Morphological Characteristics of *P. xylostella* Granulovirus and Effects on Its Larval Host Diamondback Moth *Plutella xylostella* L. (Lepidoptera, Plutellidae)

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**Abstract:** Problem statement: Diamondback moth, *Plutella xylostella* L. (Lepidoptera, Plutellidae) is the most destructive insect pest of cruciferous plants throughout the world which become resistant to wide range of chemical pesticides. PxGV is known as an effective factor on diamondback moth larval mortality. **Approach:** Some morphological characteristics, viral DNA isolation and Restriction Endonuclease (REN) analysis of *P. xylostella* granulovirus and its effects of different concentrations on its larval host Diamondback Moth *P. xylostella*, were studied. **Results:** Taiwanese isolate confirmed due to restriction pattern and genome size of PxGV Taiwanese isolate which was compared with PxGV isolates reported earlier. PxGV originally isolated in Taiwan has capsules that are ovocylindrical with a mean size of 272.84±12 by 148.27±19 nm. The virions are 168.44±16×29.57±12 nm. Results from pathogenicity test of the granulovirus to DBM using the leaf disc method shows that first, second and third instars of *P. xylostella* were significantly susceptible to infection by PxGV. Older larvae were less susceptible to PxGV than younger larvae with the same virus concentration. The LC$_{50}$ for second instar larvae was $1.39 \times 10^6$ granules mL$^{-1}$. The LT$_{50}$ values ranged between 3.813-6.946, 4.965-9.743 and 5.145-9.407 days for first to third instars in three different concentrations, respectively. **Conclusion:** Its high specificity and pathogenicity to its larval host indicate that PxGV is a good candidate as an alternative biopesticide to chemical insecticides in an Integrated Pest Management (IPM) of *P. xylostella*.

**Key world:** Diamondback moth, *Plutella xylostella* Granulovirus

**INTRODUCTION**

The diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae) (DBM), is a serious worldwide pest of *Brassica* crops. The dependence on chemical pesticides for controlling over the years has resulted in the pest developing resistance to all classes of chemical insecticides (Grzywacz et al., 2009; Roush, 1997; Sarfraz et al., 2005). To date, in addition to chemical insecticides * Bacillus thuringiensis* is also being used with considerable success (Sarfraz and Keddie, 2005). The global estimated cost of controlling this insect is US$1 billion annually. In recent years however, Granulovirus (GV) was found to be one of the key mortality factors in many populations of DBM around the world (Abdul Kadir, 1992; Talekar and Shelton, 1993; Asayama and Osaki, 1970). Studies have shown that the virus was infective to its larval host (Farrar et al., 2007; Kadir et al., 1999a; Grzywacz et al., 2002). Different isolates of PxGV were studied by Restriction Endonuclease (REN) analysis and there were no significant differences observed between isolates (Kadir et al., 1999b; Parnell et al., 2002). Hence many researches on this virus are presently being conducted with the aim of developing a GV-based biopesticide. Even though, many reports on PxGV have been published elsewhere, no study on its impact on Malaysian population of DBM has been conducted. In this study we examined morphological characteristics of GV, REN analysis and evaluated the effect of different doses on DBM mortality of different instars.
MATERIALS AND METHODS

Insect larvae: The larvae of P. xylostella were obtained from a colony that was being maintained on an artificial diet at Malaysian Agricultural Research and Development Institute (MARDI) Laboratory, Serdang, Selangor.

In vivo propagation of P. xylostella Granulovirus (PxGV): The virus tested was a Granulovirus (GV), originally isolated in Taiwan from P. xylostella larva (Farrar et al. 2007). We obtained the sample of PxGV from Mr. Hussian Kadir at MARDI. The GV was propagated using the leaf contaminated technique, with some modification (Odindo, 1981). Brassica leaves, surface-sterilized by 1% sodium hypochlorite, were contaminated with virus suspension. They were then fed to second instar larvae and reared at 23°C. Dead infected larvae were collected and stored at -20°C.

