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Susceptibility of the potato tuber moth *Phthorimaea operculella* (Lepidoptera: Gelechiidae) to the *Autographa californica* multicapsid nucleopolyhedrovirus

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Abstract:

The *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) pathogenicity was investigated for the potato tuber moth (PTM) *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae). Infection of PTM with AcMNPV viral concentration of 2.5x 10^5 PIB/ml resulted in up to 6.25 % mortality, 14.3 % less active and partially paralyzed larvae and about 14.7 % absent or not found larvae compared to the control, and assumed to be died at their very early larval instar. Observation of infected dead larvae under optical microscope revealed presence of cubic polyhedral inclusion bodies (PIBs) in different insect cells. Hybridization of extracted and digested DNA from partially paralyzed infected PTM larvae, with specific AcMNPV nucleic probe, proved the identity of the AcMNPV used in PTM infection.

Introduction

The cultivated potato, *Solanum tuberosum* L. (Solanales: *Solanaceae*), is one of the most important vegetable crops for human nutrition worldwide (Flanders *et al.,* 1999). In Egypt as in many other countries worldwide, potato crop represents one of the main vegetable cash crops. Among serious insect pests that attack potato crop, the potato tuber moth (PTM) *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae), represents one of the most dangerous pests that accompanying plant from seedling to harvest stages and storage period (Westedt *et al.*,1998), leading to severe effects on potato yield quantity and quality. PTM is widely distributed in both subtropical and temperate regions and attacks wide range of variable host plants, including tobacco, eggplant, and tomato (Fenemore, 1988 and Sporleder *et al.*,2004).

For decades, the PTM control has relied upon the use of several traditional synthetic insecticides, leading to rapid development of resistance to a wide variety of insecticides, and increasing tuber infestation rates as well (Clough *et al.*, 2008). Recently, biological control for PTM using bioinsecticides and bio-agents have gained more attention, and efforts have been exerted to evaluate them against PTM in both field and storage (Sileshi and Teriessa, 2001; Agamy, 2003 and Mandour *et al.*,2009).

To reduce infestations in stores, the soil bacterium *Bacillus thuringiensis* (Bt) and the second

member of *baculoviridae*, the *granulovirus* that named PhopGV, were used by farmers worldwide and have been proved effective in reducing PTM infestations (Von Arx and Gebhardt, 1990) and has been applied to control larval populations in the field and stored potatoes in Peru, Bolivia, Colombia, and Ecuador (Raman *et al.,* 1992 and Moscardi, 1999) and Zeddam *et al.* (1999) summarized tropical and subtropical locations worldwide from which the PhopGV were isolated.

Although several studies have demonstrated the potential of this virus to control PTM under both field and storage conditions (Raman *et al.*, 1987; Reed and Springett, 1971; Von Arx and Gebhardt, 1990; Ben Salah and Aalbu, 1992; Lagnaoui *et al.*, 1997; Das *et al.*, 1998 and Hanafi, 2005), however, several authors stated that PTM has exhibited a strong potential to develop resistance to PhopGV (Briese and Mende, 1981, 1983; Briese, 1982 and Sporleder, 2003). Briese and Mende (1983) observed a 140-fold increase in LD50 following exposure of larvae to PhopGV over six generations, two decaddes later, Sporleder (2003) also reported high levels of resistance in a PTM population exposed to the virus for 12 generations. Beside emergence of PTM resistance to the virus, the PhopGV like other member of *granuloviruses*, is highly specific to the insect host from which it was isolated except certain of its isolates that were found able to infect other insect species belong to Gelechiidae (Zeddam *et al.*, 2013).

Nucleopolyhedroviruses (NPVs) that represents the first member of *Baculoviridae* were intensively studied and commercially exploited for insect pest control due to their host specificity and safety to other plants and animals, and their infectivity to a wide range of insect pest species (Hussain *et al.*,2019). Among these viruses, the two NPVs *Autographa californica* and *Anagrapha falcifera* were found to have a relatively broad host spectrum and have been shown to infect as many as 30 different lepidopteran species from as many as 10 separate families (Adams and McClintock, 1991), therefore, they were registered in the USA and field-tested at a limited scale , showing their potentiality against a variety of crops infested with pests belonging to a number of genera, including *Spodoptera* and *Helicoverpa* (Boguslaw Szewczyk *et al.*,2011).

