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DENSOVIRUS AS INSECTICIDE

A RESEARCH PROJECT

U.S.-Israel CDR PROGRAM

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Submitted by

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U.S.- ISRAEL CDR PROGRAM

DENSOVIRUS AS INSECTICIDE

THAI GROUP

Contents

1. Introductions 1

2. Objectives 2

3. Materials and Methods 3

4. Results and Discussion 8

5. Conclusion 26

6. Workplan for the Next Period 27

7. References 28

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DENSOVIRUS AS INSECTICIDE

THAI GROUP

INTRODUCTION

Species of Heliothis and Spodoptera complex are major pests that attack at least 30 different important food and fiber crops in Thailand. The extensive and indiscriminate use of pesticides for controlling these two insect species has created many serious problems upsetting the environment and human welfare. Insect viruses due to their specificity and efficiency are receiving considerable attention to develop as microbial insecticides. Densonucleosis virus (DNV) is a unique group among the Parvoviridae family. This group of viruses includes at least 10 virus strains that isolated from different insect species. DNV's are extremely stable to the environment and exhibit a narrow species specificity allowing a selective, targeted action against certain insects without harming others. Besides causing disease in insect larvae, densoviruses offer an excellent potential vector to transduce foreign genes into insect cells. They are single-stranded DNA, small and structurally simple viruses which make them amenable to genetic manipulations and the study of their gene expression and regulation relatively easier than that of larger viruses. They are structurally similar to their family members from the mammalian parvoviruses suggesting that they may be found capable of integrating their DNA into their host genome. All these properties make DNV an excellent candidate as biological control agents.

OBJECTIVES

The present study is aiming at evaluating the potential of densoviruses (DNV) as microbial insecticide. The overall objectives include (a) the use of Galleria mellonella densovirus (GmDNV) DNA as a model system for the construction of an insect vector, and using it to transport and express foreign genes in insect larvae ; (b) isolation of DNVs which can cause disease in the agricultural important pests Spodoptera and (or) Heliothis ; (c) study the genome organization and gene expression of these DNVs as a preliminary step toward utilizing them as insect vectors.

This report covered the research work done by the Thai group during the period of May 1987 to October 1987. The specific objective during this report period include (a) isolation of the field strain of DNV which can cause disease in Spodoptera or Heliothis ; (b) mass propagation, isolation and purification of the Galleria mellonella densovirus in order to obtain a highly purified stock for further experiments ; (c) study on cytopathic effects of GmDNV for the understanding of the tissue specificity, mode of action, viral replication and the kinetic of the disease progression.

MATERIALS AND METHODS

1. Isolation of the field strain DNV

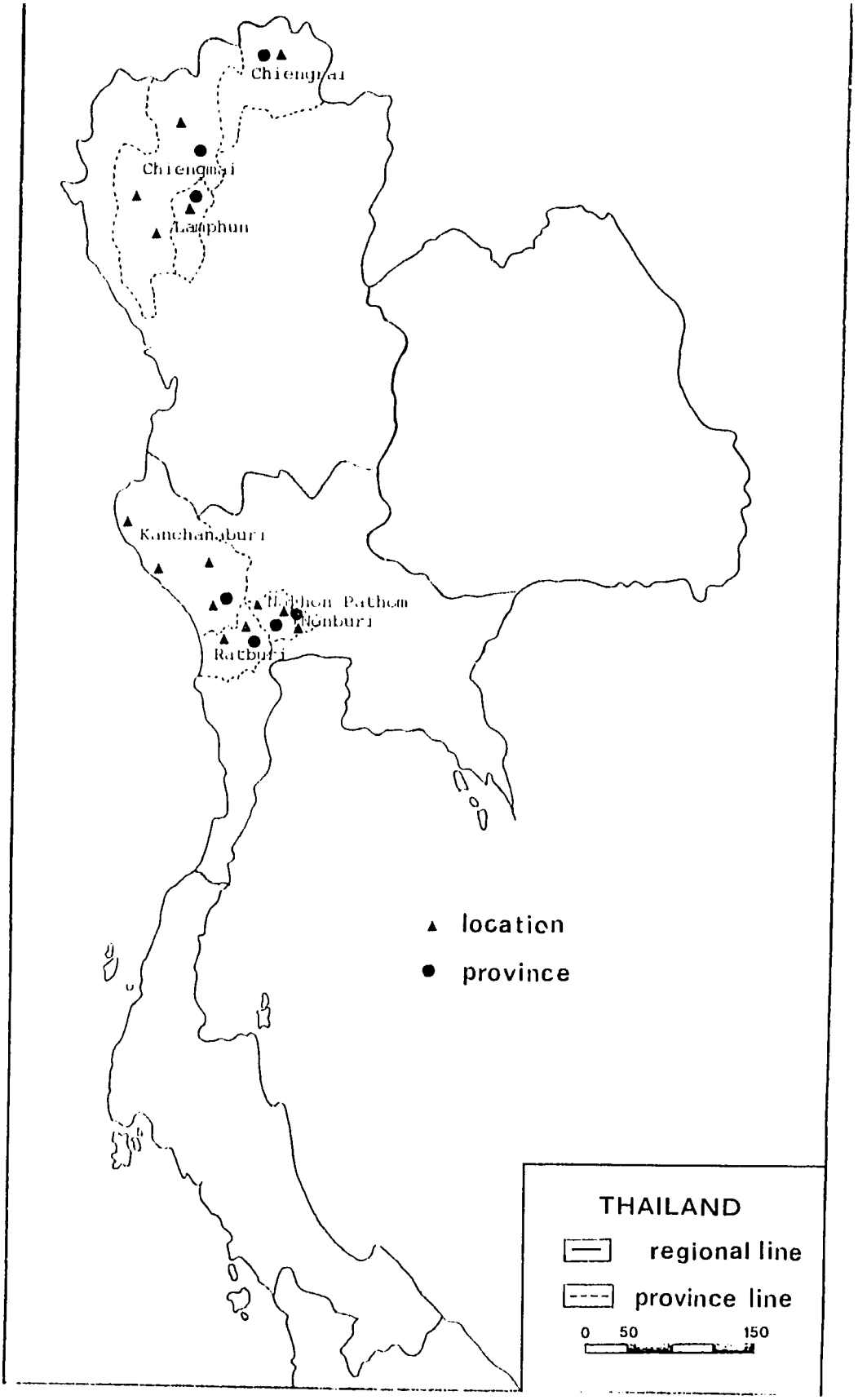
Explorationsof the field strain DNV of Heliothis armigera and Spodoptera exigua were planed by survey and collecting larvae at various locations in Thailand throughout the year according to the planting season in each part of the country. During the period of May 1987 to October 1987, surveys were made in the Northern part and the Central plain area of Thailand as indicated in Fig. 1. The name of the observed districts and number of surveys made in each location were summarized in Table 1.

