THE MASS PROPAGATION OF TWO VIRUSES OF THE SPRUCE BUDWORM, CHORISTONEURA FUMIFERANA (CLEM.) (LEPIDOPTERA: TORTRICIDAE)

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INFORMATION REPORT IP-X-1

INSECT PATHOLOGY RESEARCH INSTITUTE
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CANADIAN FORESTRY SERVICE
SAULT STE. MARIE, ONTARIO

OCTOBER, 1972

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ABSTRACT

Two viruses of the spruce budworm, *Choristoneura fumiferana*, an entomopoxvirus and a nuclear polyhedrosis virus, have been mass produced. The methods used and the yield of viruses are described.

INTRODUCTION

Three types of insect viruses have previously been isolated from the spruce budworm, Choristoneura fumiferana. They are a nuclear-polyhedrosis virus (NPV) (Bergold 1951), a cytoplasmic polyhedrosis virus (CPV) (Bird and Whalen, 1954) and a granulosis virus (GV) (Bergold, 1950). In 1970 an entomopoxvirus was isolated from the 2-year-cycle spruce budworm, C. biennis which occurred in British Columbia (Bird, Sanders and Burke, 1971) and it was found to infect the spruce budworm. A decision had been made to test budworm viruses by aerial application. The viruses selected for this test were the entomopoxvirus and the NPV. The size of the trials required that mass rearings be used for virus production which heretofore has not been attempted with the spruce budworm.

Many species of insects have been propagated either on their natural food or on artificial diet for virus production. Only 5 viruses, all NPVs, have been industrially produced, mostly for experimental field use (Ignoffo and Hink, 1971). In mass rearings for production of viruses it is desirable that one obtain the maximum yield of virus with the minimum expenditure. Wages form the largest part of a rearing budget because of the amount of manual work required. Handling of insects should therefore be kept to a minimum.

There are a number of factors of great importance which reduce handling and, therefore, costs when larvae are reared on artificial diet:

1) A sufficient volume of diet must be put in the rearing containers to maintain the insects until the appropriate stage of development has been reached or until maximum virus level in the larvae has been attained.
2) The number of larvae per rearing container must be such that overcrowding does not impede development.
3) Temperature, humidity and photoperiod must be regulated so that optimal larval growth is obtained since virus development is directly dependent on insect development. Temperature and humidity must also be suitable for maintaining the insect diet in a palatable condition. Too high a humidity encourages fungal growth and too low a humidity permits the diet to dry out.
4) Larvae must be infected at the correct stage of development so that they reach their full size but have not pupated at the time of harvesting. The virus yield is low from pupae and there is considerable wastage of effort if they reach this stage. When slow-developing viruses are being propagated, early instar larvae are fed virus-contaminated diet; for fast-developing viruses later instars are used.

5) The correct dosage of virus must be determined. If too small a dose is administered larvae will reach the pupal stage before death occurs. If too great a dose is administered death occurs before the larvae have reached maximum size and hence virus yield is reduced.

All the conditions noted had to be established experimentally. In the winter of 1970-71 the first virus production line was staffed with 5 casual assistants and 3 regular staff members and protocols were established on a trial-and-error basis. In 1971-72 with improved techniques 14 casual assistants and 3 regular staff members worked on virus production. This paper describes the methods and results obtained in the 1971-72 winter season, and a comparison with the 1970-71 production figures.

METHODS

Supply of spruce budworm larvae

Stocks of spruce budworm larvae were maintained by the methods described by Grisdale (1970, 1972) and second-instar larvae were supplied to the virus-production team in quantities frequently exceeding 100,000 per week. All larvae were reared on synthetic diet prepared according to McMorran (1965), but no formaldehyde was added because it may suppress the progress of virus infection within the host (Vail et al, 1968).

Entomopoxvirus production

The virus stocks produced in 1970-71 were later found to be contaminated in that the entomopoxvirus contained both an NPV

