Optimization of *Helicoverpa armigera* NPV *invivo* mass production and regulation of malodor associated with the process.

By
Shireen Meher K.

Submitted in partial fulfillment of
the requirements for the Degree of

Master of Science
Environmental Science and Technology

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for the Semi-Arid Tropics (ICRISAT),
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Dedicated to my grandfather,
Shri T.V.Padmanabham
[Retd. DFO, Kakinada]

CERTIFICATE

This is to certify that the thesis entitled **"Optimization of *Helicoverpa armigera* NPV *invivo* mass production and regulation of malodor associated with the process"** submitted in partial fulfillment of the requirements for the degree, Master of Science in Environmental Science and Technology of Center for Environment, Jawaharlal Nehru Technological University, Hyderabad, is a record of the bonafide research work carried out by Mr. SHIREEN MEHER K under my guidance and supervision.

No part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledged. The author of the thesis has duly acknowledged all the assistance and help received during the course of the investigation.

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CERTIFICATE

Mr. SHIREEN MEHER K has satisfactorily prosecuted the course of research and the thesis entitled "**Optimization of *Helicoverpa armigera* NPV *invivo* mass production and regulation of malodor associated with the process**" submitted is the result of original work done and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any university.

Dt: 10.4.2003

(Dr. G.V.RANGA RAO)
Project Coordinator.

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Degree:	**Master of Science in Environmental Science and Technology.**
University:	**Jawaharlal Nehru Technological University, Masab Tank, Hyderabad, India.**
Project Location:	**ICRISAT, Patancheru.**
Year:	**2002-2003.**

ABSTRACT

The indigenous production of *Helicoverpa armigera* nuclear polyhedrosis virus (NPV) has been proficiently employed by several farmers after realising its prominence as a prospective biopesticide. The only issue of concern outlined by them being the malodor associated with the recovery process, which the other farmers in the community often complained with. Also the recovery of the virus needed to be quantitatively optimized to enhance its efficiency and economy as a microbial biopesticide.

An endeavor has been made in this regard to quantify the viral recovery at different post inoculation (PI) days to obtain the maximum poly inclusion bodies (PIBs) and to regulate the malodor through several techniques. These studies were conducted at International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Andhra Pradesh.

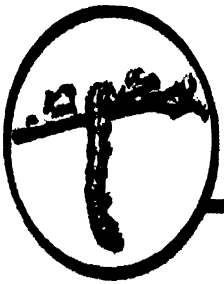
Maximum larval mortality was found to be 88% on 7th day of PI followed by 50% on 6th day of PI. The NPV yield was maximum, 0.70 LE/larva at 7th day followed by 0.64 LE/larva at 6th day of PI. The ideal period of viral harvest can be suggested to be 6th day of PI when the mortality percent and NPV yield were in accord for optimal viral recovery to avoid the constraint of malodor associated with the *Helicoverpa armigera* NPV production.

LIST OF FIGURES

LIST OF ABBREVATIONS

ABREVATION	ACRONYM
BV	Budded Virus
HNPV	*Helicoverpa armigera* Nuclear Polyhedrosis Virus
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
LE	Larval Equivalent
NPV	Nuclear Polyhedrosis Virus
PDV	Polyhedron Derived Virus
PE	Polyhedral Envelope
PI	Post Inoculation
PIB	Poly Inclusion Body

CONTENTS

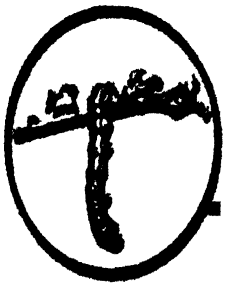


Prologue

The species of genus *Helicoverpa* have assumed the serious pest status on many important agricultural crops across the world. *Helicoverpa armigera* is the major species, causes great economic losses and thus is target of considerable insecticide application. Environmental concerns over excessive synthetic chemical insecticide use increases favor the development and marketing of alternative safer methods of pest control. In this process of the several components of integrated pest management (IPM), nuclear polyhedrosis virus (NPV) has been recognized as a promising technological solution. The virus is highly potent and host-specific which has revolutionized it as a consistent microbial biopesticide. In recent years several organizations including ICRISAT have been promoting the use of eco-friendly biopesticides including NPV.

Production of *Helicoverpa armigera* nuclear polyhedrosis virus or HNPV is majorly *in vivo* and exploited even by several farmers themselves in a domestic scale. Though these technologies were encouraging to the farmers, there are several bottlenecks that surfaced while attempting the large-scale production. Malodor was amongst them often brought about by the farming community. This constraint of malodor can be solely attributed to the byproducts of the lipid (insect mass) degradation by the contaminant bacteria. Apart from the malodor aspect, these bacteria compete with the virus for cellular mass, hence, affecting the NPV yield significantly. Hence the present study was undertaken to address the malodor problem for strengthening the existing technology.

The production procedure of HNPV has been studied experimentally to optimize the production and to provide proficient solutions to regulate the malodor. An attempt has been made to quantify the viral recovery from *Helicoverpa armigera* larvae at different intervals after inoculation so as to obtain maximum polyinclusion bodies and at the same time regulating the associated malodor.



Conceptual framework

The available literature concerning the present study is categorized and presented under following subheadings:

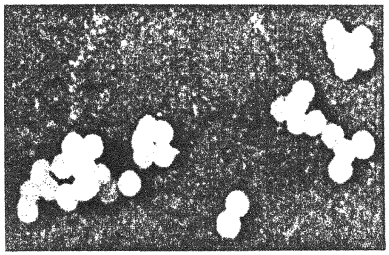
- Biology of *Helicoverpa armigera*
- Effects of *H.armigera* on various crops
- Conventional control measure of *H.armigera* and their constraints
- Integrated pest management
- Biology of nuclear polyhedrosis virus (NPV)
- NPV as biopesticide
- NPV production and quality assessment

Biology of *Helicoverpa armigera*

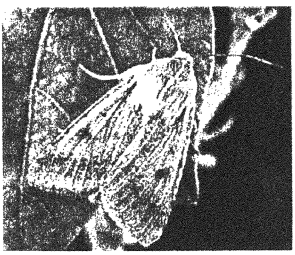
Helicoverpa armigera is the most prevalent, highly phytophagous pest in India and is of most relevance to agricultural economy. It has posed a serious threat to many crops and the apparent importance of the pest calls attention to vital biology of it.