The larvae of Heliothis armigera and Spodoptera exigua collected from planting areas of the farmers in several locations were separately reared on artificial diet in the laboratory. Diseased larvae were dissected and examined under the optical microscope for the presence of the causative organisms. Since the densovirus is very small and can not be seen by the light microscope. Diseased larvae which no detectable microorganisms were further investigated by the electron microscope. Dip preparations from disease larvae stained with 1 % uranyl acetate were examined for the presence of the densovirus. To confirm the existence of the virus in insect tissues, ultrathin sections were prepared (Materials and Methods No. 4) and observed under the electron microscope.

2. Mass propagation of the DNV of the Galleria mellonella

Currently there are no DNV isolates from Heliothis and Spodoptera. We proposed the second approach on utilization the DNV of the greater wax moth, Galleria mellonella by raising the DNV host-range mutants capable of

Fig. 1. The Map of Thailand demonstrated the provinces and districts surveyed for densovirus of *Heliothis armigera* and *Spodoptera exigua* during the period of May-October 1987.


Chiengrai
Chiengmai
Lamphun
Kanchanaburi
Nakhon Pathom
Nonburi
Ratburi
location
province
THAILAND
regional line
province line
0 50 150

9

growing in these two target insect species. While exploring the wild type strains of Heliothis and Spodoptera DNV, the GmDNV was studied concurrently in various aspects.

The DNV of the greater wax moth, Galleria mellonella (GmDNV) was obtained from Dr.Jacov Tal, the co-principle investigator from the Ben-Gurion University of the Negev, Israel. The samples received were the wax moth larvae infected with densovirus. To obtain a large amount of GmDNV for host-range mutant experiments, mass propagation of this virus was developed. The wax moth, G. mellonella were collected from wax honey comb foundations in several bee keeping areas in Chiengmai and Lamphun provinces in the Northern Thailand. Mass rearing technique of the greater wax moth was achieved using modified artificial diet. The third instar larvae were inoculated with GmDNV by diet surface contamination method. Moribund larvae at 7 days after the virus inoculation were collected. The larvae were allowed to putrefy for several days at room temperature and this preparations were kept in refrigerator until used. By the described method, adequate amount of GmDNV was obtained for further experimental uses.

3. Isolation and purification of the GmDNV

The purification procedure used was a modification of that described by Tijssen et al. (1976) and Tijssen and Kurstak (1981). The virus preparation was diluted (1 : 1) with a 0.025 M sodium phosphate buffer solution, pH 7.5 (this buffer solution was used throughout the purification procedure), then homogenized at 1,500 rpm for 5 min at room temperature. Lipid were extracted from the supernatant by vigorous shaking with an equal volume of chloroform:n-butanol mixture (1 : 1) and the phases were separated by centrifugation at 10,000 rpm for 10 min in J-21 C

Beckman centrifuge equipped with a JA-20 rotor. The supernatant was then subjected to centrifugation at 16,000 rpm for 75 min in JA-20 rotor. The pellet was suspended and centrifuged at 16,000 rpm for 75 min. The two supernatant were then pooled and pelleted at 40,000 rpm for 75 min in Hitachi 85 P-72 ultracentrifuge equipped with a RP 83 T-143 rotor. The pellet was suspended in buffer solution and centrifuged at 18,000 rpm for 30 min. The supernatant was centrifuged at 40,000 rpm for 75 min and the pellet was recovered with 0.1 x SSC buffer(1xSSC = 0.15 M NaCl plus 0.015 M Sodium citrate). The optical density of this partially purified virus preparation was measured in a Hitachi Model 200-20 spectrophotometer.

