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Standardization of laboratory mass production of *Amsacta albistriga* nucleopolyhedrovirus

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ABSTRACT: **Five experiments were carried out in the laboratory to evaluate the effect of temperature, humidity, host plant, viral dosage and age of the larvae on the yield of the nucleopolyhedrovirus that could be produced in the red hairy caterpillar, *Amsacta albistriga* (Walker). It was concluded that inoculating early sixth instar larvae with a virus inoculum of 5 x 10^8 POBs/ml, fed with castor leaves, and incubated at 30 ± 1°C with a relative humidity of 80-90 per cent gave maximum yield of *Aa*NPV. This forms the basis for mass-producing the nucleopolyhedrovirus of the red hairy caterpillar in the laboratory, which could be used as one of the major components of IPM practices for controlling *A. albistriga*.**

KEY WORDS: *Amsacta albistriga*, host plants, humidity, laboratory mass production, larval age, temperature

INTRODUCTION

Amsacta albistriga (Walker) (Lepidoptera: Arctiidae) is a seasonal and endemic pest occurring year after year in different regions of Karnataka. It feeds on a variety of crops such as groundnut, sesamum, cotton, cowpea, etc. affecting the yield adversely in these crops. In some groundnut tracts they even destroy the entire crop. In spite of several control measures such as use of bonfires, hand picking and destruction of larvae, poison baiting and pesticide application, and the pest continues to retain its notoriety as a major pest. Microbial control utilizing entomopahtogenic viruses is a non-chemical strategy to manage this dreaded pest as a large variety of lepidopteran and some hymenopteran pests are known to be susceptible to these baculoviruses.

Amsacta albistriga NPV (*Aa*NPV) with multiply occluded virions was isolated from this pest by Jacob and Subramanian (1972). Rabindra and Subramaniam (1975) reported that the migratory larvae of *A. albistriga* were highly susceptible to this virus. An experiment conducted to test the field efficacy of AaNPV in Pavagada has given encouraging results (Veenakumari *et al.*, 2005). Rabindra and Balasubramanian (1980) also reported

that a viral epizootic could be created by applying this NPV in the field, which would result in the long-term control of the pest. Biosafety studies conducted by Narayanan *et al.* (1977a, b) have shown that the common carp (*Cyprinus carpio* L.) and white mice (*Mus musculus* L.) are not susceptible to *Aa*NPV, proving its possible safety to mammals. Considering the efficacy and biosafety nature of *Aa*NPV, there is vast scope for this baculovirus as a potent biopesticide in the management of this dreaded pest (Jayaraj and Rabindra, 1989).

Homologous host insects are mandatory for mass producing any baculovirus, as these viruses are obligate pathogens. The yield of virus larva is dependent on the age of the larva, the host plant and the viral inoculum used. Apart from this, temperature and the relative humidity at which the inoculated larvae are incubated are also crucial for maximizing the yield of virus. Preliminary studies were conducted on the recovery of *Aa*NPV from larvae of different ages and under different dosage levels (Narayanan *et al.*, 1978). Baskaran *et al.* (2001) studied the effect of different host plants on the virus yield and reported that larvae fed on cotton, gave maximum yield of virus. We designed experiments to test the effect of all these parameters on the yield of virus at Project Directorate of Biological Control, Bangalore.

MATERIALS AND METHODS

Multiplication of virus

The multiply enveloped NPV was originally isolated from wild *A. albistriga* larvae in Pavagada, Karnataka in the year 2001. This isolate of *Aa*NPV, which was more virulent than the other geographic isolates tested in the laboratory, was further multiplied in the laboratory. Fifth and sixth instar larvae were collected from the endemic region of Pavagada on groundnut. These larvae were reared in 25 litre tubs (25 cm height x 40 cm diam) with lids fitted with brass mesh. The larvae were fed on castor leaves treated with *Aa*NPV for 24 hours. Thereafter, these larvae were reared on fresh bouquets of castor leaves. From the seventh day onwards all virosed larvae were collected, homogenized in sterile distilled water and filtered through a double layered muslin to separate the hairs and body tissues. The filtrate was initially centrifuged at 500 RPM for two minutes and the resulting supernatant was once again centrifuged at 5000 RPM for 15-20 minutes. The virus pellet, thus formed, was suspended in distilled water. The POBs were quantified by phase contrast microscopy (Nikon Eclipse E 400) using a double ruled Neubauer haemocytometer and the virus suspension thus standardized were stored at 4°C in the refrigerator till further use. Five laboratory experiments were conducted to evaluate the effect of different host plants, temperature, humidity viral dosage and larval age on the yield of virus that could be produced per larva.

