Ministry of Agriculture and Food Security Tanzania

The African Armyworm Spodoptera exempta nucleopolyhedrovirus (NPV) production and application manual

Wilfred Mushobozi[1], David Grzywacz[2], Flavio Moscardi[3], and Ken Wilson[4]

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[1:] Pest control Services, Ministry of Agriculture and Food Security, Arusha, Tanzania.
[2:] Natural Resources Institute, University of Greenwich, Central Avenue, Chatham Maritime, Kent ME4 4TB, UK. [3:] EMBRAPA, 86001-970, Londrina, Brazil
[4] Department of Biological Sciences, Lancaster University, LA1 4YQ, UK

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Summary

African armyworm (*Spodoptera exempta*) is a major migratory pest of pastures and cereal crops in large areas of Africa as well as parts of Arabia, Asia, Australia and the pacific. Its plagues can infest many thousands of square kilometers in eastern, central and southern Africa and are a major threat livelihoods and food security, especially to poor smallholders who lack access to effective affordable control.

The nucleopolyhedrovirus (NPV) of African armyworm (SpexNPV) is a safe specific biological control agent that shows considerable promise as a biological insecticide for the control of African armyworm. NPVs are a class of insect disease that are highly specific to insects and safe to humans they have been developed into commercial biological pesticides for the control of a number of major insect pests, including the armyworm species beet armyworm *(S.exigua),* tobacco leafworm (*S.litura,)* and the cotton leafworm *(S.littoralis).*

The methods for the cheap local production of SpexNPV have been developed by a research team based at Pest Control Services Tanzania. This involves spraying natural outbreaks of armyworm with SpexNPV and then harvesting the dead insects in which the SpexNPV has multiplied. These insects can then be formulated into an insecticide for armyworm at low cost using a kaolin formulation technique developed by EMBRAPA in Brazil. A modified form of this formulation has been successfully used in armyworm NPV.

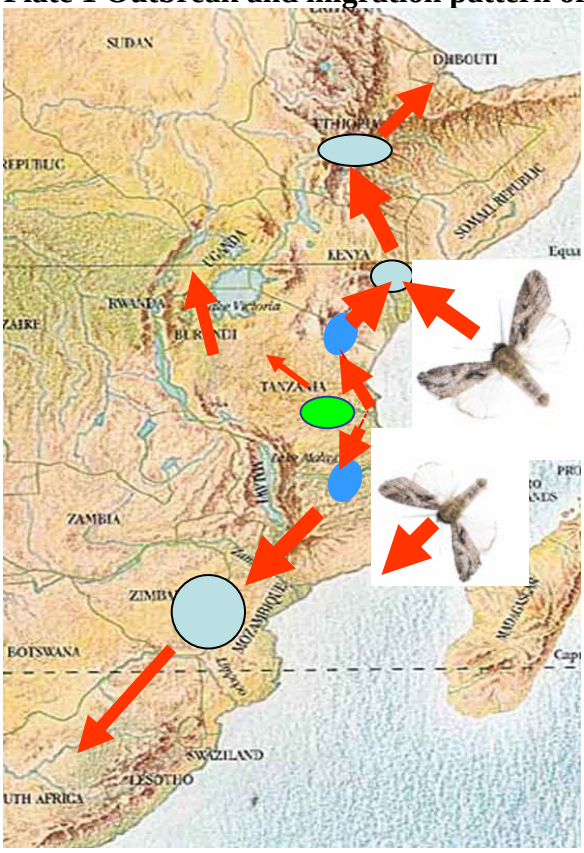
Protocols for laboratory and pilot plant scale production of this formulation are presented here as are quality control protocols and recommendations for storage and application.

Introduction

In Tanzania the Pest Control Services (PCS) of the Ministry of Agriculture and Food Security are developing a new natural biological control agent the SpexNPV to control outbreaks of African armyworm that could replace the use of expensive imported chemical pesticides to control this serious crop pest. They have been supported in this programme by a scientific team drawn from a number of research institutes including Natural Resources Institute UK, University of Lancaster UK, CAB International Africa research centre,

The African armyworm (Latin name *Spodoptera exempta*) is a major threat to basic food production in a number of east and southern African countries. This pest appears between December and May as armies of black caterpillars covering many hectares with densities of up to 1100 per square metre. As the crops grow, armyworms swarm across the countryside stripping the fields of grass, wheat, maize and other crops. In major outbreak years, 30% of grain crops are estimated to be lost; and in Maize of up to 92% loss are recorded. The outbreaks begin in Tanzania most years and the pest migrates to cause extensive damage in Kenya, Uganda, Ethiopia, and Eritrea, and may travel as far as Yemen (Rose et al 2002).

Plate 1 Outbreak and migration pattern of African Armyworm



- Outbreaks start in central Tanzania then adult moths spread to other parts of the country starting new outbreaks
- The adult moths from these outbreaks move on to other east African countries including Kenya, Ethiopia, Uganda, Sudan Eritrea and Yemen.
- Also may move southward to Malawi, Mozambique, Zambia and South Africa

This disease of armyworm is caused by a specific agent, the *Spodoptera exempta* nucleopolyhedrovirus (or SpexNPV). It has been observed since the early 1930s that late in the season, many armyworm outbreaks collapse due to the occurrence of a disease that may kill up to 98% of caterpillars. This disease was finally identified as being caused by the armyworm NPV in the 1963. However while NPV does destroy outbreaks of armyworm the NPV only appears most years after crops are damaged.

Plate 2 Armyworm larve in outbreak

Plate 3 Larva killed by NPV

However, it has been shown that by spraying NPV onto outbreaks of armyworm a rapid outbreak of the disease can be initiated that can kill larvae within 3-7 days, destroying the outbreak without use of chemical insecticides (Grzywacz et al 2006).

ARMYWORM NPV

The armyworm NPV (SpexNPV) is one of a family of natural insect diseases called baculoviruses. A number of these baculoviruses have been developed as commercial biological insecticides in USA, Europe, China, South America and India for control of several major insect pests (Moscardi 1999, Copping 2003). NPVs are completely safe because they are highly selective, attacking only specific pest insects. They have been recommended by FAO as pest control agents, and a major report concluded “baculovirus use is safe and does not cause any health hazard” (OECD 2002). This is a major attraction of NPV’s making them much safer than chemical insecticides.

It has long been known that the African armyworm have a number of natural enemies and pathogens including viruses, fungi and protozoa but the most important was reported to be the SpexNPV (Rose *et al*, 2000). This virus has been known since 1965 (Brown and Swayne 1965). Studies have confirmed that this disease is endemic in many parts of east Africa (Odindo 1983) and that this NPV is highly pathogenic to the armyworm (Odindo 1981). Cage studies on armyworm outbreaks confirmed that natural NPV can be a major cause of mortality in armyworm outbreaks (Persson 1981). However, the NPV is rarely apparent in primary outbreaks of the pest only appearing later in the season and even then it can be highly localised, affecting only small parts of the outbreak area (McKinley 1975).

The NPV’s potential as a control agent has been highlighted on a number of occasions (Tinsley 1979), but little progress to develop the NPV was made probably because cheap and effective broad-spectrum chemicals were available and considered acceptable. Trials to use crude suspensions of infected larva containing the NPV as an insecticide against armyworm had showed that this approach had promise (Brown 1966). Subsequently, the virus was

characterised (Harrap *et al*. 1977), and the first physical map of the genome completed (Brown *et al.* 1984). Cross infectivity studies with a range of NPVs have indicated that the african armyworm are susceptible only to the SpexNPV (McKinley *et al.* 1977). The SpexNPV has been safety tested following FAO/WHO recommended protocols, and no evidence of toxicity to mammals or non-target hosts was found (Harris 1973). This is in agreement with the safety review of Baculoviruses, which showed no evidence of adverse environmental impact from any use of baculoviruses as crop protection agents (OECD 2002).

NPVs are the insect viruses most studied and used commercially in pest control (Moscardi 1999, Copping 2003). They infect over 400 species of insects and are well known to cause major lethal epizootics in important lepidopteran pest species. They are commonly isolated from insects in the field and being visible with light microscopy can be detected readily if present. They also have, for viruses, a relatively fast mode of action, killing infected insects within 4-7 days.

NPVs are rod shaped double stranded DNA viruses of the family *Baculoviridae*, which infect a wide range of insect species, chiefly Lepidoptera but also some members of the Hymenoptera and Diptera. These viruses are the cause of a highly infectious lethal disease in the larvae of susceptible species, being highly host specific and most infect only a few species of closely related insects. The viruses are named after the insect in which they were first isolated and identified, such as *Spodoptera exempta nucleopolyhedrovirus* (SpexNPV).

Plate 4 SpexNPV under light microscope OB of virus seen as bright crystals

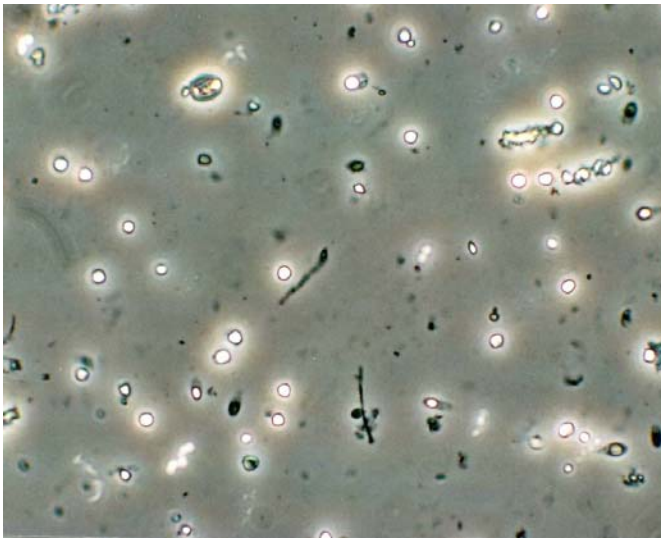
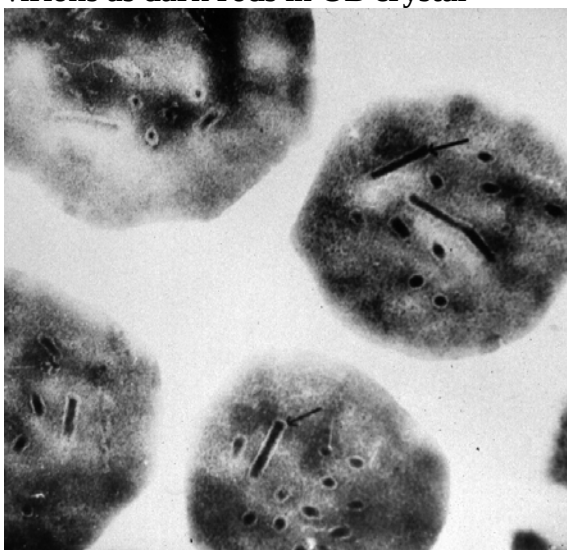


Plate 5 Electron microscope of SpexNPV showing individual infectious virions as dark rods in OB crystal



The NPVs are seen in dead or dying larvae as bright irregular crystals called occlusion bodies (OB) or sometimes polyhedral inclusion bodies (PIB) (Plate ?). These are protein crystals normally 1-7µm across that show up at x400 as bright refractive crystals, especially under phase contrast. These crystals are many sided (*polyhedra* in Latin) and are composed of a protective crystal protein called polyhedrin. Within this OB are found embedded up to 200 virus particles or virions. These virions are the actual virus infective particles and are composed of a rod-shaped DNA/protein structure called a nucleocapsid (approximately 50 x 250-400nm), inside a membrane envelope.

These OBs are the infective stage of the virus designed to transmit the infection from insect to insect. The polyhedrin crystal helps to protect the vulnerable virions from inactivation by environmental factors. Baculoviruses in this form are extremely stable and can retain infectivity for many years, if not exposed to UV light or high temperatures (>50°C).

The virus infects the insect in the form of the OB. When the polyhedra enters the mid gut of a larval host, it dissolves under the alkaline conditions (pH 9-11) releasing the virions. These virions enter the cells of the mid gut and proceed to multiply in the nucleus of the midgut cells. From this initial infection, new virions are produced which proceed to spread the infection to other body tissues such as haemocytes, tracheal cells, fat body cells and hypodermis. It is in these tissues during the later stages of the infection that polyhedra are produced in which virions become embedded. When the insect dies it ruptures releasing these polyhedra to infect other insects. Insects killed by NPV will commonly contain up to 100 million OB. In the wild, infection occurs through larvae eating OB contaminated vegetation.

Insects infected with NPV show few symptoms for the first 2-4 days after ingestion of the virus. The larvae then progressively cease to feed and become less active. During advanced stages of the infection, as the epidermis is infected, the skin becomes very fragile and ruptures easily. The larvae become wilted and the body contents become a fluidised mass of decomposed tissues and polyhedra. Just prior to death infected larvae often climb to the highest parts of the substrate on which they are located, e.g. tops of plants and attach themselves by their prolegs. On death they hang in a characteristic inverted V-shape (Plate ?)

For more detail on the biology of the baculoviruses the reader is recommended to refer to one of the standard works such as Tanada & Kaya (1993), Miller (1997) or Hunter-Fujita *et al* (1998).

NPV can be detected in light microscopy, through the bright OB in infected tissues, and electron microscopy can be helpful to determine the type of NPV (SNPV or MNPV), and other morphological characteristics (form of OB) and OB dimensions. However, to further identify and characterize these viruses to “species” one needs to look at the DNA sequence using restriction enzymes or molecular probes. Examination of the DNA using these techniques has shown that many variants of a species may exist. The existence of these genetic variants with different biological activities may have important implications for the development of biopesticides, both in the possibility to select better naturally occurring strains and as a source of material for genetic manipulation.

Production of SpexNPV.

NPVs can be produced both in live insects or tissue culture, but the latter is currently not economic for mass production and no tissue culture system that will support the replication of SpexNPV has yet been developed therefore only production methods based upon live insects are detailed here.

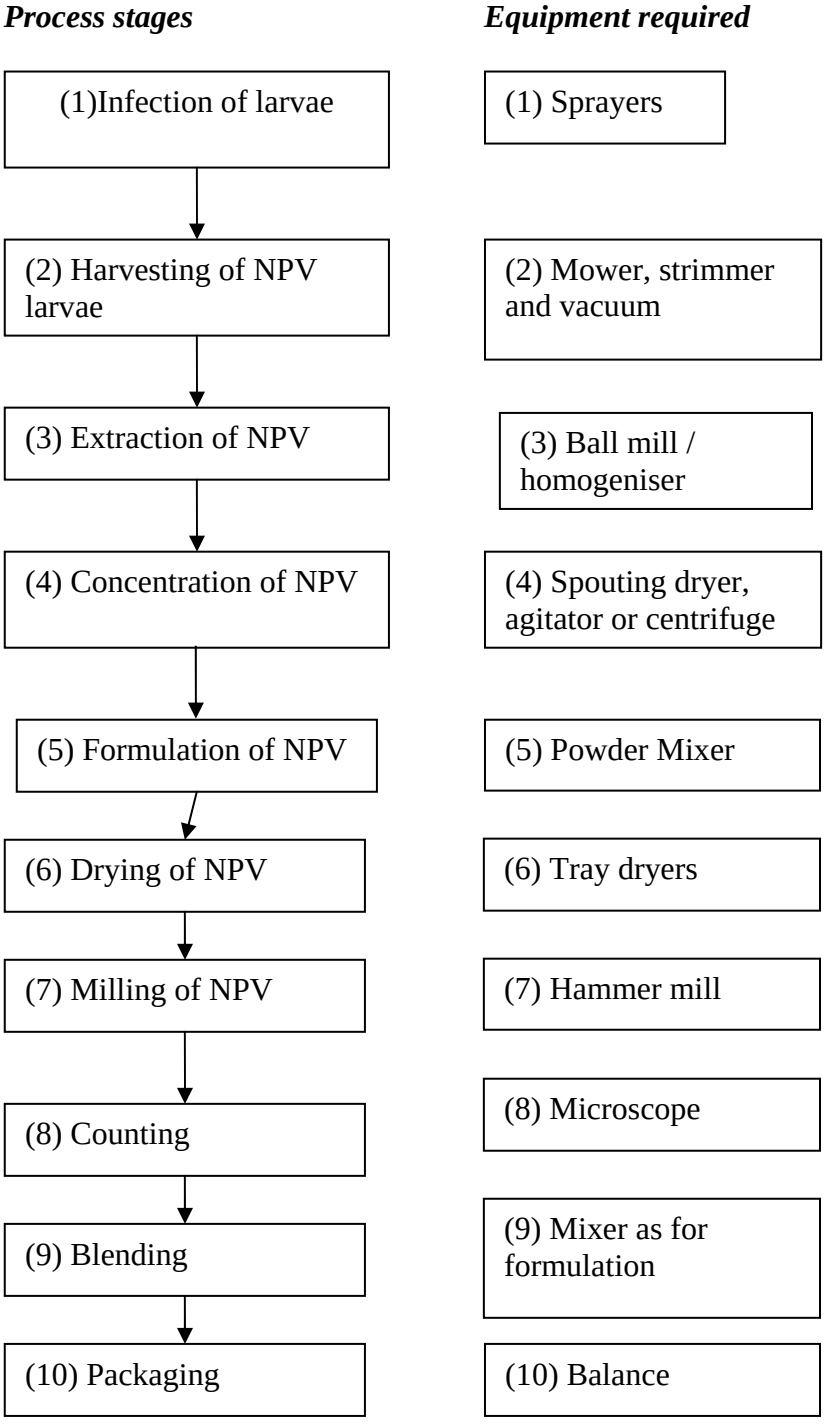
SpexNPV can only be produced only in host larvae by infecting the insect then allowing the NPV to multiply and kill the larvae. The infected or dead larvae can then be crushed to release the new NPV. This process is very efficient, as larvae infected with a few NPV infectious particles or occlusion bodies (OB) will die containing up to 500 million in the case of African armyworm (Cherry et al. 1997).