Its life cycle on several crops has been studied at several locations by many workers. Singh.H and Singh.G (1975) have studied the various stages in the lifecycle of *Helicoverpa*, described as egg, larva, pupa and adult. Fig. 1 outlines the various stages in the lifecycle. The developmental period of the various stages depend upon the weather conditions and food (Bhatt.N.J and Patel.R.K, 2001). The larvae of *H.armigera* have been reported throughout the year on cultivated plants and weeds.

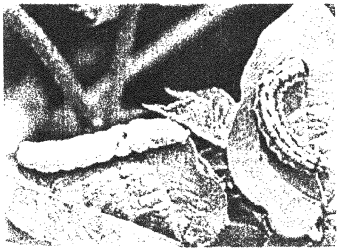
Larval stage attracts the maximum importance due to its relevance to the crop damage. Thus, it becomes the target stage for the application of control measures. The larvae pass through six instars. There is vast variation in the colour of the developed larvae. The total larval development period was around 18-20 days.



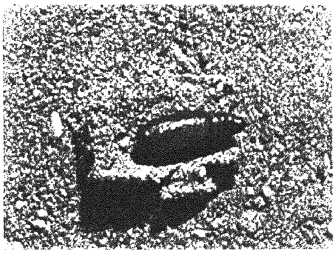
Eggs (4-6 days)



Adult moth (9-12 days)



Larvae (18-20 days)



Pupa (8-10 days)

Figure 1. Life Cycle of *Helicoverpa armigera*

Effects of *H.armigera* on various crops

In India the impact of the *Helicoverpa armigera* on the yield of various crops can differ very widely. Among several crops the importance of *H. armigera* was seen on the following crops. (Cunningham J.P. et al, 1999)

Pigeonpea (*Cajanus cajan*, Fabaceae),

Chickpea (*Cicer arietinum*, Fabaceae),

Maize (*Zea mays*, Poaceae),

Cotton (*Gossypium sp.*, Malvaceae),

Sorghum (*Sorghum bicolor*, Poaceae),

Sunflowers (*Helianthus annuus*, Asteraceae),

Soybeans (*Glycine max*, Fabaceae),

Tomatoes (*Lycopersicum esculentum*, Solanaceae),

Groundnut (*Arachis hypogea*, Fabaceae),

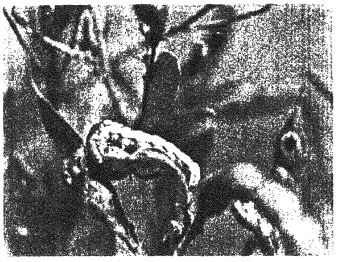
Alfalfa (Lucerne: *Medicago sativa*, Fabaceae),

Beans (*Phaseolus vulgaris*, Fabaceae),

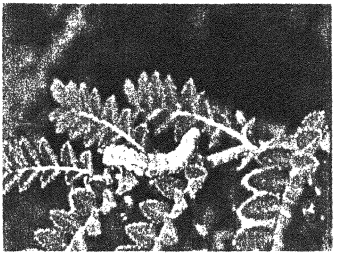
Tobacco (*Nicotiana tabacum*, Solanaceae), and

Cucurbits (Cucurbitaceae).

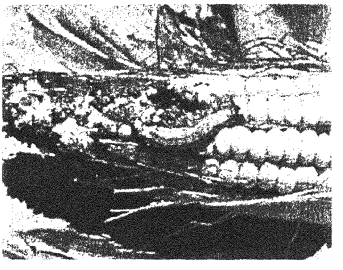
The apparent damage on various crops can be seen in the Fig. 2 (a, b, c, d, e and f).



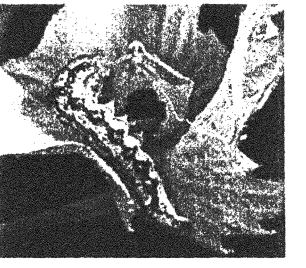
a. *Helicoverpa* larva on pigeonpea



b. *Helicoverpa* larva on chickpea

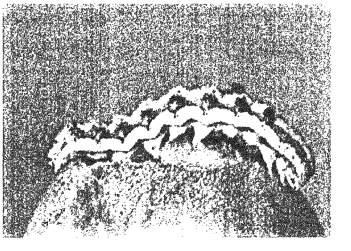


c. *Helicoverpa* larva feeding on maize



d. *Helicoverpa* larva feeding on cotton

e. *Helicoverpa* larva feasting millet



f. *Helicoverpa* larva feasting tomato

Figure 2. *Helicoverpa armigera* damage on different crops.

Conventional control measures of *H.armigera* and their constraints

Keeping in view the crop damage and agricultural economics, several control techniques have been developed and employed. Many of these have been reported to be efficient when used alone and in combination. With the emergence of snags in each technique improvisations and developments have been incorporated or new techniques have been employed. Pheromone traps have been successfully used for monitoring the pest population and farmers have been advised to carry out timely control operations against *H. armigera* (Mahajan et al, 1990). The control techniques can be broadly classified into three categories, viz. Cultural, Chemical, and Biological control.

Cultural control: These are the most primary control techniques employed by the cultivator majority of which include variation in sowing date, mixed/intercropping, handpicking and crop shaking. Last being the most effective technique in pigeonpea, which can remove 80-90% of the larval population. The most commonly employed crop shaking method is presented in Fig. 3 and intercropping in Fig. 4.

Chemical control: Chemical control is the most commonly used method hitherto, which is widely exploited by the farmers for the control of *H.armigera*. Dust as well as spray formulations of insecticides viz., carbaryl, phosvel, formothion, endosulfan and synthetic pyrehtriods like decamethrin, fenvalerate, cypermethrin etc. have been recommended (Dhruve V.R et al 1985). But, recent records (Ujagir R et al, 1997) accentuate the resistance of the pest to most of the insecticides. Also the increasing environmental concern against the insecticide pollution has promoted the researchers to explore safer and better alternatives.

