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Research Article

Granulovirus of semilooper, *Achaea janata* L. (Lepidoptera: Noctuidae): its bioefficacy and safety in mammalian toxicity tests

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ABSTRACT: Bio-efficacy of granulovirus (GV) infecting the semilooper, *Achaea janata* L., a serious defoliating pest of castor bean, *Ricinus communis* L., was established through leaf disc bioassays. Ovoid occlusion bodies (OB) of *A. janata* GV (AjGV) measuring 480±20.9 by 240±13.7 nm contained a singly embedded nucleocapsid. Estimated molecular weight of viral genomic DNA obtained with three restriction enzymes was in the range of 124 and 127 kb with a mean of 125.7±0.9 kb. Estimated median lethal concentration (LC_{50}) of AjGV mother culture to second instar larvae was $2.2\pm0.21\times10^6$ OB/ml or 85.4±9.7 OB/mm^2 leaf, while for the formulation it was $3.1\pm0.89\times10^6$ OB/ml or 123.3±35.1 OB/mm^2 leaf. Kaplan-Meier survival estimate of median lethal time (LT_{50}) for second instars was 7 days at 5×10^8 OB/ml concentration and 12 days at 5×10^6 OB/ml. AjGV mother culture and formulation were found to be non-toxic in all the mandatory mammalian toxicity tests. This study fulfills the key data requirements on identification of the AjGV Hyderabad isolate, its bio-efficacy and safety in mammalian toxicity tests.

KEY WORDS: Granulovirus, baculovirus, bioassay, *achaea janata, ricinus communis*

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INTRODUCTION

Castor, *Ricinus communis* L., is an important industrial oilseed crop in India which is the largest producer in the world with an area of about one million ha and a production of 1.2 million tonnes that meets 70% of the global demand for castor oil (Damodaram and Hegde, 2010). Pest damage is one of the major constraints in castor production. Semilooper, *Achaea janata* L. (Lepidoptera: Noctuidae) is a polyphagous pest feeding on several ornamental and fruit crops, but castor was the major host (Gaikwad and Bilapate, 1992). Semilooper causes extensive defoliation in castor during its peak vegetative growth phase and also feeds on tender capsules in developing spikes causing 30-40% yield loss (Parthasarathy and Rao, 1989). Granuloviruses are insect-specific viruses (Baculoviridae: Beta-baculovirus) exclusively found in Lepidoptera and were exploited as environment friendly pest control agents in view of their high host specificity and mammalian safety (Lacey *et al.*, 2008a). A naturally occurring baculovirus infecting *A. janata* in laboratory cultures was first isolated and reported as a mixed infection (Vimala Devi, 1992), later characterized as a granulovirus (GV) (Singaravelu and Ramakrishnan, 1998) and its symptoms of infection studied (Prasad *et al.*, 2001). However, dosage-mortality and time-response relationships have not been reported so far for *A. janata* GV (AjGV). Also, mammalian toxicity tests which are mandatory for its registration in India as per the Insecticides Act, 1968 have not been attempted. In this study, the identity of the virus occlusion bodies (OB) was ascertained by electron microscopy and by performing restriction endonuclease (REN) analysis of viral genomic DNA. We report the bio-efficacy of both the mother culture and formulation of the Hyderabad isolate of AjGV against second instar larvae along with results of mammalian toxicity tests. These findings may aid in revising the existing LC_{50} standard for AjGV.

MATERIALS AND METHODS

Insect culture

Insects for bioassay experiments were drawn from a healthy culture of *A. janata* established with field collected larvae from our research farm at Hyderabad in June, 2009. The larvae were reared on its natural host, castor leaves for three generations in the insect mass

rearing facility maintained at 27±1°C, 65±5% RH and 14:10 h (L:D) photoperiod prior to starting assays in September, 2009 and continuing into 2010.