One of the cheapest ways to mass produce NPV is by infecting out breaks of the pests in the field then collecting the larvae when they die and using the bodies to make NPV pesticide for spraying again. This system has been the basis of a highly successful programme to control velvet-bean caterpillars in Brazil. This programme collects over 40 tones of insects annually to produce a biological pesticide now used on 2 million hectares annually (Moscardi, 1999; Moscardi and Sosa-Gómez, 2000). The EMBRAPA system used in Brazil involves formulating the NPV as a low cost clay formulation, and the production costs are less than 2 US$ per hectare.

PCS is seeking to adapt this system for producing armyworm NPV in Tanzania to provide the country with a cheap, safe and effective alternative to chemical pesticides. This manual details the procedures for producing armyworm, NPV with notes on quality control procedures, storage and application.

Outline process for mass producing SpexNPV from field outbreaks

Figure 1 Flow chart of SpexNPV production

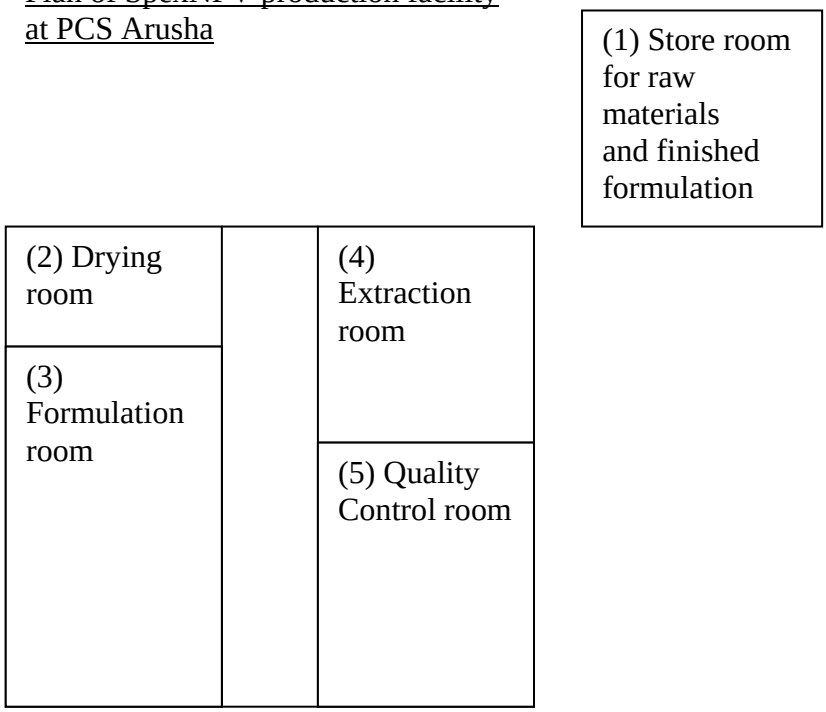


The above diagram illustrates the process of production, with key stages and equipment needs. A detailed protocol for production on a pilot laboratory scale is presented below.

Pilot plant plan
A pilot facility has been be established at PCS to produce NPV on a laboratory scale using extent facilities but it is proposed that part of the armyworm buildings at PCS Arusha be converted into a customized NPV production unit.

This will be housed in the building at the end of the PCS offices currently used as a general store.

Plan of SpexNPV production facility at PCS Arusha



List of rooms and required services and equipment
(1) Storeroom 4 x 3 m (separate building)

Freezer 900 litre x1
Refrigerator 300 litre x1
Storage shelving 20 square metres of loading 100kg per square metre

Requires mains power

(2) Drying room 2.1 x 1.5 m

Drying rack 6 level 40x150cm with 20 cm height between shelves x1
Drying trays x 24
Thermostatically controlled 1 KW heater 25-50 deg C
Dehumidifier 5 litres per hour
Expeller wall mounted fans. (x2)

Requires mains power and partition.

(3) Formulation room 2.1x 3.6 m
Benching 1.5 x 6 m
Mixer 10 litres
Hammer mill
Sieving machine
Sink

Requires mains electric and water

(4) Extraction room 2.1x 2.1 m
Homogeniser 5 litre
Fridge 100 litre
Sink

Requires mains water

(5) Quality control room 2.1 x 2.1 m
Microscope
Incubator for assays
100 litre deep freeze
100 litre fridge

Requires mains power

In addition a standby generator is available to maintain key functions, freezers etc during power cuts.

Plate 6 PCS Armyworm laboratory housing pilot NPV plant

Protocol for collecting NPV from artificially infected or natural outbreaks of armyworm.

1. Locate possible armyworm outbreaks from moth trap counts and by follow up scouting on the ground.

2. Identify outbreaks that could be used for production. The selection of outbreaks on low value pasture not adjacent to crops and near to roads is the most suitable option.

3. Perform larval counts to identify that outbreak density is suitable for production. Counts of greater than 200 larvae per m^2 are best and on grass that is adequate to support growth to late instar without the larvae having to move.

4. When outbreaks are at III-IV instars spray outbreak at 5 $x10^{12}$ OB ha. using appropriate sprayer methods detailed in application section below.

5. Monitor daily after spraying when significant NPV-killed insects appear, (say >20 dead per 0.25m^2 quadrat) commence harvesting. One would normally expect large numbers of dead to appear some 4-7 days after application depending upon conditions.

6. Using strimmers or mowers cut and collect the grass with NPV infected larvae. On long grass or uneven ground use strimmer and vacuum to collect. Place grass cuttings with larvae on in a labeled plastic bags.

7. Store bags overnight in fridge.

8. Extract next day or store collected grass in freezer to await extraction.

Protocol for small scale laboratory production of NPV from collected material.

Equipment

One 5 litre blender for extracting NPV from harvested grass
One 10 litre mixer for mixing extracted NPV with Kaolin
Drying racks for 20 trays (total 20 m^2 area)
Fan
Dehumidifier 5 litre per hour
Hammer mill.
Storage tubs 500 ml.

Procedure

1. Place 1 kg of harvested grass plus NPV in 2 litres of water in blender blend for 5 minutes.

2. Remove grass strain liquid back into blender through muslin. Add additional 1 kg of grass and blend again.

3. Repeat above four more times until 5 kg of grass has been extracted into mixer. By then approximately 1 $x10^{13}$ OBs will have been extracted into the liquid

4. Place extract overnight in fridge at 4^o C to settle out OBs. Settling may be a problem if dissolved solids from the grass thicken the extract and prevent settling. In this case dilute to 1:2 or 1:3 to reduce viscosity of extract and leave again to settle. The exact dilution settling time may vary with grass and needs to be tried and adjusted.

5. Pour off supernatant and adjust extract volume to 1 litre. Take 1 ml sample and count OB

6. Mill 1 kg kaolin, sieve through 100 mesh sieve.

7. Mix 1 Kg kaolin with 1 litre extract.

8. Pour into 1 m^2 trays at 500 ml per tray.

9. Dry in drying room at 35^o C overnight.

10. Remove cake break up and mill, sieve through 100 gauge mesh sieve.

11. Extract 10-gram sample for NPV counting then bag rest of batch.

12. When a number (3-5) of batches has been processed perform microscopic counts to determine NPV content and then bioassay against standard.

13. On basis of counts blend batches to produce uniform product of 1 $x10^{13}$ OBs per kg.

14. Pack and label. Store at -20^{o} C.

Protocol for larger pilot scale production

To be finalized after develop and evaluation trials of large scale processing equipment.

Quality control protocol.

Quality control (QC) is the key to NPV production, as the production of a consistent product of high activity is essential to the success of any pesticide. QC for chemical pesticides is relatively straightforward and uses chemical analysis to determine the active ingredient content and also to check for the absence of contaminants. In the case of a biological agent such as NPV that kills by infection of a pest insect, the procedure is different. There is a need to determine that the NPV particles are present, are the correct NPV, and are actively infectious. This cannot be carried out using any chemical analysis, so the process involves several stages (1) counting the viral particles (2) identifying them as SpexNPV and (3) testing that they are infectious to the target pest.

The techniques used are

1. Counting NPV particles
2. Bioassay
3. DNA identification

COUNTING NPV

NPV are among the easiest viruses to quantify as the polyhedra are distinctive and visible under phase contrast or dark field microscopy at x400-1000 and can be counted using standard light microscopy methods. However, this has its limitations. The first is that a good optical system and some experience is needed to do this correctly. Many counting mistakes are encountered where unsuitable, poor quality microscopes with non-phase optics are used, especially when counting impure NPV samples.

The second, more fundamental limitation is that a visible occlusion body (OB) is not necessarily an infective one. Even under the electron microscope it is not possible to determine whether the virus is infective. Only the use of a bioassay using live insects can determine whether viruses are active against the target insect.

Another limitation is that using microscopy it is not possible to identify the species or strain of NPV. To do this, molecular methods of identifying the DNA are needed.

To achieve a full assessment of the NPV activity, therefore, requires microscopic, molecular and bioassay studies on any sample. These methods are time consuming, but are essential for the successful sustained production of high-quality NPV.

Microscopy

Quality control of NPV is impossible without a good microscope. If you cannot see the product you cannot check it. With this technique, quality pays dividends; a good phase contrast microscope with correct phase contrast objectives makes picking up problems much easier. It must be remembered that it is not only what you pay for but also how you treat it that counts. A cheap but well-maintained microscope is better than an expensive one that is out of alignment or dirty. Microscopes are generally robust and give few problems, but phase contrast microscopes are soon put out of alignment by inexperienced handling, and no microscope works well if dirty or has scratched lenses.

There is no substitute for an experience in microscopy. The only way to build up this skill is through spending a lot of time looking at NPV samples and insect smears, identifying the various insect cells, bacteria, NPV, GV, microsporidia, other insect pathogens and artifacts. A routine of examinations should be established with time set aside regularly, either daily or weekly, in which product or insects are examined. Do not save up samples over weeks or months for later analysis; once a problem develops it can spread rapidly throughout the system before you get round to examining the "saved" samples. Regular "real time" monitoring, even if only on a limited scale, is better than occasional in-depth examinations. Microscopic examination of baculoviruses is described in detail in below.

Bioassay

Bioassay is the **only** way to confirm that the virus you are producing is infective. There is NO substitute. It will allow you to determine its potency and the dose level required for the inoculum. It is, with microscopy, the key technique you need to use in NPV production.

To assist in maintaining quality control, you should:

- Carry out bioassays regularly on every production batch of the virus, to ensure a consistent product.
- Carry out bioassays if you make any changes to your production system. They will help you to assess the effect on the product.
- Always compare production batches activity with that of a standard of the virus, with known high activity against the insect host. This standard should be multiplyed in enough quantity to allow comparisons with production batches for many years. Probably for three years, then go back to original stock and multiply enough virus for another three-year period.

It is important both to carry out a regular, adequate programme of bioassays, and to keep clear records of the results. A weekly bioassay of routine production batches, with the results recorded and graphed, is highly advisable. This provides a continuous check on the efficiency of the NPV production and on the quality of the NPV produced. Within a short time the normal variation in the system will become obvious and limits of "noise" in the system clear, so that any systematic loss of NPV activity can be detected. Graphs of both LD_{50} or LC_{50} and total potency ratio, (total numbers NPV produced x LD_{50} or LC_{50}), are both useful measures of productivity. Any fall in LD_{50}/ LC_{50} or potency ratio should be followed up immediately, as these may be the first indications of foreign pathogens contaminating the production system to a significant extent. Bioassay techniques are described in detail below.

DNA identification

All NPV look similar under the microscope. DNA analysis identifies the sample genetically and can tell you if the product is the correct virus. The expression of latent viruses can be detected by this method. Also, along with microscopy, it can detect contamination of the system by foreign or wild, unwanted viruses such as CPV and GV.

Uses of DNA identification to aid quality control, include:

- Making a DNA "fingerprint" of the inoculum and comparing it with the product.

- New strains can be identified and their efficacy compared with existing strains and the standard virus by bioassay.

DNA analysis is described in detail below.

COUNTING NPV

MICROSCOPES AND CHOICE OF ILLUMINATION

The light microscope is an essential tool in the production or study of NPV. The encapsulation of the baculovirus virions in a protective protein crystal makes them, unusually for viruses, visible under the light microscope. NPV at up to 5μm across can be viewed with relative ease (by the trained eye) identified and counted with a light microscope.

To see these, a microscope with a magnification of x400 (an objective of x40 - x60 and an eyepiece of x8-x10, is required). The OBs of NPV are bright highly refractile protein crystals that show up strongly as bright bodies under illumination. They can be seen clearly under bright-field illumination, but are most easily distinguished when phase contrast illumination is used with the appropriate lenses. Phase contrast illumination enhances the appearance of unstained living micro-organisms and shows up detail of cells and sub-cellular structures in unstained preparations. However, while recommended for observing NPV, it is not essential.

While NPV can be clearly seen with a light microscope no internal detail of virions or OB internal structure can be observed. To do this requires use of an electron microscope with its much higher resolving power.

COUNTING AND THE USE OF STANDARDS

Detecting the presence of NPV in an infected insect is relatively straightforward, given a well-infected insect and a good optical system. Counting accurately the numbers of OB is often more difficult for the beginner. Distinguishing between the polyhedra and such artifacts as oil droplets from the fat body, haemocytes, parasite spores, bacterial spores and other artifacts require some practice. Inability to identify NPV from such artifacts is a common problem where staff has received inadequate training or have poor microscopes. This can be especially true if the insects being examined have not reached the final stages of infection when OBs become most easily distinguished. To achieve any success requires both a good optical system and considerable care and patience.

The use of standards is invaluable. If possible a sample of pure NPV should be obtained and both counting and identification practiced for some time before attempting to examine actual infected insects or samples from production.

Counting NPV is most effectively done by examining a suspension of polyhedra in a counting chamber such as a haemocytometer that contains a known volume of liquid. Techniques do exist for counting NPV in dried stained smears (Wigley, 1980), but they are generally found to be more time consuming and less accurate than the haemocytometer technique when properly used.

COUNTING NPV USING THE IMPROVED NEUBAUER HAEMOCYTOMETER OR COUNTING CHAMBER

A haemocytometer is an essential tool used for estimating the number of micro-organisms in a sample. The Improved Neubauer Haemocytometer comprises a thick glass slide with a shallow depression in the central section divided into two halves (***figure 2***). On each side, the base of the depression has a fine ruled grid of squares (***figure 3***) which is visible under a microscope (Plate ??). The dimensions of this grid are defined. With a thickened cover slip placed over the depression a chamber is created of fixed depth.

1. Thoroughly mix the sample using a vortex mixer. Introduce 10µl of test suspension to both halves of the slide chamber from a pipette, though the precise amount is not important and an appropriately sized drop from a Pasteur pipette will do. Failure to mix the NPV sample before sub-sampling is a very common source of error.