Purification of P. xylostella GV: The purification method was conducted according to Farrar et al. (2004) with slight modification. The insect cadavers (virus stock) were placed in 1 mL of ddH2O per insect and homogenized in a tissue homogenizer. The homogenate was centrifuged at 1000 xg for 30 sec at room temperature (23°C) and debris was discarded and the homogenate was centrifuged at 5000 xg for 10 min at room temperature (23°C) and debris was discarded and the homogenate was centrifuged at 5000 xg for 10 min. The pellet (OBs) was resuspended in same volume of 0.5 M NaCl. The solution was pelleted by centrifuge at 5000 xg for 10 min at room temperature. The OBs were resuspended in small volume of ddH2O per insect equivalent and centrifuged at 5000 xg for 10 min at room temperature. The pellet was resuspended in same volume of 0.5 M NaCl. The solution was pelleted by centrifuge at 5000 xg for 10 min at room temperature. The OBs were resuspended in small volume of ddH2O. The OBs were purified by ultra-centrifuge (Beckman Coulter L-100 XP) in a 40-80% (w/w) sucrose gradient at 96,000 xg at 4°C for 3 h. The white fraction was collected and washed twice with distilled water by centrifuged at 5000 xg for 10 min. The pellet was resuspended in small volume of ddH2O and stored at -20°C. Occlusion bodies were counted by using 0.02 mm-deep counting chamber (Herber Bacteria spore counting chamber) view under dark phase illumination at × 400 magnification of Nikon microscope.

DNA extraction and Restriction Endonuclease (REN) analysis: Restriction endonuclease analysis was carried out to confirm the isolate. The method used for extraction of PxGV DNA was adopted from O’Reilly et al. (1994) with slight modification. About 80 infected larval cadavers were used to provide enough purified virus. The purified occlusion bodies were treated with Na2CO3 to a final concentration of 0.1 M and warmed at 37 °C for at least 30 min. Tris-HCl 1.0 M (pH 7.6) was added to the final concentration of 0.1 M and then centrifuged at 7000 xg for 10 min. The supernatant was mixed with 0.1 volume of disruption buffer (10 µM of EDTA, 0.25% SDS) and proteinase K to a final concentration of 500 µg mL⁻¹ and incubated overnight at 37 °C in a waterbath. Equal volume of Tris saturated phenol (pH 7.49) was added and mixed well by inverting the tube and centrifuged at 12,000 xg for 5 min at 4°C. The aqueous phase was collected and added with an equal volume of phenol: chloroform: isomyl alcohol (25:24:1), mixed well and centrifuged at 12,000 xg for 3 min at 4°C. The aqueous phase was collected and extracted with an equal volume of chloroform-IAA (24:1), centrifuge for 1 min and the aqueous phase was collected. The DNA participate was purified with 2 volumes of cold 95% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2). The DNA was centrifuged at 12,000 xg for 10 min at 4°C and washed with 70% ethanol and centrifuged for 10 min, air dried and suspended in distilled water. The DNA was stored at -20°C. Restriction enzymes, EcoRI, PstI and HindIII, were used in this study. Electrophoresis of the DNA digests was carried out at 30 V for 16 h on 0.6% agarose gels prepared with tris-acetate buffer (pH 8.3). DNA profiles of isolates present in the gels were displayed on an ultraviolet transilluminator. The PxGV DNA fragments were compared with DNA pattern of the Taiwanese isolate that were digested with PstI and EcoRI restriction endonuclease by Parnell et al. (2002) and Kadir et al. (1999b).

Morphological characteristics of P. xylostella Granulovirus: OBs were collected at 40-85% sucrose gradient and examined under a Philips 400 Scanning Electron Microscope. We placed carbon coated formvar copper grid (300 mesh) onto a drop of purified virus suspension for 5 min and blotted to dry using a filter paper. The OBs were stained with 2% phosphotungstic acid (pH 7.2) for 5 min and again blotted to dry using a filter paper. Fifty capsules of PxGV with their virions were examined and measured for their shapes and sizes.