Figure 3. Farmers performing crop shaking

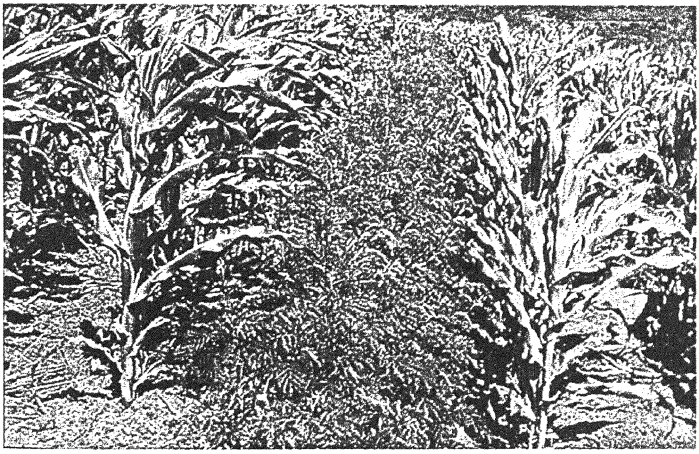


Figure 4. Intercropping of maize with a legume

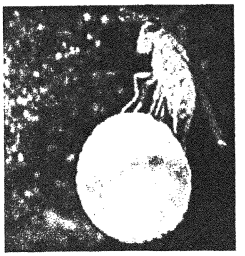
Biological control: Traditionally, biological control is defined as the action of parasites, predators and pathogens in maintaining the population of the pest at a lower level than would occur without these natural enemies. Biological control can be natural, conservative or augmentative (inoculative/inundative).

Larval parasitoids *Campoletis chlorideae* and *Eriborus argenteopilosus* have been reported causing 15-33% parasitism (Yadava C.P et al, 1985). Few of them illustrated in Fig. 5 (a, b, c and d). Other efficiently exploited species are *Bracon hebetor* and *Carcelia illota*. *Goniopthalmus halli* parasitizes the pupae of *H. armigera*. The green lacewing, *Chrysopa sceletes*; two reduviids viz. *Herpactor costalis* and *Rhinocoris squalis* and a mired bug, *Nesidiocoris tenuis* are recorded as predators of *H.armigera*.

The role of Avian predation on bollworms is also highly significant. Keeping bird perches @ 4-6 per acre facilitated large-scale exploitation of birds as predators. Drongos, Cattle egrets (*Bubulcus ibis*) being the most common bird predating on *H.armigera* (Ghode et al 1988). Bhagwat (1997) recommended bird perches to encourage predatory birds and stated that birds were found feeding on the dead virus-infected larvae. (as shown in Fig. 5 e and f)

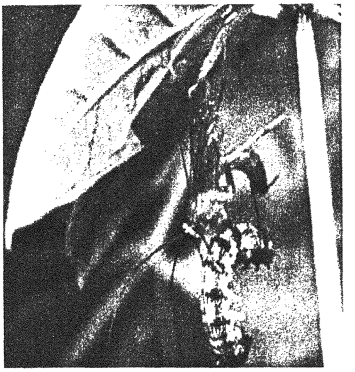
Several bacterial, viral, fungal and protozoan pathogens have been reported as successful biocontrol agents against *Helicoverpa sp.*

Bacterial pathogens	*Bacillus thuringiensis*
Viral pathogens	Nuclear Polyhedrosis Virus (NPV) and GranuloVirus (GV)
Fungal pathogens	*Metarhizium anisopliae* and *Nomuraea releyi.*
Protozoan pathogens	*Nosema* sp. and *Vairimorpha* sp.



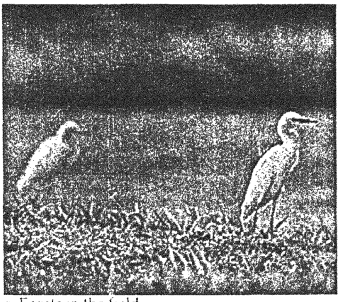
a [illegible] parasitizing prey

b Reduviid predator with *H. armigera* larva



c *Eriborus argenteopilosus* on *H. armigera* larva

d Mud wasp preying on *H. armigera* larva



e Egrets in the field

f Drongo on bird perch

Figure 5 Important natural enemies on *Helicoverpa armigera*

Integrated Pest Management

Integrated pest management (IPM) as defined by Food and Agriculture Organization (FAO) is a pest management system in the context of associated environment and population dynamics of the pest species utilizing all suitable techniques and methods in as compatible manner as possible and maintaining pest population at levels below causing unaccepted damage or loss. Thus IPM is the best combination of all possible approaches in pest management with least reliance on chemical pesticide. Increased awareness in recent years of the dangers of chemical warfare with insects, popularization of the concept "back to nature" by many environment protection groups there is an urgent need for an appreciation of value IPM and its effective implementation. The ultimate objectives of IPM are-

1. Reduce management cost.
2. Minimize environmental pollution.
3. Maintain ecological balance with minimum disturbance to ecosystem.

The goal of IPM is to manage pests and the environment so as to balance costs, benefits, public health, and environmental quality. IPM systems use all available technical information on the pest and its interactions with the environment. Because IPM programs apply a holistic approach to pest management decision-making, they take advantage of all appropriate pest management options, including pesticides, but does not mean the total elimination of pesticides.

Components of IPM: Integrated pest management is based on following different components.

- Host plant resistance
- Cultural control (Mechanical and physical control)
- Biological control
- Chemical control

Ahmed et al (1990) reviewed some recent approaches of IPM strategies to manage *Helicoverpa armigera* (Hubner). Similarly Lal (1990) recommends the use of biopesticides, Neem kernel extract, pheromone traps, advancing the sowing dates to avoid the pest, opting for resistant varieties, use of parasitoids like *Campoletis chlorideae* and pathogens like NPV in chickpea crops.

Biology of Nuclear Polyhedrosis Virus (NPV)

The nuclear polyhedrosis virus belong to the family of occluded DNA viruses, Baculoviridae. Representatives of the nuclear polyhedrosis virus (NPV) were the first insect viruses investigated. The virus is characterized by the presence of inclusion bodies, the so-called polyhedra, in infected insects. The nature and significance of these polyhedra remained a mystery for a long time until the electron microscope was available that the virus particle could be isolated and identified as the infectious viral agent. The word, "polyhedra" will designate inclusion/occlusion bodies found in the nucleus of the cells of insects as a result of virus infection.

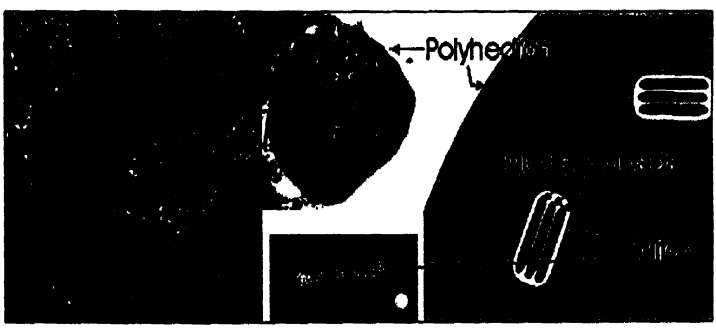


Figure 6. Cross-section of NPV polyhedron.