2. Place the cover slip over the drops and press down until fixed by capillary attraction of the drops. To tell that the coverslip is fixed correctly watch for the interference pattern of Newton's Rings seen under the part of the coverslip directly in contact with the counting chamber slide. The rings should be seen on either side of the actual counting area. **Alternatively** the coverslip is placed over the counting chamber and a drop of the suspension is placed on each side by a Pasteur pipette so as to fill both chambers, and by pressing gently the excess liquid will go to the side and middle channels of the Neubauer haemocytometer.– The choice of method it is a matter of defining a standard procedure that can be more easily performed for the conditions of Tanzania, but it has to be accurate enough). The two procedures should be tested and the final results compared to check if there are differences and the magnitude of the difference, if there is any.

3. Wait 10-15 minutes to allow particles to sediment to the chamber floor.

4. Count the contents of 10 of the larger double ruled squares in each of the two chambers, each of the large squares contain 16 small squares, on both sides of the haemocytometer. Choose a pattern of sampling both across and down the grid and use x400 phase contrast illumination. At least 300 OB per count are required to obtain a statistically valid sample and make sure that the OB are not clumped or again the count may be invalid. Count the number of polyhedra completely contained within each of the small squares plus the number touching the left and upper sides.

5. It is usual to make three separate counts on three independent sub-samples from the NPV concentration you wish to count then to average these to get the final count.

Figure 2: The Improved Neubauer Haemocytometer, with two chambers (1 and 2) for OB count.

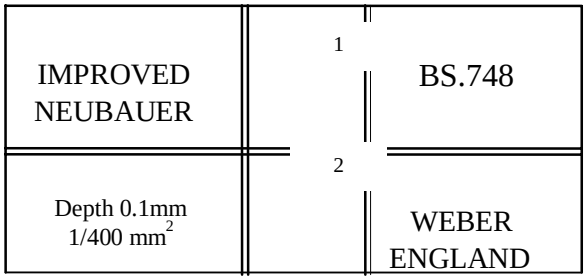
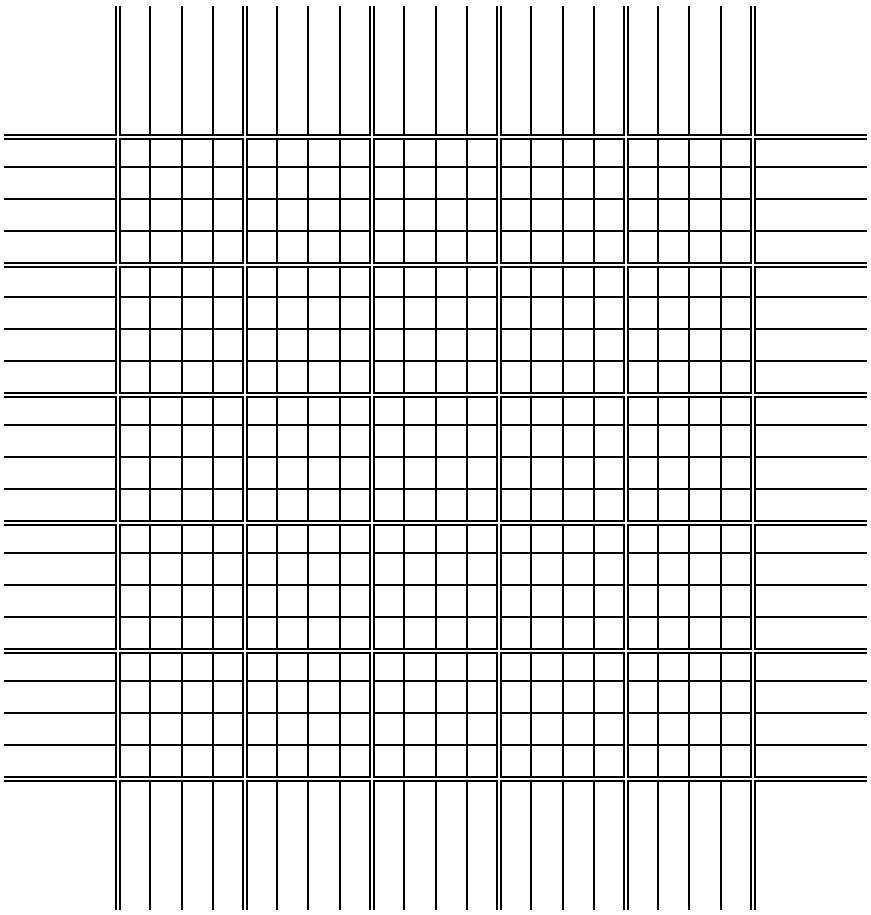


Figure 3: Grid area of central depression of the haemocytometer.

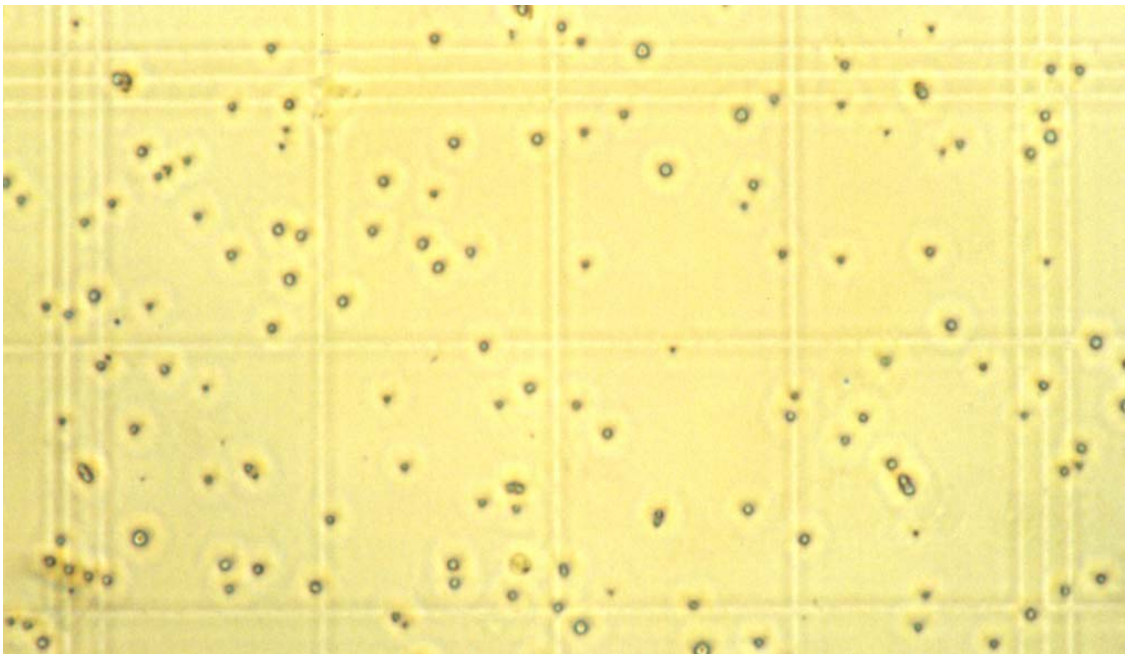


Only the specially thickened coverslips designed for haemocytometers should be used. If normal thin microscope coverslips are used these are distorted by the pressure of the air and capillary forces and so the volume of liquid over the grid is not exactly 0.0025mm^3 intended (if using a standard Neubauer chamber) and the counts will be inaccurate.

The depth of the focal field at x400 is less than the depth of the counting chamber. If all the NPV are allowed to settle to the floor they can all be seen in the focal field at once, which saves having to focus up and down. This also helps to distinguish NPV from similarly sized fat droplets which do not sediment but float to the top of the chamber.

If the sample is too crowded with OBs it can be difficult to count and the sub-sample should be diluted to a more appropriate concentration for counting. As a guide, if there are more than 10 OBs in each small square the sample should be diluted further. A standard with 10^9 OB per ml can usually be counted as a 1/100 dilution. If there are on average less than 1 OB per small square, getting a large enough number of OB counted for statistical accuracy can also be a problem and then concentrating the sample by centrifugation may be necessary.

Plate 7 NPV on a counting chamber. Eight SMALL squares of one LARGE square are shown. Magnification is X400



Either dark field or phase contrast microscopy are used to identify polyhedra. With the counting chamber under the microscope, the number of OBs in a given number of grid squares can be counted. Polyhedra touching the bottom and right sides are not counted. Since both the depth of the chamber and the grid's dimensions are known, it is then a straightforward calculation to determine the number of OBs per ml of test suspension, thus:

$$\text{Number of polyhedra per ml} = \frac{D \times X}{}$$

$$\frac{D \times X}{N \times K}$$

where:

D = dilution factor
X = total number of polyhedra counted
N = number of squares counted
K = volume above one small square in cm^3.

Area of each small square is 1/400 mm^2 = 0.0025 mm^2. Depth of chamber is 0.1mm.
Volume of liquid above a single small square is 0.0025 mm^2.x 0.1mm = 0.00025 mm^3.
To convert to cm^3 multiply by 1/1000 to get a volume of 2.5 x 10^{-7} cm^3.

Worked example:
Suppose in a sample diluted by a factor of 1000 we count 535 polyhedra in 160 small squares then:

D = 1000
X = 535
N = 160
K = 2.5 x 10^{-7} cm^3

thus: $\frac{1000 \times 535}{160 \times 2.5 \times 10^{-7}} = \frac{5.35 \times 10^5}{4 \times 10^{-5}} = 1.33 \times 10^{10}$ polyhedra / ml undiluted sample.

Provided the polyhedra are not aggregated, the distribution of counts follows the Poisson distribution and the standard error of the mean is given by:

$$\text{standard error} = \sqrt{X' / N}$$

where:

X' = mean number of polyhedra per small square
N = number of squares counted

The precision of the estimate depends on the number of particles counted. Provided the number of particles counted is greater than 50, confidence limits can be placed using the standard normal deviate "d" (1.96).

From the example above

$$X' = 535/160 = 3.34$$

$$\text{standard error} = \sqrt{3.34/160} = 0.144.$$

Since the number of polyhedra counted is greater than 50, we can use "d" (1.96) to attach 95% confidence limits to the mean count per square:

95% confidence limits = 3.34 ± 1.96 x 0.144/square
= 3.34 ± 0.28/square.

From this we can calculate the 95% confidence limits of the estimate of polyhedra per ml of undiluted sample:

95% confidence limits $= 1.33 \times 10^{10} \pm (1000 \times 0.28 \times 4 \times 10^{6})$
$= 1.33 \pm 0.11 \times 10^{10}$ pib/ml

BIOASSAY TECHNIQUES

The bioassay is a key tool in NPV production as it is only by carrying out a bioassay that one can get a true measure of the activity of an NPV pesticide. In the case of NPV, counts of OB are not reliable indicators of activity as intact polyhedra may not be infective due to inactivation or they may be defective because they were formed having few or no virions. It is only by bioassaying a measured or counted quantity of an NPV against the target pest that one can quantify its activity. In NPV work, the bioassay ranks alongside the microscope as the chief tool for for monitoring quality control in production.

The principle is to give measured doses or concentrations of the microbial agent to insects and then record the mortality induced at a standard time. A number of dilutions or concentrations of the agent are prepared and a number of insects dosed. With NPV, route of infection is through ingestion, thus the doses or concentrations are fed to the insects. The bioassay is assessed by examining all the treatments and counting the numbers of insects dead or alive at each dose or concentration daily after treatment. For comparative purposes it is usual to fix standard times after dosing for these assessments such as 5, 7 or 9 days for SpexNPV.

The bioassay is only effective, however, if the insects used and the conditions under which the bioassay is carried out, are standardised. Also, for any meaningful comparison between bioassays, each must include a standard of known concentration. For further discussion of NPV bioassays see Evans and Shapiro (1997) or Jones (2000).

Median Lethal Dose (LD_{50}) and Median Lethal Concentration (LC_{50})

It has been found that calculating the exact dose that kills 100% of a test group is both time consuming, difficult and due to the variation in dose-response, inaccurate. It is much easier and more accurate to calculate an intermediate response level, and the most commonly used one is the 50% mortality level or median lethal dose 50%, LD_{50}. This is defined as the dose of an agent that kills 50% of any group of exposed insects. To estimate an LD_{50} for an NPV, it would be necessary that the insect ingest specific doses of the virus, either incorporated in the insect artificial diet or sprayed onto its leaf surface then measure the associated mortality.

Finding the exact LD50 experimentally would be time consuming, however, it has been found that if a series of doses of different concentrations are given to groups of insects and the mortalities are plotted, it is possible to derive good estimates of LD_{50}. In order to get good estimates of partial mortality, reasonably sized samples of insects need to be used and the current recommendation is to use no less than 30 larvae for each dose.

But it is not easy or even possible to give exact doses to some types of insects, e.g. newly hatched larvae. Also, it is not often important to determine exactly the $LD_{50;}$ in many cases all that is required is to compare the activity of two agents or two samples of the same agent. In this case a comparative assay based upon relative concentrations such as median lethal concentration that kills 50% (LC_{50}) can be used and this is often much quicker than dosing

assays. In this assay mortality is determined to a range of concentrations of virus presented to the insect in diet or on leaves without determining the exact amount of OBs the larvae have consumed. While is means we cannot determine absolutely how active the NPV sample is in OB per larva it often enables us tom compare the relative activity of samples to say an NPV standard more easily. Thus it is very useful for doing quality control of production batches or efficay of formulations where one only wishes to know how active a sample is compared to a standard.

Because it can be quicker to determine LC_{50} it can also mean that larger numbers can be used so that the accuracy of the LC_{50} can equal those of the more time consuming LD_{50}. By determining the LD_{50}/LC_{50} and using it as the comparison point of a bioassay, it is possible to accurately quantify the relative activity of a number of samples of biocontrol agents.

A major consideration in bioassays is that the response of insects is variable, with age, size, instar and insect strain. Valid comparisons can only be made if a standard age and size of larvae are used. Therefore, in assays that are to be compared it is usual to use standard specified ages and sizes of insects. However, it has been found that even cultured insect stocks that are reared under apparently constant conditions and with considerable genetic homogeneity do vary in their response to standard doses and concentrations. To help to allow for this, it is common practice to include in every assay a standard strain of agent, usually one that is of high purity and genetically characterised to allow for inter assay comparisons to be made. This standard should be multiplied in a large enough amount to allow comparisons for many years

Another problem in bioassays is that the handling procedures themselves can cause death. To help estimate this, the inclusion of a control group of at least equal size to the treatment groups is essential. The mortality in this group can then be assessed, and this control mortality estimate can be used to improve the calculation of the true estimated LD_{50} or LC_{50}. For this to be accurate the control group of insects should be truly representative of all the test insects. It is not valid just to use those insects “left over” at the end of the assay, as a control. Such insects are likely to be a poor representation of the insects used in the test and so bias the control mortality and therefore the LD_{50} or LC_{50} estimate.

Bioassays must therefore:

- Always follow a standard technique.
- Use standardised disease free insects wherever possible.
- Include a known standard strain of the agent under test.
- Include a control group.

As larvae grow, it has been found that they become more different as their growth patterns diverge. Thus, using newly hatched neonates is an excellent way of improving standardisation. Another advantage of using newly hatched neonates is that they do not need rearing before the assay and so are often cheaper, particularly if large assays are being carried out. Also, it is easier to get the recommended minimum of 30 insects per dose. It is possible to use any instar but in lepidoptera with NPV, IIIrd instars are a good choice if older larvae are required. In this case, selecting larvae molting from second to third instar would be recommended, as newly 3rd intar larvae would be about the same age when they start feeding on the different treatments. Using later instars with NPV, where assessment times are 5-7 days, can be a problem as the physiological changes associated with pupation may begin to disrupt the assessment of the

results. Older larvae are also problematical as very high LD_{50} doses are required and this may be difficult in assessing many new strains of NPV, as time-consuming bulking-up of the virus would be required.

Types of bioassay

There a several types of assay appropriate for use with SpexNPV, and these include:

- Neonate (newly hatched larvae) surface assay.
- Neonate droplet assay.
- Plug or disc dosing.
- Leaf dip assay.
- Spray tray assay
- Incorporating the vírus into the host larvae diet

Any of the above assays can be used and the selection of the most appropriate depends upon the purpose, pest species and facilities available (Evans & Shapiro 1997, Jones 2002).

For routine quality control of SpexNPV in Tanzania the leaf dip assay is proposed to be adopted and the full protocol is printed below. Protocols for other bioassay techniques are given in appendix 1

Summary of main points

- Bioassay is the most important technique in virus production and quality control.
- Bioassay is only effective if the insects and conditions are standardised.
- Effective comparison between assays requires inclusion of a standard of known activity.

