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Management of Diamond Back Moth (*Plutella xylostella)* using Indigenous Isolated Granulovirus and *Azadirachta indica*

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JNA, SJNA and MAM designed and conducted the experiments, wrote the manuscript writeup and analyzed the results. SJNA and MJA critically reviewed the manuscript.

Key words
***Plutella xylostella*, Granulovirus, *Azadirachta indica*, Diamondback moth**

ABSTRACT

Plutella xylostella (Lepidoptera: Plutellidae) is a cosmopolitan pest, infesting the cruciferous crops worldwide. It has developed resistance against chemical insecticides. To overcome this problem there is dire need to explore alternative control measures which are safer to the environment and compatible with human health. The present study was conducted to investigate the insecticidal properties of native isolated *Plutella xylostella* granulovirus (PxGV) and *Azadirachta indica* (AZA) on mortality and development of *P. xylostella* under laboratory conditions. Both AZA and PxGV were applied alone and in integrated manners at LC_{20} and LC_{50} dose rates. The combination of 349.3 parts per million of AZA and 4.8×10^4 occlusion bodies per millimeter of PxGV, resulted in synergistic interaction after 3rd, 5th and 8th days post application while the additive effect was observed only after 1 day post-treatment. Moreover, combined application of PxGV and AZA at sub-lethal doses exhibited increased larval, pupal and adult longevity and decreased larval and adult weight. In conclusion, combination of PxGV and AZA at low dose rate is a viable option to control diamondback moth.

INTRODUCTION

The diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae) is of concern for most of the farmers worldwide since it causes up to 90% crop losses in the cruciferous crops (Talekar and Shelton, 1993; Kfir, 1998; Amoabeng *et al*., 2013). An estimated global control cost and yield losses due to *P. xylostella* are found between US$ 4 and 5 billion every year (Zalucki *et al*., 2012). The insect originated in the Mediterranean and South Africa and spread to the commonly cruciferous growing areas of the world (Harcourt, 1963). Neonates make small mines and feed on the young tissues of different Brassica spp. such as *Brassica campestris*, *B. napus*, *B. juncea* and *B. oleracea* (Harcourt, 1963). Conventional chemical insecticides are the mainstay of brassica growers to get rid of *P. xylostella* which cause insecticide resistance and exert hazardous effects on the environment and human health (Ahmad *et al*., 2003; Wang and Wu, 2012; Han *et al*., 2015; Huang *et al*., 2016). *P. xylostella* have developed resistance against pyrethroid and organophosphate in many areas of Punjab, Pakistan (Sayyed *et al*., 2005; Khaliq *et al*., 2007). This situation urges researchers to develop eco-friendly alternative control practices such as the use of biocontrol agents, particularly entomopathogenic microorganisms. Granulosis viruses (GVs) belonging to family Baculoviridae are a promising and environmentally friendly alternative to chemical insecticides (Mascarin and Delalibera Jr, 2012). Granulosis viruses have been successfully used against lepidopterous insect pests, but the main limitations of GVs are; their species specifity, narrow host range and slow action (Lavina *et al*., 2001; Ishii *et al*., 2003).

Azadirachta indica A. Juss, (Meliaceae: Spindales) is also a potential alternative to the chemical insecticides which have been found exerting negative effects on growth, diapauses, reproduction and molting of more than 400 insect species from different insect orders (Schmutterer, 1990; Mordue Luntz *et al*., 1998; Mordue and Nisbet, 2000; Arthurs *et al*., 2006; Kumar *et al*., 2008; Shannag *et al*., 2015). Azadirachtin (AZA) is the active ingredient present in neem based formulation, hence, derived from the natural source.

Integrated use of AZA with other control practices has resulted in enhanced insect pest control, and means to reduce the dosage of chemicals (Koppenhöfer and Kaya, 2000; Han *et al*., 2015; Nouri-Ganbalani *et al*., 2016). Combining insect pathogen with a different kind of stressor is effective strategy to increase its virulence against insect hosts (Nathan and Kalaivani, 2005, 2006; Mascarin and

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Delalibera, 2012). The combined uses of viruses and AZA have been shown to exhibit a synergistic or additive effect as reported by several researchers (Wakil *et al*., 2012; Zamora-Avilés *et al*., 2013; Shaurub *et al*., 2014). Earlier studies (Wakil *et al*., 2012; Mascarin and Delalibera, 2012) reported additive effect when a combination of viruses and AZA were applied against the lepidopterous insects. However, on the other hand synergistic effects of viruses and AZA at appropriate combination resulted in increased larval mortality (Nathan and Kalaivani, 2005, 2006: Zamora-Avilés *et al*., 2013). The present study was conducted to investigate the interaction of PxGV and AZA on the larval mortality and development of diamondback (DMB).

