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:** Taiwanian isolate confirmed due to restriction pattern and genome size of PxGV Taiwanian 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 $168.44\pm16\times29.57\pm12$ 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 1.39×10^{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 world-wide 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.

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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. Hussan 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 ddH_2O 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. The pellet (OBs) was resuspended in 1 mL of 0.1% SDS 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 ddH_2O. 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 ddH_2O 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 Na_2CO_3 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 12000 xg for 5 min at 4°C. The aqueous phase was collected and added with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), mixed well and centrifuged at 12000 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, *Eco*RI, *Pst*I and *Hind*III, 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 Taiwanian isolate that were digested with *Pst*I and *Eco*RI 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, $3.11×10^5$, $3.11×10^7$ and $3.11×10^9$ virus of concentrations, were tested on first to fourth larval instars. However, to obtain the LT_{50} and LC_{50} values, five serial dilutions of $3.11×10^5$, $3.11×10^6$, $3.11×10^7$, $3.11×10^8$ and $3.11×10^9$ 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 Taiwanian isolate: The size of the DNA fragments was estimated by comparing with fragments of lambda DNA digested with *Hind*III. Six separate and three double distinguishable bands were observed when DNA was analysed following digestion with *Pst*I and with *Eco*RI (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 Taiwanian 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).
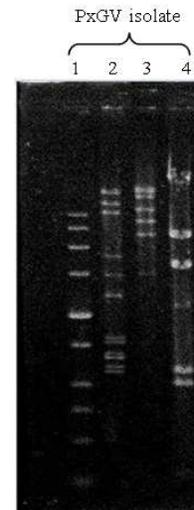
Fig. 1: Restriction endonuclease digest profiles of the DNA of *Plutella xylostella* Granulovirus (PxGV) isolate. Isolates was digested with *Pst*I, *Eco*RI and *Hind*III restriction endonuclease, fragments were separated on 0.6% agarose gel. (Lane 1) (far left of page) 0.5, 12 kb ladder molecular size standard; (Lane 2) *Pst*I, (Lane 3) *Eco*RI, (Lane 4) *Hind*III digested lambda DNA/*Hind*III marker
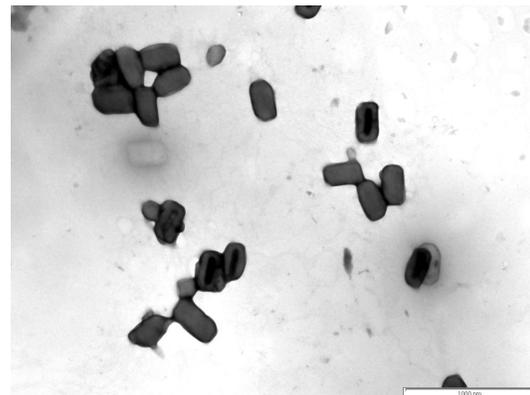
Fig. 2: Electron micrograph (Negative Staining) of occlusion bodies of *P. xylostella* granulovirus collected in purified suspension (Sucrose gradient)
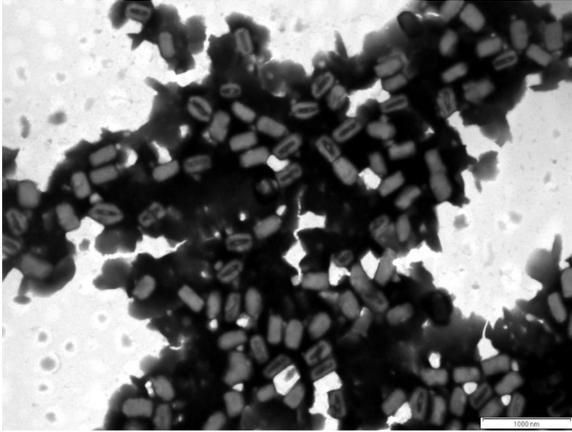
Fig. 3: The occlusion bodies of *P. xylostella* granulovirus in semi purified suspension
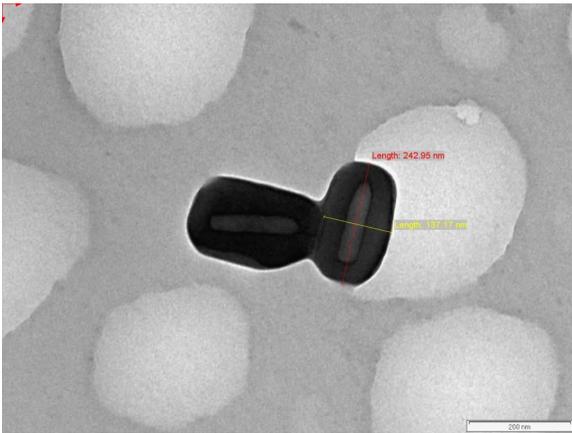
Fig. 4: The size and shape of occlusion bodies and virions of *P. xylostella* granulovirus