Size and Shape of Polyhedra: The size and shape of polyhedra varies considerably not only between the polyhedra from different insects, but often also within polyhedra of the same species. Majority of the polyhedral inclusion bodies (PIBs) of *H. armigera* NPV are spherical while some of them are irregular in shape. The size ranged from 0·6 μm to 2·3 μm averaging to 1·35 μm. The diameter of polyhedra ranges about 0.5 to 1.5 μm, depending on the insect species. Fig. 6 shows the cross-section of polyhedron of HNPV. In the boundary of polyhedron, the polyhedral envelope (PE) appears as an electron dense layer. The distance between the polyhedral envelop and polyhedral crystalline matrix is not uniform around the polyhedron. The mean volume of PIB is 1·29 ± 0·09 μm^3 and number of nucleocapsid per polyhedron is 113 ± 7·4. The fine structure of polyhedra reveals crystalline lattice of the polyhedra protein molecules, which are arranged in a cubic system. Although there is no true membrane covering the PIB, difficulties in staining PIB, the retention of their shape, and the presence of a membrane-like coat following chemical and physical treatment indicate that the exterior portion of a PIB is different for the interior portion. On the whole they are very stable and can persist indefinitely in the environment. (Gernot H. Bergold et al, 1982)

Morphology and size of viruses: Electron microscopic observations are necessary in order to investigate their exact morphology. The viruses can exist in the morphological states free virus particles isolated from polyhedra; virus particles still enclosed in polyhedra and virus particles within infected cell nuclei. In general they are rod-shaped, about 20-50 nm in diameter (incl. the developmental membrane), and about 200-400 nm long.

Virion: Infectious, rod-shaped virions are randomly occluded and singly embedded in PIBs without any apparent disruption of the lattice; an 8 nm layer separates virion from the protein matrix. Alkaline-liberated virions readily lose

their envelopes to reveal nucleocapsids each made up of a capsid surrounding a DNA core. The capsid, in turn, consists of protein subunits arranged along its long axis. The virions contain double-stranded, circular DNA molecules present super coiled and packed in the nucleocapsid.

Physico-chemical and chemical properties: It has been known for some time that acids or alkali disrupt PIBs and thus presumably destroy the viral activity. Polyhedra do not dissolve in hot or cold water, alcohol, ether, chloroform, benzol, or acetone. They dissolve, however, in aqueous solutions of NaOH, KOH, NH_3, H_2SO_4 and CH_3COOH.

=Polyhedra are heavier than water and therefore, collect at the bottom of wet mounts on microscopic slides, a characteristic, which helps to distinguish them from fat droplets, and debris, which float on top.

=Polyhedra migrate in electric field to the positive pole and have an isoelectric point of pH 5.2.

=Polyhedra consist of protein and no lipid, the two components of the polyhedra being,

- Polyhedral protein which constitutes about 95% of total weight and
- Virus particle, which amounts to 5% of the weight.

Although the field temperatures (15 to 45°C) had no effect on the stability of PIBs, viral replication was inhibited at 40°C. In water, 10min at 80°C completely inactivates the PIBs whereas 10min at 70°C reduces activity. Lyophilized preparations of PIBs at -20°Cand 5°C were very stable and lost no activity after 15 yrs.

Life cycle: Gary W. B et al, (1990) reported that the polyhedra ingested by the insect, is subjected to high pH (>10) dissolve and release the infectious virions. A unique feature of the NPV life cycle is the production of two virion phenotypes: Those virions found within polyhedra are termed polyhedra-derived virus

(PDV); the other form, found in the haemocoel of the infected host insect is termed budded virus (BV). Life cycle of NPV is represented in Fig. 7.

After rapid dissolution of the polyhedra in the midgut, the released virions (PDV) enter the host cells by fusion of the virion envelope with the microvilli of the midgut epithelial cells (Patel R.C et al, 1967). In the midgut cells, nucleocapsids are transported into the nucleus where they uncoat as early as 1hr PI and the virus undergoes a primary round of replication with progeny nucleocapsids observed as early as 8hr of PI. At 12hr PI some progeny nucleocapsids begin to bud through the nuclear membrane. In the cytoplasm the envelope acquired from the nuclear membrane is lost, and the nucleocapsid subsequently transported to and buds through the plasma membrane. These virions of the BV phenotype may infect many cells types (fat body, muscle, tracheal matrix, haemocytes, epithelial) producing a second round of replication. They may move out of the nucleus into the cytoplasm and bud through the plasma membrane, as BVs or they may be enveloped de novo in the nucleus and later occluded within polyhedra as PDVs.

Nuclear Polyhedrosis Virus (NPV) as biopesticide

The following attributes of NPV make it the most advantageous biopesticide. (Basavanna Goud et al, 1997)

- High host specificity
- High potentiality
- No effect on predatory and parasitic insects (natural enemies)
- Completely compatible with the all biologically based IPM approaches

Field trials on chickpea in India showed that HNPV at economic applications could control *H.armigera* more effectively than either chemical insecticides or commercially formulated *B.thuringiensis* (Cherry et al, 2000). It also showed that simple water suspension of unpurified HNPV itself was an effective formulation.

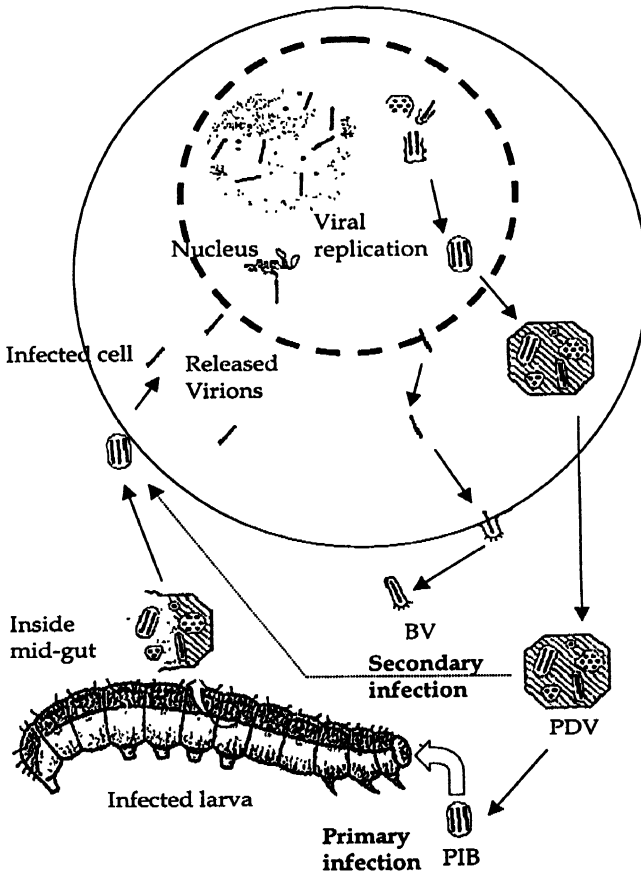


Figure 7. Lifecycle of nuclear polyhedrosis virus

NPV production and quality assessment (Bioassay)

To date the large-scale production of insect viruses has only been carried out by infection of healthy insects and harvesting of virus. Whilst *in vitro* cell culture is possible with some baculoviruses this has not yet been expanded to a commercial scale and at this stage is more expensive than *in vivo* techniques. On a smaller laboratory scale, production by *in vivo* methods is also the norm. The basic methodology is similar for large- and small-scale production; production of NPV in lepidopterous larvae will be taken as an example.

