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Transmission and effect of sublethal infection of granulosis virus (PbGV) on *Pieris brassicae* Linn. (Pieridae: Lepidoptera)

P. Sood, P. K. Mehta, K. Bhandari & C. S. Prabhakar

Department of Entomology, CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur, Himachal Pradesh 176 062, India

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Correspondence
P. Sood (corresponding author), Department of Entomology, CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur, Himachal Pradesh-176 062, India.
E-mail: pankajplp@rediffmail.com

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Abstract

A strain of granulosis virus from *Pieris brassicae* (Linn.) was isolated and characterized from the dry temperate region of Himachal Pradesh, India situated at an altitude of 2580 m above msl. The pest appears in the region during the summer months (May to September) and overwinters as pupae during the snow covered winter months. Carry over of virus inoculum as sublethal infections, could be an important mechanism for next season epidemics in the region. Effect of sublethal *P. brassicae* granulovirus infection on its host was therefore studied to understand the transmission mechanism. The third instar larvae were exposed to sublethal viral infection. The surviving larvae showed symptoms of virosis in the pupal and adult stages. The per cent pupation and adult emergence of sublethally infected larvae were significantly reduced and no adult emerged from the abnormal pupae. The mode of transmission of viruses from parents to offspring appeared to be through eggs (transovum). The emerging larvae from the eggs laid by treated females also showed symptoms of virosis. Complete mortality due to viral infection was observed at third instar stage in the offspring (F_1). The amplification of granulin gene using specific primer also showed the presence of virus in F_1 progenies of sublethally treated *P. brassicae* larvae, hence confirming vertical transmission.

Introduction

Baculoviruses have potential to be used as microbial control agents against lepidopteran pests due to their high pathogenicity, environmental stability, biosafety and host specificity. They comprise a large family of insect specific DNA viruses, divided into two genera, nucleopolyhedrovirus (NPV) and granulovirus (GV) (Moscardi 1999; Blissard et al. 2000) based on occlusion body (OBs) morphology. Baculoviruses have a long history as effective and environmentally benign insect control agents in field crops, vegetables, forests, and pastures (Moscardi 1999). Although much is known about the genetics of baculoviruses (Herniou et al. 2003), very little is known about their ecology and epidemiology, in particular their transmission which is the key to their persistence in the environment (Cory and Myers 2003). Vertical transmission or parent to offspring passage of a pathogenic microorganism is a phenomenon commonly observed with insect viruses. This type of virus transmission produces sublethal effects which could be extremely important from a pest control perspective as they offer a density-independent mechanism for retaining the virus within the pest population when numbers are low, as well as providing additional control benefits in terms of reduced host fitness (Rothman and Myers 1996). Vertical transmission of insect pathogens generally is not critical for maintaining their populations in nature nor does it correlate with disease prevalence (Fine 1984; Andreadis 1987) but rather it functions to transport these pathogens in the ecosystem and provide foci of new infections (Fuxa et al. 1992; Fuxa and Richter 1993).

Recently, a granulosis virus of *Pieris brassicae* (PbGV) has been isolated and characterized from the temperate region (situated at an altitude of 2590 m above msl, 31°25′56″ N latitude and 78°15′4″ E longitudes) of Himachal Pradesh and its pathogenecity against the pest has been proved (Sood 2004). Before this virus is exploited for the management of *P. brassicae*, there is a need to generate information on its ecology, epidemiology, transmission, etc. The present study was therefore conceptualized to study the effect of sublethal PbGV infection on its host and the transmission of the virus to the next generation. The study will be helpful for not only in effective management of the pest but also in understanding viral persistence in the absence of active host.