Bioassay: A bioassay based on Farrar et al. (2004) method with some modifications was conducted. To evaluate the dose responses and larval age susceptibility, three doses, 3.11×10⁵, 3.11×10⁷ and 3.11×10⁹ virus of concentrations, were tested on first to fourth larval instars. However, to obtain the LT₅₀ and LC₅₀ values, five serial dilutions of 3.11×10⁵, 3.11×10⁷, 3.11×10⁹, 3.11×10⁵ and 3.11×10⁹ OB mL⁻¹ were tested.
on first, second and third larval instars. Leaf discs, 4 mm diam., cut from the host plant, were treated with 5 µL of virus suspension per disc. A wetting agent, 1% Maxi green was used in all virus suspensions to spread the particles on the leaf discs. Known quantities of PxGV were pipetted on to the small leaf discs and they were dried before being fed to the larvae. For each treatment, 25 leaf discs were placed individually on in cells of a plastic bioassay tray. We used a thin layer agar in cells to keep the leaf discs fresh. One early second instar larva was placed in each cell. After 48 h of exposure and having eaten the entire leaf disc, the larva was transferred to a new bioassay tray filled with artificial diet. Mortality was recorded daily. The bioassay was replicated three times using 25 larvae per concentration.

**Statistical analysis:** The experiment was conducted following a factorial test design with an experimental unit of 25 larvae/treatment/replicate and three replications. Larval mortalities (%) caused by different virus concentrations in different larval instars were analyzed by Analysis Of Variance (ANOVA) with SAS System for Windows v6.12 software. The means were compared by Least Significant Difference test. LC$_{50}$ and LT$_{50}$ values were analyzed using by EPA Probit Analysis Program version 1.5 based on probit analysis (Finney, 1971). Cumulative larval mortality after 7 days was used for calculating the LC$_{50}$ values. LT$_{50}$ values were calculated based on virus concentrations at 3.11×10$^5$ to 3.11×10$^9$ OB mL$^{-1}$.

**RESULTS**

**REN analysis to confirmation of Taiwanese isolate:** The size of the DNA fragments was estimated by comparing with fragments of lambda DNA digested with HindIII. Six separate and three double distinguishable bands were observed when DNA was analysed following digestion with PstI and with EcoRI (Fig. 1). Restriction pattern and genome size of PxGV was similar with that of PxGV isolates reported earlier. Thus the result confirms that the PxGV used in this study was of the Taiwanese isolate.

**Morphological characteristic of PxGV and infected larvae:** The infected larva showed symptoms of swelling on each body segment followed by a change in its body color from green to pale yellow green and pale yellow or yellowish-white. In an advance stage of infection, the larval body collapsed when stimulated with a needle and white fluid often discharged from the body. The color of dead larva was brown or black at this stage of infection. Figure 2 shows separate of occlusion bodies of PxGV in purified suspension and Fig. 3 shows clumps of PxGV in a semi purified suspension. The capsules of PxGV were ovocylindrical (Fig. 4) with a mean size of 272.84±12×148.27±19 nm. The virions were 168.44±16×29.57±12 nm. The virions were rod-shaped and absent in some capsules (Fig. 2).
Influence of larval age and viral concentration on mortality: The results show that larval mortality increased with increasing virus concentration and decreased with older instars. Mortality of first instars in high concentration was significantly higher among the treated larvae than in the control. Larval mortality was significantly lower in fourth instars (3.58%) compared with the of first instars (72.17%) (Table 1).