In vivo production: During *In vivo* production of baculoviruses, the insects are reared to an optimum stage and then infected with virus. The insects are then reared for a further period and harvested just prior to, or after, death. Normally, the amount of virus produced per insect is positively correlated to larval weight. Thus conditions are optimized so that larvae reach maximum weight before dying from viral infection. Alternatively, virus can be obtained by field collection of virus-killed insects or production 'in the field' by farmers. Harvested larvae contain a mixture of virus, insect debris and contaminant microorganisms (bacteria, fungi, protozoa, etc.). Insect debris and other contaminants can alter the results of a bioassay either by affecting the virus directly, resulting in partial or total inactivation, antagonism or synergism, or by affecting the test insect/cell, resulting in death, or interfering in virus infection/replication or reducing insect feeding and hence virus uptake. These effects are often unpredictable and variable, particularly as the amount and type of contaminants can vary. Many production techniques have been designed to minimize contamination by microorganisms, for example harvesting of infected insects whilst still alive results in reduced numbers of spore-forming bacteria in comparison with harvesting after death.

It is generally important to minimize potential sources of contamination through proper preparation of insect diets, promotion of a high standard of operator hygiene and selection and maintenance of healthy insect colonies. These are also essential to ensure predictable and even growth of insects, which is essential for optimum production and accurate bioassays. Fig. 8 shows the procedure and the flow chart of *in vivo* production respectively.

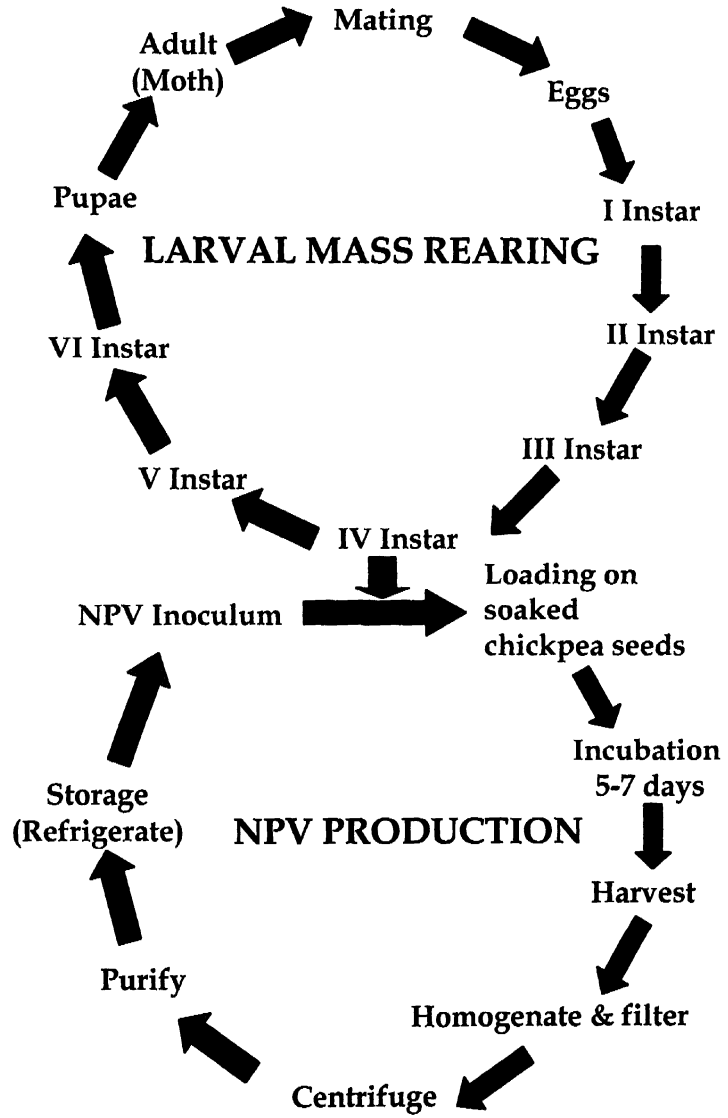


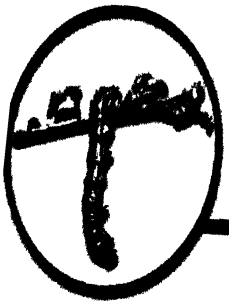
Figure 8. Flowchart of NPV production

in vitro production: As mentioned above, the large-scale production of virus *in vitro* is too expensive at present, although rapid advances have been made in recent years. *In vitro* production involves the production and infection of a susceptible insect cell line in a bioreactor. The requirements of successful *in vitro* production can be summarized as: (i) the development of robust, prolific insect cell lines that yield high pathogen titres; (ii) the availability of simple and cheap culture media; and (iii) development of plant-scale equipment and efficient, routine production procedures. Numerous cell lines are now available, along with suitable simple and serum-free media, as well as improved bioreactors and procedures. In many systems, however, there are still problems of production reverting to mutants with only budded virus. Also, further bioreactor improvements are required to achieve oxygen levels required in vessels larger than 250 litres. Cell culture of NPV is the best established, with a number of cell lines capable of supporting the replication of *Spodoptera* and *Heliothis* NPVs. A necessary feature of cell culture systems is a high level of sterility, so the contamination problems encountered with virus produced *in vivo* do not occur, but of course this requires the availability of facilities that allow sterile handling of equipment. Ideally, virus suspensions that are to be bioassayed should be purified so that other material or microorganisms present do not interfere with the infection process. However, in a number of cases the aim of a bioassay is to test the effect of these materials on viral potency. Even in such cases, it is desirable, if not essential, to also include a purified virus sample in the assay for comparison. A number of purification techniques are available which have different efficiencies; some of these methods themselves may also affect virus activity.