MATERIALS AND METHODS

Tested insect

The larvae of DBM used in this experiment were collected from cabbage plantations in different areas of Faisalabad, Punjab, Pakistan and transferred to Integrated Genomics Cellular Developmental and Biotechnology (IGDMB, Lab), Department of Entomology, University of Agriculture, Faisalabad. The rearing conditions were maintained at 25±2 °C, 60±5% relative humidity and 8:16 (D: L) h photoperiod. The field collected larvae were transferred in plastic vials (base radius 2.8 cm × height 7.0 cm) and fed on artificial diets consisting of kidney bean 50g, choline chloride 0.05g, L-cysteine 0.2g, cholesterol 0.25g, wesson salt mixture 0.5g, sucrose 2.0g, ascorbic acid 2.0g, casein 3.5g, dried yeast 20.0g, agar 18.75g, wheat germ 50.0g, dried kale powder 5.0g, agar 5.0g, linoleic acid 0.17ml, propionic acid 0.8g, distilled water 340.0g and streptomycin 0.75g (Htwel *et al*., 2009). The pupae were placed in Petri dishes (9cm-diameters) kept in wooden cages (2ft l × 2ft w × 2ft g). After adult emergence, equal ratio of male and females were allowed to mate and oviposit in wooden cages. Moths were provided with 10% honey-water solution by soaking a cotton swab in plastic cups (7.8cm × 5.5cm) containing 50mg kale leaf powder on the bottom of rearing cage as an oviposition substrate for 24h.

Preparation of PxGV

The PxGV infected last instar larvae of *P. xylostella* were collected from cabbage growing areas of Faisalabad and identified using molecular tools and sequenced. Distilled water was used to macerate the larvae and obtained suspension was sieved through cheesecloth to remove insect large debris. Further centrifugation of the filtered suspension was done for 45 minutes at low speed (3000 rpm) and the resulting supernatant suspension was centrifuged at high-speed (16000 rpm) for 10 min. The resulting pellets were washed three-times and suspended in distilled water (Shapiro *et al*., 2005; Green *et al*., 2006). The concentration of virus was measured by using Neubauer hemocytometer. Different concentrations of PxGV: 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8 and 1×10^9 POB mL^{-1} were prepared by diluting with distilled water (Nathan and Kalaivani, 2006).

Azadirachta indica

The commercial formulation of *A. indica* Margosom® 0.15%, provided by Agri Life, Hyderabad, India was used.

Treatment with PxGV and Azadirachta indica

Lethal concentration of PxGV and AZA against 1st, 2nd and 3rd instars larvae of DBM were determined by the contaminated surface of the diet. Different concentrations of AZA (1300, 1250, 1000,870, 810 and 700 ppm) and PxGV (1×10^2 and 1×10^9 POB mL^{-1}) were spread on a small piece of artificial diet in a plastic vial (base radius 2.8cm × height 7cm) (Zamora-Avilés *et al*., 2013). Twenty larvae of each instar were fed on treated diets for 48 h and then shifted to the fresh untreated diet on individual basis. Larvae were observed daily during nine days to determine virus mortality. The experimental conditions were maintained at temperature 25±2 °C, R.H 70±5 % and a photoperiod of 8:16 (D: L) h in an incubator. Larvae continued to feed until pupation or death. Twenty 2nd instar larvae of DBM were individually placed in the plastic vials (base radius 2.4cm × height 6cm) containing a part of artificial diet treated with PxGV at LC_{20} (3.2×10^6 POB mL^{-1}) and LC_{50} (4.8×10^4 POB mL^{-1}), and AZA at LC_{20} (349.3ppm) and LC_{50} (701.77ppm), alone and in all possible combinations. For control treatment, larvae were fed on untreated artificial diet. The whole bioassay was repeated three times.

Effect of PxGV and Azadirachta indica *on development*

Sub-lethal effects of PxGV and AZA on the development of DBM were determined. Twenty, neonates of DBM were fed on diet treated with sub-lethal doses of AZA at 349.3ppm and PxGV at 4.8×10^4 POB mL^{-1} alone and in combination. Survivors were monitored daily until pupation. The developmental parameters estimated were larval weight, its life span, pupation rate, and pupal weight.

Data analysis

The mortality data were taken after 1st, 3rd, 5th and 8th day post-treatment. The control was adjusted by using Abbott's formula (Abbott, 1925) (Table II) and further, analysis of data was done by one-way analysis of variance (ANOVA) using Minitab (Minitab, 2003). Mean mortalities were compared by using Tukey-Kramer (HSD) test at α=0.05

Table I. Concentration mortality responses of different larval instars of diamondback moth to AZA and PxGV.

