

CONTENTS

CONTENTS	3	
INTRODUCTION		
1. ASEPTIC TECHNIQUE	7	
STERILISATION: DISINFECTION: Micro-biological growth media. Laboratory glassware Small pieces of equipment: Laboratory benches WHATEVER YOU DO.	7 7 7 7 7 7	
2. STERILIZATION TECHNIQUES		
Dry heat or oven sterilization Autoclave or pressure cooker Points to remember when using a pressure cooker or autoclave Using the pressure cooker Using a manual autoclave Using a manual autoclave Using an automatic autoclave:	8 8 9 9	
3. AGAR		
Tap Water Agar (TWA) Potato Carrot Agar (PCA) Antibiotic Solution Malt Extract Agar (MEA) Potato Dextrose Agar (PDA) Sabouraud Dextrose Agar (SDA) Ready-mixed Powdered Agar Products Pouring Agar Plates Preparing Slopes in Bottles	9 10 10 10 10 10 10 1	
4. SAFETY IN THE LABORATORY		
Responsibilities	11 11 11 11	
6. MICROSCOPY	19	
Microscopes Using a Microscope Making Microscope Smears	12 12	
7. MISCELLANEOUS TECHNIQUES	13	
Spore Counting Using an Improved Neubauer Haemacytometer Diluting formulated spores in oil Preparing the haemacytometer Counting the spores Calculating the concentration Calculating the concentration Obtaining the required concentration Germination Test	13 14 14 14 14 14	
8. BIOASSAY OF FUNGAL ENTOMOPATHOGENS	16	
Why do bioassays? Insect bioassay Inoculation Recording Grasshoppers - special points Analysis Bioassays against other insects	16 16 17 17 17	
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INTRODUCTION

This section describes a number of techniques which will be useful to insect pathologists working in the laboratory. Most of the techniques will be familiar to plant or medical pathologists. The reader is strongly advised to seek advice and assistance from pathologists trained in other fields.

The techniques described assume that the

pathologist has access to a reasonably well-equipped laboratory, including autoclave or pressure cooker, microscope, glassware, and chemicals. A list of essentail basic equipment and some suggested suppliers is given in Appendix IV.

For guidance on the identification of the most common fungi affecting insects, please see Section 2.

1. ASEPTIC TECHNIQUE

Aseptic technique is essential for all pathology work and must be thoroughly practiced and mastered.

STERILISATION:

Eliminates all viable microbes from a material.

DISINFECTION:

The removal of potentially infective microbes, but does not render the object sterile.

Cultures of insect pathogenic fungi or bacteria for insect pathology must be pure. This means that they must be free of any living microbes other than the one required. The presence of unwanted fungi or bacteria is known as CONTAMINATION and the microbes responsible are referred to as CONTAMINANTS.

We use aseptic technique so that we can handle, or manipulate microorganisms without introducing contaminants into the culture. Aseptic technique also protects the operator from potential infection from pathogenic organisms.

ALWAYS use aseptic technique when handling microorganisms and also when preparing micro-biological media in which to grow these organisms.

ALL equipment and media to be used during the handling of the microorganism must be sterile.

THERE IS NO SUCH THING AS MOSTLY STERILE

There are many different methods of sterilization. The method you use depends on the equipment you have and what it is you are sterilizing. As a general rule, the following methods are most appropriate.

MICRO-BIOLOGICAL GROWTH MEDIA

Wet heat sterilization, usually using an autoclave although a domestic pressure cooker will do just as well (see lab techniques).

1 Raise the temperature to 121° C and the pressure in the closed chamber to 15 psi for 15-20 minutes.

2 DO NOT over fill vessels containing liquid, leave a large space at the top of all bottles to allow for expansion and boiling of the liquid on heating in the autoclave.

3 Loosen screw caps before autoclaving.

LABORATORY GLASSWARE

Sterilize as above using wet heat sterilization or dry heat sterilization in an oven (see lab techniques) at 160°C for 1-2 hours. If you have no autoclave, pressure cooker or oven, you can use certain chemical agents such as strong acids or alkalines, phenols or ethylene oxide. All chemical methods are potentially hazardous to the operator and should be avoided where possible.

Methods for chemical sterilization can be found in the Plant Pathologists Pocketbook (see Appendix II). SMALL PIECES OF EQUIPMENT:

Sterilize glass rods and metal tools by dipping them in 70% ethanol (alcohol) and then flaming to burn off the alcohol.