Material and methods

Rearing of experimental insect

The initial culture of *P. brassicae* was started from field collected eggs. The eggs were kept in sterilized Petri-plates (7.5 cm diameter) over a UV irradiated filter paper moistened with sterile distilled water (SDW) to prevent desiccation under laboratory conditions (Temp. 25 ± 2°C and RH 75–80%). Newly hatched larvae were transferred to fresh cabbage leaves surface sterilized with aqueous solution of sodium hypochlorite (0.05%) followed by three washings with SDW. The cabbage leaves were kept in ethanol washed and UV sterilized cage ($15 \times 15 \times 15$ cm^3). The first three larval instars were reared in the small cages ($15 \times 15 \times 15$ cm^3) while the later instars were reared in large cages ($45 \times 45 \times 55$ cm^3). Caterpillars in cages were provided with surface sterilized fresh cabbage leaves daily. The full grown caterpillars were transferred to the new cages for pupation. Two days old pupae were detached from the walls of cage and were kept in a batch of 20 pupa in each cage ($60 \times 60 \times 70$ cm^3) over a thick layer of UV irradiated filter paper for adult emergence. The adults were held in cages ($60 \times 60 \times 70$ cm^3) provided with cotton swabs soaked in honey solution, SDW and some flowering shoots of mustard as pollen source. Potted cabbage plants were kept in each cage for egg laying whenever needed.

Virus strain

The *Pieris brassicae* granulovirus (PbGV) used in the study was the local strain isolated and characterized from the dry temperate region of Himachal Pradesh by Sood (2004). The virus was multiplied in the host larvae in the Postgraduate Laboratory of Department of Entomology, CSK HP Agricultural University, Palampur, India (1200 m amsl). The virus was purified and the concentration of OBs in stock suspensions was determined by direct count using Helber bacteria haemocytometer (0.02 mm depth). A stock solution of PbGV with strength of 2×10^{12} OBs/ml (approximately) was prepared for transmission studies and stored in amber coloured bottles at 4°C. Serial dilutions (2×10^3 to 2×10^9 OBs/ml) were prepared from the quantified virus stock solutions in sterile distilled water.

Standardization of sublethal concentrations

A leaf disc bioassay technique, as described by Ballard et al. (2000), was used for standardization of sublethal concentrations of PbGV. Cabbage leaf discs (diameter 4.5 cm) were cut from fresh cabbage leaves collected from an insecticide free plot and washed with 70% ethanol. The discs were treated with different virus concentrations (2×10^3 to 2×10^9 OBs/ml) and shade dried. The treated discs were singly kept in the UV irradiated Petri dishes. Twenty-five pre-starved larvae (24 h) each of second, third and fourth instars were released on treated cabbage leaf discs for each treatment separately. Each treatment was replicated thrice and an untreated control (DSW) was also kept. After 24 h of feeding, the larvae were transferred to untreated cabbage leaves. The mortality counts were recorded after 72 h of release and thereafter. In case of uncertainty, larvae were prodded with a needle in order to test the characteristic disintegration of the larval tissue. The concentration indicating minimum percentage of larval mortality was determined as sublethal dose of PbGV.

Effect of PbGV sublethal infection

Three concentrations one above and one below the sublethal dose were used to study the effect on *P. brassicae* larvae and its further transmission to next generation.

Bioassay methodology

Cabbage leaf discs (4.5 cm diameter) were surface sterilized with ethanol and treated with 100 μl suspension of three PbGV concentrations, i.e. 2×10^4, 1×10^4 and 1×10^3 OBs/ml and shade dried. Treated discs were singly kept in the UV irradiated Petri

dishes. Twenty-five pre-starved (24 h) larvae of third instar were released on each disc for each concentration separately. Each concentration (treatment) was replicated four times and an untreated control (treated with sterilized distilled water) was also kept. After 24 h of feeding, the larvae were transferred to ethanol washed untreated cabbage leaves and were reared individually for further development. The leaves were changed daily and larval development was monitored during the course of studies. The larvae which showed symptoms of virus infection were separated out. The larvae that survived against virus challenge were reared in separate cages ($15 \times 15 \times 20$ cm^3) until pupation. The number of healthy and diseased pupae were recorded from both virus treated as well as control treatment. The pupae from each treatment were then kept separately in cages ($45 \times 45 \times 55$ cm^3) for adult emergence. The data on adult emergence was recorded daily for all treatments.