Virus production

AjGV Hyderabad isolate was propagated *in vivo* by administering a dose of 5×10^4 OB/ml to healthy 6 day old larvae. Diseased larvae were harvested between 7 and 10 days after infection in Single Distilled Water (SDW) and kept frozen till further use. Thawed samples were homogenized and processed through differential centrifugation at 400g for 5 min to remove insect host debris and 11,000g for 30 min to pellet the virus and suspended in sterile distilled water (SDW). Virus serial dilutions were enumerated using a haemocytometer with 0.02 mm depth (Weber Scientific International Ltd, UK) under dark field at 20x magnification (350BR, Olympus, Japan). Mean OB/ml in the stock was calculated from three independent estimations. An aqueous suspension formulation was prepared as described earlier (Prasad *et al.*, 2010) containing 0.02% AjGV active ingredient, 0.05% sodium salt of alkyl aryl sulfonate as anionic surfactant and 5×10^9 OB/ml count.

Confirmation of virus OB by both scanning electron microscopy (JSM 5600, JEOL Ltd., Japan) and transmission electron microscopy (H-7500, Hitachi, Japan) and also identity of viral DNA by its fragment pattern with EcoRI, BamHI and HindIII restriction enzymes were carried out as per the method of Singaravelu and Ramakrishnan (1998). Restriction endonuclease analysis (REN) was repeated thrice and molecular weights of fragments estimated using Vision-capt software, Vilber Lourmat, Japan.

Bioassays

Clean castor leaves were cut into leaf discs of 80 mm diameter to give 5028.6 mm^2 surface area. Using a micropipette 100 µl of virus dilution was applied on the adaxial surface and spread uniformly with a bent glass rod and air dried before applying another 100 µl of virus similarly on the abaxial surface. Log serial dilutions tested were 5×10^4, 5×10^5, 5×10^6, 5×10^7, and 5×10^8 OB/ml to give an approximate concentration of 1.9, 19, 198, 1988 and 19886 capsules per mm^2 leaf inclusive of both surfaces. Control discs were treated with SDW containing 0.02% Tween-80®. Treated leaf discs were placed in sterile petri dishes (9 cm diameter) and starved second instar larvae were released and incubated at 27±1°C, 65% RH, 14:10 h (L:D) photoperiod in an environmental test chamber (Sanyo Co., Japan). Thirty to fifty larvae were used in each treatment. In all six independent bioassays were carried out involving three each for mother culture and formulation of AjGV. Fresh untreated leaves were offered after 48 h to larvae transferred to 1 liter jars. Daily mortality was recorded. Dead larvae were removed and wet smear was microscopically examined to ascertain the cause of death.

Estimation of leaf consumption by virus infected larvae

Thirty second instar larvae were tested in three replications of ten each by administering 5 serial dilutions of virus (5×10^4 to 5×10^8 OB/ml) as described earlier. Extent of feeding was measured using a leaf area meter (LI-COR, USA). Percentage reduction in leaf consumption per replication was computed over control as [(C-T)/C] ×100, where, C and T are percentage leaf area fed per larva in control and virus treatment, respectively. Data was analyzed using one-way ANOVA (SAS Institute, 2009).

Estimation of LC_{50} and LT_{50}

Log transformed virus test concentrations (OB/ml or OB/mm^2 leaf disc) were regressed on cumulative mortality data adopting Probit analysis to estimate LC_{50} value at 95% confidence limits (Finney, 1971) using SPSS Base 16.0 software (SPSS Inc., 2007). Mortality in control group was included in the Probit analysis. Time-mortality response of larvae in bioassays with virus formulation till death of larvae or pupation (until censored) was analyzed to estimate LT_{50} at 95% confidence limits through Kaplan-Meier product limit survival function analysis using PROC LIFETEST (SAS Institute, 2009), and the probabilities of survival were plotted over time. Log-rank test was used to test the homogeneity of different virus concentrations, while significant differences between pairs of virus concentrations were found using Sidak's adjustment for multiple-comparison test.