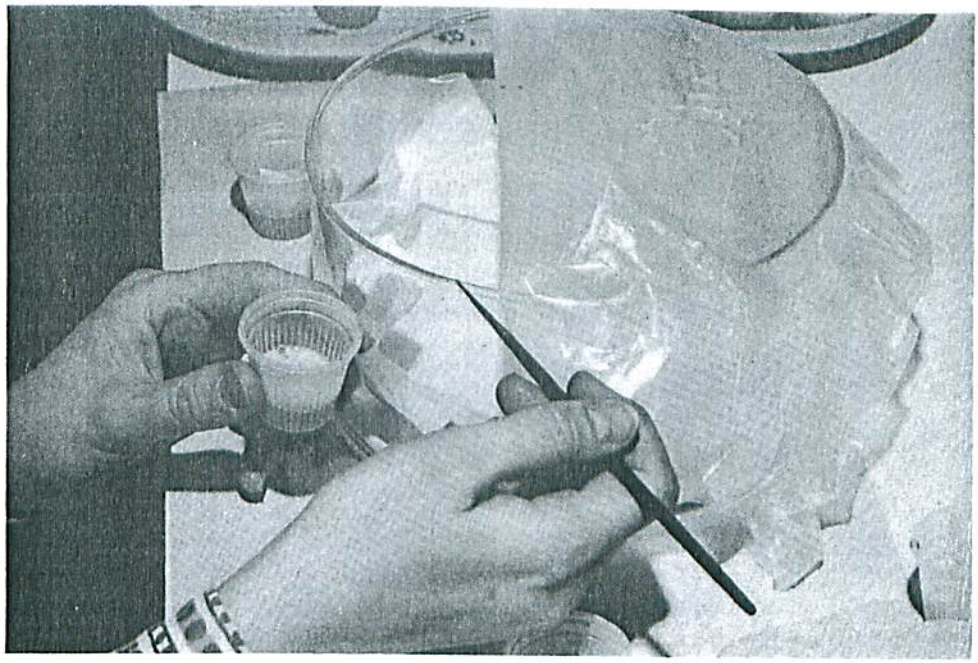

Fig. 1. Second-instar larvae emerging from their hibernacula are transferred on to diet using a camel hair brush.

Fig. 2. Cream cups on disposable trays are stacked in environmental cabinets.

and a CPV contaminant which showed up in the field trials. At that time a part of the virus produced in the mass rearing was used as seed-inoculum to reinfect more larvae. In retrospect this was considered to be an unwise procedure and a supply of virus inoculum was produced in a separate room. The original virus inoculum was obtained from a virus-killed C. biennis larvae. Second-instar spruce budworm larvae were infected by placing them individually in 3/4 oz. cream cups[1] sprayed with 0.15 ml virus suspension containing 10^6 inclusion bodies/ml. They were reared in a "Conviron" cabinet[1] at 75°F[2], 50% R.H. and 15 hrs. light. After 25 days each larva was examined microscopically to ensure that only entomopoxvirus and no NPV or CPV were present. These larvae were then freeze-dried and used as an inoculum for mass propagation.

The inoculum was prepared by triturating one larva in 200 ml of distilled water using a tissue grinder. The concentration of this suspension was estimated using a Petroff-Hauser bacteria counter and was adjusted to 10^6 inclusion bodies/ml. Artificial diet (16 ml) was poured into 3/4 oz. creamer cups, 78 of these were placed on a 14" x 18" pressed board cafeteria tray[1] and the surface of the diet sprayed with the virus suspension at a rate of 0.15 ml/cup using a Wood's #66 atomizer type sprayer[1].

