individual action. These finding stand parallel with the findings of Reddy and Manjunatha, (2000); and Kalantari *et al.,* (2014) who observed additive interaction from most of combinations. Liu *et al*. (2006) observed additive and antagonistic effect by combining NPV with Cry1Ac toxin against *H. armigera*. Kalantari *et al*. (2014) reported synergistic action by combining *Bt* at lower concentration and HaSNPV at higher concentration. Combination of Cry1Ac and CPV has yielded additive and synergistic interaction by Marzban *et al.* (2009) and Marzban (2012). Larvae of *H. armigera* have been infected synergistically by Matter and Zohdy (1981) by the use of two pathogens, *Bt* and NPV, on *H. armigera*.

The synergy between these two agents may come from the gut being the common site of infection. *Bt* toxins get adhered to specific binding sites in insect's midgut lining where they unsettle the cytoplasmic membranes

Fig. 1: Different steps in treatment application and rearing of *H. armigera* (A: commercial formulation of NPV, B: formulation of *Bt*, C: *Bt* culture, D: *H. armigera* eggs placed for incubation, E: artificial diet, F: neonates reared on normal diet, G: diet mixed with *Bt* and NPV formulations, H: treatment application, I, J: mortality recorded for treatments, K: pupation, L: adult emergence)

making the way to cell lysis. This distortion of epithelial lining renders insect unfit to continue feeding, making it lethargic and ultimately leading to death (Marzban *et al*., 2009). NPV works by binding of polyhedra to the midgut membranes. The epithelial membrane gets degenerated and liberates nucleocapsids into the cytoplasm. The midgut cells are principal initial target tissues which are basis for replication of the nuclear polyhedrosis virus are attacked after ingestion by a susceptible host, followed by transmission of infection from cell to cell (Liu *et al*., 2006). The virus multiplies rapidly and eventually fills the body of the host with virus particles resulting in insect's death. The *Bt* in lower concentration retards the growth of larvae offering an opportunity for the virus to propagate and show its lethal action. Thus combined action of both agents surrounds insect physiology from various ways leaving no way for escape. The most authentic hypothesis in this regard is that in the presence of *Bt*, number of larvae that can survive the NPV infection is reduced. Thus a series of changes happening in insects feeding results in delayed onset of resistance (Hesketh and Hails, 2008). Salama *et al*. (1993) discussed another possibility for the phenomenon that early damage by *Bt* supports later invasion by NPV. The combined action of *Bt* and NPV also improves the speed of kill as evidenced by histopathological study of Knaak and Fiuza (2005) who reported that the infection of NPV was intensified 6 h after NPV-*Bt* combined application against lepidopteran caterpillars. The magnified action was symptomized by rapid vacuolization of the cytoplasm in insect's midgut resulting cellular disorganization. The physiological abnormalities aroused by the joint action of both agents were evidenced by Duraimurugan *et al*. (2009) that proved suppression of detoxification enzymes in cotton bollworm larvae when challenged by combined action of NPV and *Bt*.

Bt insecticides kill target insects rapidly after ingestion as it acts as a highly host specific but NPV is fairly slow acting and takes several days or weeks to kill an insect and during that time insect continues to feed. A reduction in the application of broad-spectrum pesticides increases the potential for IPM and can allow the build-up of natural enemies. In this context, the use of narrow-spectrum microbial products that do not interfere with a conserved natural enemy complex can be of added value in pest management.

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