Mammalian toxicity tests

Mammalian toxicity tests with mother culture and formulation samples of AjGV were conducted following the guidelines approved by the Central Insecticide Board – Registration Committee (CIB-RC), India for bio-pesticides [Baculoviruses, Nucleopolyhedrovirus (NPV) and Granuloviruses (GV)] (http://cibrc.nic.in/guidelines.htm) at the International Institute of Bio-technology and Toxicology (IIBAT), Padappai, Tamil Nadu, India which is a certified test facility (http://indiaglp.gov.in/TestFacilities.html).

RESULTS AND DISCUSSION

Ovoid virus OB measured 480±20.9 by 240±13.7 nm (mean±SE) in TEM with a singly embedded nucleocapsid (Fig. 1). REN analyses of viral genomic DNA yielded consistent profiles of 12, 18 and 20 fragments (Fig. 2) with corresponding genome size estimates of 127, 124 and 126 kb with BamHI, EcoRI and HindIII, respectively (Table 1),

with a mean genome size of 125.7±0.9 kb. REN fragment pattern with BamHI, EcoRI and HindIII were similar to the pattern reported earlier by Singaravelu and Ramakrishnan (1998).

Microscopic examination of smears from dead larvae in the bioassays revealed OB in all the cases. Probit analysis of dose-mortality response data for AjGV-Hyderabad isolate after adjusting mortality in control larvae showed that the rate of virus induced death was different at various time periods. In each case the time selected for calculation of LC_{50} values was the point (in days) at which the sigmoid cumulative mortality-time response curve flattened off (Bliss, 1952). This happened with cumulative

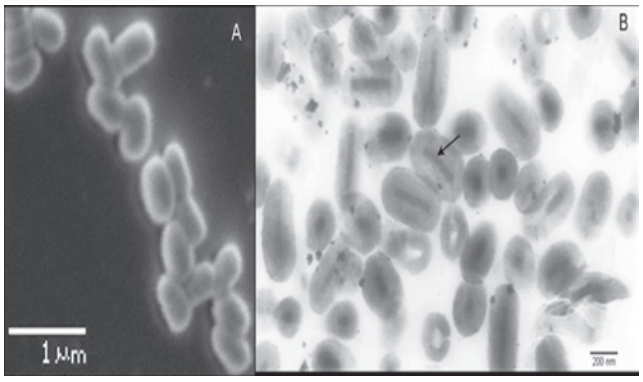

Fig. 1: (a) SEM image of ovoid virus occlusion bodies of *Achaea janata* GV, (b) TEM image showing singly enveloped nucleocapsid (arrow)