Table 1: Percentage mean mortality (interaction) different doses × different larvae instars (DMR test)

Treatment (OB mL^{-1})	Dose (mortality %)	Larvae instar (mortality %)
3.11×10^9	70.667^a	72.167^a
3.11×10^7	49.33^b	57.417^b
3.11×10^5	42.917^c	32.250^c
Control	1.66^d	3.583^d

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).
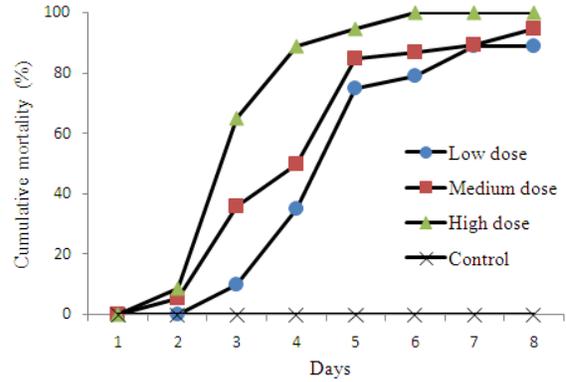
Fig. 5: Cumulative mortality (%) of first instar larvae DBM at different doses (dose1 = 3×10^5 OB mL^{-1}, dose2 = 3×10^7 OB mL^{-1}, dose3 = 3×10^9 OB mL^{-1})

Table 2: Percent mean mortality of different larvae instars of *Plutella xylostella* treated with different concentrations of PxGV

Doses	1st instar	2nd instar	3rd instar	4th instar
3.11×10^9	98.33^a±1.07	96.66^a±1.69	73.31^a±2.93	14.33^a±3.65
3.11×10^7	91.00^a±3.05	79.66^b±2.78	29.00^b±2.37	0.00^b±0.00
3.11×10^5	89.33^a±2.60	53.33^c±0.89	26.66^b±1.55	0.00^b±0.00
Control	1.66^b±0.00	1.66^d±0.00	0.00^d±0.00	0.00^b±0.00

Means followed by same letters in same column are not significant (LSD)

Virus concentrations did not significantly affected 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 (LC$_{50}$) in second instars was 1.39×10^6 OB mL^{-1}. The LC$_{50}$ value for the third instars increased to 13.66 folds compared to that of the second instars (Table 3). The LT$_{50}$ 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).
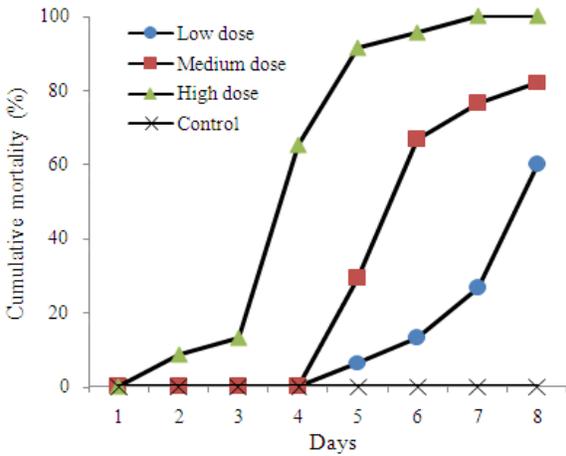
Fig. 6: Cumulative mortality (%) of second instar DBM larvae at different doses (dose1 = 3×10^5 OB mL^{-1}, dose2 = 3×10^7 OB mL^{-1}, dose3 = 3×10^9 OB mL^{-1})