Sterilize inoculating loops and needles by holding in a flame until red hot.

LABORATORY BENCHES

Swab the working surface with 70% alcohol or chemical disinfectant to prevent the introduction of contaminants.

NEVER allow anything which is sterile to come into DIRECT contact with the bench.

Other methods of sterilization are available.

1 Ultraviolet radiation: can be useful for benches and clean rooms.

2 Gamma radiation: is used for the sterilization of plastics in industry.

3 Filtration: using filters of a maximum pore size of 1nm, generally used to sterilize small quantities of liquids which are unstable at high temperatures.

However these methods are not often used in insect pathology.

USE ASEPTIC TECHNIQUE DURING ALL MICROBIAL TRANSFERS

Make sure that ALL equipment used is properly sterilized using the most appropriate method.

WHATEVER YOU DO

Use the flow bench only for inoculations and sterile work.

Do not use the flow bench for general laboratory procedures.

Always clean out the flow bench before and after use.

Keep the bench clear! The outsides of bottles and bags are sources of contamination!

Always make sure that there is a free passage of air between the air filter and your work. Objects placed in front of the filter will contaminate the air which passes over them.

Always sterilize inoculating loops and the necks of bottles before and after each procedure using a flame.

Remember in sterile procedure: EVERYTHING you use MUST be sterile.

Autoclave everything yourself!

Always autoclave 1 ml pipette tips before you use them.

REMEMBER Your hands are not sterile. The bench is not sterile. The outsides of bottles and bags are not sterile.

YOU ARE ONE BIG CONTAMINANT

Relax ! sterile technique takes time. Do not try to hurry !

SHORT CUTS = CONTAMINATION

2. STERILIZATION TECHNIQUES

Metal

Sterilize needles, wire loops etc. by heating them in a flame until red hot.

Glassware

Can be sterilized in a hot air oven, a domestic oven will do. Do not pack glassware too tightly.

Media

Wet heat sterilize in an autoclave or pressure cooker for forty (40) minutes.

DRY HEAT OR OVEN STERILIZATION

Using the oven method of sterilization, glassware will be sterile as follows:

Oven temperatures and time for sterilization

- 120°C 8 hours
- 140°C 3 hours
- 160°C 1 hour
- 180°C 20 min.

AUTOCLAVE OR PRESSURE COOKER

You can use an autoclave or pressure cooker at 15 psi for 15 minutes, this is the standard recommended for most microbiological media. Under certain circumstances, you may be required to alter the temperature/pressure for sterilisation. The table below gives the temperatures which will be achieved at various pressures in pounds per square inch (psi), the time required for sterilisation should be stated in the instructions given:

Autoclave pressures and temperatures:

- 5 psi 107°C
- 7 psi 110°C
- 10 psi 115°C
- 15 psi 121°C
- 20 psi 126°C

POINTS TO REMEMBER WHEN USING A PRESSURE COOKER OR AUTOCLAVE

Use distilled water (if available) in pressure cookers and autoclaves.

Ensure that all bottles and containers are heat-proof.

Never fill bottles and containers to the top, always leave a gap between the liquid and the lid to allow the liquid to expand and boil. Loosen the caps of all bottles and containers before placing in a pressure cooker or autoclave, this allows the steam to enter the containers and sterilise the contents.

Always read the manufacturers instructions before using a pressure cooker or autoclave (the instructions for using pressure cookers and autoclaves below are only meant as guidelines).

USING THE PRESSURE COOKER

1 Put a little water in the bottom of the pressure cooker.



2 Put the media in heat proof containers or in sterilisable plastic bags.

3 Put the containers in the pressure cooker on the trivet* and make sure that the lids of bottles

are loose.

4 Close the pressure cooker but do not put the weight on top on the steam valve. Put the pressure cooker on the heat.

5 Once the water in the pan starts to boil, steam will come out of the open valve. Allow steam to pass out of the valve for about 5 minutes, then put the weight on top of the valve.

6 The steam builds up in the inside of the pressure cooker until it reaches the correct pressure. The steam then lifts the weight and starts to escape. The weight acts to regulate the pressure inside the pan.

7 As soon as the steam starts to escape, time the sterilization from the point at which the steam starts to escape. Turn down the heat during sterilization so that the steam is only just escaping, NOT rushing out.