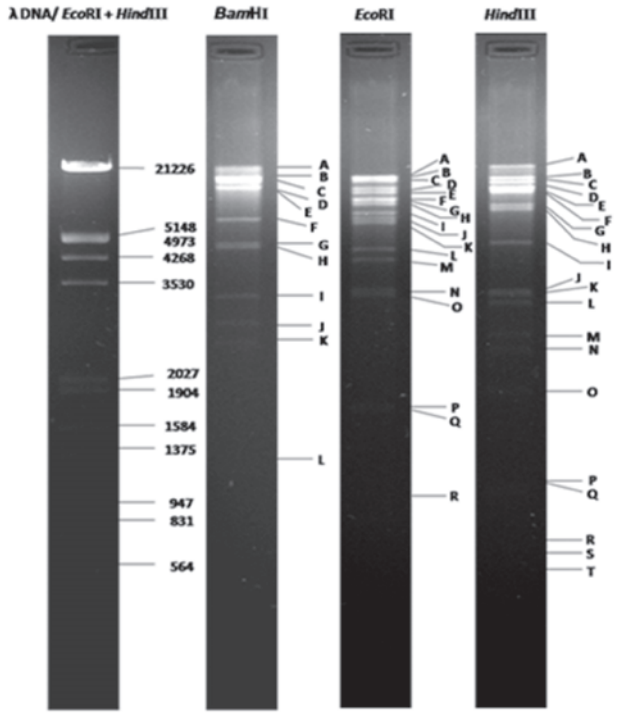

Fig. 2: Restriction endonuclease profiles of *Achaea janata* GV genomic DNA

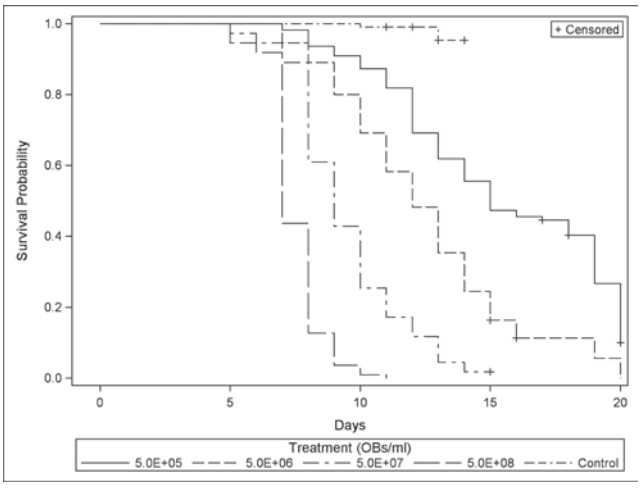

Fig. 3: Survival probability over time for *Achaea janata* second instar larvae exposed to GV

mortality between 10 and 12 days after infection in all the assays. With mother culture, LC_{50} ranged between 2.0×10^6 and 2.4×10^6 OB/ml (mean±SE of $2.2\pm0.21\times10^6$ OB/ml) which is equivalent to a concentration of 76.4 to 95.7 OB/mm^2 (mean of 85.4±9.7 OB/mm^2) on treated leaf discs (Table 2). In case of aqueous formulation, LC_{50} ranged between 1.6×10^6 and 4.7×10^6 OB/ml (mean of $3.1\pm0.89\times10^6$ OB/ml) which is equivalent to a concentration of 64.6 to 186.0 OB/mm^2 (mean of 123.3±35.1 OB/mm^2) on treated leaf discs (Table 2). In all the cases, Pearson c^2 goodness-of-fit test significance was greater than 0.15 and no heterogeneity factor was required to calculate the confidence limits. Slope values for mother culture ranged between 0.90 and 1.02 (mean of 1.05±0.21) while the same values for the formulation was between 0.79 and 1.36 (mean of 1.14±0.18) (Table 2). These values were within the range for pathogens that do not rely on a toxic mode of action (Burges and Thomson, 1971).

Kaplan-Meier survival function analysis (Fig. 3) yielded LT_{50} values of 7, 9, 12 and 15 days with confidence intervals of 7-8, 9-10, 11-13 and 14-18 days for 5×10^8, 10^7, 10^6 and 10^5 OB/ml concentrations, respectively. Cumulative mortality at 5×10^4 OB/ml was less than 50% and hence not included in the analysis. Log-rank test showed significant difference ($c^2 = 375.55$, df = 3, $p < 0.0001$) among virus concentrations tested. Further, Sidak's adjustment for multiple-comparison test showed significant differences between all the pairs of virus concentrations ($p < 0.0001$). Dose related time-mortality response observed with AjGV (Fig. 3) was similar to that observed with other baculoviruses (Van Beek *et al.*, 1988).

Table 1: Molecular weights of AjGV genomic DNA restriction fragments in kilobase pairs (± SEM)

Fragment	*Bam*HI	*Eco*RI	*Hind*III
A	39.3 ± 0.1*	16.2 ± 0.3	20.3 ± 0.2
B	17.3 ± 0.1	14.4 ± 0.6	16.0 ± 0.3
C	16.2 ± 0.1	12.5 ± 0.4	14.2 ± 0.0
D	14.9 ± 0.4	11.0 ± 0.4	12.1 ± 0.3
E	13.3 ± 0.2	9.6 ± 0.2	10.9 ± 0.1
F	6.9 ± 0.4	8.5 ± 0.1	9.9 ± 0.3
G	4.9 ± 0.0	7.8 ± 0.2	8.6 ± 0.4
H	4.7 ± 0.1	7.2 ± 0.2	7.8 ± 0.3
I	3.3 ± 0.1	6.6 ± 0.1	4.8 ± 0.1
J	2.8 ± 0.1	6.0 ± 0.1	3.4 ± 0.0
K	2.5 ± 0.1	5.3 ± 0.6	3.3 ± 0.1
L	1.2 ± 0.1	4.4 ± 0.2	3.1 ± 0.1
M		3.9 ± 0.4	2.6 ± 0.1
N		3.4 ± 0.0	2.4 ± 0.0
O		2.7 ± 0.9	1.9 ± 0.1
P		1.7 ± 0.0	1.2 ± 0.0
Q		1.5 ± 0.4	1.1 ± 0.0
R		1.0 ± 0.0	0.8 ± 0.1
S			0.7 ± 0.0
T			0.6 ± 0.0
Total	127.3 ± 0.8	123.8 ± 2.8	125.8 ± 1.1