Acetone co-precipitation: This was first developed for *Bacillus thuringiensis* (Dulmage and Rhodes, 1971), but was subsequently adapted for use with viruses (Ignoffo and Shapiro, 1978). Aqueous lactose and acetone solution is slowly added to a virus suspension. This causes the virus to precipitate from the suspension, although bacteria will also precipitate. The suspension is then filtered and washed with sterile water. This technique removes, for instance, insect lipids, protein, as well as killing some vegetative bacteria. However, a number of workers have found that it also reduces the potency and shelf life of baculoviruses (Ignoffo and Couch, 1981).

Density-gradient centrifugation: This has been described by Harrap *et al.* (1977) and Hunter *et al.* (1984) for baculoviruses, and is the most often-used method for producing highly purified suspensions. Infected larvae are macerated in 0.1% (w/v) sodium dodecyl sulphate (SDS), filtered through a double layer of muslin and centrifuged at 100 *g* for 30 s to remove gross debris. The supernatant is then centrifuged at 2500 *g* for 10 min, to remove soluble material, lipids, and other contaminants. The resulting pellet is resuspended in 0.1% SDS, layered on a 45–60% (w/v) sucrose gradient and centrifuged at 50,000 *g* for 1 h. The purified virus forms an opaque band at a sucrose density of 54–56%. The band is removed with a syringe or pipette, diluted in 10 times the volume of 0.1% SDS in sterile water, repelleted, as described above, and finally washed in distilled water by suspension and repelleting three times. Extra centrifugation steps may be included to improve purity (Hunter-Fujita *et al.*, 1998). Although this process results in a highly purified virus, it also results in almost half of the virus being lost (Cherry *et al.*, 1997) and, if used for commercial production, can increase costs fourfold (Jones, 1994); it is therefore more suitable for small-scale laboratory applications.

Semi-purification: Semi-purification of virus, which removes large insect debris and some contaminant microorganisms, can be used if the equipment is not available for density-gradient centrifugation. Semi-purification is also more suitable for large-scale production procedures, as long as the number and type of contaminant microorganisms is monitored (Grzywacz *et al.*, 1997; Grzywacz, 1998). The following methodology for semi-purification of NPV has been successfully used on a laboratory scale by F.R. Hunter-Fujita and K.A. Jones (1998) in Thailand and results in a suspension that can be quantified using a haemocytometer. Bioassays techniques for entomopathogenic viruses and other virus groups, based on centrifugation, are summarized by Evans and Shapiro (1997).



Materials and methods

Cell-wells:

These are synthetic tray like structures that provide several compartments for isolated rearing of *Helicoverpa* larvae; which otherwise can enrage upon each other due to cannibalism.

Oviposition Cages:

Cylindrical, transparent containers, which are provided with a ventilated lid [45cm * 30 cm dia]. These were used to keep the moths (adults). Muslin liners are placed along the length of the cage hanging, which act as supporting base for the female moths to lay eggs after they mate and fertilize. Feed is placed in small cells containing cotton soaked in honey/sucrose solution, which are replaced regularly (alternate days).

Figure 9. *Helicoverpa armigera* moths in oviposition cage.

Jars:

Small synthetic containers [5cm * 7cm dia] that are used to rear the just hatched larvae. Usually the liners containing the eggs and young larvae are kept in these jars till they develop to second instar after which they are shifted to cell-wells. These jars internally coated with a layer of synthetic diet to support the young larvae.

Field-collection and rearing of larvae

Larvae of different stages were collected on various crops like pigeonpea, chickpea and occasionally from weed, *Lagashia* {during the off-seasons}. The collection was done in specialized cell-wells. Prior to the use these cell-wells are sterilized using 1% clorex solution and exposing them to UV light. Small pieces of the synthetic diet were placed in each of the wells proportionately.

Cell-wells were placed in the incubator (at 25°C ± 2°C with 12:12 photoperiod) to regulate the growth of the larvae. The diet was replaced regularly and ensured sufficient food supply and sanitation. The details of the artificial diet used in the study is given in the appendix.

Once the larvae pupated they were transferred into clean jars. Fungal infected pupae are disinfected in 1% clorex solution and dried before they are kept in separate jars. These pupae later metamorphosed into moth (adult).

Thus obtained moths are placed carefully in oviposition cages along with the feed (honey). The moths laid their eggs on the liners provided in the cage. These liners were regularly checked and replaced. Liners loaded with eggs were carefully placed in clean sterilized jars finely coated with a layer of diet. The eggs hatched into young larvae and when they grew to second instar stage, they were transferred into cell-wells provided with diet. Larvae were regularly monitored for their growth and once they attained the desired size i.e., forth instar (average weight 0.14 g) they were isolated for the inoculation.

Inoculation

The obtained forth instar larvae were transferred to clean cell-wells. The inoculum used was indigenously (ICRISAT) prepared NPV. Chickpea seeds soaked overnight were used as the medium for inoculation, which were air-dried and coated with the inoculum. The concentration of the inoculum was 0.1ml/100 seeds [1ml = 1LE = $6 * 10^9$ PIBs] and each larva was supplied with 2 seeds. Homogenous distribution of the inoculum was ensured through thorough mixing followed by air-drying.

Thus inoculated larvae were kept under constant monitoring and checked for developments.

Harvest

The inoculated larvae were harvested at different intervals viz.

2^{nd} day ,

3^{rd} day ,

4^{th} day ,

5^{th} day,

6^{th} day and

7^{th} day post-inoculation, PI.

For each harvest the larvae were isolated into a clean container and ensured that no diet or other debris was present with the isolation. The mortality percent was determined prior to the actual harvest process. Then the larvae were ground or blend thoroughly to acquire a homogenate. Distilled water was added wherever required to get proper homogenate.

It was then filtered through double layered muslin cloth to filter out the left-out cadaver pieces of the larvae and debris. The filtrate was then distributed evenly into centrifuge tubes and centrifuged initially at 1000rpm for 30-60 sec. This would precipitate the remaining cadaver masses that have got through filtering; it is discarded and recentrifuged at 5000rpm for 15 min. The pellet thus obtained was carefully collected after discarding the upper liquid containing water and lipids. The NPV thus obtained was labeled and stored in a cool place.

Acetone extraction

To the homogenate obtained from blending the larvae, 10% acetone solution was then added; dissolving the lipids present in it. Thus, lipid free NPV is obtained which was much clearer in counting and devoid of the malodor.