Finally, as the use of alternative environmentally safe means for controlling insect pests is highly recommended and encouraged in organic agriculture and other safe food production systems. Hence, considering the importance of PTM as a key insect pest and based on earlier studies that revealed that *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) was able to multiply *in vitro* on an established cell line derived from *P. operculella* embryos (Lery *et al.,* 1998), therefore, the pathogenicity of AcMNPV to PTM *in vivo* was examined as a primary work aims to evaluate possible potentialities of other available baculovirus isolates to be employed in PTM control programs.

Materials and methods

1. Virus:

An AcMNPV strain was previously obtained as a gift from Dr. Croizier (INRA, France). To conserve the initial properties, the virus strain was regularly multiplied in the laboratory on the pink boll worm *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) (Vail *et al.,* 1972). The polyhedral inclusion bodies (PIBs) were isolated as follow; using a sterile pestle and mortar, the collected infected larvae were homogenized in 50 mmol/l Tris.Hcl and 2 mmol/L sodium dodecyl sulfate (SDS), pH 7.8, and filtrated

through a cheese cloth, then the filtrate was centrifuged at 1000 x g for 2 minutes to remove the larger particles. Finally, the obtained suspension was purified by centrifugation through a 30%-70% sucrose gradient for 20 minutes at 25,000 x g. then band containing the purified PIBs was removed to a clean centrifuge tube, then diluted by completing the its volume with 50 mmol/l Tris. Hcl and re-centrifuged. A pellet containing PIBs was re-suspended in sterile distilled water, observed through phase contrast optical microscope, counted using a haemocytometer then stored at 4 ^{0}C for further investigations.

2. Insect rearing:

The PTM used in this study were derived from a PTM colony maintained at the Plant Protection Research Institute (PPRI) that is affiliated to the Agricultural Research Center, Egypt. At the beginning, received pupae were kept under laboratory conditions of 27 ± 1 and L16:D8 photoperiod as described by Zeddam *et al.* (2013). For PTM egg production, disinfected pupae were sexed, then transferred to adult box at 1:1 ratio (Male: female) then adult box was covered by a circular piece of white paper (or filter paper), sitting on top of a black fine mesh net fitted tightly over the opening with an elastic band and the box was provided with 10% honey solution, as a source of feeding for emerged adult, through a tissue paper soaked in the honey solution. Papers containing PTM laid eggs were cut off into sections, of 100 eggs each, and then kept in plastic ventilated cylinder boxes for further use under the same controlled conditions. By the appearance of larval dark head capsules, the eggs were surface disinfected in 10% sodium hypochlorite for 1 minute, rinsed in distilled water, dried on filter paper, then transferred to small potato tubers at a ratio of (2gr potato per 1 PTM larva) and incubated at same conditions until pupation. Finally, new formed pupae were regularly used to re-initiate PTM new laboratory rearing cycles.

3. Larval infection:

To examine the pathogenicity of extracted AcMNPV PIBs produced on *P. gossypiella*, the polyhedral suspension were mixed with triton-X100 at final concentration of 0.1% and used as infectious suspension, then small round potato tubers calculated as 2 gr potato per one PTM larva, were covered with the infectious suspension then treated tubers were air dried and placed inside a small ventilated plastic containers. Finally, PTM eggs with black head capsules, prior to hatch, were placed on contaminated tubers and incubated in plastic boxes at 27^{o}C and observed every other day. For control treatment, larvae were feed on potato tubers treated with sterile distilled water mixed with triton x100 0.1%, and larvae were observed for viral infection and resulted mortality up to 14 days post inoculation.

4. Examination of AcMNPV infected PTM larvae through optical microscope:

Smears from dead and abnormal dissected AcMNPV infected PTM larvae were prepared and stained with methylene blue, then examined using the oil immersion lens of the optical microscope to detect presence of PIBs.