Effect on fecundity, hatchability and larval development of F_1

The females emerged out from virus treated as well as control treatments were monitored for oviposition and the number of eggs laid on potted plants kept in cages ($45 \times 45 \times 55$ cm^3) was recorded. The egg masses from each treatment were clipped along with leaves and kept separately on a thick moistened filter paper in a Petri-plate (7.5 cm). Observations on hatchability were recorded periodically to work out the per cent hatchability. The neonate larvae were separately maintained on cabbage leaves for further development and their developmental parameters were recorded. The larvae that died of sublethal virus infection during the course were kept in Eppendorf tubes under refrigerated conditions after initial microscopic examination of OBs for final confirmation of PbGV by molecular techniques using primer specific to granulin gene (Burden et al. 2002). The primer sequences used were:

Consensus F primer: 5′ CAAGATCAAGGAATTCGCACCCGACGTA3′

Consensus R primer: 5′GTTCTAGTTCCTTAAGCGTGGGCTGCAT3′

Extraction of genomic DNA and PCR amplification

Total genomic DNA of PbGV was extracted following the procedure of Murray and Thompson (1980). For extracting total genomic DNA, healthy fifth instar larvae, adults (control), sublethally treated fourth instar larvae, adults and their off-springs (second instar larvae) were taken, washed with deionized water and then dried on filter paper. The larvae were then ground individually in pestle and mortar with liquid nitrogen and maintained in separate tubes for extraction of virus DNA. About 100 mg powder from these tubes was transferred to Eppendorf tube containing 680 μl of CTAB extraction buffer maintained at 60°C and mixed thoroughly by turning the tubes up and down. The tubes were then incubated at 60°C for 1 h with gentle mixing after every 10 min. To each tube equal volume (680 μl) of chloroform: isoamyl alcohol (24 : 1) was added. The contents were mixed gently and centrifuged at 10 000 ***g*** for 10 min in high speed refrigerated centrifuge (REMI, India) at 4°C. Aqueous phase was transferred to new tubes, 450 μl prechilled isopropanol was added and kept at −20°C for 20–30 min to precipitate the DNA. Tubes were then spun at 10 000 ***g*** for 10 min and supernatant was decanted. The DNA pellet was washed three times with 70% ethanol, dried and dissolved in 100 μl of Tris EDTA buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0). RNase at 10 mg/ml (MBI Fermentas) was added and the emulsion was incubated for half an hour at 37°C. DNA was stored at −20°C for further use.

Amplification of virus DNA

The PCR amplification was carried out for confirmation of transmission of PbGV in survivors of virus challenge using granulin gene specific primers (Burden et al. 2002). The PCR reaction was performed in 0.2 ml PCR tubes with 25 μl reaction volume containing 20 ng of DNA template, 20 pmol of each primer in 25 mM $MgCl_2$, 10 mM of each deoxyribonucleoside triphosphate (Fermentas), five units of *Taq* polymerase [Life Technologies (India), Pvt. Ltd, Delhi, India] and 10 × reaction buffer. Amplifications were performed using thermal cycler (GeneAmp PCR system 9700; Applied Biosystems, Foster City, CA) with an initial denaturation step of 2 min at 94°C followed by 40 cycles at 94°C for 30 s, 65°C for 1 min, 72°C for 30 s and a final elongation step at 72°C for 10 min. The product was separated in a 1.2% (w/v) agarose gel in TAE buffer (40 mM Tris–acetate, 1 mM EDTA). DNA ladders of 100 bp (Bangalore Genei, Peenya, Bangalore) and lambda DNA/*Eco*RI–*Hin*dIII double digest (MBI Fermentas, Burlington, ON, Canada) were used as molecular weight markers. The gels were run at 80 V for 1 h using Bangalore Genei power pac system. The gels were

stained with ethidium bromide (0.5 μg/ml) for 10 min after electrophoresis. The gels were viewed and images were captured using gel documentation system (AlphaImager 2200; Alpha Infotech Corporation, San Leandro, CA).

Statistical analysis of data

The data recorded for different parameters in the present study were analysed statistically by using the technique of analysis of variance for Completely Randomized Design as described by Gomez and Gomez (1984). The treatment means were compared at 5% level of significance by least significant difference test described by Gomez and Gomez (1984).