NPV quantification

The NPV obtained was quantified by counting the polyhedra, which were distinctive and visible under phase contrast or dark field microscopy at 40X resolution. The NPV obtained from centrifugation was highly concentrated and hence was diluted to 1000 folds i.e., 0.1ml NPV in 100ml distilled water to render the counting process more feasible.

A Petroff chamber, employed in association with Haemocytometer was used for the purpose of counting. The chamber has 25 major grids, which were further divided into 16 cells each thus, in total 400 cells were present. Number of polyhedra in each cell were counted randomly and the final count was hence determined.

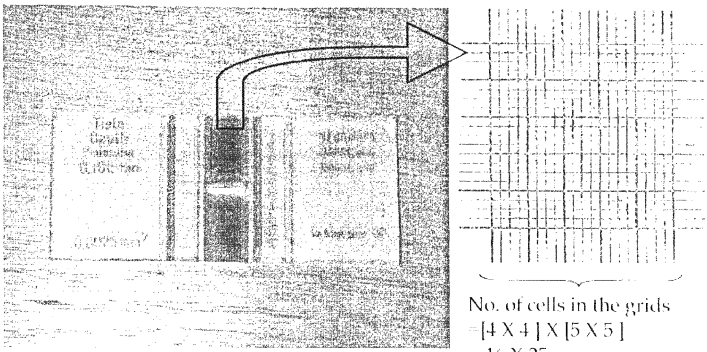


Figure 10. Neubauer chamber (slide) of haemocytometer.

No. of cells in the grids
= [4 X 4] X [5 X 5]
= 16 X 25
= 400 cells

And the final yield was calculated by following equation.

$$\text{POLY INCLUSION BODIES [PIB/ml]} = \frac{D \times N}{X \times K}$$

where **D**= Dilution factor

N= No. of PIBs

X= No. of Cells counted

K= Constant ($2.5X\ 10^{-7}$)

FINAL COUNT [LE/ml] = PIB / *6x10⁹

* 6×10^9 PIB = 1 Larval equivalent (LE)

FINAL COUNT [LE/larva]= FINAL COUNT [LE/ml] / No. of larvae harvested

Worked-out example:

For the values of

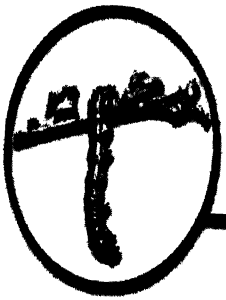
D= 1000
N= 210
X= 100

$$\text{Count} = \frac{1000 \times 210}{100 \times 2.5 \times 10^{-7}} = \frac{2100}{2.5 \times 10^{-7}} = \frac{840}{10^{-7}}$$

$$= 840 \times 10^{7}$$

$$= 8.4 \times 10^{9}\ \text{PIB/ml}$$

$$\text{Final count} = \frac{8.4 \times 10^{9}}{6 \times 10^{9}} = 1.4\ \text{LE/ml}$$



Results

Initial larval mortality was observed two days after inoculation and progressed gradually with 50% mortality on 6th day followed by 88% on 7th day. The progress was recorded as 14% on 3rd day, 23% on 4th day, 30% on 5th day, 50% on 6th day and 88% on 7th day. This suggested about 30% mortality in the first 5 days PI followed by 58% in the next two days (Table 1, Fig. 11)

Observation on viral production revealed 87.3% loss in yield when all the larvae were processed on 2nd day PI followed by 78.9% loss on 3rd day, 66.1% loss on 4th day, 48.5% loss on 5th day, 8.4% loss on 6th day PI. This provided good clue that on processing larvae at 50% mortality on 6th day one need not sacrifice much of virus. At this stage only 50% larvae were dead and the remaining were active. (Table 2, Fig. 12)

The problem of malodor starts with the dead larvae as contaminant bacterial activity increases and the purification of body material starts. At this juncture one has to draw a line without sacrificing the quantity of the product (viral yield). Since 50% larvae on 6th day were alive the problem of malodor was reduced significantly, while maintaining the viral yield.

During storage: To address the problem further the processed virus suspension was treated with 10% acetone solution (v/v). This helped in further cleaning the lipid mass and the left over contaminant bacteria in the preparation. This not only provided better clarity to the virus suspension for taking counts but also had significantly regulated the malodor problem. Literature showed that it had no ill effect on the quality of virus. Preliminary observations one month after acetone treatment did not effect the mortality of larvae in bioassay. Hence, further studies are required in this area to demonstrate the persistence of the virus after acetone treatment.

Table 1. Mortality of *Helicoverpa* larvae at different intervals after NPV inoculation

Post Inoculation Day	Total no. of larvae inoculated with NPV	No. of Larvae dead	Mortality [%]
2	102	1	1
3	102	15	14
4	106	24	23
5	102	30	30
6	106	53	50
7	105	92	88

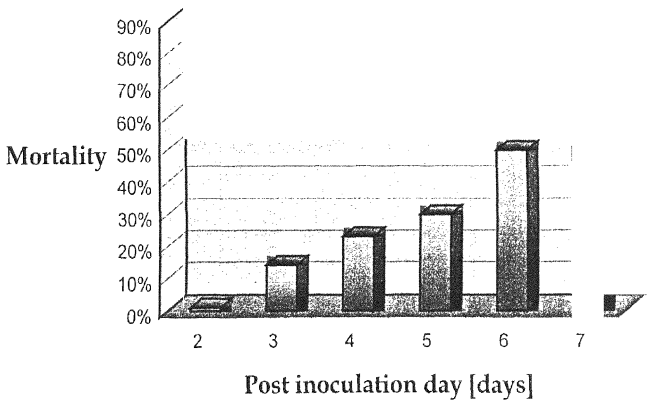


Figure 11. Mortality of *Helicoverpa* larvae at different intervals after the NPV inoculation

Table 2. Yield of NPV from *Helicoverpa* larvae at different intervals after NPV inoculation

Post Inoculation Day	Total no. of larvae inoculated with NPV	Count [P.I.B/ml]	Final Count [LE/larva]	Reduction in virus [%]
2	102	$1.08X10^9$	0.09	87.3
3	102	$1.80X10^9$	0.15	78.9
4	106	$3.00X10^9$	0.24	66.1
5	102	$4.38X10^9$	0.36	48.5
6	106	$8.10X10^9$	0.64	8.4
7	105	$8.76X10^9$	0.70	0.0