1 Appendix I
2 Appendix II

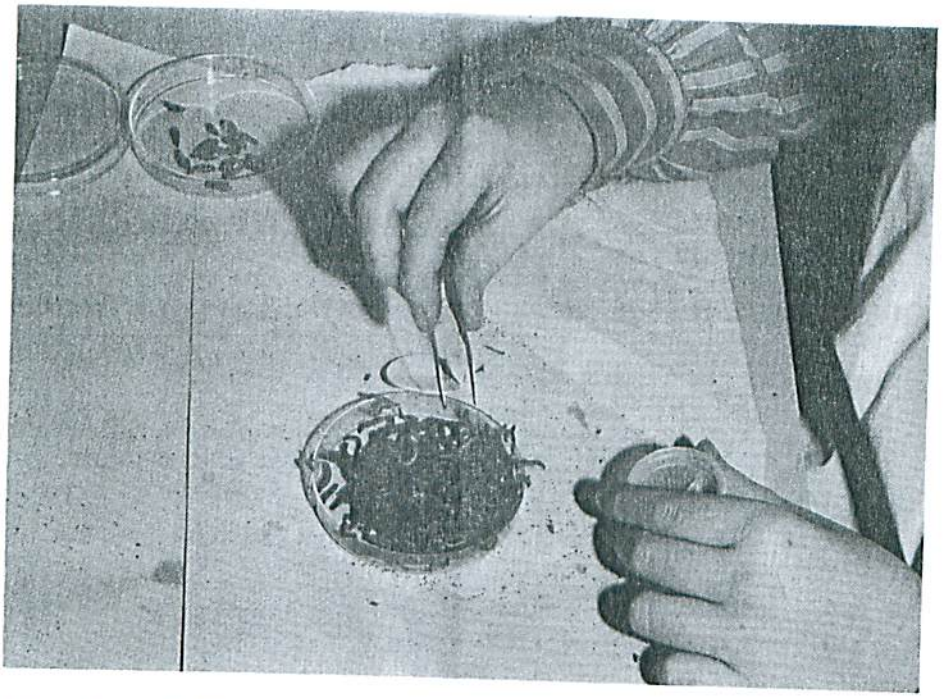

Fig. 3. Infected larvae are removed and placed in disposable plastic petri dishes.

Fig. 4. Infected larvae are freeze-dried.

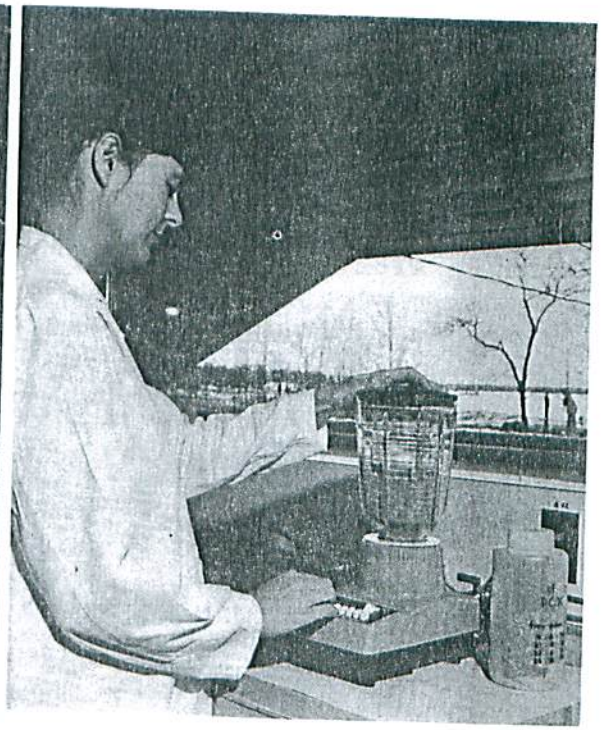

Fig. 5. Freeze-dried larvae are ground to a fine powder.

A crew of 5 people using camel hair brushes transferred second-instar budworm larvae into the sprayed cups and 7 larvae per cup was found to be the optimal number (Fig. 1). The trays were stacked in piles up to seven high in environmental chambers[1] (Fig. 2) and the temperature maintained at 75°F, the humidity at 50 to 55% and the photoperiod at 15 hours. Because the lights were mounted on the rear wall of the environmental chambers, the stacks of trays were rotated three times per week to ensure equal illumination and even insect development.

Previous experimentation had shown that all larvae were infected and that maximum virus yield occurred 25 days after inoculation; the larvae were harvested after that time period. Nearly all the larvae were alive and heavily infected and only a few pupae had formed. The exact time is not critical in entomopoxvirus production as heavily infected larvae remain alive for longer periods and the 25 day period could safely be extended to 30 days. Four people removed the larvae and pupae from the cups with lightweight forceps and placed them in 6" plastic disposable petri dishes (Fig. 3). The larvae and pupae were placed in separate dishes and were then kept at -20°C.

The frozen larvae and pupae were freeze-dried (Fig. 4). At first intact insects were put in the freeze-drier but it was found that they took 3-4 days to dry completely. A large backlog