LEAF DIP BIOASSAY

Given the limitations of the plug-dosing assay, for the majority of purposes concentration based methods for estimating LC_{50} are preferred. Among the most widely used is the leaf dip assay. In this technique, leaves from a suitable host plant are dipped in different concentrations of NPV, and the leaves are then fed to the target pest insects. The insects are usually exposed to the dipped leaf for a given time, commonly 24 hours and then transferred to clean leaves or artificial diet. The advantage of this assay is simplicity, especially where host plants grow naturally. However the assay can be affected by variation in the amount of virus suspension retained by the leaf, changes in the nutritional quality or acceptability of the leaves and (if these plants are not grown in isolation) contamination with insect pathogens such as viruses or bacteria.

Procedure

1. Select some appropriate leaves, (older, tougher leaves may be unattractive to larvae or may contain high levels of toxic/repellent chemicals, and so new young leaves are preferred).

1. Pour out 100ml of each appropriate virus dilution into an 200 ml plastic pot. A sample set of fivefold dilution is shown below but the exact range may be adjusted depending upon experience.

    Dilution 1 = 2.0×10^7 PIB/ml
    Dilution 2 = 4.0×10^6 "
    Dilution 3 = 8.0×10^5 "
    Dilution 4 = 1.6×10^5 "
    Dilution 5 = 3.2×10^4 "

One pot will also contain the **control dose** of distilled water.

3. Add 100µl of a wetting agent to each pot. Close the lid firmly and swirl to mix thoroughly.
4. Using strong forceps, dip a leaf into the appropriate virus suspension until thoroughly wetted. Five leaves should be treated with each dilution, including the control solution, giving a total of 30 leaves.

5. Allow the excess to drip off and hang or place each leaf to dry.

6. Place the dry, treated leaves into 25 ml pots, 1 leaf per pot.

7. Using a second pair of forceps, carefully place a single larva into each pot.

8. Put on the lid firmly and pierce with a mounted needle to allow ventilation. Write the concentration of the treatment dilution on the lid.

9. Leave the pot at 25-27°C for 24 hours.

10. Take 30 clean 25 ml pots and add a cube of diet to each pot.

11. Transfer each larva individually from the leaves to a clean pot with diet.
12. Close the lid and pierce with a needle. Write the virus concentration or treatment number on each pot lid.

13. The mortality at each level should be assessed daily for 10 days.

Spray tray assay

This is not a routine bioassay, as for quality control of production batches. Rather, it is valuable for testing formulations, their persistence on leaves, adjusting dosages of the virus etc. When evaluating formulated product an appropriate way to do this under field conditions is sometimes to actually spray it onto host plants grown in trays then infest them with insects and evaluate its performance. This approach may also be used to test new formulations or look at the effectiveness of the NPV on different crops or under different field conditions.

Plate 8 Trays of Maize being used for spray tray assay

Procedure

1. Rear maize plants from seed in seed trays of suitable dimensions e.g.50x 25 cm. Rear until at least 12 cm high, do not use plants larger than 30 cm as these can be too fibrous for newly hatched larvae to feed on. You should aim to have 5-9 trays per sample to be assayed or treatment to be tested.

2. Larvae of a suitable age from newly hatched to III instar can be used for the assay. These may be bred in the laboratory, preferably, or field collected. At least 30 larvae should be used for each sample or treatment but a lager number 50-100 is prefereable.

3. An area for application should be marked out (e.g. 20 x 20m) and the maize trays laid out in a regular pattern to allow unimpeded movement of the sprayer for good coverage trays should not be placed within 2 metres of the edge of the plot. All trays should be marked with treatment and replicate number with labels in indelible ink.

4. The sample treatments of SpexNPV should then be made up in the required volume of carrier to give the desired field application rate. As an example, a standard treatment 1 x 10^{12} OB per ha delivered using a lever operated sprayer at 170 litres per hectare to a 20 x 20 metre using powdered SpexNPV at 3x10^{12} OB per g requires 6.8 litres at a concentration of 0.883 g SpexNPV per 15 litres + 15 ml of Triton-X (i.e. 0.1%). See appendix for sample calculations.

5. Trays are then sprayed using appropriate spray equipment. During spraying coverage should be checked by placing water (for aqeous applications) or oil (for ULV applications) sensitive papers in amongst plants. Wind speed and temperature should be recorded.

6. After spraying trays are moved to shade or into laboratory. When dry, larvae should be placed on trays. The larvae should be distributed equally between treatments and trays so all trays have the same number of larvae. A suitable number would be 200 per m^2 or 75 per tray of 0.375 m^2.

7. On each day following application examine trays and count numbers of larvae alive and dead of NPV. Record numbers. Note counting and finding newly hatched larvae can be difficult for the first few days of the trial. Record max-min temperatures during this period.

Example Calculations for concentration of SpexNPV-2004 required to achieve dosage of 1 x 10^{12} OB per ha:

Concentration of PCS-NPV-2005 powder = 3.0 x 10^9 OB per g

Calibrated sprayer uses 170 litres per ha.
Each tank holds 15 litres
So, require 170/15 tanks of NPV = 11.33 tanks

Therefore - Amount (g) of PC-NPV-2005 powder required for a 1 ha plot (at a dosage of 1 x 10^{12} OB per ha) = 1 x 10^{12} / 3 x 10^9 g
= 333 g
= 333/11.33 g per 15 litre tank
= 30 g NPV per 15 litre tank

Calculations for amount of PC-NPV-2004 required to achieve dosage of 1 x 10^{12} OB per ha on a 20 m x 20 m plot:

Assume - Calibrated sprayer uses 170 litres per ha. (= 10,000 m^2)
A 20 m x 20 m plot = 400 m^2

Therefore, to spray a 20m x 20 m plot, need to spray 170 x 400/10000 litres = 6.8 litres of NPV, at the concentration calculated above

ANALYSIS OF BIOASSAY DATA

Once mortality estimates for each treatment have been made, standard methods exist for estimating the control mortality. From the data LD_{50}/LC_{50} can be obtained by **(a)** longhand calculation using the probit method of Finney (1971); **(b)** graphically by plotting probit mortality against the logarithm of the NPV concentration, **(c)** by using a specific computer software package based upon probit or logistic methods. Most statistical packages in current use provide probit analysis procedures. The probit and logistic procedures are mathematical functions aimed at transforming the sigmoid mortality concentration response curve into a

straight-line response for ease of plotting and calculation. This makes the statistical analysis of mortality data much easier.

In practice the graphical method **(b)** is nearly as accurate as the calculation method, and so is usually adopted where computer methods are not available.

The probit packages produce output giving LC50 and can be set up to give other LC_{50} estimates LC_{90} etc if specified. It also includes the 95% fiducial (error) limits for the LC_{50} the slope of the fitted line and some estimate of heterogeneity as a Chi-squared or other statistic. For discussion of LC_{50} analysis and detailed interpretation refer to Jones (1997) or Evans and Shapiro (1997).

Graphical method for estimation of the Median Lethal Concentration (LC_{50}).

The graphical method for estimating LC50s has largely been superceded by the use of computer programmes and is rarely used in practice. However having an understanding of this technique is still useful in ensuring staff can understand how the computer packages work and can help them recognize when the key assumptions that underlie the analysis are not met by the data. All staff analyzing or interpreting bioassay data should be able to perform the graphical method.

1. From the bioassay mortality data calculate the % mortality at each sample dilution.

2. Also calculate the % natural mortality in the control treatment.

3. Adjust the % mortality at each dilution for natural mortality according to Abbott's formula:

$$\text{Adjusted mortality} = \frac{[\%\ \text{mortality} - \%\ \text{control mortality}] \times 100}{100 - \%\ \text{control mortality}}$$

For example:

If 23 larvae in a sample of 30 died after treatment with a dilution:

$$\text{then \% mortality} = \frac{23}{30} \times 100\% = 76.6\%$$

then, if in the same bioassay 2 out of 30 larvae died in the control treatment:

$$\text{control mortality} = \frac{2}{30} \times 100\% = 6.6\%$$

Using Abbott's formula:

$$\text{adjusted mortality} = \frac{[76.6 - 6.6] \times 100}{} = 74.9\%.$$

4. Using a standard table for transformation of percentages to probits, (see ***7.6.2***), find the appropriate probit value for % adjusted mortality at each dilution.

5. Calculate the logarithm of the virus concentration at each dilution. Thus for example, 2 x 10^7 has a logarithm of 7.301.

6. Plot the results on ordinary graph paper with log dose on the horizontal axis and probit mortality on the vertical axis. Ignore probits outside the range 2.5 - 7.5.

7. Draw the best fit straight line through the plotted data points.

8. Find the logarithm corresponding to probit 5 and calculate the antilogarithm. This is the approximate LD_{50}.

Table 1 Transformation of percentages to probits (from Finney, 1971)

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
	0.0	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

IDENTIFICATION OF INSECT VIRUSES WITH RESTRICTION ENDONUCLEASES

INTRODUCTION

When we talk about *Spodoptera exempta* NPV (SpexNPV) we are usually naming it from the host the virus was first identified in. Accurate identification of different species and strains of NPV is possible only by analysis of the viral DNA. Under the microscope it is impossible to tell the difference between all the NPV found in the wide variety of hosts now identified. The differences are not visible, but can be detected in the DNA of the virus. The most commonly used technique for identifying NPV is to visualise a DNA profile using Restriction Endonuclease analysis (REN), also known as Random Fragment Length Polymorphism (RFLP).

This technique has many uses including:

1) In quality control, to check that the progeny virus is the same as the inoculum. Larvae can contain a latent virus that may be expressed in addition to or instead of the inoculated virus.
2) In some places licence to use the products will require restriction analysis to confirm the identity of the virus.
3) Continuous collection and assessment of new strains is still necessary in order to develop products which are more virulent or which have the potential to control several other species. Virus isolates can be profiled to differentiate between strains.

DNA PROFILING

The DNA genome of NPV is from 80 to 200 kilobase pairs long. To differentiate between viruses a DNA profile or "fingerprint" is made of the DNA extracted from a sample and cut with a restriction enzyme. In the method of virus identification known as restriction endonuclease analysis, REN, or DNA fingerprinting, this long chain of DNA molecules is cut in several places. The DNA of each virus species or strain will have a different sequence.

The cutting is done through the action of restriction endonucleases. These are enzymes extracted from bacteria, e.g. *E. coli*. They exist in these organisms as a defence mechanism against invasion by foreign DNA. They work by recognising a particular foreign DNA sequence, **GAATTC** in the case of *E. coli*, and cutting the DNA at this site, called a restriction site, thereby making it harmless. For *E. coli* the cut occurs after the first **G**.

The enzyme recognises a specific short sequence within the whole DNA strand and cuts the strand wherever this sequence occurs. Every copy of the genome will be cut in the same places. The result of the action of the enzyme is to produce several fragments of various lengths from each strand of DNA. When the cut DNA is inserted into a gel and subjected to an electrical field, the different size strands will move at different speeds through the gel, smaller fragments moving faster than large ones, and therefore separated out. The number and length of the fragments depends on how many times the short, recognised sequence appears within the complete sequence.

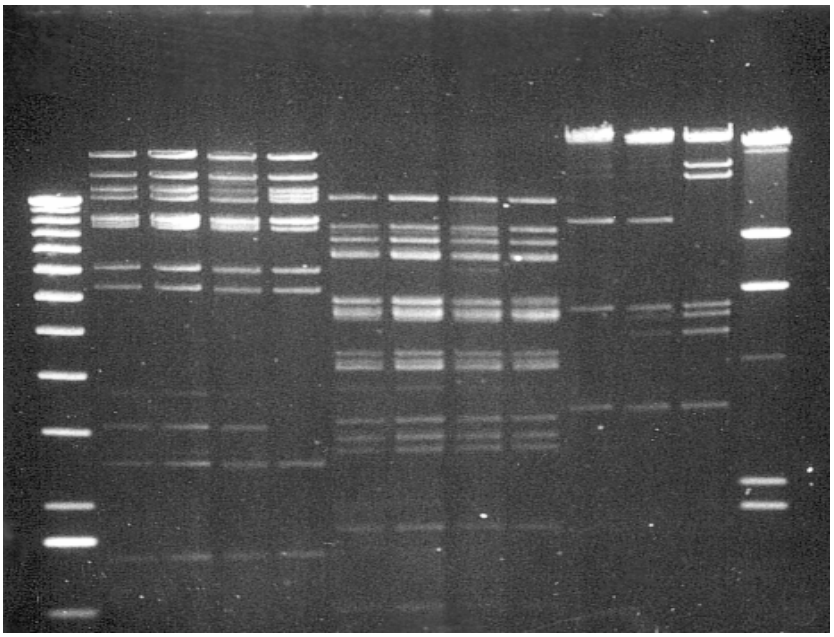
In practice this is achieved through running the fragments through an agarose gel subjected to electrophoresis. When these fragments are injected into an agarose gel and an electric current is passed through the gel, (electrophoresis), the smaller fragments will move faster and the larger fragments remain near the injection end of the gel. By staining the DNA in the gel with a UV luminescent chemical (ethidium bromide) the fragments can be visualised and photographed. The pattern produced after electrophoresis by the different size fragments of DNA can therefore be recorded and compared with that produced from other samples of virus. The same pattern will be reproduced each time the same virus is cut.

Let us take *H. armigera* NPV as an example. It has a chain of about 145,000 molecules; 145kb. At four places on this chain the DNA occurs in the sequence **CTGCAG**. This is the sequence recognised by the restriction enzyme found in *Providencia stuartii,* **Pst1** for short. If this enzyme is added to a sample of *H. armigera* virus DNA, it will cut the DNA chain wherever it finds this sequence, i.e. four times. This leaves us with five pieces of DNA instead of one long piece. Of course this cutting is happening to every chain of virus DNA that is present in the sample, providing that there is enough enzyme. The sizes of the cut pieces happen to be about 65,000, 60,000, 10,500, 6,000 and 3,500 bp. All we need to do now is put the cut DNA onto the gel and pass an electric current through it and the five strands will be separated according to their length, the small ones travelling faster through the gel than the large ones.

In order to see these fragments, ethidium bromide is added to the gel, which attaches to the DNA and is visible under UV light. The stained gel is then photographed and this gives a permanent record of the virus, ready to compare with any future virus produced or any virus suspected of being a contaminant.

Different viruses and even different strains of virus will be cut at different points and therefore will produce a different pattern.

Plate 9 DNA profiles of four different strains of an NPV cut with *Hin*DIII, *Eco*RI and *Pst*I restriction enzymes



DNA EXTRACTION AND IDENTIFICATION PROCEDURE

Extraction and dissolution of virus

1. Place one larva in a microtube and add distilled water up to 1ml. Squash gently with pestle or pipette tip and vortex for 10 seconds. Aliquot if necessary. (*Skin may be removed carefully with the tip of a pipette, but avoid contaminating the outside of tube. If pure virus is used, begin at step 6.*)

2. Centrifuge for 2-3 seconds to pellet insect debris.

3. Transfer the supernatant carefully with filter tip to clean microtube. (*Heavily virused larvae may pellet some NPV at this point on top of the insect debris layer. This can also be removed with the supernatant*).

4. Centrifuge for 10 minutes at 12,000 rpm. (*NPV will pellet in less time than GV*).

5. Remove and discard the supernatant. (*The pellet of NPV will be seen as a light coloured area and any dark layer above it can be discarded with the supernatant. It is possible to **gently** loosen the top dark debris layer with the tip of a pipette to assist removal*).

1. Make up to 1ml with distilled water. Vortex to re-suspend the pellet.

7. Centrifuge for 5 minutes at 12,000 rpm. (10 minutes for GV).

8. Remove the supernatant and add 120µl distilled water. Vortex to re-suspend the pellet. (*Reduce this volume to between 50 and 100µl if sample is very small. Reduce EDTA, proteinase K, Na_2CO_3 and SDS in steps 9, 10 and 11 proportionally*).

9. Add 25µl **0.5M EDTA** + 3µl of **proteinase K** (20mg/ml). Incubate for 1½ hours at 37ºC. (See ***Note 1, Section 8.3.8***).

10. Add 75µl (approx. half volume) of **1M Na_2CO_3** and incubate for 15 minutes at 37°C. (*The pH of the suspension should be >9.3 for dissolution to take. When this happens the liquid becomes clear instead of milky. If this does not occur add another 15µl Na_2CO_3*).