Results

Effect of sublethal infection

Larval mortality, pupation and adult emergence

Sublethal infection of PbGV in third instar larvae of *P. brassicae* at three concentrations i.e. 2×10^4, 1×10^4 and 1×10^3 OBs/ml resulted in 63.9%, 61.5% and 55.6% larval mortality, respectively (table 1). The larvae which tolerated the sublethal dosages resulted in 49.2%, 37.0% and 19.8% abnormal pupae in the three treatments respectively, in comparison to 1.2% in case of control. Afterwards, the normal pupae from sublethally treated larvae at three doses resulted in 35.5%, 19.6% and 9.9% abnormal adults in comparison to 1.8% in case of untreated control. Sex ratio of adults emerged were found to deviate slightly though non-significantly in favour of females in the treated ones compared to the control. The hatchability of the eggs laid by females in different treatments was observed to be 83.6%, 82.4% and 85.8%, respectively which were significantly lower in comparison to 94.5% in case of control (table 2). The larval mortality of the off-springs at the three tested dosages was observed to vary from 29.8% to 34.3% and 86.0% to 89.0% in the first and second instar, respectively. However, complete mortality at third instar stage due to virosis was observed in the sublethally treated *P. brassicae* off-springs.

Table 2 Effect of sublethal infection of PbGV on egg hatchability and percent larval mortality in the F_1 generation

Concentration (OBs/ml)	Egg hatchability (%)	Corrected larval (%) mortality in F_1 generation over control		
		First instar	Second instar	Third instar
2×10^4	83.6 ± 1.63	32.9	89.0	100
1×10^4	82.4 ± 1.58	34.3	86.0	100
1×10^3	85.8 ± 1.81	29.8	88.9	100
Control	94.5 ± 1.80	–	–	–
$LSD_{0.05}$	3.21			

$LSD_{0.05}$ = Least significant differences test between treatment at 5% level of significance.

Developmental attributes of P. brassicae

The data presented in table 3 reflects the effect of sublethal infection of PbGV on developmental attributes of *P. brassicae*. The sublethal inoculation of third instar larvae of *P. brassicae* at three concentrations prolonged the duration of fourth instar (4.30, 4.42 and 4.42 days) significantly in comparison to control (3.15 days). Fifth instar duration was also observed to be prolonged by 1.25, 1.17 and 1.00 days in comparison to control. The larvae surviving the sublethal dosages showed increased pupal duration of 1.49, 1.89 and 1.79 days than the control and increased adult longevity (7.47–7.65 days) in comparison to control (6.00 days). The incubation period of the eggs in F_1 was also increased significantly in all the three concentrations (6.00–6.27 days) in comparison to 4.1 days in control. Likewise, the duration of first as well as second

Table 1 Effect of sublethal infection of PbGV on per cent mortality, pupation and adult emergence of *P. brassicae*

Concentration (OBs/ml)	Corrected mortality[1] (%)	Pupation (%)	Normal pupae (%)	Adult emergence (%)	Normal adult (%)	Sex ratio (female:male)
2×10^4	63.9	36.1 ± 1.41	50.8 ± 1.80	15.5 ± 1.29	64.5 ± 2.12	1 : 0.64
1×10^4	61.5	38.5 ± 1.80	63.0 ± 2.20	20.5 ± 1.17	80.4 ± 0.91	1 : 0.62
1×10^3	55.6	44.4 ± 1.96	80.2 ± 1.15	30.5 ± 1.80	90.1 ± 1.34	1 : 0.66
Control	–	84.5 ± 1.50	98.8 ± 1.92	83.5 ± 1.5	98.2 ± 0.98	1 : 0.72
$LSD_{0.05}$		3.67	3.38	3.70	4.04	NS

$LSD_{0.05}$ = Least significant differences test between treatment at 5% level of significance.
[1]Based upon n = 100, NS, non-significant.

Table 3 Effect of sublethal infection of PbGV on developmental attributes of *P. brassicae*

	Duration (in days)[1]							
				Adult longevity		F1 generation		
Concentration (OBs/ml)	Fourth instar	Fifth instar	Pupae	Male	Female	Incubation period	First instar	Second instar
2×10^4	4.30 ± 0.96	5.65 ± 0.73	12.15 ± 0.73	7.47 ± 0.45	10.57 ± 0.11	6.22 ± 0.58	5.12 ± 0.87	5.12 ± 0.34
1×10^4	4.42 ± 0.21	5.57 ± 0.88	12.55 ± 0.50	7.65 ± 0.78	10.77 ± 0.86	6.00 ± 0.35	5.10 ± 0.19	5.12 ± 0.29
1×10^3	4.42 ± 0.22	5.40 ± 0.31	12.45 ± 0.26	7.60 ± 0.96	10.97 ± 0.34	6.27 ± 0.23	5.15 ± 0.54	5.17 ± 0.18
Control	3.15 ± 0.34	4.40 ± 0.50	10.66 ± 0.97	6.00 ± 0.23	9.17 ± 0.25	4.10 ± 0.68	3.15 ± 0.18	3.82 ± 0.28
$LSD_{0.05}$	0.80	0.81	1.01	1.15	1.21	0.85	0.49	0.54

[1]Mean of 10 observations with four replications.