1 Appendix I

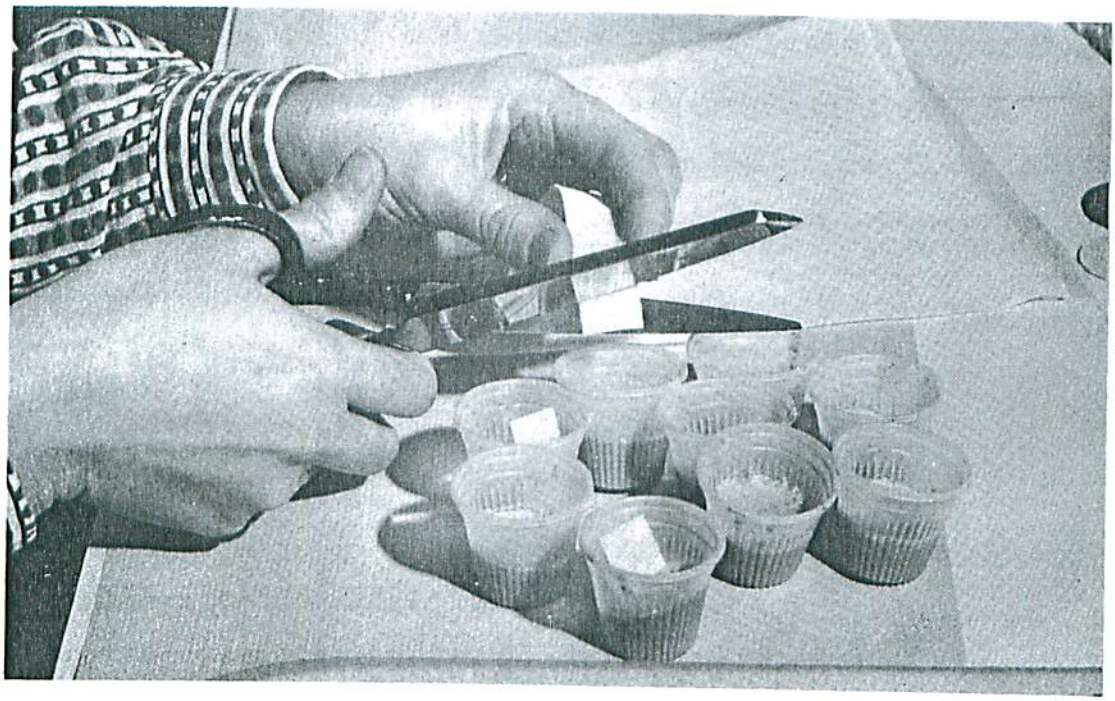

Fig. 6. Squares of muslin containing second-instar larvae are put in cups intended for NPV production.

Fig. 7. Fifth-instar larvae are transferred on to diet sprayed with NPV.

of material soon accumulated and it was found that the time could be reduced to 24 hours if the integument was broken. Therefore the frozen larvae were put in plastic bags, pounded with a rubber mallet until the larvae were fragmented, ground in a Waring blender and finally freeze-dried. After freeze-drying the larvae were ground to a fine powder using a Waring blender (Fig. 5). Each day's production was stored separately and then bioassayed on test insects to confirm the absence of contaminating viruses.

Nuclear polyhedrosis virus production

After field trials the NPV produced in 1970-71 was found to be contaminated with CPV and a pure culture was maintained independent of the mass rearings. Basically the same procedures were used for NPV as for entomopoxvirus production, diet, temperature, humidity and light being the same. Fifth-instar larvae are the best size to infect for production of NPV and two methods were used. In the first method second-instar larvae were placed on uncontaminated diet, 15 ml per cup, and reared to the fifth-instar. Without opening the cups a syringe was used to layer 0.5 ml of NPV suspension containing 2×10^7 inclusion bodies per ml on the surface of the diet. The diseased larvae were collected after 8-10 days.

Although very economical in terms of labour and artificial diet this method was abandoned. The larval development was very uneven and while the majority of larvae were fith-instar, third-,

fourth- and sixth-instars were also present. Hence there was considerable wastage as maximum virus production was only achieved in some of the larvae.

The second method, which was considered superior, involved setting up on diet (12 ml/cup) second-instar larvae still in their hibernacula but ready to emerge. This was done by Grisdale's technique (Grisdale, 1971) where larvae are stored spun-up in layers of muslin. Squares (1 cm) of muslin containing approximately 25 larvae were placed in each cup (Fig. 6). They were reared until the first fifth-instar larvae appeared. The cups were examined daily for 3 or 4 days and as more larvae reached fifth-instar they were removed and placed on fresh infected media (7 per cup on 10 ml diet sprayed with an NPV suspension of 2×10^7 inclusion bodies per ml) (Fig. 7). When first larval mortality occurred in 8-10 days all the remaining larvae in the batch were collected and frozen. The harvesting time is more critical for NPV as larvae in the terminal phase of NPV undergo complete tissue breakdown. If the fragile integument is ruptured a milky suspension of viral inclusion bodies and tissue debris leaks out.