11. Add 25µl of **10% SDS** and incubate for 30 minutes at 37°C.

12. Centrifuge for 1 minute at 10,000 rpm to pellet undissolved polyhedra or any remaining debris. Remove the supernatant to a clean tube. (*At this point some of the liquid in the tube can be very viscous. This should be retained with the rest of the supernatant*).

Phenol extraction of DNA

1. Add an equal volume of **tris-saturated phenol**. Put the pipette tip through the tris layer to the liquid phenol layer at the bottom, to pipette up the phenol**.** ***(Handle phenol carefully and wash off immediately if in contact with skin).***

2. Agitate **gently** for at least 5 minutes.

3. Centrifuge at 12,000 rpm for 5 minutes. This will result in a viral DNA layer at the top, phenol at the bottom and often a visible white protein interface.

4. Carefully transfer the upper phase to a clean microtube, taking care not to disturb the interface. (*If the tip of the pipette is cut diagonally to produce a larger opening this will help to prevent a surge of liquid dragging the interface upwards*).

5. Repeat the procedure in steps 1 to 4 using an equal volume of **25:24:1, tris-saturated phenol:chloroform:isoamyl alcohol**. If the interface is still not clear this step should be repeated.

6. Repeat steps 1 to 4 again, but this time using an equal volume of **24:1, chloroform:isoamyl alcohol** and without cutting the tip. (*Put the tip into the centre of the upper layer in this step*).

7. The DNA is now in solution and ready for dialysis or ethanol precipitation.

Dialysis

1. To prepare a microtube for use in dialysis, cut the cap with its hinge from the tube. Cut the top 5mm off the tube and discard the lower section.

2. Cut off a 20mm length of dialysis membrane and trim off both sides to make two pieces 20 x 20mm in size. Soak the pieces in **x1 tris-acetate buffer** for 5 minutes. (*The membrane must be handled carefully to avoid too much contamination with salts, etc. from the skin, but any gloves used should not have powder on the outside*).

3. Pipette the extracted DNA (no more than 250µl) into the microtube cap, as prepared above.

4. Lay a single piece of dialysis membrane across the cap and carefully fit the 5mm section of tubing over this, enclosing the DNA. Push down evenly to avoid tearing the membrane. (*There will usually be some trapped air bubbles. This does not matter*).

5. Place the cap assembly into a large beaker (e.g. 600-1000ml for 10-20 samples) of **x1 tris-acetate buffer** with the membrane uppermost. Submerge the cap to ensure contact of the membrane with the buffer and while submerged, carefully turn the assembly over to float with the cap uppermost and membrane below.

6. Dialyse at 4°C for at least 36 hours, changing the buffer three times, **or** for 12 hours followed by ethanol precipitation. (*x1 buffer for the changes should be kept at 4°C*).

8. To change the buffer, pour off the old buffer whilst retaining the samples in the beaker. Pour in the fresh buffer and repeat the process of turning the samples to ensure contact. (*Wear gloves but avoid touching the samples if possible*).

7. After dialysis is complete remove the assembly from the beaker using forceps and dab the membrane surface **very gently** with a tissue.

8. Nick the membrane with a scalpel and pipette out the DNA into a clean-labelled microtube. Discard the used scalpel. (*It is only necessary to make a very small cut which can be enlarged with the pipette tip*).

9. The DNA is now ready for digestion with restriction endonuclease enzymes. Store at 4°C.

Ethanol extraction as an alternative to dialysis

N.B.: This procedure should be used in conjunction with a 12-hour dialysis or to concentrate DNA.

1. To the extracted DNA solution add 1/5th volume of **3M sodium acetate** followed by 2.5 volumes of cold **ethanol absolute**, taken straight from the freezer and kept on ice during this procedure. (*The DNA will become visible as white strands, often looking like cotton wool)*

2. Place samples in the freezer for one hour to ensure complete precipitation. (*Overnight for RNA*).

3. Centrifuge at 10,000 rpm for 5 minutes to pellet the precipitate. (*Even if a precipitate is not clearly visible at this stage there may be enough DNA to digest*).

4. Pour off the ethanol and drain for a few minutes on a tissue. Add 500µl of 70% ethanol and gently wash the pellet. Centrifuge as in step 3. Pour off or pipette off the ethanol.

5. Leave on the bench for at least one hour to evaporate any residual ethanol, or preferably place under low vacuum for about half an hour. This will result in a very dry pellet of DNA. (*If any ethanol is left it can inhibit the RE analysis*).

6. Re-suspend the DNA pellet in about 20µl **x1 tris buffer** or **sterile distilled water**. (*The volume can be increased if there is a large amount of DNA*).

7. The DNA is now ready for digestion.

Digestion

1. Pipette 25µl of DNA solution into a clean microtube. (see ***Section 8.3.8, Note 2***)

2. Take enzymes and their buffers from the freezer and place the enzymes on ice.

3. Add **x10 enzyme buffer** to the DNA. The amount required will be equal to 1/10 of the final volume in the tube, i.e. DNA + buffer + enzyme. For 25µl of DNA add 2.9µl of buffer. (*To ensure the buffer and enzyme are all transferred and well mixed, pipette directly into DNA solution*).

4. Add a quantity of **R. E. enzyme** equal to 1/2 the volume of buffer used, i.e. 1.5µl. (*Hold the enzyme container carefully by the top only and return to the ice immediately, and to the freezer as soon as all samples have been completed*). See ***Section 8.3.8, Note 3.***

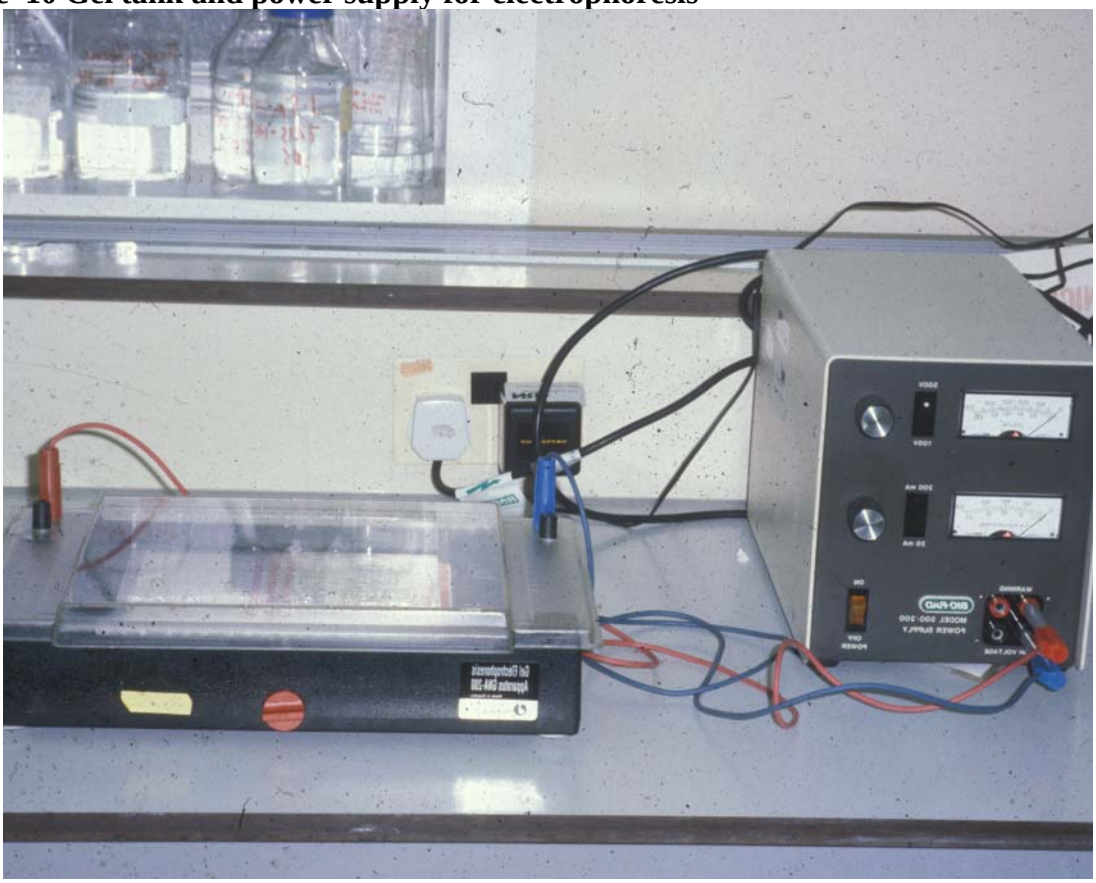
5. Stroke the side of the tube gently to mix.

6. Incubate at 37°C for 2 to 4 hours.

7. At the end of digestion add bromophenol-blue stopping mix equal to 1/10 of the volume in the tube, i.e. 3µl. Stroke the side of the tube to mix. (*Blue is used to visualise the extent of movement*. See ***Section 8.3.8, Note 4***)

8. The sample is now ready for electrophoresis and should be stored at 4°C until needed.

Plate 10 Gel tank and power supply for electrophoresis



Electrophoresis

1. Make a 0.6% gel by adding **x1 tris-acetate** buffer to **low melting point agarose** (molecular biology grade) in a 500ml conical flask. (*250ml and 1.5g is sufficient for a gel 200 x 150 x 8mm.*)

2. Bring to the boil in a water bath, over a bunsen burner or in a microwave and boil gently until all of the grains have dissolved. Stir occasionally during heating. (*Do not use microwave at maximum power or the liquid will "volcano"*).

3. Cool slightly, stirring or "swirling" occasionally to prevent differential cooling and then add 1-2µl of **10mg/ml ethidium bromide** for each 100ml of gel. This will bind to the DNA and make it visible under UV light. (***CARE*** *- powerful mutagen. Use gloves when handling).* See ***Section 8.3.8, Note 5.***

4. Wipe the plastic gel mould with ethanol and if not provided with end plates make the walls by fastening adhesive tape along the two open sides, sticking it to the base and to the end of the side walls. (*Sellotape, masking tape or labelling tape can be used*). The mould should be placed flat on a polystyrene (thermocool) sheet. (See ***Section 8.3.8, Note 6***). (*The level should be checked with a spirit level if available*).

5. Cool the gel to about 60°C then pour into the mould ensuring that there are no air bubbles. Any bubbles or loose particles can be carefully moved to the sides of the mould with a pipette tip.

6. Immediately place the required comb in position at one end of the gel to create wells. (*Make sure the comb is clean or it may tear the gel when it is removed*).

7. Allow to set for 30 minutes to 1 hour, and then gently remove the comb and tape. Use as soon as possible to prevent drying. If necessary the gel can be kept in the tank of buffer for a short time if not being used immediately. (*Use gloves when handling the gel and mould because ethidium bromide has been added*).

8. Pour sufficient **x1 tris-acetate buffer** into the tank to cover the flat base.

9. Place the mould with the gel on it into the tank, making sure it slots in correctly, and with the wells at the negative end (indicated on the tank). Top up with buffer to cover the gel by about 3-5mm.

10. Using a fine pipette tip, carefully place digested DNA into the wells. The sample should be released slowly from the pipette when the tip is in the buffer and just above the well. The sample will sink to the bottom of the well. Discard the tip after each sample. (See ***Section Note 7***)

11. Into one well pipette the molecular weight marker, e.g. **digested lambda DNA** or 2µl of a **1 kb ladder**. (*Either of these will provide a reference scale for determining the size of fragments in each band. Reduce the amount if a small gel is being used*).

12. Replace the lid on the tank and connect to the power supply (-ve to blue, +ve to red).

13. If a large tank is being used, set the power level to 35V and run overnight. A small gel can be run at a higher voltage for about 5 hours.

14. The furthest extent of sample movement through the gel will be indicated by the position of blue dye. Do not allow the dye to run beyond the end of the gel or some bands may be lost.

15. Switch off the power supply and remove the mould and gel from the tank. Gloves must be worn.

16. Place the assembly on a UV transilluminator to see if the bands are visible. (*Wear UV protection goggles or visor when viewing the bands*).

17. If more staining is needed, prepare a solution of 2µl of **10mg/ml ethidium bromide** per 100ml of **x1 tris-acetate buffer**. Slide the gel very carefully from the mould into this solution and leave for 30 minutes preferably on a rocking table. (**Gloves**!).

18. Take the gel from the staining solution using a gel scoop and place briefly in a tray of distilled water to remove the excess ethidium bromide solution.

19. Take the gel from the water and place on the UV transilluminator surface (Plate 24). View through protective eye shields or photograph as required (Plate 25). If there is too much 'background' the gel can be destained in fresh x1 buffer for 30 minutes to several hours. (*'Background' results from ethidium bromide being absorbed by the gel*).

20. To photograph place a photographic hood carefully over the gel and put the Polaroid camera on the top. For a 200 x 150mm gel, using a large hood, the aperture and time for a non-negative Polaroid photograph should be *f* stop 5.6 for ½ or 1 s. For a small gel, using a small hood, the settings will be 5.6 for 1/8 s. .

8.3.7 Preparation of solutions

SOLUTIONS

Tris-acetate buffer:	Tris-acetate EDTA buffer, pH 8.3 usually supplied as x10 liquid or powder, to be made up to x1 with distilled water. Store at 4°C.
0.5M EDTA:	Use a heated stirrer if possible. This solution may re-crystallise, but will re-dissolve on heating. Make up with distilled water. Store at room temperature.
Proteinase K:	Supplied as a powder. Make up at 20mg/ml with distilled water. Store at 4°C.
1M Na_2CO_3:	Should be made up fresh at least each week. Store at room temperature or 4°C.
10% SDS:	Sodium dodecyl sulphate made up at 10% (w/v) with distilled water. Store at room temperature. Re-crystallises at low temperatures, but can be re-dissolved by gentle warming.
Tris-saturated phenol:	Make up fresh as required and keep dark. Half fill a McCartney bottle with tris-acetate buffer. Add phenol until bottle is ¾ full and shake to dissolve. The phenol will form a layer at the bottom and is drawn off with a fine pipette. Use gloves and fume cupboard if possible. Discard unused saturated phenol via safety officer. Store phenol crystals at -20°C. Keep solution out of light at 4°C. (*8-hydroxyquinoline can be added to a final volume of 0.1%, which will extend the time it can be kept before the phenol darkens and becomes unusable*)
Chloroform/ isoamyl alcohol:	Make up at 24:1 in a McCartney bottle. The isoamyl alcohol prevents foaming. Keep solution at 4°C. Add to saturated phenol as required for 25:24:1 mix.
Enzymes and buffers:	Each enzyme is supplied with its appropriate buffer. A chart is available to indicate the correct combinations to use. Store at -20°C.
Stopping mix:	25% Ficoll (w/v), 0.1M EDTA, 0.25% bromophenol blue (w/v), made up with distilled water. Store at room temperature.
High mol. wt. marker or lambda + HindIII:	15µl water or buffer, 1.7µl stopping mix, 2µl marker. Store at 4°C.
1 kb ladder:	20µl stock solution of ladder, 20µl stopping mix, 80µl x1 tris buffer. Store stock at -20°C and working mix at 4°C.

ADDITIONAL NOTES

1. EDTA disrupts the host insect enzymes and proteinase K breaks up host protein.

2. The amount of DNA sample to be digested depends on the size of the wells in the gel and the concentration of DNA in the dialysed solution. (*This can often be estimated by the pellet size in* ***extraction step 5***).

3. **Alternative method of adding RE enzyme:** If the DNA solution is quite concentrated and several samples are to be digested with the same enzyme, a bulk mix of the enzyme can be made to reduce the number of tips used.

   For 10 samples:

   30.0µl water (to increase volume for pipetting accuracy)
   32.0µl buffer
   15.0µl enzyme.

   (*In practice it is always best to make a mix for one more sample than you need because of pipetting errors. If the DNA is very concentrated the volume of enzyme used can be doubled to ensure complete digestion*).

   Put 22µl of each DNA solution into separate tubes. Pipette 7.4µl of the mix into each tube and continue from ***stage 5*** of the ***digestion procedure***.

4. **Stopping mix:** EDTA stops the reaction, Ficoll or sucrose increases the density in order to hold it in the wells and bromophenol blue marks the samples in the well and through the gel. See ***Solution section*** for quantities.