Table 3: The LC$_{50}$ values of *P. xylostella* granulovirus on different instars of *P. xylostella*

Instars	No. of larvae used	LC50	95% confidence limits	
			Lower limit	Upper limit
1st	173	>1.20×10^3	-	-
2nd	210	1.39×10^6	3.52×10^5	3.63×10^6
3rd	179	1.90×10^7	1.58×10^6	1.51×10^9

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*

Instars	Dose	No. of larvae used	LT$_{50}$ (days)	95% confidence limits	
				Lower	Upper
1st instar	3.11×10^9	63	3.813	3.396	4.210
	3.11×10^7	54	4.965	4.482	5.462
	3.11×10^5	56	5.145	4.760	5.519
2nd instar	3.11×10^9	61	4.419	4.177	4.656
	3.11×10^7	56	6.726	6.286	7.318
	3.11×10^5	54	7.771	6.728	10.278
3rd instar	3.11×10^9	65	6.947	6.325	7.910
	3.11×10^7	53	9.743	8.485	12.846
	3.11×10^5	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 (3.11×10^7 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 (3.11×10^9 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.
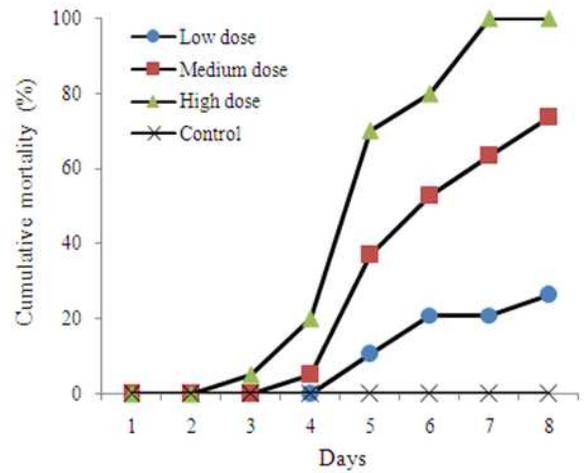
Fig. 7: Cumulative mortality (%) of third instar DBM larvae at different doses (dose1 = 3×10^5 OB mL^{-1}, dose2 = 3×10^7 OB mL^{-1}, dose3 = 3×10^9 OB mL^{-1})

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 *Pst*I and *Eco*RI 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 2.36×10^6-

3.95×10^7 Occlusion Bodies (OB) mL^{-1} for second instar DBM compared with the LC$_{50}$ values of 1.55×10^7 OB mL^{-1} for the *Plxy*GV-Tw. The obtained LC$_{50}$s range for neonate larvae were from 3.82×10^5-3.42×10^6 OB mL^{-1} as well as 2.06×10^6 OB mL^{-1} for second instar larvae (Kadir *et al*., 1999a). Kadir *et al*. (1999a) suggested that similarity in the LC$_{50}$ values in different larvae 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 LC$_{50}$ calculated by Kadir *et al*. (1999a) for second instar larvae, biological activity of Taiwanian isolate was 1.5 fold more virulent than those isolate used by Kadir *et al*. (1999a). Several factors had been reported to affect LC$_{50}$ 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 Van Beek, 1997; Cory and Myers, 2003). Other factors that could lead to differences of calculated LC$_{50}$ 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 (LT$_{50}$) decreases with increasing dose (Van Beek *et al*., 1988; Toprak *et al*., 2006). The LT$_{50}$ at dose 10^6 OB mL^{-1} (with mean LC$_{50}$ 1.85×10^6) 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 (3.11×10^7) 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 Taiwanian 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.

ACKNOWLEDGMENT

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