$LSD_{0.05}$ = Least significant differences test between treatment at 5% level of significance.

Table 4 Effect of sublethal infection of PbGV on reproductive parameters and fecundity of *P. brassicae*

	Duration of reproductive phase						
Concentration (OBs/ml)	Females that laid eggs (%)	Pre- mating period (days)	Mating period (minutes)	Pre-oviposition period (days)	Oviposition period (days)	Post-oviposition period (days)	Average fecundity/female
2×10^4	20.25 ± 1.40	2.10 ± 0.04	62.95 ± 1.50	4.32 ± 0.09	3.35 ± 0.03	1.07 ± 0.009	21.40 ± 1.09
1×10^4	21.25 ± 1.52	2.15 ± 0.06	62.70 ± 1.01	4.37 ± 0.02	3.37 ± 0.07	1.05 ± 0.008	21.50 ± 1.50
1×10^3	22.00 ± 1.01	2.32 ± 0.07	62.82 ± 2.52	4.42 ± 0.05	3.27 ± 0.05	1.12 ± 0.004	22.80 ± 1.45
Control	31.00 ± 1.60	2.12 ± 0.04	34.67 ± 1.44	2.07 ± 0.01	4.25 ± 0.08	1.07 ± 0.007	32.50 ± 1.23
$LSD_{0.05}$	3.7	NS	6.87	0.73	0.70	NS	4.7

$LSD_{0.05}$ = Least significant differences test between treatment at 5% level of significance. NS, non-significant.

instar in the off-springs indicated significant prolongation as compared to control.

Reproductive parameters and fecundity of P. brassicae

The data on effect of sublethal effect on reproductive parameters and fecundity of emerging adults revealed that 20.25–22.0% of females in F_1, laid eggs as compared to 31% in control though the difference with respect to pre-mating and post-oviposition periods were found to be non-significant (table 4). The mating period (62.7–62.9 min), pre-oviposition period (4.32–4.42 days) and oviposition period (3.27–3.35 days) were observed to differ significantly as compared to control (34.37 min, 2.07 and 4.25 days), respectively.

PCR amplification of granulin gene

The surviving normal larvae and adults derived from the sublethally inoculated larvae were investigated to ascertain the presence of viral DNA in survivors. The PCR amplification of genomic DNA with specific primers confirmed the presence of PbGV DNA (a product of approx. 460 bp as expected) in surviving normal larvae, adult and F_1 larvae (fig. 1).

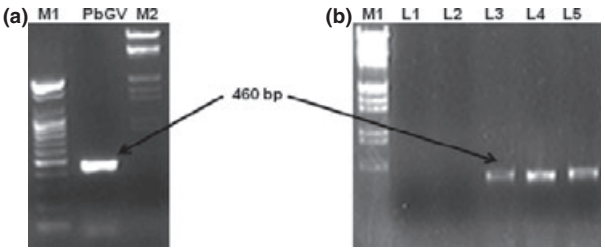

Fig. 1 Agarose gel electrophoresis of PbGV granulin-specific PCR product showing the amplification of granulin gene from (a) GV infected *Pieris brassicae* larvae and (b) healthy larval (L1) & adult (L2) DNA Showing no amplification of granulin gene, and L3, L4 & L5 sublethally GV infected larvae (L3), adult (L4) and second instar F_1 larval (L5) DNA (showing granulin gene amplification). (a) M1:100bp Ladder, M2: λ DNA *Eco*RI/*Hin*dIII double digest; (b) M1: λ DNA *Eco*RI/*Hin*dIII double digest.