[a]Means of three independent determinations, * Fragment size determined by digesting eluted fragment with *Eco*RI.

Type II GVs like *Cydia pomonella* GV are highly infectious to their hosts with a median lethal dose (LD_{50}) of 1-5 OB/larva (Payne, 1986), while others like *Agrotis segetum* GV have an LD_{50} of 10^4 OB for neonate larvae (Allaway and Payne, 1984). *Spodoptera litura* GV reported from India had an LC_{50} value of 1740.1 OB/mm^2 diet against fourth instar larvae and the estimated LT_{50} was 14 days at the LC_{50} concentration (Subramanian *et al*., 2005). We have observed *A. janata* larvae to be voracious feeders. For this reason, it was important to assess the extent of leaf feeding in virus treated larvae. There was no significant difference in leaf area fed across all the virus concentrations at 1 and 2 days after treatment. However, virus tested at or above LC_{50} value caused significant reduction in feeding by larvae (F=12.26; df=4,10; P=0.001) ranging from 72.9 to 86.6% at 6 days after exposure (Fig. 4). Similar levels of decrease in food consumption have been reported for several GVs (Tatchel, 1981; Bhandari *et al.*, 2010). Healthy full grown *A. janata* larvae weigh over 1000 mg by 10 days at 27°C and measure over 7 cm in length. Although, the range of LC_{50} and slope values reported here for AjGV mother culture and formulation were closer to that reported for *Plutella xylostella* GV-Taiwan isolate against second instar larvae(Abdul Kadir *et al*., 1999), the LT_{50} value of 12 days for AjGV at 5×10^6 OB/ml (nearest to LC_{50} value) was 2.4 times longer at 27°C. The difference in speed of action may lie in the range of larval tissues infected by a virus with higher virulence associated with a broader range of tissues infected (Lacey *et al*., 2008b) which needs to be investigated for AjGV. Observed prolongation in larval duration in AjGV infected survivors up to 23 days at or below LC_{50} concentration (10^6 OB/ml) was also observed for other GVs (Hunter-Fujita *et al*., 1998).

AjGV mother culture and formulation were found to be non-toxic and non-virulent in acute oral, pulmonary

Table 2. Bioefficacy of GV mother culture and formulation to second instar *Achaea janata* larvae

AjGV preparation	Sample size (n)	LC_{50} x 10^{-6}(OB/ml)	Slope	SE	χ^2	LC_{50}(OB/mm^2)
Mother culture	35	2.1 (1.2-3.8)	1.02	0.13	0.40	84.1 (46.5-150.8)
	35	2.4 (1.3-4.5)	0.90	0.11	1.50	95.7 (50.4-178.5)
	35	2.0 (1.1-3.5)	0.99	0.13	2.05	76.4 (41.4-137.6)
Formulation	50	4.7 (3.1-7.3)	1.23	0.14	4.40	186.0 (120.9-288.6)
	30	3.0 (1.4-6.3)	0.79	0.11	3.04	119.2 (56.0-248.2)
	30	1.6 (1.0-2.9)	1.36	0.20	1.30	64.6 (37.3-112.5)