Treatment	Larval instar	LC_{20} (95% Fiducial limits)	LC_{50} (95% Fiducial limits)	Slope ± SE
AZA	1st	184.29(33.4 – 324.6)	542.59(292.40 – 674.24)	1.04±0.27
	2nd	349.3 (202.7 - 458.0)	701.77 (576.457 - 781.687)	1.62±0.28
	3rd	577.37 (503.42 - 634.76)	821.64 (771.892 - 862.243)	3.21±0.32
PxGV	1st	2.1×10^3 (8.8×10^2- 4.4×10^3)	1.3×10^5 (8.0×10^4 - 2.1×10^5)	0.27±0.01
	2nd	4.8×10^4 (2.1×10^4 - 9.5×10^4)	3.2×10^6 (2.0×10^6 - 5.0×10^6)	0.26±0.01
	3rd	2.2×10^5 (8.5×10^4 - 4.7×10^5)	3.8×10^7 (2.1×10^7 - 7.2×10^7)	0.21±0.01

[a]LC_{50} values are in ppm; [b]LC_{50} values are in OB mL^{-1}.

(Sokal and Rohif, 1995). LC_{20} and LC_{50} were calculated using Probit Analysis. The interaction between binary treatments was estimated following the co-toxicity co-efficient equation CTF= (Oc-Oe)/Oe ×100 described by (Mansour *et al*., 1966). Oc was observed and mortality calculated at the different interval after combined treatment, where Oe was the summation of each individual treatment used in the bioassay. The interaction between treatments was considered synergistic if CTF value was ≥ 20, the ≤ -20 indication of antagonistic, while a value between 20 and -20 was an additive effect.

RESULTS

Treatment with PxGV and Azadirachta indica

Single AZA treatment was effective against larvae of DBM. Further increasing the host stage (larval instar) tended to decrease susceptibility. For neonates, the LC_{50} value was 542.59 ppm and this value increased to 701.77 and 821.64ppm for 2nd and 3rd instar respectively which were higher than that of the 1st instar. Similarly, DBM larvae showed resistance to infection of PxGV, as the larval instar progressed. LC_{50} of PxGV were calculated as 1.3×10^5 OB mL^{-1} to the 1st instar larvae. This dose rate significantly increased to 3.2×10^6 and 3.8×10^7 OB mL^{-1} up to the 2nd and 3rd instar larvae, respectively (Table I). A significant anti-feedant effect was observed at high concentrations of AZA treatments. Artificial diet ingestion was proportionally lower by the DBM larvae, compared with that of low doses or control treatment. The infection of PxGV affected larvae exhibited some distinct symptoms, like the puffy, color change from dull green to pale-yellow, elongated integument, and high mortality rate. LC_{50} doses against 2nd instar revealed delayed effect of PxGV when compared for the LT_{50} values of AZA and PxGV (11.50 days) in comparison to AZA (4.70 days).

Interaction of AZA and PxGV

The bioassay testing on 2nd instar DBM larvae was conducted to study the interaction of AZA and PxGV. Results demonstrated that single PxGV treatment had no toxic effect on the larvae at LC_{50} doses until 120 h after treatment and it required 180 h to reach a peak mortality of 22.4±4.81. The AZA treatment showed mortality of about 19.0±5.0 at an LC_{20} concentration in the first 36 h. It is important to note that, no larval kill was observed after 96 h. The investigation demonstrated that AZA at LC_{20} dosage and PxGV at LC_{50}, increased the larval mortality significantly. The typical curve showed peaks at 36 h after treatment and another at 180 h (Fig. 1).

Table II. Median lethal time for 2nd instar larvae of diamondback moth to AZA and PxGV at LC50 doses.

Treatment	Larval instar	LT_{50}(days) (95% (Fiducial limits)	Slope±SE
AZA	2nd	4.7 (4.3 - 4.9)	0.33±0.11
PxGV	2nd	11.5(9.7 - 12.5)	-0.87±0.39