Maintenance of healthy host culture

Adults of *A. albistriga* were collected from Pavagada (Karnataka) - an endemic area - by setting up light traps. These moths were brought to the laboratory and confined in acrylic cages (28 x 28 x 30 cm). Ten per cent honey solution was provided as food for the moths. They were also provided with brown paper strips (10 x 1.0 cm) for laying eggs. The egg patches were collected separately in a plastic container. The larvae that hatched from these egg patches were released on bouquets of castor leaves in 5 litre plastic containers (21cm height x 15 cm diam) with lids fitted with brass mesh. Fresh castor leaves were provided daily as feed to these larvae. Once the larvae reached third instar, they were shifted to 25 litre plastic tubs (25 cm height x 40 cm diam).

Standardization of different larval instars

Out of seven larval instars of *A. albistriga*, the late fourth, early fifth, late fifth, early sixth and late sixth instar larvae were inoculated with 5×10^8 POB/ml of *Aa*NPV to estimate the percentage mortality and the yield of POB per larvae. Castor leaf discs of 3.2 cm diameter were cut using a metallic leaf cutter and then washed thoroughly in water containing 1 per cent streptomycin and air dried under a fan. Aliquots of 10 ml of *Aa*NPV (5×10^8 POBs/ml suspended in 0.1 % Teepol) were spread homogenously on both sides of the leaf disc using

a blunt end of a glass rod. The leaf discs were shade dried and placed in 50 ml plastic containers along with a larva of *A. albistriga* (depending on the treatment). The larvae were fed on virus treated leaf discs and later with castor leaves as described earlier. Each treatment was replicated four times with ten larvae per replication. Larvae fed on untreated castor leaves served as control.

Standardization of host plants

Different plants such as castor (*Ricinus communis* L.), cowpea (*Vigna unguiculata* (L.) Walp.), calotropis (*Calotropis gigantea* R. Br.) and groundnut (*Arachis hypogaea* L.) were used as host plants. These plants were raised in pots housed in a net house (except for *Calotropis*, which was collected from the field). Aliquots of 10 ml of *Aa*NPV (5×10^8 POBs/ml suspended in 0.1 % Teepol) were spread homogenously on both sides of the leaf disc of all plants using a blunt end of a glass rod. Fifty larvae constituted five replications for each of the host plants. Similarly, untreated leaves were used as the control. The larvae were allowed to feed on the entire treated leaf disc and then fed regularly on leaves untreated of the respective host plants.

Standardization of temperature

Early sixth instar larvae of *A. albistriga* were inoculated with *Aa*NPV @ 5×10^8 POBs/ml using castor leaf discs as mentioned earlier. These larvae were incubated at room temperature (28± 1°C, during July-August) and in BOD incubators maintained at different temperatures: 20 ± 1°C, 25 ± 1°C and 30 ± 1°C. All the treatments were replicated five times with ten larvae in each replication. The control larvae were fed untreated castor leaves.

Standardization of humidity

Early sixth instar larvae of *A. albistriga* were inoculated with *Aa*NPV @ 5×10^8 POBs/ ml and incubated at ambient humidity in the laboratory (76-80%) and in environmental chambers at 60 ± 2, 70 ± 2, 80 ± 2, 90 ± 2 per cent relative humidity. All the treatments were replicated five times, with each replication having ten larvae. A control was also maintained where the larvae were fed untreated leaves.