Virus concentrations did not significantly affect the first instar mortalities and as expected neonate larvae were susceptible to all viral concentrations. The mortalities in the highest virus concentration (3.11×10^9 OB mL⁻¹) were 98.30, 96.60, 73.30, 14.30% from first to fourth instars, respectively. There were significant effects of virus concentration on the second and third instar mortalities. However no mortality occurred from all doses on fourth instars, except in the highest concentration (Table 2). The results show that mortality in highest concentration on first and second instars started on the second day and caused 100% mortality within 5 and 7 days, respectively (Fig. 5 and 6). The mortality in high concentration on third instars started in third day and caused 100% mortalities within 7 days (Fig. 7). Larval mortalities in low concentration (3.11×10^5 OB mL⁻¹) started on the third day on first instars with 89% mortality and on the fifth day on second and third instars with 53 and 26.3% mortalities, respectively (Fig. 5-7). The median lethal concentration (LC₅₀) in second instars was 1.39×10^6 OB mL⁻¹. The LC₅₀ value for the third instars increased to 13.66 folds compared to that of the second instars (Table 3). The LT₅₀ values in lowest concentration (3.11×10^5 OB mL⁻¹) increased from 5.14 on the first instars to 9.41 days on the third instars (Table 4).
Fig. 6: Cumulative mortality (%) of second instar DBM larvae at different doses (dose1 = 3 \times 10^5 OB mL^{-1}, dose2 = 3 \times 10^7 OB mL^{-1}, dose3 = 3 \times 10^9 OB mL^{-1})

Table 3: The LC_{50} values of P. xylostella granulovirus on different instars of P. xylostella

| No. of larvae used | LC_{50} | 95% confidence limits |
|--------------------|---------|-----------------------|
| Instars            |         | Lower limit | Upper limit |
| 1st                | 173     | >1.20 \times 10^5 | -         | -         |
| 2nd                | 210     | 1.39 \times 10^6 | 3.52 \times 10^5 | 3.63 \times 10^6 |
| 3rd                | 179     | 1.90 \times 10^7 | 1.58 \times 10^6 | 1.51 \times 10^9 |

Chi-square for heterogeneity 2nd instar = 1.323

Table 4: The LT_{50} values of P. xylostella granulovirus in different concentrations on different instars of P. xylostella

| No. of larvae used | LT_{50} (days) | 95% confidence limits |
|--------------------|-----------------|-----------------------|
| Instars            | Dose            | Lower | Upper |
| 1st instar         | 3.11 \times 10^7 | 63    | 3.813 | 3.396 | 4.210 |
| 2nd instar         | 3.11 \times 10^7 | 54    | 4.965 | 4.482 | 5.462 |
| 3rd instar         | 3.11 \times 10^7 | 56    | 5.145 | 4.760 | 5.519 |
| 2nd instar         | 3.11 \times 10^7 | 61    | 4.419 | 4.177 | 4.656 |
| 3rd instar         | 3.11 \times 10^7 | 56    | 6.726 | 6.286 | 7.318 |
| 3rd instar         | 3.11 \times 10^7 | 54    | 7.771 | 6.728 | 10.278 |
| 3rd instar         | 3.11 \times 10^7 | 65    | 6.947 | 6.325 | 7.910 |
| 3rd instar         | 3.11 \times 10^7 | 53    | 9.743 | 8.485 | 12.846 |
| 3rd instar         | 3.11 \times 10^7 | 58    | 9.407 | 8.378 | 11.779 |

The LT_{50} values increased 1.8 fold for the first to the third instars. In second concentration (3.11 \times 10^7 OB mL^{-1}) LT_{50} increased from 4.97-9.74 days (1.9 fold for first to third instars). The LT_{50} in high concentration (3.11 \times 10^9 OB mL^{-1}) increased from 3.81-6.95 (1.8 fold for first to third instars). The results show that with increasing dosage, mortality increased and also first, second and third instars of P. xylostella were significantly susceptible to infection by PxGV, respectively, while the fourth instars larvae were less susceptible to PxGV at given concentrations.