The partially purified virus was further purified by centrifugation in a linear sucrose gradient 8, 15, 25, 37 % in 0.1 x SSC buffer solution. Virus samples (0.5 ml) in 0.1xSSC were layered on top of the gradient and centrifuged at 30,000 rpm for 80 min in Hitachi RPS 65T swinging bucket rotor. The fractions were collected and dialyzed against 0.1xSSC buffer at $4^{o}C$ overnight. This virus preparation were negatively stained with 1 % uranyl acetate on formvar-coated grids and examined with a JEOL JEM-100S electron microscope.

4. Pathology of the GmDNV

Cytopathic effects of the GmDNV were studied from ultrathin sections of the infected tissues. Fourth instar larvae of the greater wax moth were fed a diet contaminated with GmDNV. Diseased larvae were collected after 4 days and pieces of tissue were dissected approximately 3-5 mm in diameter. Tissues were then fixed for 3 hr in 2.5 % glutaraldehyde in 0.1 M

phosphate buffer, pH 7.2. Following a 15 min wash in buffer solution, tissues were post-fixed in buffered 1.0 % osmium tetroxide, washed and dehydrated through an ethanol series to propylene oxide, and embedded in Epon-Araldite mixture. Ultrathin sections, 600-700 A^0 thick were cut and stained with 1 % uranyl acetate and 1 % lead citrate. Observations were made with a JEOL-JEM-100S electron microscope operated at 80 kv.

RESULTS AND DISCUSSION

1. Isolation of the field strain DNV

Fourteen districts in 7 provinces in the Northern and the Central Plain area of Thailand were investigated for the field strain densoviruses of Heliothis or Spodoptera. Larvae of these two insect species were collected from 60 locations of onion, cotton and tomatoes planting areas as indicated in Table 1. Microscopic observations were used to determine the causative microorganisms in diseased larvae. The results indicated that the major mortality factor is the bacterial diseases. Other diseases include viral disease caused by occluded viruses and protozoa disease caused by microsporidia. Parasitization and insecticide were also considered as the causes of the death of the larvae. During this report period, the densovirus of these two insect species has not been discovered. However many samples are awaiting for investigation and the results have not yet been concluded. Chao et al.(1985) reported a newly isolated DNV from Pseudoplusia includens, a Lepidopteran insect from the genus Noctuidae, which is homologous to Heliothis and

Table 1. Number of locations in several districts in the Northern and Central plain area of Thailand explored for the naturally occurring strains of densovirus of *Heliothis armigera* and *Spodoptera exigua*.

Province	District	No. of locations surveyed
Nakornpathom	Banglane	3
	Kamphaengsaen	16
Ratburi	Dummernsadauk	4
	Banpong	2
Nonburi	Bangbuatong	3
Kanchanaburi	Tarmaung	8
	Muang	2
	Tongpapoom	4
	Saiyok	6
Lamphum	Muang	2
Chiengmai	Fang	4
	Chiengdown	2
	Sarapee	2
Chiengrai	Jadeeyai	2

Spodoptera. Therefore we hope that the DNV of these two insect species do exist in the nature. We speculate with high degree of assurance that the GmDNV has serological relationship with other DNV's. Once the GmDNV is purified, attempts will be made to develop a rapid diagnostic of the diseases in the larvae, for example, the ELISA technique. More surveys and explorations for the field strains DNV are continuing in the other parts of the country.

2. Mass propagation of the Galleria mellonella DNV

The greater wax moth, G. mellonella has never been reared in our laboratory. Attempts were made to raise G. mellonella culture on artificial diet. The rearing medium used were modification of the diet described by Yendol,1 Two formula of the modified diets as shown in Table 2 were experimented. The baby food supplement (Produced by Food Research and Development Institute, Kasetsart University) in the second formula was used in substitution to the mixed cereal (Nestum) in the first formula. The mixed cereal is very expensive. The results revealed that both formulas are equally good. The greater wax moth undergoes normal life cycle (Fig. 2). White creamy eggs were laid in cluster. Eggs hatch within 5-7 days after laying. There are 5 instars larvae. The larval stage is 25-32 days and the pupa stage is 10 days. The life span for the female and the male moth are 7 days and 10 days respectively. To date, five generations were raised successfully in continuous in the laboratory. This experiment was quite significant since more economic diet is used with satisfactory results. Current larval

14

Table 2. The composition of the diet for rearing larvae of the greater wax moth, *Galleria mellonella* (Linnaeus)

Original Medium		Modified Medium (in g)			
Yendol's diet (in g)		Formula I		Formula II	
Pablum Mixed Cereal	293	Mixed Cereal (Nestum)	200	Baby Food Supplement	300
Kellog's Special K	20	Dry Milk	15	Dry Milk	15
Carnation Instant	23	Honey	50	Honey	50
Honey	142	Glycerine	60	Glycerine	60
Glycerine	127	Vitamin stock*	15	Vitamin stock*	15
Water	50	Water	20	Water	100
Pollen	38	Formalin 40 %	5	Formalin 40 %	3
				Choline chloride	0.5

* Vitamin stock contained (in g) niacin, 6 ; calcium pantothenate, 6 ; thiamine (B1), 3 ; riboflavin (B2), 3 ; pyridoxine, 1.5 ; folic acid, 1.5 ; biotin, 0.12 ; vitamin B12, 0.012 ; inositol, 10 ; choline chloride, 25 in 1000 ml of distilled water.

