In vivo Production of a Nuclear Polyhedrosis Virus Utilizing Tobacco Budworm and a Multicellular Larval Rearing Container[1, 2]

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ABSTRACT The effects of viral inoculum and incubation period on virus yield in a method of *in vivo* production of the multicapsid nuclear polyhedrosis virus of *Heliothis armigera* (Hübner) were examined utilizing *Heliothis virescens* larvae reared in multicellular containers. Trays of diet were surface contaminated with virus levels ranging from 54 to 2708 polyhedral inclusion bodies (PIB) per mm^2 of diet at 7 days after egg infestation. The virus was harvested either at 6 or 7 days after treatment. Within the ranges of this study, the greatest quantity virus was obtained when the diet was inoculated with the 54 PIB level, and the virus was harvested at 7 days. This combination resulted in an average production of 2919×10^9 PIB per tray, with 5.7×10^9 PIB per larvae and 523 collectable larvae per tray. It also represented an increase in polyhedra of $2.43 \times 10^5\times$ compared to the inoculum. The costs of the production were estimated based on prevailing wages and current costs of materials.

KEY WORDS Tobacco budworm, *Heliothis virescens*, NPV, production, nuclear polyhedrosis virus.

At the present time, there are only four entomogenous baculoviruses that are registered for use as microbial pesticides in the United States. Although registered, none of these viruses are presently in commercial production. The baculovirus (nuclear polyhedrosis viruses) of the cotton bollworm, *Heliothis zea* (Boddie), has been one of the most extensively studied (Ignoffo and Couch 1981), and was last available as a commercial product from Sandoz, Inc. Some nuclear polyhedrosis viruses (NPV) were produced in cell culture and results have been promising (Hink 1982). However, the technology for practical commercial *in vitro* production is not yet available and virus for field studies to determine their effectiveness as microbial insecticides is normally produced *in vivo* using mass reared insect hosts (Ignoffo 1966, Vail et al. 1973). Shapiro (1982) discussed many of the factors that have to be considered in virus production and although many advances have been made in commercial *in vivo* production, more research and development is needed on various individual and limited rearing systems. Research that has been conducted on production was often due to the lack of commercial availability of various individual baculoviruses for laboratory or field testing. This lack of materials could

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[2] This article reports the results of research only. Mention of a proprietary product does not consititute endorsement or a recommendation for its use by USDA.

impede the investigation of promising viruses for use as microbial insecticides. One of the research objectives of this laboratory is to evaluate insect pathogens and develop application methods for control of noctuid pests of field crops. Future research and demonstration programs could require large quantities of baculovirus. The following study was therefore conducted to evaluate one method of *in vivo* production of the multicapsid NPV of *H. armigera* (Hübner) for utilization in research studies.

Materials and Methods

The system used to rear larvae for virus production was a modification of the system reported by Raulston and Lingren (1972) and was described by Hartley et al. (1982). The larval rearing unit consists of a fiber glass tray (64.4 × 34.4 × 2.8 cm), a polystyrene insert with 903 cells, and a 125 um mesh polypropylene cover. These trays hold ca. 3.8 l of a soyflour-wheat germ diet with low agar content. An egg-infesting device (Hartley et al. 1982) is used to deposit 2 to 4 eggs from tobacco budworms, *H. virescens* (F.) per cell. Trays were prepared and held at 29.5°C for 6 days per the described rearing procedures, then held for 1 day at 26°C prior to inoculation of the virus and thereafter. The majority of the larvae were in the 3rd-4th instar at the time of inoculation.

The virus used was the China variant of the multiple-embedded NPV (MNPV) from *H. armigera* (*Ha*MNPV) originally obtained from Dr. John Hamm, USDA-ARS, Georgia Plains Experiment Station, Tifton, GA. The China isolate of *Ha*MNPV was shown to be genotypically distinct from the Russian isolate (Gettig and McCarthy 1982) and could be of future commercial importance. Based on preliminary tests, 6 ml of virus suspension was applied as a spray on the diet surface of each tray. Five concentrations of inoculum that resulted in treatment levels of ca. 54, 135, 270, 1354, and 2708 polyhedral inclusion bodies (PIB/mm^2 of diet surface were used. Each test was composed of 2 trays of each of the 5 treatments and was set up in a RCB design with a factorial arrangement. Untreated rearing trays from the test dates were checked for virus contamination as a control. Larvae were manually collected on the 6th and 7th day following application of the virus (1 tray for each treatment for each day) and the numbers of live or dead recorded. The total weight of the live larvae was recorded. Dead larvae which could not be removed by forceps were collected using a suction tube attached to a trap flask. The polyhedra were then harvested and the total virus from each tray was determined using methods as described by Vail et al. (1971). Briefly, larvae are homogenized in a blender, coursely filtered through 4 layers of cheesecloth, and purified by multiple centrifugation (ca. 5,000g for 15 minutes). The samples are then brought to a known volume and the concentration of polyhedra determined by multiple counts using a hemacytometer. The entire production procedure was replicated 8 times. The first replication also contained inoculum concentrations of 13 and 5417 PIB/mm^2 of diet surface. However, the 13 PIB/mm^2 concentration resulted in the pupation of ca. one third of the larvae present and survival of most larvae at 7 days, and the higher level resulted in mortality and total breakdown of the larval integuments as small larvae, making them uncollectible by our procedure. Since neither of these trays resulted in significant virus productions, those inoculum levels were not included in the study.