Discussion

Exposure of third instar larvae of *P. brassicae* to sublethal PbGV dosages resulted in larval mortality ranging from 55.6% to 63.9% and the impacts were also evident in the surviving pupae and adults. The findings are in agreement with the report of

Easwaramoorthy and Jayaraj (1989), who observed that sublethal infection of granulosis virus on sugarcane shoot borer, *Chilo infuscatellus*, caused reduction in per cent pupation and adult emergence. Melamed-Madjar and Raccah (1979) also reported that pupae derived from sublethally treated *Sesamia nonagroides* larvae had higher rate of incomplete pigmentation due to SnGV infection and no adults emerged from these pupae. The rate of vertical transmission of NPVs in eight species belonging to *Mythimna*, *Pseudoplusia, Spodoptera, Lymantria*, and *Trichoplusia* varied from 0.5% to 57.1% under laboratory conditions (Kukan 1999).

Vertical transmission rates of around 12% was reported by Zhou et al. (2005) in *H. armigera* when HaSNPV was applied in field, however rates increased to around 30% when HaSNPV was applied under laboratory conditions. This might be due to the lower virus-induced mortality among F_1 progeny from paired bollworm adults with either both parents or one parent treated with HaSNPV.

The sex ratios of adults emerged from sublethally treated third instar larvae though deviated slightly in favour of female in comparison to control in the present study, however the differences were non-significant. The earlier findings of Easwaramoorthy and Jayaraj (1979), reported deviation in sex ratio of sugarcane shoot borer, *Chilo infuscatellus* from expected 1:1 to 1:5.70 (Female:Male). Melamed-Madjar and Raccah (1979) had also reported similar deviation in sex ratio (1:5) of corn borer *S. nonagroides* due to GV infection. The change in sex ratio pattern in present findings to that of earlier reports could be attributed to species, which need to be further elucidated.

The findings on substantial prolongation in larval, pupal and adult life after sublethal viral infection are in concurrence with Hua Lu et al. (2001), who reported that developmental duration of virus fed larvae of diamond backmoth, *Plutella xylostella* was lengthened due to GV infection. The results of Tatchel (1981) reporting extended larval instar duration of *Pieris rapae* due to GV infection also support the present findings.

The data on average fecundity of sublethally inoculated *P. brassicae* manifested that 20.25–22.00% of viroid females laid eggs as compared to 31% in control with significant reduction in average fecundity. The present findings draw considerable support from the findings of Melamed-Madjar and Raccah (1979), who also reported that sublethal inoculation of SnGV on corn borer *Sesamia nonagroides* larvae caused substantial reduction in fertility rate of emerging adults. Matthews et al. (2002), also observed a 15% reduction in the number of eggs laid by adults developed from sublethally treated larvae of tomato moth, *Lacanobia oleracea.* The reduction in fecundity could be attributed to the hormonal and enzymatic changes associated with virus infections affecting the production or development of eggs and sperms (Cory et al. 1997).

The reduction in corresponding hatchability of eggs laid by the surviving females and complete mortality in the third instar stage could be attributed to virosis. Easwaramoorthy and Jayaraj (1989) too observed 50% diseased *C. infuscatellus* larvae from the infected parents. The findings indicated the vertical transmission of GV from infected adult to offspring through eggs. Laur and Quenin (2003) also reported 50.8% larval mortality in the F_1 population of codling moth *Cydia pomonella* obtained from CpGV infected parent. However, David and Taylor (1976) observed that PbGV failed to transmit vertically from one to next generation in *P. brassicae.* Similarly, Matthews et al. (2002) and Etzel and Falcon (1976) contradicted on transovum transmission of granulosis virus and observed the larval mortality of F_1 generation not to vary significantly in comparison to control. The lower concentrations used in these studies could be the reason for not transmitting the virus inocula to the next generation. It might have not caused mortality in the F_1 population as optimum level of inoculum is required to cause larval mortality. The amplification of virus DNA in the off-spring in the present study without doubt confirms the vertical transmission of PbGV in survivors of virus challenge. The findings are in line with those of Burden et al. (2002), who detected RNA transcripts for the *Plodia interpunctella* GV granulin gene in F_1 larvae of *P. interpunctella* obtained from adults raised through sublethally treated larvae. Under field conditions, egg masses produced infected larvae at rates ranging from 2% to 80%, whereas decontaminated egg masses yielded only 0.1–9% infected larvae (Kukan 1999). In view of implications of transmission mechanism of PbGV, there is a need to further investigate the true mechanism of transmission of sublethal infection for its subsequent utility in the management of pest.

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