-15-

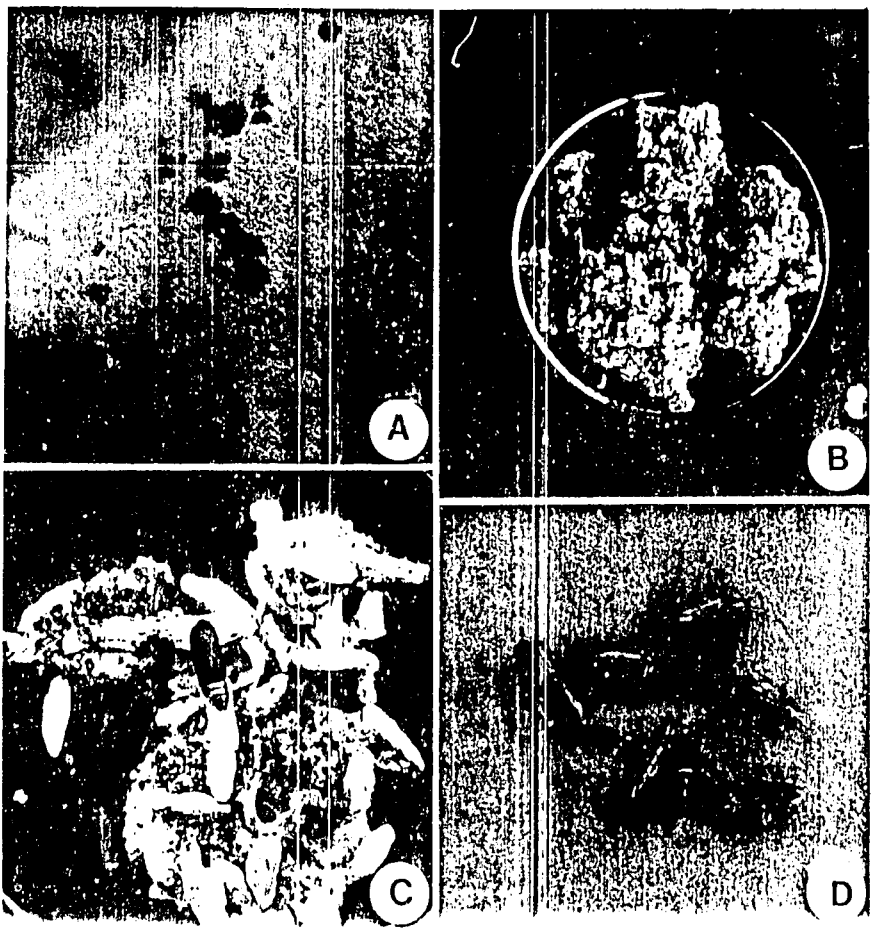
Fig. 2. The greater wax moth, Galleria mellonella reared on artificial diet in the laboratory.

A. The egg masses on filter paper.

B. Young larva feeds on artificial diet.

C. Pupae usually form inside the shelter or cocoon.

D. Adult male and female wax moth.



-17-

production technology provides adequate insect hosts for GmDNV production.

Mass propagation of the GmDNV was accomplished by using the greater wax moth as insect hosts. Diet surface contamination method caused 90 % mortality in fourth instar larvae. GmDNV harvested by allowing the diseased larvae to putrefy several days at room temperature created many contaminants which then make the purification procedure even more difficult. Freezing the diseased larvae right after harvesting and homogenizing them collectively is another trial which expects to improve the purity of the virus preparation. To avoid the impurity probleme and to obtain the optimal production of GmDNV, *in vitro* production was investigated. Primary cultures, both ovarian culture and hemolymph-cell culture were established by the method of Goodwin (1975) and using Grace's insect culture medium (Grace, 1962). Once the insect cell cultures were established, the studies on viral DNA synthesis and viral replication, morphogenesis and cytopathology can be made in both *in vivo* and *in vitro* systems.

3. Isolation and purification of the GmDNV

Highly purified preparations of GmDNV are needed for host-range mutant experiments and other serological studies. After lipid extraction and differential centrifugation, the partially purified virus was negatively stained and observed by electron microscope. The preparations comprised small, spherical particles measured 23 nm in diameter (Fig. 3). In his review paper on densovirus, Kawase (1985) stated that although all densovirus seemed to have diameters of the order of 19 to 24 nm, some variation existed. He also mentioned that Vago *et al.* (1964, 1966) reported the semipurified Galleria DNV stained with 2 % PTA had a diameter of 23 nm, but those in sections of insect tissues were 19-20 nm. Our present study is in an

agreement with that reported by Vago et al. In uranyl acetate stained preparations, many virus particle with black spot inside were observed. (Fig. 3 C,D). It was suggested they are probably empty particles which stain penetrated.