All virus collected in this study was then pooled and processed. The processing was done by adding lactose to form a slurry which was then lyophilized and ground into a powder in a ball mill (Vail et al. 1971). This final product was bioassayed to determine the LC_{50} in PIB/ml by incorporating known concentrations of virus in larval diet using methods similar to those described by Dulmage et al. (1976) as modified by Bell and Romine (1986). All statistical analyses were conducted using the procedures of the Statistical Analysis System (SAS Institute 1985). Production data was subjected to ANOVA and means were examined by the method of least significant difference (LSD), and the median lethal concentration (LC_{50}) and 95% confidence interval was estimated from the bioassay data utilizing the PROBIT procedure.

Results and Discussion

The numbers and weights of larvae collected and the virus yields are given in Tables 1 and 2, respectively. Examination of untreated trays indicated no NPV contamination in the controls. With the same dosage of virus, there were generally greater numbers of live larvae collected on day 6 than day 7, as would be expected, and greater numbers of dead larvae on day 7. There were significant differences in the average numbers of live or dead larvae due to collection day. Although the greatest numbers of dead larvae and of total larvae collected per tray (406 and 594 larvae, respectively) occurred in trays contaminated with the greatest virus concentration (6×10^8 PIB/tray) and collected on day 6, this combination of treatment and collection day resulted in one of the lowest yields of virus ($1794 \times 10_9$ PIB/tray). In this treatment the larvae could have died during younger instars (resulting in lower PIB production) and the greater numbers of total larvae might be due to less cannibalism of the dead or dying larvae by the living but diseased larvae.

Trays harvested at 7 days after treatment produced both the greatest and the least virus production (2919×10^9 PIB/tray and 1610×10^9 PIB/tray) (Table 2). There was a significant overall increase in virus from trays collected on day 7 compared to those collected on day 6, averaging 2348×10^9 and 1963×10^9 PIB/tray, respectively. The most virus produced in this study was obtained by using an inoculum level of 54 PIB/mm^2 (1.2×10^7 PIB/tray) and harvesting at 7 days after treatment. This production represented averages of 0.55 larval equivalents (LE) per larvae from 236 live larvae and 1.33 LE per larvae from 287 dead larvae collected per tray, or 486 LE/tray, an increase in virus of 2.43×10^5 compared to the inoculum. A larval equivalent equals 6×10^9 PIB (Ignoffo 1966). Ignoffo and Shapiro (1978) found that more polyhedra were collected from dead larvae than from living larvae. They further demonstrated that virus processed from dead larvae was ca. 4-7X more active than virus from living larvae. Therefore, the ratio of virus collected from dead larvae and living larvae may be of significant importance. The percentage of virus collected from dead larvae in this study averaged 73% and ranged from 61 to 85%, with 75% of the virus harvested from the 54 PIB/mm^2 treatment coming from dead larvae. The bioassay of the pooled and processed virus, utilizing polyhedra incorporated in the diet, indicated LC_{50} of 559 PIB per ml diet (95% C.L.: 421 - 723 PIB per ml diet).

The cost of production is always an important factor, both in production for research and demonstration programs, and more importantly for future consideration of

Table 1. Comparison of larval collections of *H. virescens* from multicellular rearing trays at 6 and 7 days after inoculation with various levels of a nuclear polyhedrosis virus.

Treatment†, ‡ (PIB/mm²)	No. Live Larvae/Tray	No. Dead Larvae/Tray	Total Larvae/Tray	Avg. Wt. for Live Larva (mg)
		Day 6		
54	291	216 a	508 ab	282 a
135	267	207 a	473 ab	311 a
270	211	245 a	456 ab	313 a
1350	141	274 a	416 b	319 a
2708	188	406 b	594 a	245 a
Avg.	220	270	490	294
		Day 7		
54	236 *	287 b	523 a	310 a
135	178 *	357 ab*	534 a	309 a
270	183	381 a*	565 a	311 a
1354	129	372 a*	501 ab	287 a
2708	78 *	353 ab	432 b	295 a
Avg.	161 *	350 *	511	302
LSD virus treatment	49.2	78.8	94.0	57
LSD collection day	22	35	106	26

† Means in each column and collection date followed by the same letter are not significantly different (P = .05; LSD procedure).