Standardization of inoculum dose of *Aa*NPV

Early sixth instar larvae of *A. albistriga* were inoculated with six serial dilutions of *Aa*NPV ranging from 5×10^8 to 1×10^9 POBs/ ml. The different dilutions were 5×10^8, 6×10^8, 7×10^8, 8×10^8, 9×10^8 and 1×10^9 POBs/ ml. The larvae were inoculated using castor leaf discs as described earlier. Each treatment was replicated three times and each replication contained ten larvae. A control was also maintained with untreated castor leaves.

For all experiments, observations were recorded daily on *Aa*NPV induced larval mortality. Yield of virus per larva and per gram of larva were also calculated using a double ruled improved Neubauer haemocytometer.

Estimation of virus production per larva

The dead larvae were collected individually using a metal spatula. They were weighed individually and preserved at 4°C in a refrigerator. The viral occlusion bodies were recovered by crushing each dead larva in 10 ml sterile distilled water. The yield of the virus in each larva was enumerated using a double ruled Neubauer haemocytometer. The samples were diluted as per requirement and counted using a phase contrast objective at x400 magnification (Nikon Eclipse E 400) to determine yield of virus per larva.

Data on larval mortality due to *Aa*NPV and yield of virus (influenced by host plants, age of larvae, temperature and humidity) were subjected to statistical analysis of variance.

RESULTS AND DISCUSSION

i. Standardization of larval instars

There was 100 per cent mortality 8 DAT when late fourth instar larvae were inoculated with 5×10^8 POB/ml. The mortality rate decreased as the larval age increased. Least mortality of 56.67 per cent was observed when late sixth instar larvae were inoculated (Table 1). The remaining larvae pupated

successfully. There was 80 per cent mortality in early sixth instar larvae. In the laboratory the mean larval cadaver weight ranged from 201.24mg from late fourth to 611.40mg in late sixth instar larvae. But we could recover NPV infected late instar larvae weighing as much as 1196 (891-1569) mg from NPV sprayed fields in Pavagada, yielding as much as 1.021×10^{10} (3.5×10^{9} - 1.36×10^{10}) POB/ml.

Maximum yield of 4.32×10^{9} POB/larva was recovered from a late sixth instar larva while minimum yield of 0.83×10^{9} POB was obtained from a late fourth instar larva. Yield of POBs per larva was directly proportional to the age of the larva, which in turn was positively correlated to the weight of the larva. Similar observations were also made by Narayanan *et al.* (1978). They reported that a maximum yield of 5.8×10^{9} POBs/g of larva was obtained when sixth instar larvae of *A. albistriga* were inoculated with 8.05×10^{7} POBs/ml of *Aa*NPV. In the present study, yields of 6.98×10^{9} POBs and 7.06×10^{9} POBs were obtained per gram of larva when early sixth and late sixth instar larvae, respectively were inoculated for virus production. Pawar and Ramakrishanan (1971) on the other hand reported that there is no correlation between the weight of the larva and number of polyhedra recovered from *Spodoptera litura* (Fabricius) larvae.

When both the per cent larval mortality and the yield of POBs were considered, it was found that inoculating early sixth instars followed by late fifth instar larvae were more beneficial for maximum production of virus.

ii. Standardization of host plants

There was no significant difference in larval mortality when the larvae were fed with different host plants. Larvae that fed on *Calotropis* weighed the least (361.23 mg), whereas those larvae that fed on other host plants ranged from 455.38 - 482.38 mg. There was a significant difference in the yield of virus between those larvae that fed on *Calotropis* and other host plants. The yield of virus was least in those larvae that fed on *Calotropis* leaves (1.91×10^{9} POB/ larva), whereas in others the yield ranged from 3.29-3.48 x 10^{9} POBs/ larva (Table 2). Considering the availability of castor plants which frequent many vacant plots and wastelands and also yield of virus/larva when used as a host plant, castor seems to be the most appropriate choice as a host plant for the mass production of *Aa*NPV.