DISCUSSION

The results of this study in many ways corroborated with works that had been carried out by Asayama and Osaki (1970). Even though the symptom of the infected DBM larvae were generally similar, however the sizes the capsules and the virions were relatively smaller than those obtained by Asayama and Osaki (1970) and Wang et al. (2005). The differences in the size could be due to several factors. One of the factors might be related to the environmental and physiological conditions of the host cells at the time of infections (Kioukia et al., 1995). Despite of smaller sizes, the virus maintains its DNA and virulence against its host. The DNA profile obtained through gel electrophoresis after a digestion process with PstI and EcoRI produced a profile similar to that of viral DNA that was studied earlier by Parnell et al. (2002) and Kadir et al. (1999b). The virus was also effective against Malaysian DBM. The larval susceptibility to PXGV increased with the increased in the dose and decreased significantly with increasing larval age. This pattern of susceptibility has been shown in many studied (Duan and Otvos, 2001; Sporleder et al., 2007; Ignoffo, 1966; Evans, 1981).

The LC_{50} values of PxGV on P. xylostella larvae recorded from this study showed some variations from two related studies by Grzywacz et al. (2002) and Kadir et al. (1999a). Grzywacz et al. (2002) reported that all isolates of PxGV were pathogenic to Kenyan strains of DBM with LC_{50s} varying from 2.36 \times 10^9-
3.95×10⁷ Occlusion Bodies (OB) mL⁻¹ for second instar DBM compared with the LC₅₀ values of 1.55×10⁷ OB mL⁻¹ for the PlxyGV-Tw. The obtained LC₅₀ range for neonate larvae were from 3.82×10⁷-3.42×10⁶ OB mL⁻¹ as well as 2.06×10⁶ OB mL⁻¹ for second instar larvae (Kadir et al., 1999a). Kadir et al. (1999a) suggested that similarity in the LC₅₀ values in different larval instars might be due amount of virus ingested by older larvae which consequently caused increasing level of susceptibility to infection. Generally the PxGV isolate used in this study was more virulent than that of the Kenyan (Grzywacz et al., 2002). In comparison with LC₅₀ calculated by Kadir et al. (1999a) for second instar larvae, biological activity of Taiwanese isolate was 1.5 fold more virulent than those isolate used by Kadir et al. (1999a). Several factors had been reported to affect LC₅₀ values in bioassays. One of the factors was the bioassay protocol. Kadir et al. (1999a) used droplet method for bioassay tests and suggested that using leaf disc or diet assays is more precise than droplet feeding assays. Although we used leaf disc assays for bioassay tests, moreover during in vivo propagation, several passages of PxGV on Malaysian DBM larvae can be responsible for increasing isolate pathogenicity (Kolodny-Hirsch and Myers, 2003). Other factors that could lead to differences of calculated LC₅₀ were different temperatures during bioassay, different range of virus doses and overlap of larval instars and mixed larval ages used for tests. Many research works indicated that the median survival time (LT₅₀) decreases with increasing dose (Van Beek et al., 1988; Toprak et al., 2006). The LT₅₀ at dose 10⁶ OB mL⁻¹ (with mean LC₅₀ 1.85×10⁶) for neonate, second (measured at 27°C) and third instar larvae (measured at 23°C) were determined 5.99, 4.98 and 5.51 days respectively (Kadir et al., 1999a). Kadir et al. (1999a) did not show the dose dependency. Thus the variation could be due to other environmental conditions such as different temperature during bioassay. The median lethal time presented here in median concentration (3.11×10⁷) for first, second and third instar larvae were 4.96, 6.72 and 9.72 days respectively. Even though these results were not in agreement with those reported by Kadir et al. (1999a) but they were consistent with those of other studies which suggested the presence of dose dependency.

**CONCLUSION**

These findings demonstrated that Taiwanese isolate of PxGV has high potential for controlling of Malaysian population of DBM. As expected, the younger larvae were more susceptible to all virus concentrations. Thus application of PxGV should be targeted to the early stages of larval development so as to obtain the full potential the virus. Future research must be focussed on formulation, commercialization and field application.

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