‡ PIB = polyhedral inclusion bodies.

* Significant level of difference compared to corresponding treatment on Day 6 data.

commercialization. The cost is also in a constant state of change, based primarily on improving rearing methods and prevailing local labor and overhead costs. The method reported here produced virus at an initial estimated cost of $0.0167-$0.0210 per LE, not including building and labor overhead costs. This figure also does not include the cost of storing the virus and quality control procedures that would be required in commercial manufacture. The cost of the materials for maintaining the colony and test trays was only $0.0035 per LE and the remaining ca. 80% of the estimated cost was labor. These figures were estimated based on a production capacity of this facility of 30 trays per day, occupying 400 ft² of floor space, and producing 102,060 LE per 7-day week. Although the described method of production resulted in a basic cost of only ca. $0.50-$0.80 per 0.4-ha-treatment and produced quantities sufficient for field research studies, the method would not be the best available for commercial production. A more automated method of rearing and virus production in *Heliothis* is presently available. This method utilizes heat-formed disposable plastic trays (Ignoffo and Boening 1970, Ignoffo

Table 2. Yields of nuclear polyhedrosis virus from *H. virescens larvae* reared in multicellular rearing trays and collected 6 and 7 days following inoculation.

Treatment†‡ (PIB/mm²)	Total PIB from Live Larvae (x 10^9)	Total PIB from Dead Larvae (x 10^9)	Total PIB/ Tray (x 10^9)	Number§ L. E./Live Larva	Number L. E./Dead Larva	Average Number L. E./Larva	Average¶ Yield Increase (×)
			Day 6				
54	747 a	1186 a	1932 a	.43 a	.97 a	.62 bc	1.61×10^5
135	667 a	1373 a	2040 a	.43 a	1.18 a	.70 ab	6.80×10^4
270	671 a	1321 a	1991 a	.51 ab	.92 a	.70 ab	3.19×10^4
1354	549 a	1509 a	2059 a	.64 b	.94 a	.81 a	6.86×10^3
2708	534 a	1260 a	1794 a	.47 a	.52 b	.49 c	2.99×10^3
Avg.	634	1330	1963	.50	.91	.67	
			Day 7				
54	738 a	2181 a*	2919 a	.55 a	1.33 a*	.95 a	2.43×10^5
135	602 ab	1872 a	2474 ab	.56 a	.86 b	.76 b	8.25×10^4
270	677 a	2024 a*	2701 ab	.61 a	.88 b	.80 ab	4.50×10^4
1354	433 b	1601 ab	2034 bc	.56 a	.72 b	.67 b	6.78×10^3
2708	241 b*	1370 a	1610 c	.54 a	.64 b	.62 b	2.68×10^3
Avg.	538	1810	2348*	.56	.89	.76*	
LSD: Treatment	236.8	608.4	752	.16	.29	.18	
LSD: Day	106	272	336	.07	.07	.08	

† Means in each column and collection date followed by the same letter are not significantly different (P = .05; LSD procedure).

‡ PIB = polyhedral inclusion bodies.

§ L. E. = Larval equivalent (6×10^9 PIB).

¶ Yield increase = total virus collected per tray/virus inoculum per tray.

* Significant level of difference compared to corresponding treatment on day 6 data.

and Couch 1981). Such automated equipment is presently being used in the USDA boll weevil rearing facility at Mississippi State University. Although the disposable trays are more expensive compared to the resuable multicellular trays, the reduction in labor costs by automation of set-up and collection should compensate for the increased cost, producing ca. 12 times as much virus with the same amount of labor. The automated equipment forms the tray, injects the larval diet, infests eggs, and seals the unit in one operation at a speed of 168,960 larvae per 8-hour day. Although the estimated cost per LE of virus with the automated equipment would still be in the same approximate range as with the multicellular equipment, after the initial cost of the equipment, the estimated capacity is greatly increased and the anticipated overhead costs should be less than with the multicellular.

This study demonstrated a method of production of a nuclear polyhedrosis virus using resuable multicellular trays. Inoculation of diet surface with the lowest level of virus used in the study (54 PIB/mm^2) produced the maximum yield. Although an inoculum of 13 PIB/mm^2 was found to be too low, the exact inoculum for maximum yields might be between those levels. The quantity, quality, and cost of the baculovirus produced in this study indicated the method was efficacious, especially for producing quantities needed for field trials of candidate viruses for insect control.

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