5. Preparation of non-radioactive nucleic probe and hybridization:

A cloned fragment that concludes the polyhedrin gene, gift from Dr. Croizier (INRA, France), was used for the detection of AcMNPV genome among total extracted DNA from partially paralyzed and abnormal infected PTM larvae by applying a non-radioactive Dig DNA labeled nucleic probe which prepared according to the supplier protocol. The sensitivity of the prepared nucleic polyhedrin probe was

tested using different amount (Dilutions) of extracted AcMNPV DNA. For AcMNPV viral DNA detection, a randomly chosen individual representing dead, abnormal, and partially paralyzed PTM larvae were separately homogenized in TE buffer (0.01mmol/l Tris and 1mmol/l EDTA pH 8.0 then diluted up to 1/100. Then 10 **µ**l of diluted total insect DNA were subjected to the DNA dot blot hybridization with the prepared labeled probe.

6. Extraction and analysis of AcMNPV infected PTM total larval DNA:

The total insect DNA of abnormality, and partially paralyzed infected PTM larvae was separately extracted using techniques derived from Malone and McIvor (1995) as follow: Larvae were grinded in a buffer consisting of four parts of the homogenization solution (100mM/l NaCl, 200 mM/l sucrose, 10mM/l EDTA, and 30 mM/l Tris. HCl pH 8.0) and one part of lysis solution (250 mM EDTA, 2.5% SDS, 500mM/l Tris. HCl , pH 9.2) and incubated for 30 min. at 65^{O}C. Proteinase K was added to a final concentration of 1 µg /ml. Then the suspension was incubated at 37 ^{O}C for 3 hrs. Then potassium acetate was added to final concentration of 3 mM and the suspension incubated at 0 ^{O}C for 30 min. the final suspension containing total DNA was centrifuged in a microfuge for 1 min. at 13000g to remove insect cellular fragments and the DNA was precipitated with 2 volumes of 100 % glacial ethanol. Total infected larval DNA was digested to completion with *Pst*I endonuclease and electrophoretic mobility through 1% agarose gel was realized according to Maniatis *et al.* (1989), then separated digested DNA was transferred to a nitrocellulose membrane and immobilized by UV-cross linking. Finally, southern blot hybridization was realized according to Southern (1975) and Solanas and Escrich (1997), using the prepared DIG- labeled probe based on polyhedrin cloned sequence.

Results and discussion

As existence of a plant crop with a single economically important pest is unlikely to occur, developing of a complex multi species-baculovirus formulations that are able to control several pests simultaneously is highly recommended and require continuous search among available baculovirus species (Or isolates) to find the most effective one (s) for each pest in each region (Santiago Haase *et al.*, 2015). The *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) is a reference model for viral genetic research, biotechnological applications and biological control of several insects and can cause pathogenicity for a large number of lepidopteran species belonging up to twelve families and often representing important pests and wide geographically ranges (Groner, 1986). This virus can be laboratory mass produced and several of its host can be reared industrially on artificial diets (Vail *et al.*, 1972).

Preliminary trails to infect PTM larvae with AcMNPV at 10^4PIB/ml revealed neither appeared viral infection nor late disease symptoms, however, increasing the viral concentration to 2.5x 10^{5} PIB/ml resulted in up to 6.25 % mortality, 14.3 % less active and partially paralyzed larvae beside about 14.7 % absent or not found larvae compared to the control, and assumed to be died at their very early larval instar (Table 1).

Table (1): Infectivity of *Autographa californica* multicapsid nucleopolyhedrovirus to potato tuber moth *Phthorimaea operculella* larvae when applied at 2.5x 10^5 PIB/ml. Percentages of dead, less active and partially paralyzed abnormal larvae were counted based on alive larvae obtained from the control.