Many small globular structures were observed in all preparations (Fig. 3 C,D). Nakagaki and Kawase (1982) proposed the twelve capsomer model for the capsid structure of the densonucleosis virus of the silkworm, Bombyx mori. They suggested the globular structures were possible the capsomers from disrupted virus particles. This structure was also found in Galleria DNV reported by Tijssen and Kurstak (1979). To confirm this suggestion, we will separate this structure by sucrose density gradient centrifugation and determine its ultraviolet absorption spectrum.

Another structure frequently observed in partially purified preparations is the long thread-like structure which supposed to be flagella of the bacteria (Fig.3 D). However in negatively stained preparation from the healthy wax moth larvae, similar structure was also observed (Fig.3 A). Therefore it could be the structure derived from the insect host. However more observations have to be done to prove this speculation.

This present study showed that subsequent sucrose density gradient centrifugation gave rise to more clear preparation, but not enough to eliminate other contaminants. Further isopycnic centrifugation of the purified virus preparation in $CsCl_2$ is under investigated with the specific objective to improve the purity of the preparation. The partially purified

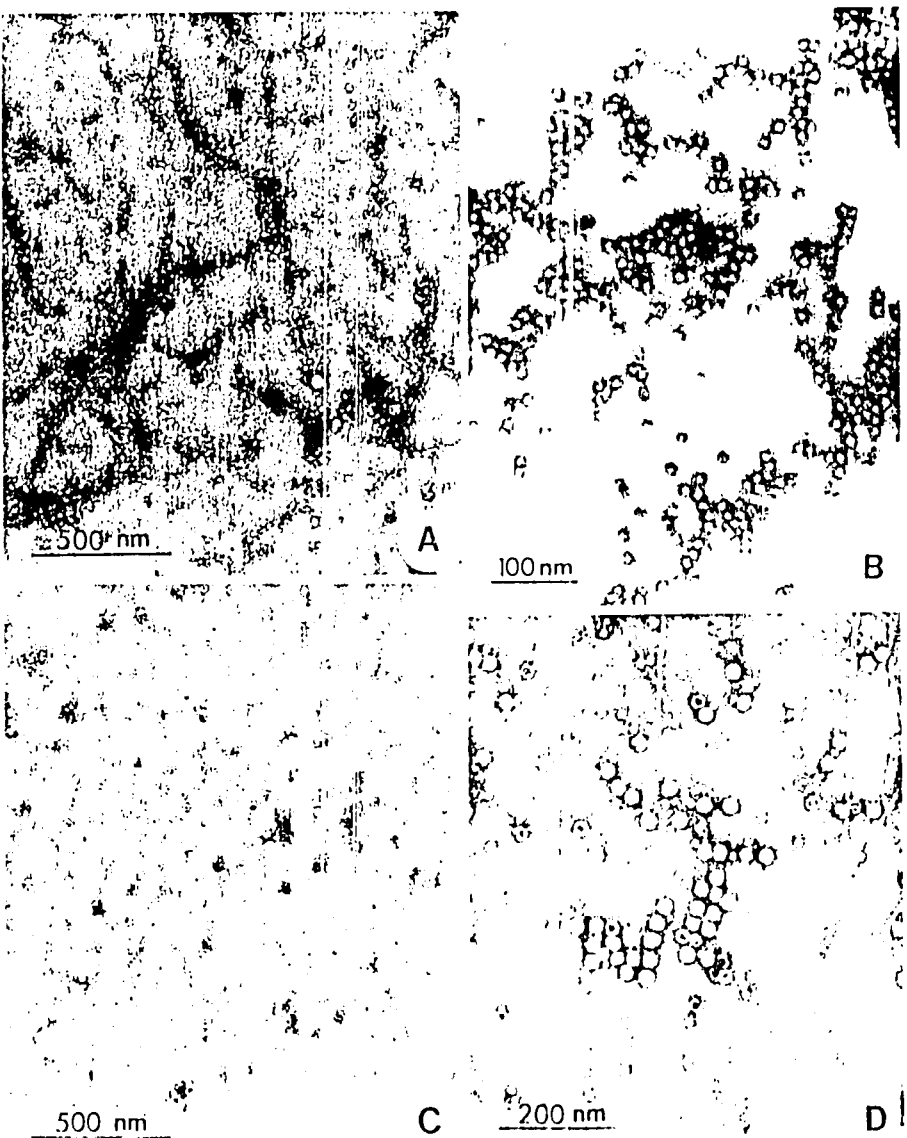
Fig. 3. Electron micrograph of the densonucleosis virus of the greater wax moth, Galleria mellonella

A. Negatively stained preparation from healthy larva.

B. Original purified preparation obtained from Israel, 2 % PTA stained.

C. Partially purified virus preparation using differential centrifugation, 1 % uranyl acetate stained.

D. Partially purified virus preparation using differential centrifugation and sucrose density gradient centrifugation, 1 % uranyl acetate stained.


500 nm
A
100 nm
B
500 nm
C
200 nm
D

GmDNV obtained by the described method had an ultraviolet absorption spectrum characteristic of a nucleoprotein with the ratio of absorbance at 260 nm to that at 280 nm = 1.447 (Fig.4).