5. Ethidium bromide is not essential at this stage, but it is occasionally useful to be able to see the progress of the DNA fragments through the gel. If not used now the gel will need to be stained after electrophoresis for 30 minutes in tris-acetate buffer, containing 2µl of 10mg/ml ethidium bromide in each 100ml of buffer.

6. The polystyrene (thermocool) sheet is to ensure even cooling of the gel to prevent distortion of the profiles.

7. Liquid placed in the wells should come just below the top of the gel surface to prevent it "streaming" over the edge of a well. Too much DNA will result in streaked or non-discreet bands.

8. Two molecular weight markers may be needed to cover the range of fragment sizes. High molecular weight marker, lambda Hind111 digested and 1 kb ladder are the markers most commonly used.

9. Two different films can be used. 665 will give you a negative, e.g. for enlarging, as well as a positive print. 667 produces a positive only, but gives good resolution in the ½-1 second exposure time. 665 needs an exposure of 2-3 minutes to visualise the weaker bands. However, the gel will need re-staining for a second photograph and too much exposure to UV can degenerate the DNA.

Storage of SpexNPV

Liquids suspensions

Liquid suspensions of NPV should be stored in deep freeze at -20 deg C.

Freeze dried powders

NPV in freeze-dried powder form is stable for moderate periods of one to three months at room temperature. It will however absorb moisture from the air if bottles are left open so containers should be tightly closed at all times. For long-term storage it is best stored in a deep freeze at -20^{o}C or refrigerator at about 4^{o}C.

Clay formulations

These types of formulations may be stable for six months at room temperature, however they can be stored for over a year in refrigerators or for over three years in freezers. If storage space is a problem, the concentrated suspensions of NPV can be maintained in freezers to be defrozen and formulated just before use in the field.

Application of SpexNPV

NPVs in general can be applied successfully using any commonly used method of insecticide application including hydraulic sprayers (motarised or hand lever operated), motarised mistblower and ULV sprayer. ULV applications though require the use of oil based spray formulations and these are not currently planed to be produced for SpexNPV as yet.

Plate 11 Ground application of SpexNPV with motorised knapsack sprayer

Plate 12 Aerial application of NPV to armyworm

The recommended application rate is $1x10^{12}$ OB per ha. This can be in the form of aqueous suspension, freeze-dried powder or clay formulation. All are compatible with standard sprayers hydraulic or motarised knapsack sprayers but not ULV sprayers.

Ground spray with hydraulic lever knapsack sprayer

Materials needed

NPV
Lever operated knapsack sprayers fitted with fine spray nozzle such as Hardi 15.
Triton (added at 0.02% to final spray liquid)
Clean water (This should be of pH 7±1 as using water that is too basic or acid pHs lead to lower field activity)
Spray paint to mark sprayer tracks
Clean plastic cups with lids to collect larvae

Marker pen to label cups	Protective clothing
Gloves	Site square
Soap bar	Stopwatch
Tape measure	Anemometer
Measuring cylinder	Pre-weighed virus powder
Notebook and pencil	Marker posts

Method

1. An area of maize or grass selected for the trial on the basis of scouting is identified

2. When 1^{st}-2^{nd} instar armyworm larvae are found in the maize, the treatments will be applied the following day. Lever operated knapsack sprayers, will be used to apply the treatments at an equivalent volume application rate of 170 litres per hectare.

3. Prior to the trial sprayers will be calibrated. Previously tests showed that a track spacing of 60 cm and a walking speed of 1.24 m/sec with a fine nozzle gave a volume application rate of 170 litres per hectare.

4. NPV treatments will need 1.0×10^{12} OB per hectare. This will be made up from appropriate stocks in 0.1% Triton or other surfactant.

5. The larvae will be counted immediately before spraying (day 0), then after spraying the mortality will be assessed daily until mortality ceases usually 7-14 days after application.

To be noted when carrying out the treatments:
Vegetation height
Planting density
Weather conditions (wind speed and direction, sun)
Time of day and date
Volume sprayed
Area sprayed

Ground spray with motorised mistblower

Equipment

NPV
Motarised mistblower e.g. Solo 412 aster.
Triton (added at 0.02% NPV)
Clean water (pH 7±1)
Spray paint to mark sprayer tracks
Clean plastic cups with lids to collect larvae

Marker pen to label cups	Protective clothing
Gloves	Site square
Soap bar	Stopwatch
Tape measure	Anemometer
Measuring cylinder	Pre-weighed virus powder
Notebook and pencil	Marker posts

Method

1. Identify and mark out plots as described in protocol for knapsack sprayer.

2. Do initial larval count prior to application.

3. Spray plots with an application rate of 50litres/ha with the nozzle 30-50 cm from the canopy and a swath width of 8 metres and a walking speed of 1 m/sec with the flow set to position 3 gave the required volume application rate of 50 litres per hectare.

To be noted when carrying out the treatments:
Vegetation height
Planting density
Weather conditions (wind, sun)
Time of day and date
Volume sprayed
Area sprayed

Aerial spray

Equipment
Virus
Ground to air radio
Flags for marking
Site square prism
GPS (to log position of trial)
Camera (for write up)
Anemometer
Tape measure
Stopwatch

Method

Area required - the minimum area that can be reliably treated with an aeroplane fitted with boom and nozzle equipment at a rate of 10-50 litres per hectare is 4 ha. This will require 40-200 litres of virus formulation. However the dead liquid in the plumbing system is around 15 litres so extra should be added to allow for this (check with spraying company).

1. Discuss with pilot the sprayer to establish track spacing, forward speed and flow rate.

2. Collect pre-spray insects.
.
3. Mark out 4 hectares of land infested with larvae, using flag posts that can be seen by the pilot. A moving marker held at both ends of the plot will help the pilot to spray the correct track distance.

4. Prepare and fill the aircraft at base airfield with volume needed for the trial plot.

5. Put some spray sampling papers out on vertical sticks and on the ground within the block.

6. Measure and note meteorological parameters, wind speed, direction, and temperature.

7. Call the pilot by radio to do the spray treatment.

8. Record time of start.

9. Spray the block.

10. Record time of finish, and any information on the spraying such as blockages, track errors or spray running out early or not being finished at the end of the block.

11. Sample larvae after application to assess effectiveness at 3-7 days post spraying using larval sampling protocol below.

Use counting quadrats and record total larvae and virus-infected larvae.

Assessment of all the trials is carried out through estimating larval populations within treated plots using 50x50cm quadrat counts the method most commonly used for assessing armyworm control trials (Rose *et al*, 2000). Population counts are carried out prior to spraying on the day of application then at predetermined intervals for up to 14 days post application. Depending upon temperatures and vegetation population counts are not made after 10-14 days as by this time larvae in control treatment plots usually have consumed all suitable vegetation and will have migrated away from the plots.

Plate 13 Technique of counting larvae using a quadrat and a silly hat.

Plate 14 Close up of larval counting

The counting technique consists of placing quadrats onto the ground in treated areas and the larvae in each square are counted and larval stage recorded. In small plot trials quadrats are thrown randomly from the centre of the plot.

In large plot trials however quadrats are counted at regular intervals along transect lines to ensure sampling over these larger areas was representative. For each replicate 30 separate quadrat counts are made on each assessment day. Prior to trials a pre-spray assessment is made to provide base-line data after which further assessments are made on selected days post application. The numbers of insects infected with SpexNPV are also estimated visually based on clear symptoms of SpexNPV infection (immobility, darkening of cuticle, loss of turgor, obvious lesions).

Rearing S.exempta for Bioassays (adapted from Smith 1999)

The African armyworm has a typical Lepidopteran life cycle (Rose et al 2003) as illustrated in Fig 3 below.

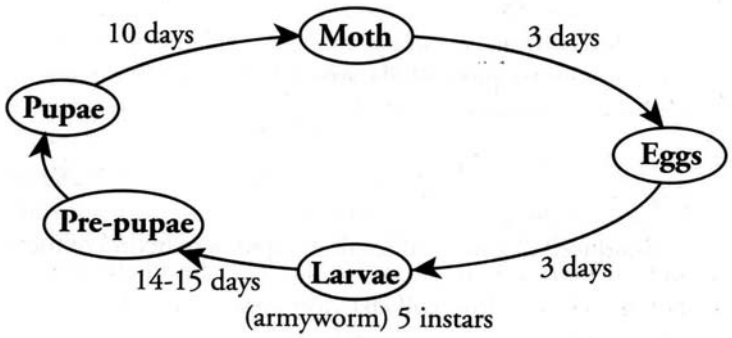


Fig 3 Life cycle of African armyworm when reared at a constant 25-26^{o} C

The larvae of armyworm in the gregarious form are velvety black are restlessly active and feed voraciously. The solitary form in contrast tends to be sluggish and is seldom seen in the wild.

Plate 15 Two larval forms Solitary green on left and gregarious black on right

Ideally larvae should be reared under controlled conditions and a constant temperature in the 24-27^{o} C is often recommended with a 12/12-hour light dark cycle. Adults are sensitive to flickering light so under artificial conditions any lights showing signs of failure should be removed immediately.

In the tropics armyworm can of course be reared under ambient conditions and simple shaded areas with sides clear to allow ventilation can be effective. If reared indoors it is important to

prevent high daytime temperatures as these can stress larvae and adults. This can be very important if rearing for virus bioassays, as stressed larvae are highly susceptible to infections and not suitable for bioassaying.

Moths

The adult moths are best held in cylindrical breeding cages, 30 cm high and 23 cm in diameter, the bases of which are lined with 24 cm diameter filter paper. Two strips of bonded viscose tissue ('nappy liner') or filter paper are taped to the sides of the cage to provide a surface on which emerging moths can cling while their wings expand and dry, and to provide a site for egg laying or oviposition.

Plate 16 Adult moths of *S.exempta*

Two or three small pots of dilute honey or sugar solution (2% honey or sucrose in distilled water), are provided as food and replaced daily. Each pot is filled with a small amount of cotton wool to prevent the moths from crawling into it and drowning. Approximately 15-20 pairs of moths are held in each cage. The colony can he maintained at a relatively low level by setting tip two cages each week, but this can be varied to meet production requirements. Under the stated temperature regime, the adults emerge about 10 days after pupation.

The sexes can be differentiated by the colour and pattern of the forewings. The females are a uniform dark brown whereas the males are grey-brown with lighter patches. The female has a

tuft of black hair-scales at the tip of the abdomen which is visible with the naked eye (Rose et al 2002).Young adults can be particularly active. In order to avoid losing any of them, the lid of the cage should be lifted very carefully when new food pots are added and liners are attached to the sides (see Appendix 2, Daily tasks).

Eggs

1. Egg laying begins 3 days after mating and continues for 5-6 days; peak laying occurs on the third or fourth night. The eggs are laid on the nappy liners in tightly packed batches of 100-400 eggs. They are covered by the black hair-scales from the tip of the female's abdomen. Later batches are smaller, more scattered around the liner and less well covered. Females can lay up to 1000 eggs over 5-6 nights.

2. Each day, the nappy liners are removed from the cages, the egg masses cut out and the number of batches recorded. The eggs are then placed in an 250 ml plastic pot, labeled with the day and date, and placed in an incubator at 24-26 ^{o}C. Under these conditions, the eggs hatch after 3 days.

3. The egg masses will need to be sterilized by soaking them, while still attached to the liners, in a 0.2 % solution of sodium hypochlorite (commercial bleach will do) for 5 minutes. The bleach solution is drawn off using a funnel and the eggs rinsed thoroughly in distilled water. The liners should be left to dry before being placed in 8 ox pots and stored in an incubator at 25 ^{o}C.

Larvae

Newly hatched

1. The newly hatched larvae are transferred to clean 200 ml plastic pots each containing cut fresh wheat or maize seedlings.

2. Each day, 40-50 larvae are gently tapped from the nappy liners on their silken threads into each of two or three pots. A sable (fine) paint brush can be used to assist this process.

3. The pots are labelled with the date of hatching and the date on which they need to be replaced. Ventilation holes are made in the pots using a suitable implement (e.g. a pill or needle).

Older larvae

The rearing of the larvae from approximately 6 days old (3rd instar onwards) is the crucial stage as this is when the risk of disease is at its highest. It is particularly important to maintain the strictest standards of hygiene.

Fresh maize is used to rear the larvae but an artificial diet combined with fresh, good-quality wheat seedlings (approximately 200 cm tall and 2 weeks old) has proved to be the successful.

Five days after hatching the larvae, mostly at 3rd instar, are transferred to a larger container a

sandwich box being commonly used.

Approximately 16-20 larvae are removed from the two 250 ml pots (8- 10 from each), using forceps sterilized in 0.5% sodium hypochlorite solution/bleach (or 1% Virkon disinfectant), and placed in a plastic sandwich box (23 x 12 x 6 cm) lined with a 24 cm filter paper. Fresh wheat seedlings or cut maize is added to the box. Fresh food is provided daily, the quantity increasing as the larvae grow. The boxes are labelled with the date of hatching, the date of transfer and the number of larvae transferred. As the larvae are reared in groups, they turn velvety black on top with pale lines on each side and a pale greenish-yellow underside (Rose et al 2002).

When they reach 10 days old, the larvae need to be placed on vermiculite for pupation. The filter paper and larvae are lifted gently out of the box and vermiculite is added to a depth of about 1 cm. A semi- circle of filter paper is placed on top of the vermiculite. Fresh wheat seedlings are added and the larvae counted back into the box. A note is made on the label of the day and date the vermiculite was added and the number of larvae transferred.

Cleanliness is still extremely important in order to maintain the health of the colony. Prior to the pre-pupal stage, the boxes containing the larvae need to be cleaned out daily to prevent the build-up of debris and the formation of moisture and mould. The soiled paper and larvae are lifted out of the box and a clean semi-circle of paper placed on the vermiculite. The diet is replaced and fresh wheat seedlings are added. The larvae are put back into the box using sterilized forceps as before.

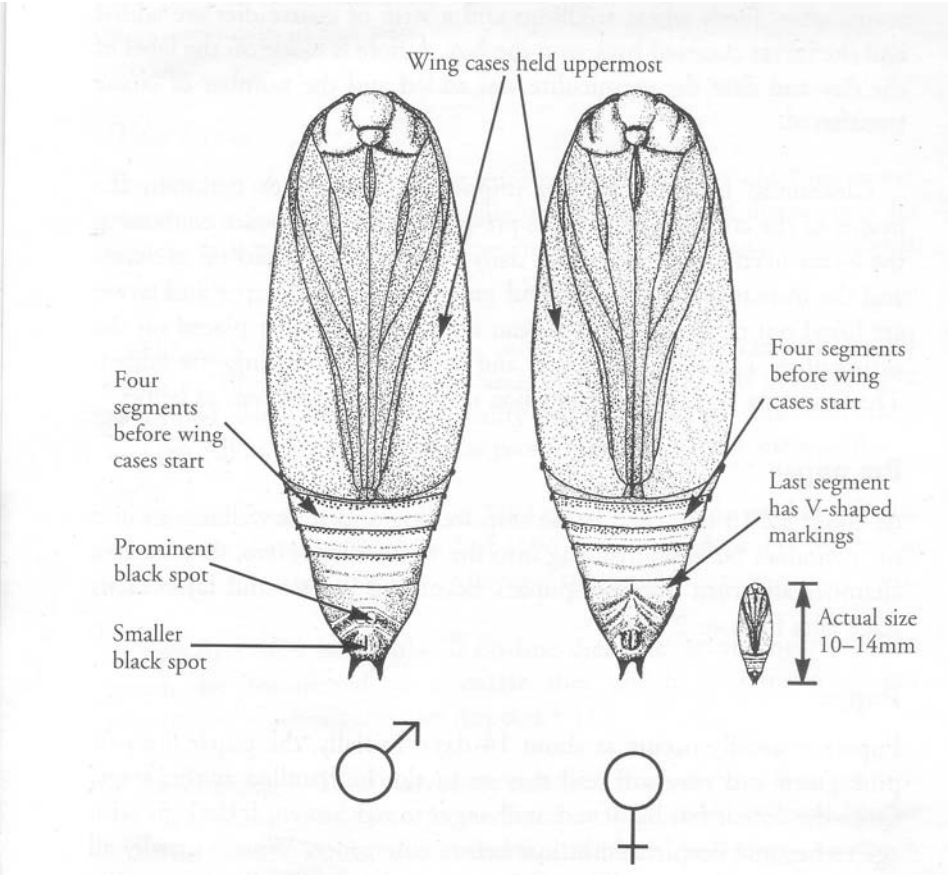
Pre-Pupae

At about 12-13 days, the larvae stop feeding and start walking around the container before burrowing into the vermiculite. Here, they make a chamber and turn into ‘pre-pupae’, becoming shorter and fatter. This stage lasts for 1 or 2 days.