Nº of Rep.	Nº of tested PTM	Nº of Dead Larvae 2nd & 3rd instar	Nº of abnormal aged larvae	Total dead and abnormal Larvae	Nº of Alive	Nº of absent Individuals	Nº of individuals based on control	% of Dead	% of abnormal aged larvae	% of Total dead & abnormal	% of absent Individuals
1	200	8	26	34	101	25	160	5	16.25	21.25	15.625
2	200	13	20	33	107	20	160	8.125	12.5	20.625	12.5
3	200	9	23	32	102	26	160	5.625	14.375	20	16.25
Control	200	0	0	0	160	0	160	0		0	0
MEAN								6.25	14.375	20.625	14.7916667
total PTM	800	30	69	99							

Examination of discharged body fluid from dead PTM individuals through optical microscope, showed the presence of polyhedral inclusion bodies of nucleopolyhedro type similar to the used one for infection (Figure 1). However, examination of partially paralyzed and abnormal larvae by optical microscope, didn't give a clear configuration on the viral infection due to the limited amount of observed PIBs therefore, a Dig-labeled nucleic probe based on the AcMNPV polyhedrin gene sequence, was prepared and found to have detection sensitivity up to 5ng of viral DNA. Subjecting randomly chosen PTM larvae that represent dead, partially paralyzed, less active and abnormal larvae to dot blot hybridization with the prepared nucleic probe, revealed the presence of AcMNPV viral DNA in testing larvae of different categories (Figure 2).

Concerning the PTM partially paralyzed, and less active and abnormal larvae, the total insect DNA was extracted, digested with *Pst*I endonuclease then subjected to southern blot hybridization to confirm identity of the produced virus that multiplied at low rate in partially paralyzed larvae. The fact that PTM larva is a small sized insect, it was difficult to use standard techniques for extraction, purification and analysis of the viral DNA. To minimize the viral DNA loss, extraction steps were reduced by elimination of purification steps based on centrifugation as described in the original protocol and the total insect DNA was also extracted and caused interference with electrophoresed viral endonuclease restricted fragments, therefore, obtained fragments (bands) were directly transferred and hybridized the with AcMNPV polyherdin nucleic probe. In this way, it was possible to reveal a viral infection from infected larvae and to prove the identity of the multiplied AcMNPV in PTM larvae (Figure 3).