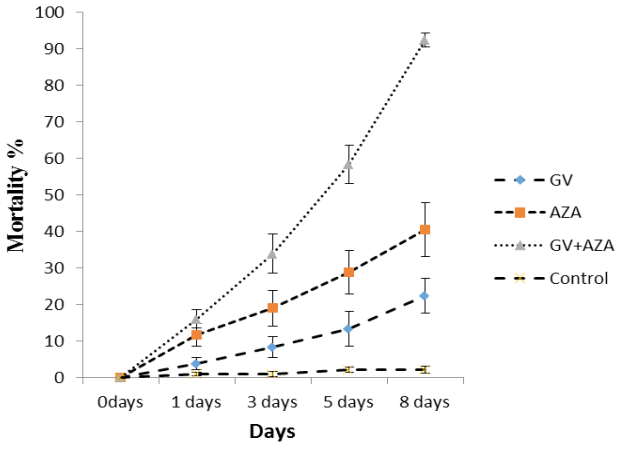

Fig. 1. The mean mortality (% ± SE) of 2nd instar larvae of diamondback moth treated with GV and AZA alone and in combination. Error bars represent standard Error.

The comparison between observed mortalities of combined treatment with the corresponding expected

mortalities revealed that combination of AZA at LC_{20} dose with PxGV at LC_{50} concentration further increased larvae death. The comparison between observed mortalities of combination treatment with the corresponding expected mortalities revealed that combination of AZA at LC_{20} dose with PxGV at LC_{50} concentration further increased larvae death CTC values were 27.77, 40, and 41.88 at 48-h, 96-hr and 144-h time point, respectively. These revealed synergistic effect against the larvae. Additive effect was shown at the period of 24 h after treatment (CTC=3.57).

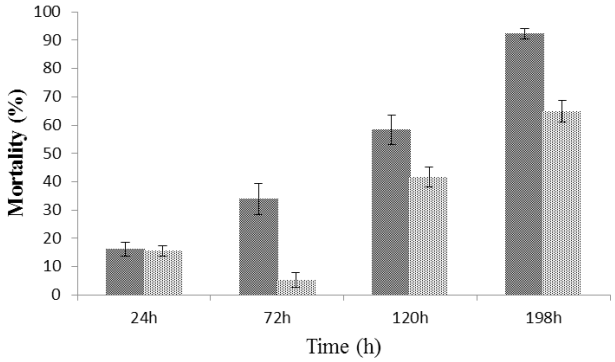

Fig. 2. Interaction effects of AZA and PxGV on 2nd -instar larvae of diamondback moth. Expected mortality based on average of individual effects, where the summation of each individual treatment represents observed mortality. Observed mortality (dark gray color) and Expected mortality (light gray). Error bars represent Standard-Error (±SE).

Effect of PxGV and Azadirachta indica *on development*

The significant effects of sub-lethal concentrations of AZA and PxGV on the growth and development of DBM were observed. Exposing the larvae to sub-lethal doses of AZA and PxGV significantly changed larval duration, its weight, pupal period, and mass, adult longevity and its mass (larval duration: $F_{3,35}$= 43.3, p<0.01; larval weight $F_{3,35}$= 9.10, p<0.01; pupal duration: $F_{3,35}$= 11.5, p<0.01; pupal mass: $F_{5,35}$=4.05, p<0.01; adult longevity ($F_{3,35}$=10.8, p<0.01); adult weight ($F_{3,35}$=8.36, p<0.01). In general, an increase in larval and pupal interval along with a decrease in larval weight was seen in all the treatments tested. The current study also confirmed that the adult lifespan and weight were substantially reduced. Profoundly highest detrimental effect was observed at the combined application of AZA and PxGV followed by the sole application of AZA and PxGV (Fig. 1 and Table III).

DISCUSSION

Frequent uses of synthetic insecticides have caused insecticide resistance in insect pests and posed environmental and human health-related issues (Nathan and Kalaivani, 2006). These situations forced researchers to develop biological and environmentally friendly control measures (Wood and Granados, 1991). Among different families of insect viruses, members of family Baculoviridae offer a biologically safer alternative to the conventional chemical insecticide (Sun and Peng, 2007). Recently, identification of nucleopolyhedroviruses (NPV) to control *Spodoptera litura* (Ahmad *et al*., 2018), insect pests and their management (Ahmad *et al*., 2019) have been done in Pakistan. Several researchers have reported the successful use of PxGV against larvae of DBM (Nakahara *et al*., 1986; Parnell *et al*., 2002; Fahimi *et al*., 2008; Muthamia *et al*., 2011). The slow killing speed of PxGV has limited their commercial production worldwide (Sun and Peng, 2007; Han *et al*., 2015). Laboratory bioassays also showed that single treatment of PxGV alone against DBM was nontoxic in early two days and required 5 days to kill the host. These results inferred that PxGV is slower in action than AZA and could not be used as a sole application by the farmer. Efficacy and killing speed of viruses against lepidopteron insects increases by combining with boric acid, chitinase, optical brighteners, *Bacillus thuringiensis*, nematode and neem components as reported by (Mascarin and Delalibera Jr, 2012). Neem with active ingredient Azadirachtin, (complex tetranortriterpenoid molecule) is another potent alternative to the chemical insecticides which affects growth, reproduction, and molting of more than four hundred insect species (Mordue Luntz *et al*., 1998). Susceptibility of DBM larvae depends on the concentration of azadirachtin, and insect species and age. Also, together with baculovirus, it has a synergistic or additive effect. In present study, the lower and combined application of PxGV and Azadirachtin resulted in synergistic effect (CTC >20). The synergistic interaction between PxGV and AZA was decreased with increasing dose rate and the antagonistic or additive effect was due to a higher dose rate of Azadirachtin application which affects the insect gut and resulted in bad ingestion of GV.