Pupation usually occurs at about 14 days. Initially, the pupae are pale pink-green and very soft and they must not be handled at this stage. Once the cuticle has hardened, it changes to red-brown. It darkens with age to become deep chestnut just before emergence. When virtually all the larvae in the container have pupated, the vermiculite is carefully sifted and the pupae removed. The number taken from each box should be recorded so that percentage pupation can be calculated. Under good conditions, this should be above 80%. If it drops to about 50%, the rearing routine must be examined. Any deformed pupae should be discarded.

New cages should be set up twice weekly. It is advisable to place the pupae removed over 3-4 days together, i.e., Monday’s (including Saturday’s and Sunday’s) and Tuesday’s pupae, and pupae removed from Wednesday to Friday, are kept together. The pupae can be sexed by observing the ventral side. ‘When held with the wing cases uppermost, the last abdominal segments show the distinguishing features (see Figure 4).

Figure 4 Distinguishing marks of male and female *S. exempta* pupae



Between 15 and 20 pairs of pupae are placed on damp vermiculite (moistened with distilled water) in 250 ml pots (without lids) and each pot is placed in a cylindrical plastic breeding cage (described above). Each cage is labelled with the day and date it was set up and the number of pairs of pupae it contains.
The cycle begins again when the adult moths start to emerge after 9 or 10 days.

Hygiene and disease control

Strict cleanliness in the breeding area is essential. Working surfaces should be regularly washed with 2-5% sodium hypochlorite solution. Forceps should he left in a 0.5% bleach solution or 1% Virkon disinfectant.

The three pathogens most likely to cause problems are:

- viruses - NPV
- microsporidian Protozoa - *Nosema* species
- fungi and bacteria.

The entry of viruses and Microsporidia can be prevented by cautious introduction of new larvae or pupae from the field. The entry of fungi and bacteria usually results from poor hygiene, poor diet preparation, and too high a humidity during the larval stages. Control of all three can be achieved through strict hygiene.

Pots and sandwich boxes containing diseased insects must not be opened but must be deep—frozen for at least 12 h. The contents can then be discarded, ideally wearing disposable gloves, and the boxes left soaking in 1% Bleach or Virkon for 10 minutes before rinsing. It is useful to mark those boxes that contained diseased larvae so that when they are re-used the

development of the larvae can be checked carefully; if they remain healthy, the container can be classified as “clean”. All other containers and paintbrushes, etc. must be similarly treated with Bleach or Virkon.

Spodoptera exempta Nucleopolyhedrovirus (SpexNPV)

SpexNPV infection is most evident during the larval stage. Death can occur with in a few hours with the body contents rapidly liquefying. Infection can be recognized by dark stains on the filter paper, indicating that a larva has liquefied, or the appearance of “hanging limp bodies”. Dead larvae are often eaten by larvae that may be infected but are still actively feeding, so a decrease in the number of larvae per box can also indicate the presence of disease. Routine sterilization of eggs, pupae, equipment and re-usable containers should help to ensure a virus-free colony. Virus infection is difficult to eradicate, but if it is not dealt with rapidly, the entire culture will be lost.

Microsporidia

Infection of a culture by *Nosema* spp. can be more of a problem because it can exist unnoticed for several generations. The parasite is unaffected by the surface sterilization of eggs and pupae because it is contained within the insect and transmitted trans-ovarially. The early symptoms of disease, which can be mistaken for inbreeding depression, include increased development times and reduced fecundity and fertility over several generations. Severely affected larvae stop feeding, lose weight and appear shrivelled; they remain motionless and eventually die when their body contents liquefy. Good hygiene and selection of only the healthiest insects should help to reduce the incidence of *Nosema*.

Fungi and bacteria

Fungal and bacterial infections generally result from contamination of the artificial diets. They can be reduced by good hygienic practices during the preparation of the artificial media, and by the addition of an antibiotic to the diet ingredients such as methyl-4-hydroxybenzoate (see Appendix I). Condensation in the larval containers, caused by temperature fluctuations or high humidity in the rearing rooms, will increase the chances of infection.

Allergen containment

Staff who work with insect cultures over a long period may develop skin and/or respiratory allergic reactions. Allergy to locusts is particularly well known, but the large quantities of hairs and scales shed by moths may present some risk under prolonged exposure. Simple protective dust masks should be worn when handling the adult moths. Scales should he removed from the rearing room by the use of a vacuum cleaner, and surfaces should be wiped down with a damp cloth. An exhausting fan would also help reducing the amount of moth scales in the laboratory air. A laboratory coat and hair-covering cap worn only within the insectary area should help reduce the risk of dust being transferred to other parts of the building.

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Appendix 1 Alternative bioassay protocols

SURFACE DOSE BIOASSAY TO DETERMINE LC_{50} IN 1ST INSTAR LARVAE

In this technique, artificial diet is used to overcome some of the criticisms of leaf dip assays. Hot artificial diet is dispensed into a small 25ml pot or tissue culture well, which is allowed to cool and solidify. If a special low solid content agar diet is used a completely flat diet surface is formed and on to this can be dispensed a fixed amount, (100-200µl), of a known concentration test agent, which is then swirled or spread over the surface to form a continuous layer. When this has dried, 10 newly hatched larvae can be placed in the pot to feed and left in the pot for 5 or 7 days, and the mortality assessed. As newly hatched larvae are sensitive to handling stress it is common practice to check mortality in all pots after 24 hours, and for the presence of NPV, which will not have any effect in this time. Those dead can then be deducted from the mortality estimation. This assay has the advantage that it is easy to set up and many treatments can be tested by a single worker in a day. Also, many pots can be prepared in advance, without virus, and stored in the refrigerator for up to a week for use when required.

Procedure

1. Using a stock suspension of $1x10^8$ polyhedra per ml, make six 5-fold serial dilutions in the following way:

    - Transfer 4ml of 1% brilliant blue in distilled water into each of six bottles, capable of holding 5ml or more.
    - Add 1ml of the stock suspension to the first bottle containing brilliant blue solution, and mix thoroughly.
    - Using a clean pipette, add 1ml of this first dilution to the second bottle, mix thoroughly.
    - Repeat this procedure for the remaining bottles.
    - Label each bottle with the dilution it contains.
    - Check the dilution concentrations by doing microscope counts.

2. For each dilution set out an artificial diet pot, ensure the diet surface of each pot is completely smooth and that no diet is left on the side of the pot.

3. Mix the first dilution well and remove 50µl of suspension. Add this to the first pot of the set of six artificial diet pots, ensure the whole diet surface is covered by the suspension, i.e. all of it is blue. Repeat this for the other five pots.

4. Place five larvae in each pot, replace the lid and label the pot with the dilution.

5. Repeat this procedure for each of the other dilutions in the series.

6. Keep the insects in these pots for 24 hours, if possible in a temperature controlled environment.

7. After 24 hours, transfer the larvae singly from the dosed pots to fresh pots containing un-dosed artificial diet. Label each pot with the appropriate dilution and return them to a temperature controlled environment.

8. Record the mortality at regular intervals until the seventh day.

DROPLET BIOASSAY

Another excellent, very quick assay for newly hatched larvae is the droplet assay (Plate ??). In this NPV concentrations are made up in brilliant blue dye (1%). Drops of these concentrations are then placed on parafilm and neonate larvae are released near them. Larvae coming into contact with the drops will drink from them and those that have done so, and therefore taken a dose of virus, can be recognised by their blue colour. After 30 minutes or so these blue larva are removed and placed at a rate of 10 per 25ml pot containing artificial diet. The assay relies on the finding, confirmed by radio-isotope and fluorescent tracer studies, that newly hatched larvae are very consistent in the amount they drink.

This is a very good, easily set up assay, for those species in which the larvae drink and using this it is possible to do more samples in a day than with any other assay. However, not all lepidopteran larvae drink and a number of important pest species including *H. armigera* appear not to drink and for such species this assay cannot be used.

The method below has been adapted from a technique described by Hughes & Wood (1981). The droplet bioassay is a method used at NRI to assay NPV samples in first instar (neonate) larvae. From the assays lethal concentration (LC) data of the samples can be obtained.

Sample preparation

The concentration of the sample to be assayed must be known, therefore NPV counts of the top concentration of each dose series must be made using a haemocytometer. An appropriate dose series of 5 different concentrations of NPV suspended in 1% brilliant blue is used.

When dealing with NPV samples a 5-fold dilution series should be prepared for bioassay that will produce a dose response from approximately 90 to 10 percent with gradual reduction in mortality from the top dose to the lowest. The dose series required to produce a dose response is as follows:

2×10^7 PIB/ml
4×10^6 PIB/ml
8×10^5 PIB/ml
1.6×10^5 PIB/ml
3.2×10^4 PIB/ml

A total of 800µl of each dose is perfectly adequate to provide enough inoculum for the assay.

1. Calculate what volume of stock sample is required to deliver 2×10^7 OBi.e. if the stock suspension = 1.0×10^8 PIB/ml then 200µl is required.

2. Place out five Eppendorf tubes in a rack and label them 1 to 5.

3. Treat the stock sample in an ultrasonic bath for 1 minute, then whirlimix for 30 seconds. To *tube 1* deliver the calculated volume of stock sample (for the example here it would be 200µl). Make the volume in *tube 1* up to 500µl with distilled water.

4. To the remaining 4 tubes deliver 400µl of distilled water.

5. Take *tube 1*, close the lid tightly and mix thoroughly for 30 seconds using a whirlimixer.

6. From *tube 1* remove 100µl and deliver it in to *tube 2*. Whirlimix this suspension and remove 100µl delivering it to *tube 3*. Repeat this for all tubes.

7. *Tube 5* should be treated the same way but discard the 100µl of suspension removed from it.

8. All tubes should now contain 400µl of suspension that is double the concentration of the final dose required. To each tube add 400µl of 2% brilliant blue. Each tube now contains 800µl of suspension in 1% brilliant blue and is at the concentration required.

9. The dose series is now complete, creating 5 treatments. For precision, three counts must be made of the top concentration. The tubes are then labelled with the respective concentrations obtained from the counts of the top dose.

Administration of dose

In a droplet bioassay 30 to 50 neonate larvae are dosed per treatment. The treatments (doses) are presented to the larvae in liquid form. The larvae will dose themselves by drinking some of the suspension. Dosed larvae can be detected by their blue colour, hence the use of the brilliant blue (Plate 21). A control is always used in any bioassay and in this type of bioassay it is 1% brilliant blue (BB). If several samples are to be bioassayed over a period of time a standard dose series is also used. This will account for variation in response of the colony of insects and facilitates the calculation of an average dose-response.

Plate 2 Droplet dosed first instar larvae (blue stomachs)

1. Calculate how many diet filled pots are required. Ten larvae can be placed per pot, with 3 to 5 pots per treatment.

2. Place the pots onto the trays to allow any condensation present to evaporate.

3. Cut a strip of parafilm and place it on the bench. Take the control solution (1% BB) and deliver five, 5µl drops of it on to the parafilm in a ring formation. The ring of drops must have a radius smaller than that of the rim of a 30g plastic pot.

4. Take a sterile paint brush and collect a group of approximately 100 neonate larvae from the tub. Place these in the centre of the ring of drops. Then place an empty 30g pot over the top to prevent larvae from escaping from the ring of drops.

5. Take the standard set of dose series and treat in the ultrasonic bath for 1 minute. Whirlimix the lowest concentration for 30 seconds. Deliver five, 5µl drops of it onto a separate sheet of parafilm in the same manner as above. Repeat this procedure for the remaining doses of the series.

6. Place 100 neonate larvae in to the centre of each ring of drops and cover them with a 30g pot.

7. Take the dose series of your sample and treat it in exactly the same way as the standard.

8. When all the dose series have been placed out they must be left for 30 minutes to allow a sufficient number of larvae to dose themselves.

9. During this time set up a beaker full of 1% Virkon (commercial disinfectant) or 0.1% bleach and three beakers full of distilled water. Place the paint brushes in the Virkon.

10. After 15 minutes remove the brushes and rinse them in the distilled water.

11. When sufficient larvae have been dosed, take a paint brush and remove 30 or 50 dosed larvae from the control. Place 10 larvae in each diet filled pot (Plate 22).

Plate 3 Collecting dosed larvae with an artist's paintbrush.

12. When the required number of larvae has been collected place a piece of filter paper over the top of the pot and snap the lid on. Label the pots appropriately.

13. Repeat this procedure for all of the dose series.

14. Fill in an experiment sheet.

15. If a large number of dose series are to be assayed then it is desirable to place out dose series, two at a time. This prevents larvae from being exposed to the dose for too long.

16. Mortality is recorded on day 1, 5 and 7. The results obtained are then fed into a computer programme (MLP). The programme carries out probit analysis of the results and provides the LC_{50} and LC_{95} of the samples. The programme also provides other information such as the slope.

Artificial diet for polypots

2. This is a diet suitable for neonate insects used in bioassays. It contains no added vitamins or minerals, is thin and easily pourable.

2. The quantities of ingredients listed below form a stock of ready prepared dry material from which quantities can be taken to prepare polypots for bioassays.

3. The following quantities should be weighed out and then mixed thoroughly to provide base dry-material:

    300g ground wheat germ
    270g dried yeast
    50g table sugar
    50g casein

4. When preparing polypots for an assay, 50g of the ready prepared material will provide enough for 200 polypots. For every 50g of material 2.5g of methyl-4-hydroxybenzoate must be added.

PREPARATION OF 200 POLYPOTS

2. Prepare the following ingredients: 50g dry material (see above), 2.5g Methyl Paraben, 750ml distilled water, 8.4g agar

3. Measure out the water and mix the agar with it. Dissolve the agar fully by boiling the mix.

4. Once boiled add the dry material and Methyl Paraben and mix thoroughly with an electric mixer. Bring back to the boil and simmer for 45 minutes.

5. When cooked dispense the mixture into the polypots while it is hot (Plate 23). Carefully fill the pots to a depth of approximately 4mm. Allow the diet to set then use as required.

6. Any polypots not used in the assay can be stored in the refrigerator for later use. Storage can be up to 2 weeks.

DIET PLUG BIOASSAY TO DETERMINE LD_{50}

The nearest assay to the true LD_{50} assay used with insects is the plug or leaf disc-dosing assay. In this technique plugs of artificial diet or discs of leaf or food tuber are cut and measured doses of agent are put on each disc or plug. These are then given to larvae to feed on for a specific time period, usually overnight or 24 hours. All those larvae that have consumed the disc/plug, and therefore the dose, are then transferred to plain diet and monitored. Because cutting very small discs and keeping them from drying out is difficult, this assay is normally used with the older larvae of third instar or later. It is also relatively time consuming and is therefore only used where obtaining exact LD_{50}s is valuable. Its advantage is the high certainty that as the entire dose is taken in over 24 hours, it gives a true indication of the LD_{50}. Its disadvantage is that it is very time consuming and so is unsuitable for experimental use or routine monitoring on any scale where many samples need to be assessed.

Procedure

1. Prepare serial dilutions from the stock suspension in the following way:

a. take six screw-capped bottles and add 4ml of distilled water to each;
b. take 1ml of the stock suspension, add it to the first bottle and shake thoroughly;
c. with a clean pipette take 1ml of the first dilution and add it to the second bottle, shake thoroughly;
d. repeat this process for the remaining bottles;
e. label bottles with the appropriate dilution; and check dilution concentrations.

2. Cut 30 plugs of artificial diet, using a 5mm-cork borer, and place them on a sheet of aluminium foil or parafilm. Repeat for each dilution. The plug must be of a size that will be consumed entirely by a single test larva in a 24-hour period.

3. To each plug add a fixed volume of dilution (usually 1-10µl) and allow it to soak in completely. The actual dose applied can be calculated.

4. Treat an additional 30 plugs with distilled water alone to act as controls.

5. With as little handling as possible, place the treated plugs into microtubes or small vials, and add a single 3rd instar larva to each. Close the microtube lids and pierce a ventilation hole into the lids, or plug the vials with cotton wool. Label each tube/vial with the appropriate dilution.

6. If possible transfer the containers to a constant temperature environment or incubator (25-27°C is ideal).

7. After 24 hours, transfer the larvae singly from the treated plugs to small bottles or pots containing clean artificial diet. Label the pots with the correct dilutions and return them to a constant temperature environment.