This finding does not support the findings of Baskaran *et al.* (2001) who reported maximum larval mortality (91.1 %) in *Calotropis* fed fourth instar

Table 1. Influence of different larval instars on production of *Aa*NPV

Instar	Larval mortality (%)	Average larval weight at harvest (mg)	Yield of *Aa*NPV x 10^{9}	
			POBs/larva	POBs/g of larva
Late IV	100.0^{a}	201.2^{d}	0.83^{e}	4.12
Early V	93.3^{ab}	298.4^{c}	1.68^{d}	5.63
Late V	86.7^{ab}	452.1^{b}	2.88^{c}	6.37
Early VI	80.0^{b}	498.8^{b}	3.48^{b}	6.98
Late VI	56.7^{c}	611.4^{a}	4.32^{a}	7.06

Figures followed by the same alphabet are not significantly different from each other (p=0.05).

Table 2. Influence of host plants on production of *Aa*NPV

Host Plant	Larval mortality (%)	Average larval weight at harvest (mg)	Yield of *Aa*NPV x 10^9	
			POBs/larva	POBs/g of larva
Castor	83.3	468.2[a]	3.32[a]	7.09
Groundnut	86.2	482.4[a]	3.29[a]	6.82
Calotropis	73.3	361.2[b]	1.91[b]	5.28
Cowpea	76.7	455.4[a]	3.48[a]	7.64

Figures followed by the same alphabet are not significantly different from each other (p=0.05).

larvae. He also reported a virus yield of 2.12 x 10^9 POB/ larva (*Calotropis*) and 1.83 x 10^9 POB/larva (castor). This may be attributed to difference in the age groups of the larvae that were used in the experiment. In groundnut fields in Gujjanadu (Pavagada taluk) intercropped with castor, *A. albistriga* larvae preferred castor (3.63/ plant) to groundnut (2.06) and *Calotropis* (which grew wild on the bunds).

Acquisition of the virus by an insect depends on the type of host plants that it feeds on. This is because the pH of the leaf tissues has either an antagonistic or additive effect on the acquisition rate of the virus (Benz, 1987). It was observed that susceptibility of *Lymantria dispar* (Linn.) larvae to baculovirus varied significantly on the host plants used (Keating *et al.*, 1988). Richter *et al.* (1987) reported that there was a 10-fold difference in susceptibility of *Spodoptera frugiperda* J. E. Smith when different host plants were used for infecting the larvae.

iii. Standardization of temperature

There was significant difference between the treatments when virus inoculated larvae were incubated at different temperatures. Larval mortality was lowest (10 %) at 20°C, while the remaining larvae pupated successfully after an average of 18.65 days. Larval mortality was highest at 30°C (84 %) but was not significantly different from those at room temperature (28 ± 1°C)(82 %). Maximum yield of 7.21 x 10^9 POB/g of larvae was obtained at an incubation temperature of 30°C (Table 3).

Table 3. Influence of different temperatures on production of *Aa*NPV

Temperature	Larval mortality (%)	Average larval weight at harvest (mg)	Yield of *Aa*NPV x 10^9	
			POBs/larva	POBs/g of larva
20 ± 1°C	10.0[c]	198.2[c]	1.14[a]	5.75
25 ± 1°C	76.9[b]	436.4[b]	2.68[b]	6.14
30 ± 1°C	84.0a	482.4[a]	3.48[c]	7.21
Room temperature (28 ± 1°C)	82.0[b]	478.2[a]	3.32[c]	6.94

Figures followed by the same alphabet are not significantly different from each other (p=0.05).

Although no work has been done on the effect of incubation temperature on the yield of virus on *A. albistriga*, lots of work has been done on other species of insects. Taun *et al.* (1998) have reported 30°C to be the ideal incubation temperature (for early fifth instar larvae) for the mass production of *S. litura* NPV. Wen *et al.* (2003) have reported that the incubation temperature of 28 ± 1°C resulted in maximum yield of *S. exigua* NPV. El-Saadany *et al.* (1992) reported that with increase in the larval incubation temperature from 25° to 30°C, there was increase in larval mortality and yield of virus/ larva in *S. littoralis*.