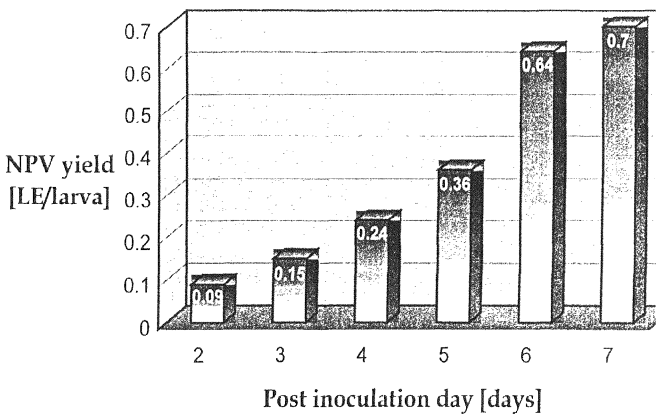
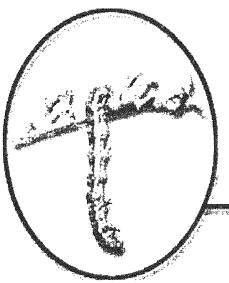


Figure 12. Yield of NPV from *Helicoverpa* larvae at different intervals after NPV inoculation



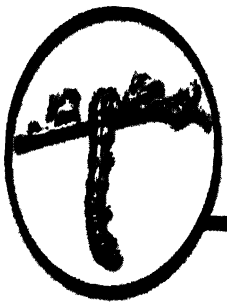
Discussion

The most ideal period for harvest can be suggested to be 6th day PI when the mortality percent and NPV yield both are in consensus with the optimal production requirements.

Since, the mortality percent at 6th day PI is much lesser than at 7th day PI, the microbial contamination is much lesser hence, mitigating the malodour caused by the microbial degradation of lipids. Harvest at 5th day PI would significantly reduce the NPV yield and the same at 7th day PI would radically increase the contaminant microbial population effecting the NPV production and causing malodour. Ignoffo and Shapiro 1977 have also earlier reported the radical increase in microbial contamination. Also the increasing population of contaminant bacteria would pose as a competitive constraint for the NPV multiplication (Cherry et al. 1997). This is because for the NPV to multiply it requires cells to infect and reproduce within. It is evident by the relative difference in the NPV yield is hardly 8% inferring that the NPV replication has nearly ceases after the death of the larva. The bacteria, which are usually opportunistic normal flora of the larvae, can only infect and multiply after the death of the larva. Though as mentioned by Ignoffo 1977 that there is significant difference in the viral yield between dead and live larvae, the sacrifice of the minute(~8%) percent of yield is accountable to the regulation in the contaminant bacterial population.

The Acetone precipitation, which has been tried, has a significant effect on the NPV yield and actually has double advantage. Acetone being a potent anti-microbial agent it regulates the bacterial infection and being a lipid solvent, removes the lipid (fat cells) from the larval homogenate, thereby inhibiting the bacterial lipid degradation (Ignoffo et al 1977). Hence, in both the ways regulates the bacterial lipid degradation and in turn the malodour.

The only constraint in this process, which needs appraisal, is the effect of Acetone on virulence and shelf life of NPV. Though theoretically acetone doesn't affect the polyhedral envelope, periodical bioassay of acetone precipitated HNPV has to be performed to comment and conclude upon its effect.



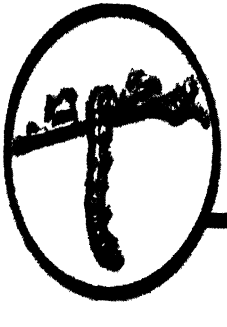
Epilogue

A more productive approach of the problem would be to reduce the initial burden of the contaminant bacteria in harvested insects by improved hygiene and by using a more selective method of harvesting less contaminated larvae rather than by trying to clean up already contaminated material.

Acetone extraction is a promising option to reduce the contamination and thereby the malodour. But, in this regard appropriate "Bioassay tests" have to be carried out to assess the effect of acetone on virulence and shelf life of NPV.

A critical disadvantage associated with NPV (as biopesticide) is the delayed time of action, takes nearly 5-6 days to kill the insect. This is a serious concern because the larvae can cause significant damage before the virus can render the larvae inactive. Intense research has been in course and a few appreciable outcomes suggesting the genetic manipulation of NPV. The viral genome is incorporated with certain insect toxin genes (extracted from Spider and other venomous insects) that reduce the time of action several folds. Similarly several other genetic engineering techniques are being employed and studied to improve the potency of the virus.

Another critical aspect of concern is the UV sensitivity. The virus is rendered inactive by the sunlight and due to this the field application of the virus is effected. Several UV absorbers and protectants have been used in association with the virus to overcome the flaw. Further research in the genetic aspects of the virus can aid in conquering this problem efficiently.



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Appendix

Diet for *Helicoverpa armigera*

Larval diet

Ingredients:

Ingredient	Single batch
a. Chickpea flour	300.0 g
b. Ascorbic acid	4.7 g
c. Methyl-p-hydroxybenzoate	5.0 g
d. Sorbic acid	3.0 g
e. Auromycin powder	11.5 g
f. Linseed oil	12.0 g
g. Vitamin stock solution	10.0 g
h. Water	450.0 ml
i. Yeast	48.0 g
j. Agar	17.3 g
k. Water (for yeast & agar)	800.0ml

Preparation:

Weigh out all dry ingredients and have all wet ingredients in appropriate measuring cylinder. Use a large bowl. Add ingredients a - g in the bowl, add water (h) and mix thoroughly using hand-held mixer.

Meanwhile heat water for yeast/ agar in saucepan on hotplate and boil. Add yeast and mix thoroughly with mixer. Sprinkle in agar while stirring and mixing thoroughly all the time. Remove from heat and pour into the ingredients in the plastic bowl. Mix continuously until an even consistency is obtained.

Pour hot diet into stainless steel trays on a level surface maintaining 5mm of uniform diet depth. Set slightly, carefully and leave to cool completely in a

laminar flow cabinet. When cool, place tray in plastic bag, exclude air and store in refrigerator.

Cut into one inch cubes when required and place in appropriate dishes for larvae. The prepared diet comes to around 3-4 trays and would serve nearly 300 larvae (third instar) for two days.

Adult diet

Ingredients:

Sucrose	50.0 g
Methyl-4-hydroxybenzoate	1.0 g
Vitamin stock solution	10.0 ml
Water	500.0 ml

Vitamin stock solution composition:

Nicotinic acid	1.528 g
Calcium pantothenate	1.528 g
Riboflavin	0.764 g
Aneurine hydrochloride	0.382 g
Pyridoxine hydrochloride	0.382 g
Folic acid	0.382 g
D-biotin	0.305 g
Cyanocobalamine	0.003 g
Water	500.000 ml