RESULTS AND DISCUSSION

In the winter of 1970-71, with 4.4 man years labour a total of 4,185 gm of freeze-dried virus-infected larvae was produced and in 1971-72, with 7.4 man years labour a total of 24,434 gm was produced. Hence the efficiency of the 1971-72 operation was

more than triple that of the previous year.

Entomopoxvirus-infected larvae grow much larger than normal healthy larvae (Bird, Sanders and Burke, 1971) and it was found that a gram of freeze-dried material contained about 30 larvae. In contrast there were about 66 NPV-infected larvae per gram. Hence it is easier to produce entomopoxvirus in large quantities.

The larval equivalent (i.e. the number of inclusion bodies from one larvae) of the entomopoxvirus was found to be 6×10^8 inclusion bodies and the NPV was 0.5×10^8. These figures compare unfavourably with larval equivalents of some other species cited by Ignoffo and Hink (1971). For example the larval equivalent of Trichoplusia ni NPV was calculated to be $60\text{-}80 \times 10^8$ in one experiment and 130×10^8 in another, and Heliothis zea was found to be 150×10^8. The larvae of both these species are, however, much larger than the spruce budworm.

The number of larvae infected and the quantities of freeze-dried virus-infected material are given in Table 1 for the 1970-71 operation and in Table 2 for the 1971-72 production.

Table I. Spruce budworm virus production in 1970-71

	Entomopoxvirus	NPV
Number of larvae infected	222,000	222,000
Gm. freeze-dried larvae	1,885	2,300

Table II. Spruce budworm virus production in 1971-72

	Entomopoxvirus	NPV (method 1)	NPV (method 2)
Number of larvae infected	991,000	397,378	372,897
Gm. freeze-dried larvae	16,838	2,861	4,745

On the basis of 30 larvae per gm it can be calculated that in the course of entomopoxvirus production there was a considerable loss of insects during production. With no loss of larvae a total of 33,000 gm could have been produced from 991,000 larvae, but in fact, only 17,000 gm was obtained. Of this 48% loss about 1% was due to fungus, 20% to pupation (the pupae were not counted in the total) and the remainder to loss of small larvae. When handling tiny second-instar larvae a considerable loss is largely unavoidable. In the 1970-71 entomopoxvirus production about 30% of the infected material was collected as pupae but this figure can be reduced to as low as 5% by adjusting the dosage of virus and the rearing temperature.

The second method of NPV production used in 1971-72 involving the selection of fifth-instar larvae was about 40% more efficient than the method of inoculating the cups and was definitely worth the extra manpower. The losses by this method were very small as only fifth-instar larvae were counted and handled. It was not possible to determine the loss of small larvae while rearing them to fifth-instar. After setting up fifth-instar larvae there was

a 9% loss due to pupation and a further 9% loss to unknown causes.

This programme is continuing and methods are constantly under review.

ACKNOWLEDGEMENTS

We wish to thank Dr. J. M. Cameron for his support of this project and Dr. T. A. Angus for his assistance in arranging the facilities, and the encouragement and advice he has given. We also wish to thank Mr. D. E. Anderson and Mr. E. R. Rayner for taking the photographs.

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APPENDIX 1

Manufacturers of equipment and materials

Creamer cups (3/4 oz.)

Portion Packing Ltd.,
26 Tidemore Ave.,
Rexdale, Ontario.

Creamer caps

Standard Cap & Seal (Canada) Ltd.,
Burlington, Ontario.

Pressed board cafeteria trays

Keyes Fibre Company,
Waterville, Maine 04901.

Woods No. 66 Sprayer

G. H. Wood & Co. Ltd.,
P. O. Box 34,
Toronto 18, Ontario.

"Conviron" cabinet

Controlled Environments,
661 Madison St.,
Winnipeg 21, Manitoba.

Environmental cabinets

Ross Engineering of Canada Ltd.,
Ville Lasalle,
Quebec.

APPENDIX II

The effect of temperature on the development of entomopoxvirus in spruce budworm larvae

Temperature °F	Number of larvae	% virus infection in larvae	% Pupae	Number of days until harvested
80	431	29	62	20
75	316	93	5	25
70	330	100	0	32

Second-instar larvae were placed 7 per cup on diet sprayed with 0.15 ml virus suspension containing 10^6 inclusion bodies per ml. A temperature of 75°F was selected as being optimal; the small percentage of unwanted pupae is compensated by the reduced length of time to harvesting.