An incubation temperature of 30 ± 1°C or room temperature (28 ± 1) °C is therefore ideal for the mass production of *Aa*NPV in the laboratory.

iv. Standardization of humidity

Maximum larval mortality of 86 per cent was recorded when the larvae were incubated at 90 per cent humidity. But there was significant difference in larval mortality only between 60 per cent and rest of the treatments. Kelly and Entwistle (1988) suggest that increased humidity may improve levels of infection and virus yield. Moawad (1986) for *S. litura* and Fuxa *et al.* (1999) for *Trichoplusia ni* reported that increase in humidity favoured larval mortality due to viral infection. Epizootics of NPV of the African army worm, *Spodoptera exempta* Walker were noticed at those sites where there was high relative humidity, wide range of temperatures and high larval density (Odindo, 1983).

Yield of virus was higher when the inoculated larvae were reared at 80 and 90 per cent relative humidity. The yield of POBs reduced significantly when the inoculated insects at 70 and 60 per cent relative humidity (Table 4).

v. Standardization of dosage of *Aa*NPV for mass production of *Aa*NPV

Different dosages of *Aa*NPV ranging from 5 x 10^8 to 1 x 10^9 POB/ml were tested to determine the optimum dose of virus inoculum for maximizing the virus yield. There was no significant difference either in larval mortality or yield of virus between the doses tested (Table 5). Yields of virus in all the treatments ranged from 3.10 – 3.41 x 10^9 POBs/ larva. Senthil Kumar *et al.* (2005) have reported that beyond a threshold level, there was a negative correlation between the inoculum dose and yield of virus in *S. litura*. This they attributed to the death of larvae due to higher doses of virus, before they could attain their full size. Cherry *et al.* (1997) also reported that virus yield per larva of *Spodoptera exigua* (Hübner) increased with increasing dose, but once the threshold inoculum of 1 x 10^4 POBs/ cell was crossed, increase in viral yield per larva was limited. Therefore for *A. albistriga* a dosage of 5 x 10^8 POBs/ml acts as the threshold level (or as standard dose) for inoculating sixth instar larvae for the mass production of virus in the laboratory.

Table 4. Influence of different humidities on production of *Aa*NPV

Humidity (%)	Larval mortality (%)	Average larval weight at harvest (mg)	Yield of *Aa*NPV x 10^9	
			POBs/larva	POBs/g of larva
60 ± 2	60.0[b]	372[c]	2.06[a]	5.53
70 ± 2	72.0[a]	424[b]	2.71[b]	6.38
80 ± 2	84.0[a]	470[a]	3.19[c]	6.78
90 ± 2	86.0[a]	471[a]	3.38[c]	7.16
Room humidity (76-80%)	85.8[a]	458[a]	3.21[c]	7.01

Figures followed by the same alphabet are not significantly different from each other (p 0.05).

Table 5. Influence of dosage on production of *Aa*NPV

Viral dose (POB_s/ml	Larval mortality (%)	Average larval weight at harvest (mg)	Yield of *Aa*NPV x 10^9	
			POBs/larva	POBs/g of larva
$1x10^9$	83.3	502.3	3.41	6.79
$9x10^8$	83.3	498.3	3.38	6.78
$8x10^8$	83.3	512.1	3.14	6.13
$7x10^8$	83.3	502.1	3.18	6.33
$6x10^8$	80.0	496.3	3.10	6.24
$5x10^8$	80.0	503.2	3.26	6.47

Considering all these factors we can conclude that inoculating early penultimate instar larvae with a virus inoculum of 5 x 10^8 POBs/ml, fed with castor leaves, and incubated at 30°C with a relative humidity of 80-90 per cent will give maximum yield of *Aa*NPV. With sufficient training this could be an efficient production system, where even farmers can mass-produce the virus. In such cases however the product should be sent to government approved centers for quality testing. It will be highly cost effective as i) plenty of homologous host larvae are available in the endemic areas (collection of which would also act as mechanical control), ii) they do not need any synthetic diet for rearing the larvae as they can be efficiently reared on castor leaves, iii) no expensive equipments are required for incubating the inoculated larvae, as they can be reared efficiently under ambient conditions, iv) even though labour intensive, women folk and unemployed youth in the village can be engaged to culture the insects, which provide additional employment, and v) cottage industries can be set up.

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