4. Pathology

The objective of this study is to understand the cytopathic effects and the morphogenesis of the GmDNV. Similar phenomenons would expect to occur in other DNVs. Once the local strain or host range mutants DNV of H.armigera or S.exigua were obtained, their pathology studies will be facilitated by this background informations.

Densovirus of G. mellonella multiplies in almost all of the insect tissues with the exception of the mid gut (Kawase, 1985). The most obvious pathological changes occur in cells of the fat body and trachea. However the hemocytes, gonads and hypodermal cells are shown to be affected. The first ultrastructural change of the infected cells is the hypertrophy of the nucleus (Fig.5C). Electron-dense area or virogenic stroma are observed in the nucleus. The virus particles, measuring 20 nm in diameter (average from 50 particles) are found dispersing among the virogenic stroma (Fig.5D). The cellular chromatin and the nucleolus are pushed to the periphery of the nucleus and disintegrated finally. The virus particles multiply rapidly, accompanied by the progress in nuclear hypertrophy. At the last stage of infection, virogenic stroma are replaced by the virus particles which occupy the whole nucleus. The viral mass ruptures the nuclear membrane at several points which allows the passage of the virions to the cytoplasm (Fig.6 A).

In some of the infected cells, we observed the accumulation of the virions into paracrystalline array (Fig.6 E,F,G). Garzon and Kurstak, 1976

Fig. 4. Ultraviolet absorption spectra of the partially purified densovirus of the greater wax moth, Galleria mellonella in 0.025 M Sodium phosphate buffer, pH 7.5 .

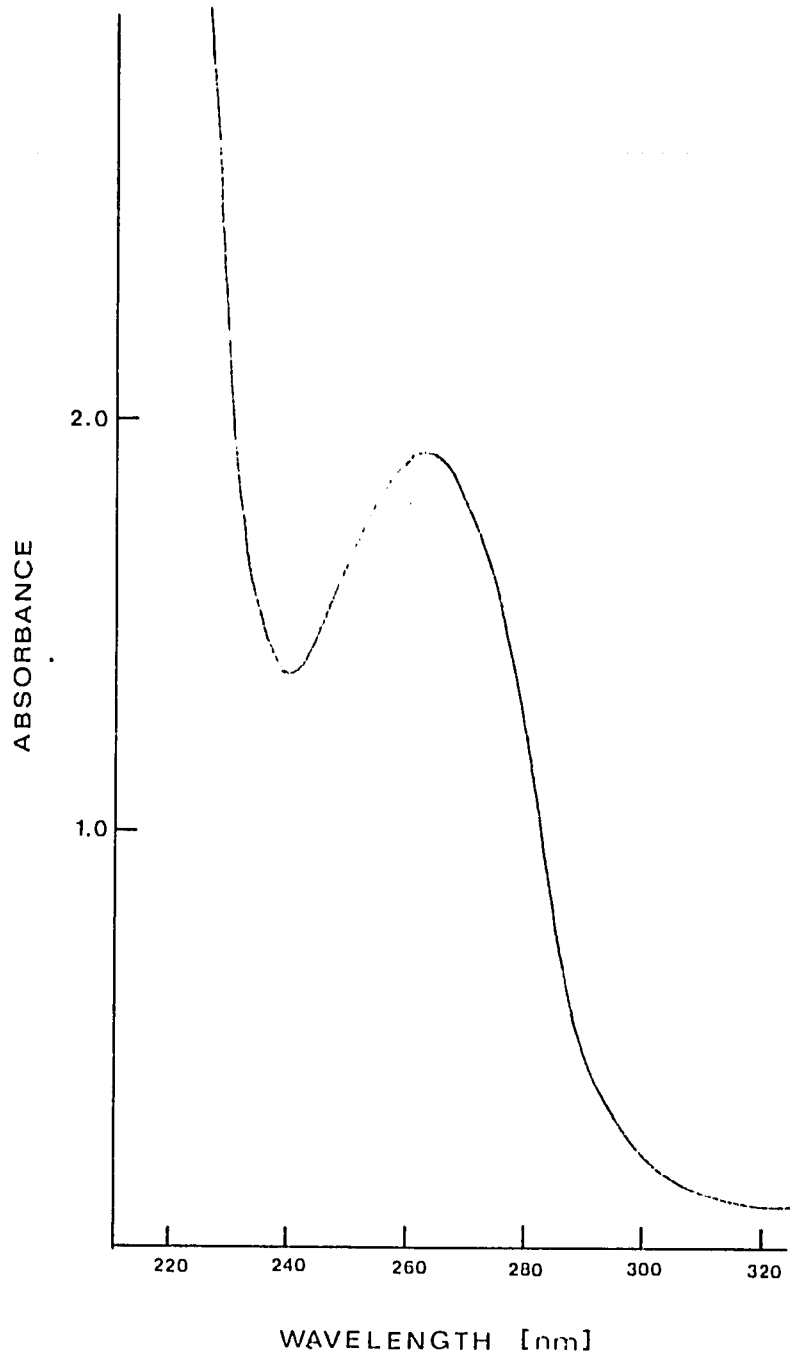

2.0
1.0
ABSORBANCE
220
240
260
280
300
320
WAVELENGTH [nm]

Fig. 5. Electron micrographs of healthy cells and densovirus infected cells of the greater wax moth, Galleria mellonella

A. Healthy gut cells demonstrated the compact and uniformly dense nucleolus (NU) and dispersed cellular chromatin (CH).

B. Healthy fat cell with patches of cellular chromatin.

C. Hypertrophied nucleus of the hemocyte. Note the cellular chromatins were pushed to the periphery.

D. Hypertrophied nucleus of the tracheal cell occupied by the virions dispersed among the virogenic stroma. N = nucleus, T = trachea cell.