[a] Figures in parentheses are lower and upper 95% confidence limits; 3 df for Pearson Goodness-of-Fit test χ^2

and intravenous mammalian toxicity and pathogenicity tests (Table 3). Also, mother culture was non-infective with no cytopathic effects in cell culture tests and the formulation was non-toxic in dermal and eye irritation tests which were observed for several baculoviruses (Burges *et al*., 1980). Two nucleopolyhedrosis viruses (NPVs) infecting *Helicoverpa armigera* (Hubner) and *Spodoptera litura* (Fabricius) are the only two registered microbial insecticides of viral origin in India. Laboratory bioassay procedures for three granuloviruses namely, AjGV, *Chilo infuscatellus* GV (CiGV) and *Plutella xylostella* GV (PxGV) have been incorporated in the guidelines for registration of baculoviruses by CIB-RC. The recommended LC_{50} standard for AjGV formulation by leaf disc method to second instars is <4 OB/mm^2 (http://cibrc.nic.in/guidelines.htm). The range of LC_{50} values obtained in our study suggests a revision of this standard to 123±35 OB/mm^2 which is the mean LC_{50} value from three independent determinations for formulation. It is also essential that the counting method for AjGV be revised to using a shallow counting chamber of 0.02 mm depth due to smaller size of OB (<500 nm) as against the existing CIB-RC guideline of using Neubauer haemocytometer (0.1 mm depth) for both NPVs and GVs. Accordingly, the 'K' factor in the enumeration formula, would be 7.81×10^{-8} as against the existing 2.5×10^{-7} cm^3.

This is the first laboratory assay report on susceptibility of *A. janata* to its granulovirus. An integrated approach for its field use in semilooper management in castor is required by identifying a 'control window' as suggested for other baculoviruses by Evans (1994). In the semi-arid tropics, *Trichogramma chilonis* Ishii (Hymenoptera: Trichogrammatidae) is a key natural egg parasitoid of *A. janata* with field parasitization levels up to 30 to 40% (Lakshminarayana, 1992) early in the crop season, while *Snellenius maculipennis* (Szepligeti) (Hymenoptera: Braconidae) is an important larval endo-parasitoid, which is active during the mid-season. Use of chemical insecticides adversely affected the activity of *S. maculipennis* due to quick mortality (<3 days) of host *A. janata* larvae (Basappa and Lingappa, 2002), while field application of AjGV was safe (Prabhakar and Prasad, 2005) probably due to its host specificity and completion of the endoparasitiod development inside the host prior to virus induced kill occurred. Similar observations on compatibility of an endoparasitiod, *Microplitis croceipes* (Hymenoptera: Braconidae) and a nuclear polyhedrosis virus of *Heliothis zea* (Lepidoptera: Noctuidae) has also been reported (Eller *et al*., 1988). However, the virus-parasitoid interactions could be complex and warrant detailed studies to model the long-term impact of AjGV use.

This study meets the key data requirements for registration of AjGV with CIB-RC under the Insecticides Act, 1968. The findings are likely to aid in revising the standards for AjGV as suggested.

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Table 3: Results of mammalian toxicity tests with AjGV mother culture and formulation

Culture	Test	Result
Mother culture	Toxicity and pathogenicity study in cell culture (Human WI-38 & African Green Monkey CV-1 cell lines)	No cytopathic effects, Non- infective
	Acute oral toxicity / pathogenicity study in albino mice	Non-toxic, non-virulent
Formulation	Acute oral toxicity / pathogenicity study in Swiss albino mice	Non-toxic, non-virulent
	Acute Dermal Irritation / Corrosion in New Zealand White Rabbits	Non-toxic, non-virulent
	Acute Eye Irritation study in New Zealand White Rabbits	Non-toxic, non-virulent
	Study on the Container-Content Compatibility	Non-toxic, Safe
Both mother culture and formulation	Acute oral toxicity/ pathogenicity study in Wistar rats	Non-toxic, non-virulent
	Acute pulmonary toxicity / pathogenicity study in Wistar rats	Non-toxic, non-virulent
	Acute intravenous toxicity / pathogenicity study in Wistar rats	Non-toxic, non-virulent

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