8. Measure the mortality at regular intervals until the seventh day at least but normally to pupation or moth emergence.

Appendix 2: Example Insect rearing schedule for Spodoptera exempta.

THIS SCHEDULE MUST BE FOLLOWED EXACTLY CONSULT BEFORE MAKING ANY CHANGES TO THE ROUTINE

This is a daily routine, allowances must be made for weekends when no staff are at work. .

1. Collect egg masses each day from the adult cages. The egg masses are collected by cutting out the paper on which the egg mass has been laid. All egg masses should be surface sterilised by washing in 0.5% bleach solution (5 ml bleach in 1000 ml water, do not use perfumed bleach); immerse egg masses in an beaker containing the bleach solution for 15 minutes. The eggs should then be washed thoroughly with distilled water and left to dry. Once dry the egg masses should be placed in an 8 oz pot labelled with the date of collection and left in the controlled temperature room for three days.

2. After three days four egg masses should be separated to provide insects for the rearing colony. Larvae from these egg masses will be reared on natural diet (maize or wheat seedlings).

3. The four separated egg masses should be placed on a tray of maize/wheat seedlings and left to hatch. Four other egg masses should be attached to the lids of an 8 oz plastic pots, one egg mass to each lid. The lids should then be placed onto pots containing cut seedlings and the pots should be left for the eggs to hatch.

4. Check the egg masses in pots on the fourth day after collection; they should have hatched. If hatched add new seedlings. If egg masses have not hatched by the fifth day after collection they should be discarded.

5. The density of larvae that have hatched should be assessed and the numbers adjusted so that there are approximately 50-100 larvae per pot The pots can then be left in the CT room for a further 6 days, when the larvae will be seven days old (III instar). Check and add fresh seedlings daily.

6. The rearing colony larvae should be allowed to feed en-masse on the tef/wheat seedlings for five days. After this time they must be transferred to individual polypots. Each day 100 five day old larvae should be transferred individually to polypots. The polypots should be placed on a plastic tray labelled with the date that the larvae hatched. The remaining larvae can be discarded. Larvae in polypots should be fed with wheat seedlings which should be replaced daily. The larvae should remain in the polypots until pupation.

7. Polypots containing larvae that are 20 days old or more should be checked on a daily basis for pupation. All pupae should be removed from the polypots and placed together with those collected on previous days.

8. Every Monday the pupae that have been collected during the previous week should be sexed and placed in pots (one for the males and one for the females) containing 2% bleach solution (20 ml bleach in 1000 ml water). The pupae should be left in the bleach solution for 10 minutes after which they are removed and left to dry on a paper towel.

9. Once the pupae are dry they should be placed into adult cages. Place 20 female and 20 male pupae on the surface of sterilised damp sand placed in a plastic pot. This should be then

placed in the adult cage. Ten cages should be prepared in this way. Any remaining pupae can be discarded.

10. Check the cages on the following Monday and thereafter daily, if moths have emerged add a pot of adult food (see appendix 1 for details) and place egg papers around the sides of the pot containing the pupae and hanging down the sides of the cage (two strips). Papers on which eggs have been laid should be replaced with new strips and the eggs collected. The adult food must be changed daily.

11. The adult moths should be kept for 2 weeks after which they should be discarded and the cages cleaned.

12. All working surfaces should be washed daily with 2% bleach. Forceps and other equipment should be placed in 2% bleach for two hours then rinsed in water before use. Plastic pots and adult cages should be sterilised by immersing overnight in 2% bleach solution. Metal and glass equipment should be sterilised weekly by autoclaving or placing in an oven set at 160°C for 2 hours.

13. **STAFF WORKING IN THE INSECTARY SHOULD NEVER ENTER THE VIRUS PRODUCTION SUITE. EQUIPMENT ETC. FROM THE VIRUS SUITE SHOULD NEVER BE TAKEN INTO THE INSECTARY. ONLY STERILISED PRODUCTION TRAYS SHOULD ENTER THE DIET PREPARATION AREA. STAFF WORKING IN THE VIRUS PRODUCTION SUITE SHOULD NEVER ENTER THE INSECTARY OR THE DIET PREPARATION AREA**.

DAILY ROUTINE

1. Check adult cages, remove papers with egg masses and replace with new paper. Replace maize or wheat with fresh. Place new adult diet pots and egg papers in cages with newly emerged moths.

2. Cut out eggs and wash in 0.5% bleach. Separate four egg masses for breeding colony. Place egg masses in pots labelled with date of collection.

3. Place three day old egg masses on tef/wheat seedlings and over diet in pots.

4. Check egg masses that were placed on diet the previous day. If hatched label with date of hatch. Adjust numbers in pots to 50-100. Place unhatched egg masses with current days egg masses. Discard egg masses that are more than five days old (unless the room temperature has been less than 25°C for a long period).

5. Place 100 five day old larvae from the maize/wheat seedlings individually in polypots. Place on tray and label with date of hatch.

6. Feed all larvae in polypots with wheat seedlings.

7. Check all polypots with larvae aged 20 days or more and remove pupae.

8. Send to the virus production suite all 6 day old larvae that have been reared on artificial diet.

9. Clean benches and instruments.

WEEKLY ROUTINE

1. Every Monday prepare diet for production trays and rearing pots that will be required for the week. .

2. On Friday sex and wash all pupae. Prepare 10 cages of 40 pupae (20 male and 20 female). Label cages with the date they were prepared. Discard adult moths in cages prepared 2 weeks ago. Wash cages. Wash floors with 2% bleach.

Diet Preparation

Natural diet for larvae

Both wheat and maize can be used for rearing *S. exempta* larvae. Seeds must be untreated. They are grown under artificial light on cotton wool in plastic trays measuring 56 x 28 x 8.5cm. Wheat seedlings are used at two weeks old and tef seedlings at one week.

Cotton wool is laid out in trays allowing a border of 2.5 cm on all sides and then wetted thoroughly. The border prevents larvae escaping from trays. Wheat/maize seeds are sprinkled over the cotton wool and the tray is then covered with a perspex sheet to encourage germination. Water as necessary.

Before use seedlings should be surface sterilized by exposure to UV light overnight.

Diet for adult moths

Prepare a solution of 10% sucrose in distilled water (10gms sugar in 100ml water). Pour into polypots containing sterilised cotton wool. Place 2-3 pots in each large adult cage and replace daily. Prepare new sucrose solution every week and keep in the refrigerator.

DAILY SCHEDULE

MONDAY

1. Check all the adult cages and collect all egg masses which have been laid over the weekend. Replace oviposition paper as necessary. Wash eggs in 0.5% bleach then in tap water and allow them to dry.

2. Prepare new 10% sucrose solution for feeding adults and replace all old pots with fresh solution in each of the adult cages containing moths.

3. Place about 4 egg masses, or sufficient to give at least 100 larvae for your breeding colony, onto a tray of sterilised wheat clearly labelled with the date of oviposition and later, with the date of hatch.

4. Transfer 100 of your oldest group of larvae from the wheat in a tray to individual rearing pots. Place pots on clean trays which must all be clearly labelled with the date of hatch of those larvae. Discard any further larvae in that age group along with the wheat they were growing on.

5. Replace wheat in **all** the individual rearing pots regardless of its condition as it will be at least 60 hours old. You must use UV-sterilised wheat (sterilised last Thursday night).

6. Collect all pupae from the individual rearing pots and place them in a clearly labelled pot.

7. Wash and dry all pupae (in 2% bleach) that have been collected from the previous Friday, Saturday, Sunday and from today. Separate them according to sex.

8. Empty and clean the oldest group of adult cages (unless they are still producing lots of eggs). These will be two weeks old today.

9. Prepare new adult cages from the pupae you have just washed. Label each cage clearly with the date and number of pupae.

10. Water wheat in greenhouse and insectary.

11. At the end of the day place a tray of wheat under the UV light for use tomorrow. DO NOT EXPOSE YOUR EYES TO UV LIGHT FOR MORE THAN A COUPLE OF SECONDS.

DAILY SCHEDULE

TUESDAY

1. Check adult cages and collect all egg masses which have been laid over-night. Replace oviposition paper as necessary. Wash eggs in 0.5% bleach then in tap water and allow them to dry.

2. Replace all sucrose solution in adult cages. Check for emergence in the newer cages and place pots of sucrose solution if any adults have emerged.

3. Place about 4 egg masses, or sufficient to give at least 100 larvae for your breeding colony, onto a tray of sterilised wheat clearly labelled with the date of oviposition and later, with the date of emergence.

4. Pass all surplus egg masses to the virus labs in a pot labelled with the number of masses and the date of oviposition.

5. Transfer 100 of your oldest group of larvae (5 days old) from wheat in a tray to individual rearing pots. Place pots on clean trays which must all be clearly labelled with the date of emergence of those larvae. Discard any remaining larvae in that age group along with the wheat they were growing on.

6. Check all solitary larvae and replace wheat as necessary in all individual rearing pots. You must use UV-sterilised wheat.

7. Collect all new pupae from the individual rearing pots and place them in a clearly labelled pot.

8. Prepare artificial diet.

9. Water wheat in greenhouse and insectary if necessary.

10. At the end of the day place a tray of wheat under the UV light for use tomorrow. DO NOT EXPOSE YOUR EYES TO UV LIGHT FOR MORE THAN A COUPLE OF SECONDS.

DAILY SCHEDULE

WEDNESDAY

1. Collect all egg masses which have been laid over-night. Replace oviposition paper as necessary. Wash eggs in 0.5% bleach then in tap water and allow them to dry.

2. Replace all sucrose solution in adult cages. Check for emergence in the newer cages and place pots of sucrose solution if any adults have emerged.

3. Place about 4 egg masses, or sufficient to give at least 100 larvae for your breeding colony, onto a tray of sterilised wheat clearly labelled with the date of oviposition and later, with the date of emergence.

4. Pass all surplus egg masses to the virus labs in a pot labelled with the number of masses and the date of oviposition.

5. Transfer 100 of your oldest group of larvae (5 days old) from wheat in a tray to individual rearing pots. Place pots on clean trays which must all be clearly labelled with the date of emergence of those larvae. Discard any further larvae in that age group along with the wheat they were growing on.

6. Replace wheat in **all** individual rearing pots regardless of its condition. You must use UV-sterilised wheat.

7. Collect all new pupae from the individual rearing pots and place them in a clearly labelled pot with Tuesday's pupae.

8. Water wheat in greenhouse and insectary if necessary.

9. At the end of the day place a tray of wheat under the UV light for use tomorrow. DO NOT EXPOSE YOUR EYES TO UV LIGHT FOR MORE THAN A COUPLE OF SECONDS.

DAILY SCHEDULE

THURSDAY

1. Collect all egg masses which have been laid over-night. Replace oviposition paper as necessary. Wash eggs in 0.5% bleach then in tap water and allow them to dry.

2. Place about 4 egg masses, or sufficient to give at least 100 larvae for your breeding colony, onto a tray of sterilised wheat clearly labelled with the date of oviposition and later, with the date of emergence.

3. Replace all pots of sucrose solution in the adult cages. Check for emergence in the newer cages and place pots of sucrose solution if any adults have emerged.

4. Pass all surplus egg masses to the virus labs in a pot labelled with the number of masses and the date of oviposition.

5. Transfer 100 of your oldest group of larvae (5 days old) from wheat in a tray to individual rearing pots. Place pots on clean trays which must all be clearly labelled with the date of emergence of those larvae. Discard any further larvae in that age group along with the wheat they were growing on.

6. Check all solitary larvae and replace wheat as necessary in all individual rearing pots. You must use UV-sterilised wheat.

7. Collect all new pupae from the individual rearing pots and place them with Tuesday's and Wednesday's pupae.

8. Wash (in 2% bleach), dry then sex all pupae from Tuesday, Wednesday and today.

9. Empty and clean the oldest group of adult cages (unless they are still producing lots of eggs). These will be two weeks old today.

10. Prepare new adult cages from the pupae you have just washed. Label each cage clearly with the date and number of pupae.

11. Sow approximately 6 trays wheat and water the wheat in greenhouse and insectary if necessary.

12. At the end of the day place 2 trays of wheat under the UV light for use tomorrow and Monday. DO NOT EXPOSE YOUR EYES TO UV LIGHT FOR MORE THAN A COUPLE OF SECONDS.

DAILY SCHEDULE

FRIDAY

1. Collect all egg masses which have been laid over-night. Replace oviposition paper as necessary. Wash eggs in 0.5% bleach then in tap water and allow them to dry.

2. Replace all pots of sucrose solution in the adult cages. Place sucrose solution into any cages where adults are likely to emerge over the weekend.

3. Place about 4 egg masses, or sufficient to give at least 100 larvae for your breeding colony, onto a tray of sterilised wheat clearly labelled with the date of oviposition and later, with the date of emergence.

4. Pass all surplus egg masses to the virus labs in a pot labelled with the number of masses and the date of oviposition.

5. Transfer 100 of your oldest group of larvae (5 days old) from wheat in a tray to individual rearing pots. Place pots on clean trays which must all be clearly labelled with the date of emergence of those larvae. Discard any further larvae in that age group along with the wheat they were growing on.

6. Replace wheat in **all** individual rearing pots in preparation for the weekend. You must use UV-sterilised wheat.

7. Collect all new pupae from the individual rearing pots and place them in a clearly labelled pot.

8. Water wheat in greenhouse and in insectary.

9. Clean all surfaces and floor with bleach.

DAILY SCHEDULE

SATURDAY AND SUNDAY

1. Collect all egg masses which have been laid over-night. Replace oviposition paper as necessary. Wash eggs in 0.5% bleach then in tap water and allow them to dry.

2. Replace all pots of sucrose solution in the adult cages.

3. Place about 4 egg masses, or sufficient to give at least 100 larvae for your breeding colony, onto a tray of sterilised wheat clearly labelled with the date of oviposition and later, with the date of emergence.

4. Pass all surplus egg masses to the virus labs in a pot labelled with the number of masses and the date of oviposition.

5. Check larve in pots and add wheat to individuals requiring more food.

Virus Laboratory Technician Schedule

Initially, your main responsibility will be to maintain the cleanliness of the virus laboratories and office and the equipment. You will also be involved in rearing larvae from egg to 3rd instar on artificial diet (but this job is likely to transfer to the insectary later) and eventually you will be involved in all aspects of the process of virus production.

IT IS OF EXTREME IMPORTANCE THAT YOU DO NOT ENTER THE INSECTARY OR HANDLE MATERIAL TO BE USED IN THE INSECTARY. ALL POSSIBLE ACTION MUST BE TAKEN TO SAFEGUARD THE INSECT COLONY AGAINST CONTAMINATION.

Rules

1. Laboratory coats must be worn at all times while working in the labs. They must be removed before leaving the building.

2. Wear rubber gloves when handling bleach and when washing equipment in bleach.

3. Always wash your hands before leaving the building and especially before eating or drinking.

4. They must be no food or drink in the laboratories or stored in the fridge/freezer.

5. Always inform Head of unit if you are going to be absent so that arrangements can be made to cover for you.

Daily schedule

Place 1st instars onto diet in pots at approximately 50 larvae per pot. Any instruments used should be sterilised in 5% bleach then rinsed in water before and after use. Also wipe the laminar flow cupboard with 5% bleach before and after use.

Sterilise all dirty and contaminated plastic equipment (trays, pots, tubes) by washing in detergent then soaking overnight in 2% bleach. Drain and allow to dry naturally. Sterilized diet trays and pots should be sent to the insectary in a clean bag while still wet with bleach and allowed to dry there. This is the only occasion when materials can pass from the virus laboratories to the insectary. Ensure that you lab coat is removed and your hands clean. Do not enter the insectary yourself.

Sterilise dirty glassware and metal equipment/instruments by washing and then either by autoclaving (for 15 minutes after pressure is reached) or by heating at 160^0C for 2 hours in the oven.

At the end of every day sweep floors and empty bins. Wipe all bench surfaces in both laboratories with 2% bleach.

Weekly schedule

Lab coats must be changed every Monday. All dirty coats from the previous week must then be washed with bleach and detergent powder. Wash towels also.

On Mondays make new 2% bleach solution in the dustbin. Add 1 litre of bleach to a full load of water. Make new 5% bleach solution in the wash bottle and instrument pot. Use 12ml bleach in the wash bottle and 50ml bleach in the instrument pot and fill each with water.

On Fridays mop the floors in both laboratories and the office with 2% bleach and detergent.