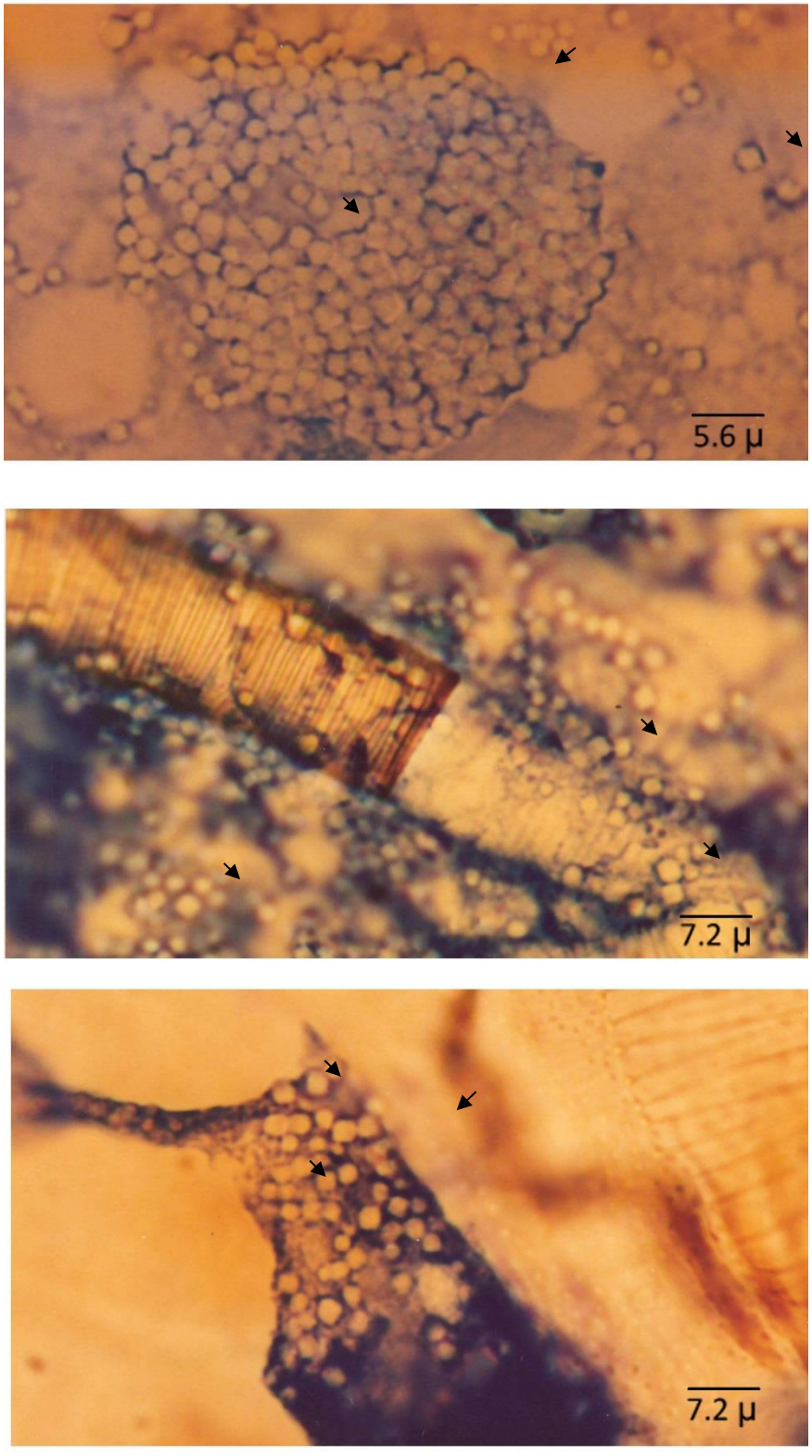

Figure (1): Micrograph of different tissues prepared from *Autographa californica* multicapsid nucleopolyhedrovirus infected PTM larvae and observed under optical microscope using oil immersion lens. Arrows indicate the formed polyhedral inclusion bodies .

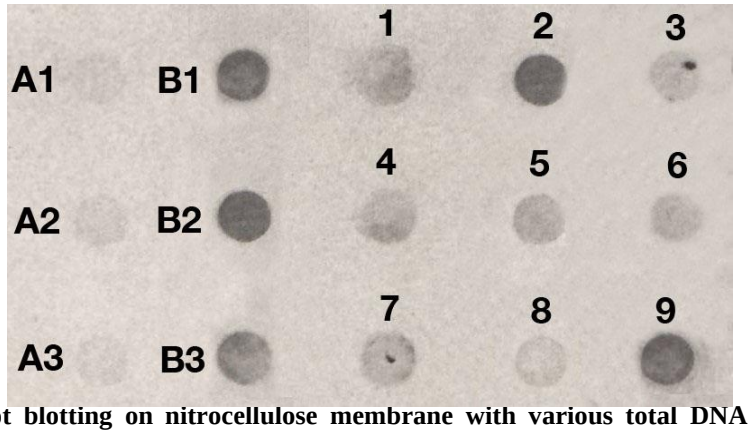

Figure (2): Dot blotting on nitrocellulose membrane with various total DNA extracted from *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) (B1, B2 and B3) used as positive control, and from healthy and non infected PTM larvae (A1, A2 and A3) used as negative control. Numbers from 1 to 9 represent total DNA from randomly chosen dead, abnormal, and partially paralyzed larvae showing different level of AcMNPV DNA presence.