Similarly, low dose rate of Azadirachtin and pathogens resulted in high larval mortality of *Lymantria dispar* multiple nucleopolyhedrovirus (Cook *et al*., 1996), *Spodoptera frugiperda* (J.E. Smith) (Zamora-Avilés *et al*., 2013), *S. litura* (SpltNPV) (Nathan and Kalaivani, 2005, 2006; Shaurub *et al*., 2014), *Helicoverpa armigera* (HaMNPV) (Kumar *et al*., 2008; Wakil *et al*., 2012), *P. operculella* (Zeller) (Mascarin and Delalibera Jr, 2012) and *Pieris brassicae* (PbGV) (Bhandari *et al*., 2009). Mascarin and Delalibera Jr (2012) also reported higher larval mortality of *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) (86.7%) when DalNeem was combined with Granulosis Virus as compared to their

Table III. Effect of GV and AZA alone and in combination on the development of diamondback moth. Column with the identical letter is not significantly different at P<0.01. The values in the table are mean ±SE.

	Larval duration (days)	Larval weight (mg)	Pupal duration (days)	Pupal weight (mg)	Adult Longevity (days)	Adult weight (mg)
Control	14.05±0.19 d	6.5±0.32 a	4.94±0.24 c	5±0.23 a	16.1±0.41 a	3.04±0.18a
GV	16.11±0.16 b c	5.7±0.35 ab	5.88±0.32 ab	4.61±0.28 ab	15.1±0.40 ab	2.8±0.19 ab
AZA	15.27±0.22	4.8±0.41 bc	5.33±0.14 bc	3.9±0.58 ab	14±0.28 bc	2.2±0.14 bc
GV+AZA	17.38±0.26 a	4.0±0.38 c	6.44±0.13 a	3.3±0.36 a	13.5±0.23 c	1.9±0.17 b
d.f	3,35	3,35	3,35	3,35	3,35	3,35
F	43.3	9.10	11.9	4.05	10.8	8.36
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

GV: Granulovirus; AZA: *Azadirachta indica.*

alone treatment. Senthil Nathan and Kalaivani (2005) observed that the insect's gut lining or the peritrophic matrix is affected by the AZA treatments, enabling the easy penetration of pathogen. For the completion of life-cycle, insects require good growth and development. Any interruptions due to biotic or abiotic factors can delay their growth and development and make them more susceptible to natural enemies and environmental regimes. The combination of AZA mixtures increased larval duration by making them more vulnerable to virus infection. Similar results were reported previously using neem extracts in combination with LdMNPV (Shapiro and Dougherty, 2006). Like previous reports, reduction in food consumption, reduced larval and pupal weight, the longevity of the adult stage was observed under combined treatments of PxGV and AZA. In this bioassay, the low dose rate of AZA with PxGV resulted in synergistic effect against the second instar larvae of *P. xylostealla*. These findings are different from the findings of Zamora-Avilés *et al*. (2013), who observed, SfMNPV and AZA produced synergistic interactionagainst *S. frugiperda*. In contrast Mascarin and Delalibera, 2012 reported the additive effect of GV and Dalneem against the potato tuber worm. The same effect was also reported by many scientists for NPV with different bio-rational insecticide against insect pest (Wakil *et al*., 2012; Nathan and Kalaivani, 2005, 2006).

CONCLUSION

The results from present study showed that sublethal dose rate of PxGV and AZA mixture under laboratory condition caused higher larval mortality of DBM. Furthermore, detrimental effects on survival, growth, and development of *P. xylostella* were observed when PxGV and AZA were applied at sublethal dose rate. Integration of PxGV and AZA is good strategy toward controlling DBM but further field studies are required for validation of AZA-OB mixtures in commercial growing condition.

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Statement of conflict of interest

The authors have no conflict of interest.

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