A
B
C
D
T
1μm
1μm
1μm

reported the presence of the paracrystalline viral inclusions in the cytoplasm and nucleus. In our study we detected this arrangement of the virions only in the cytoplasm. This study revealed that the virions tend to stay individually at random in the nucleus. Once get into the cytoplasm they tend to arrange into a paracrystalline array. Several plans of crystallization can be observed. It is interesting to note that in some infected cells there is no obvious cytopathic effects in the nucleus, however many paracrystalline array of the accumulated virions are observed in the cytoplasm (Fig. 6 C). Virus particles arranged in paracrystalline array are frequently observed to be enclosed in microbody-like structure (Fig. 6 G). Further pathology study will be concentrated on the kinetic of the disease progression.

Fig. 6. Electron micrographs of cells infected with densovirus of the greater wax moth, Galleria mellonella.

A. Hypertrophied nucleus of fat cell filled with virus particles. The membrane is ruptured releasing virions into the cytoplasm (arrow).

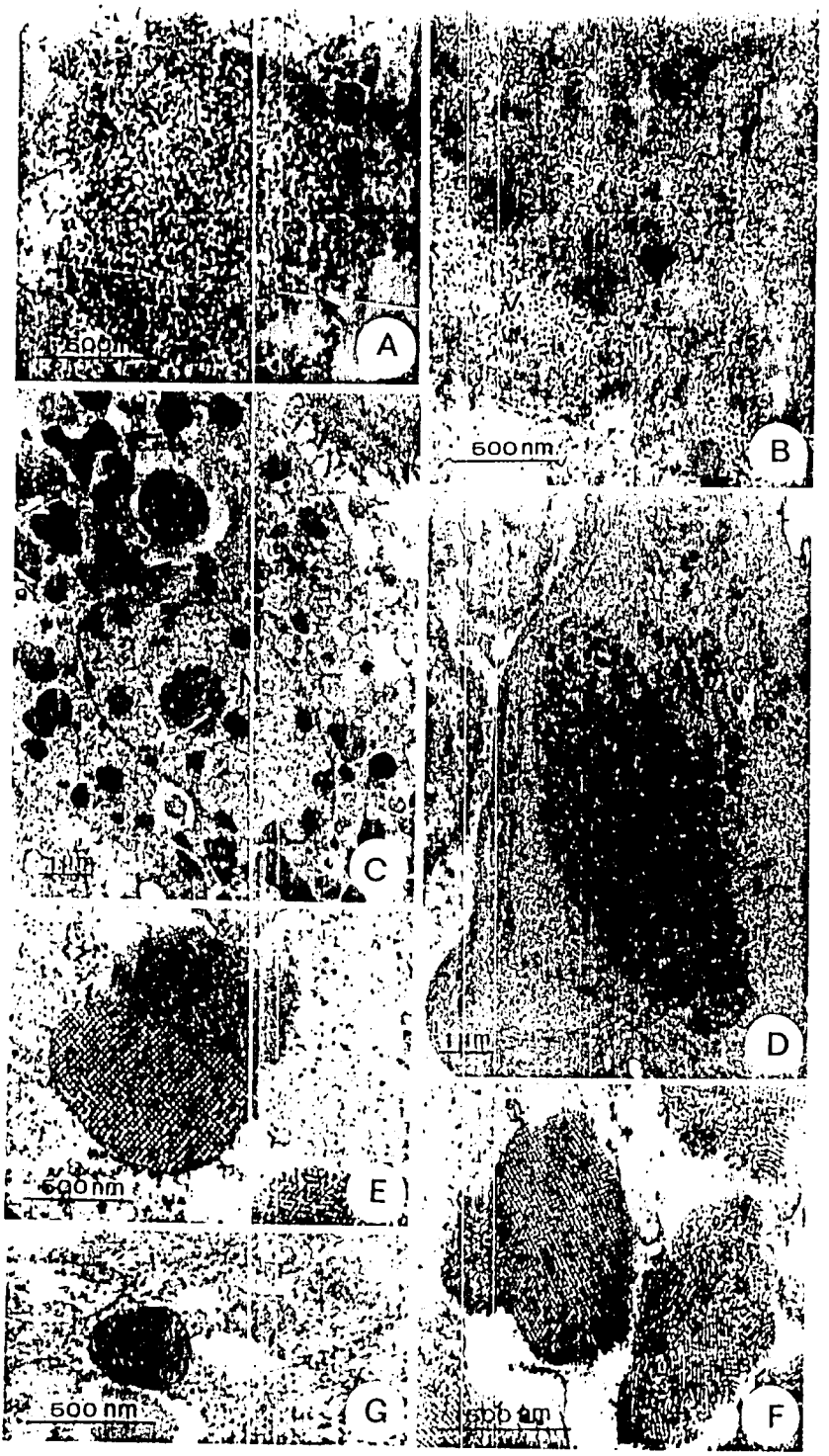
B. Virus particles (V) accumulated inside and around virogenic stroma (VS).