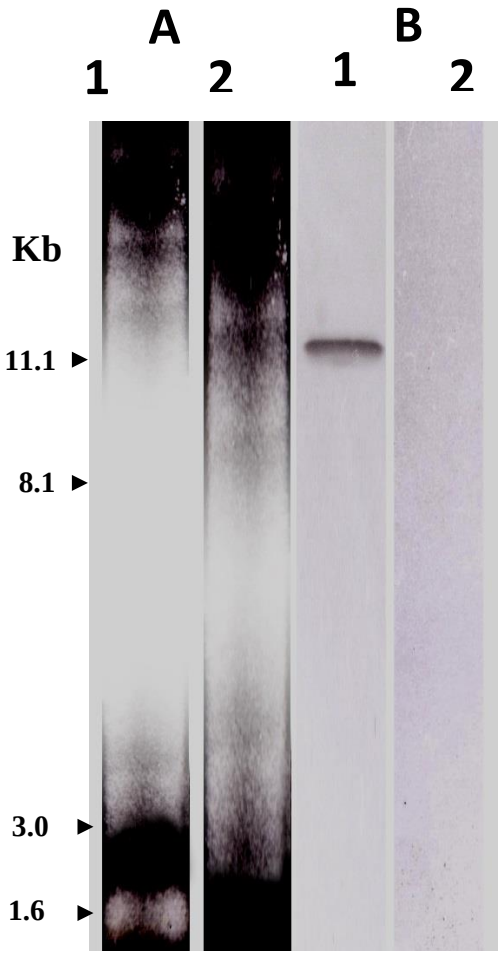

Figure (3): Southern blot hybridization using labeled *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) polyhedrin probe:
(A): Ethedium bromide stained gel after electrophoresis of *Pst I* digested total DNA extracted from AcMNPV infected potato tuber moth *Phthorimaea operculella* (PTM) (1) and from non-infected (Healthy) PTM larvae (2) Arrows indicate DNA fragments size.
(B): *Pst I* digested total DNA extracted from AcMNPV infected PTM (1) and from non-infected (healthy) PTM larvae (2) hybridized with the dig-labeled polyhedrin nucleic probe.

The obtained *in vivo* low pathogenicity of AcMNPV for PTM that fed on virus contaminated tubers was not correlated with the *in vitro* multiplication obtained with the same virus on different *P. operculella* cell lines resulting in high titers of the virus (Lery *et al.,* 1998). This contradiction between *in vitro* and *in vivo* results could be due to one or more of the following reasons; firstly, the fact that no artificial diet is available for PTM laboratory rearing, presented a big barrier that hinders the accurate determination of viral lethal dose. The insect behavior may also have certain effects because newly hatched larvae may be exposed early , during their crawling on tuber surface, to an optimum number of polyheda during the first hours after hatching and may die during this early stage, this probability could participate in explaining reasons for absence of about 14.7% of PTM larvae from treated replicates compared to the actual number of PTM larval individuals obtained from the non treated replicate (Control), while other larvae that penetrate the tubers and their ingested viral particles were not sufficient to initiate a lethal infection could explain the resulted abnormal and partially paralyzed aged larvae. The fact that death occurred on 1st and 2nd larval instars, while only abnormalities were found in advanced larval instar also could be due to the existence of different levels of susceptibility among different larval instar to the viral infection, and among the insect population itself.

In this regard, Sporleder (2003) found that susceptibility of PTM larvae to PhopGV decreases rapidly with larval age, and Salama *et al*. (1995) reported that the virulence of the bacterium *Bt* is similarly host age dependent. Finally, this phenomenon could be simply related to the used viral dose in relation to larval behavior. Carrera *et al.* (2008) stated that precise evaluation of Baculoviruses biological activity by the usual droplet method is not appropriate for boring insect larvae like the potato tuber moths because it remain on plant surfaces for a limited time that could not exceed several minutes before penetrating the substrate and get protected from viral inoculums on tuber surface. Also, when PhopGV biological activity was evaluated by immersing PTM eggs or the potato tubers in viral solutions, Sporleder *et al.*(2005) and Zeddam *et al* . (1999) found that both methods didn't allow the precise determination of the granule concentration presented on the potato surface and high variability between experiment´s data were obtained.

In conclusion, baculoviruses could represent an attractive solution to supplement and/or in certain cases replace chemical insecticides in IPM programs and several insect viruses, mainly belongs to Baculoviridae were registered as bio-insecticides to control pests in crops, forests, and pastures worldwide (Xiu-lian and Hui-yin, 2007). The NPVs are commonly isolated from infected insects collected from the fields. In case of PTM, only granuloviuses was isolated and successfully used, however, resistance to the virus has been recently documented. Therefore, the presented work aimed to initiate regular investigation for other possible potent baculoviruses that could overcome certain baculovirus technical application problems in field such as their relative slow speed of action, their restriction and specificity to their insect host, and relatively low virulence particularly for advanced larval instars. So, this work could propose a possibility to obtain a naturally recombinant candidate that expressing the advantages of narrow host range,

the PhopGV, and the speed of killing of AcMNPV.

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