C. Healthy looking nucleus in which the cytoplasm is filled with virus particle accumulated into a paracrystalline array (arrow). N = nucleus

D. Hypertrophiednucleus of tracheal cell filled with virus particles which are in close association with virogenic stroma . N = nucleus

E. and F. Higher magnification showing the formation and arrangement of a paracrystalline array. Different planes of crystallization can be recognized.

G. Virus particles arranged in paracrystalline array are enclosed in "microbody-like" structure.



29

CONCLUSION

The work has been done according to the activity plan we proposed. It is unfortunately that the field strain of densovirus of the target insect species has not been found. Extensive surveys and exploration for the local strain will be continued. However we have already investigated the second approach on trying to raise the host range mutant strain. Successfully reared the greater wax moth in the laboratory will enable us to obtained sufficient amount of virus for experimental uses. When the Israeli group success in construction the GmDNV infections clone, it will be sent to us for replication in the wax moth larvae. Work will be concentrated on purification procedure in order to obtained highly purified virus for serological study. Primary insect cells were cultured in the laboratory at present. We aim to establish the in vitro system for viral production and other biochemical study of the densovirus. Useful informations and results from the experiments conducted by the Thais and Israeli have been exchanged and the work will be incorparated in close relationship.

WORKPLAN FOR THE NEXT PERIOD

1. Exploration and isolation the naturally occurring DNV strain of *Heliothis armigera* or *Spodoptera exigua* in other parts of the country will be continued.

2. Improve the densonucleosis virus purification procedure by modification of the described technique and using isopycnic centrifugation in $CsCl_2$.

3. Once the highly purified GmDNV preparation was obtained, antibody will be raised and the serological studies will be conducted which include the ELISA technique for rapid diagnostic of the diseased larvae. The ELISA work will followed the method described by Tijssen *et al.*(1982).

4. DNV host-range mutants will be raised in the laboratory by both per os inoculation and haemocoelic injection of the highly purified GmDNV.

5. When the insect cell cultures are established successfully, *in vitro* production of DNV will be developed. Other investigations include the studies on viral DNA synthesis, viral replication using immunofluorescence and autoradiography will be followed. We aim to study in comparable both *in vitro* and *in vivo* system.

6. Determine the potential hazard of the DNV local strains and the host-range mutants to the silkworm, *Bombyx mori*.

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U.S.- ISRAEL CDR PROGRAM

DENSOVIRUS AS INSECTICIDE

ISRAELI GROUP

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for the period 1.4.87 - 30.9.87
Grant No.936-5544-G-00-7016-00

Densovirus as Insecticide

10 November 1987

1. Introduction

The overall objective of this research is the construction of a genetic vector for transient expression in insects. Toxin genes, toxic to insects, will be fused into the vector DNA molecule and will be used for purposes of biological/genetic control of pests. The vector will be based on the genome of densovirus (DNV), a virus belonging to the *parvoviridae* family. The genome is single stranded DNA with an approximate size of 5900 base pairs, and like all other parvoviruses contains terminal palindromic structures amounting to 5-10% of the genome.

At this stage, we are working toward two short range specific aims. (i) we are aiming at determining the entire nucleotide sequence of the *Galleria mellonella* virus (GmDNV) DNA. (ii) in parallel and in collaboration with the |Thai group, we are attempting to obtain host range mutants of GmDNV, which will replicate in insects of agricultural importance such as *Spodoptera* and *Heliothis*.

2. Materials and Methods.

For specific aim 1, The sequencing method of Sanger, employing dideoxy nucleotides, is being used in conjunction with M13-based plasmids.

For specific aim 2, we are attempting to feed heterologous larvae with GmDNV virus, or inject the virus using a syringe. Larvae which develop disease will be analyzed by electron microscope to determine which larval tissues are affected.

3. Results

As a preliminary stage for sequencing, a detailed restriction map is being constructed. It now consists of over 100 cleavage sites. A sequencing strategy has been designed and subcloning of fragments is under way.

The isolation of host range mutants of GmDNV is being carried out in collaboration with the research group of Dr. Amos Navon from the Volcani Institute for Agricultural Research in Beith Dagan, near Tel-Aviv. Dr. Navon is an insect pathologist and his laboratory maintains cultures of Spodoptera and Heliothis. The experiments in this direction have just started, but we have already grown a virus stock for these experiments. It should be noted, however, that one preliminary injection experiment was done with encouraging results. Spodoptera larvae were injected with GmDNV and mid-gut sections were

35

visualized in EM. The pictures show abundant quantities of particles in the nuclei, but there is no proof yet that these particles are virus particles. In the future, sections will be sent to Dr. Attathom for analysis.

4. Discussion

The working plan proceeds along the lines described in the grant proposal. The collaboration with the group at the Volcani Institute is very promising, since we will be able to benefit from their expertise in handling live larvae for experimentation. Various larval instars will be examined for susceptibility to viral disease as well.