8 Turn off the heat after the recommended sterilisation time.

9 Leave to cool before removing the weight (or opening the valve).

10 Wait until pressure is completely reduced then lift the weight off the valve. Any remaining steam will escape and the pressure cooker is then safe to open.

N.B. Never open a pressure cooker or autoclave until the valve has been opened to release the pressure.

USING A MANUAL AUTOCLAVE

- 1 Put in sufficient water.
- 2 Load the articles to be sterilized into the autoclave.
- 3 Screw down the lid.
- 4 Open the steam valve.

5 Switch on. If there are high and low switches on the autoclave make sure both_are switched on.

6 Let steam come out for at least five (5) minutes before closing steam valve. Continue heating until the pressure is up to 15 psi.

7 Adjust pressure and turn the heat down or the high switch off.

8 Leave to steam for the appropriate time then turn off the autoclave.

9 Leave to cool to reduce the pressure to zero.

10 Open the steam valve to release any remaining pressure.

11 Wait five (5) minutes before opening the lid.

USING AN AUTOMATIC AUTOCLAVE:



1 Open the autoclave and fill with water to the right level.

2 Set the timer and switch on the heat.

3 Set the "power" switch to the "off" position as soon as the alarm sounds, or light flashes.

 $4\,$ Wait until the pressure falls and the temperature reaches 80°C or less.

5 Open the autoclave.

3. AGAR



Agar is used to solidify nutrient media for growing bacteria and fungi, as it becomes liquid at 100°C and sets at 40°C. Agar can be obtained either as pure agar powder for adding to nutrient solutions which are prepared in the

laboratory or ready mixed as a nutritive substrate in powder form with nutrients added according to specific recipes.

Sabouraud dextrose agar (SDA) and Malt extract agar (MEA) can be bought ready prepared or can easily be made up in the laboratory using pure agar powder plus the raw ingredients.

N.B. Ready mixed agar products are generally more expensive than purchasing the pure agar powder and the media components separately.

Agar powder will only dissolve in boiling water, once dissolved, the solution will remain liquid until it has cooled to 40° C. It will then solidify into a firm gel.

If required, agar can be reheated by steaming or autoclaving and will become liquid again at 100°C.

Agar can be used in Petri dishes (plates) or in bottles (slopes). If agar is to be used in Petri dishes, it should be sterilised in a large bottle/bottles and distributed after sterilisation.

=If it is to be used for making agar slopes, it should be distributed into the small bottles once it has dissolved and sterilsed in the individual bottles.

Several different recipes based on agar are given below:

TAP WATER AGAR (TWA)

1 Put 15 g pure agar and one (1) litre of tap water into a conical flask.

- 2 Boil the agar in the water until dissolved.
- 3 Sterilize at 15 psi for 20 minutes.

POTATO CARROT AGAR (PCA)

This is a very weak medium used for storing cultures and can be made with antibiotic solution added for making isolations from insects.

1 Grate 20 g potato and 20 g carrot (wash the potatoes and carrots well before grating).

2 Boil grated potato and carrot in one (1) litre tap water for one (1) hour.

- 3 Strain through a fine sieve DO NOT press.
- 4 Add 20 g agar to the strained liquid.

5 Heat the solution until the agar is dissolved, then add water to make up to one (1) litre.

6 Sterilize at 15 psi for 15 minutes.

N.B. Antibiotic solution may be added to PCA for use in isolations from insects or cleaning up cultures after bacterial contamination.

ANTIBIOTIC SOLUTION

1 Weigh out 0.05 g of Chloramphenicol powder into a clean, dry Universal bottle.

2 Add 10 ml of 90 - 95% alcohol (ethanol). Do not sterilise this solution.

3 Add antibiotic solution to the sterilised agar at the rate of 1ml in 100 ml agar medium (i.e. 10 ml in 1 litre).

4 Gently invert the bottle several times to distribute the solution evenly throughout the medium, but don't shake the bottle as this will create air bubbles.

5 Now autoclave the medium again for just 10 min at 10 psi and 115°C (this can be done in a pressure cooker using the small part of the weight only) to ensure total sterility.

N.B. Wear gloves at all times when handling concentrated antibiotics. Chloramphenicol is <u>poisonous.</u>

MALT EXTRACT AGAR (MEA)

1 Boil 20 g malt extract in one (1) litre water until dissolved.

2 Add 20 g agar.

3 Boil until agar is dissolved.

4 Sterilize at 15 psi for 15 minutes.

POTATO DEXTROSE AGAR (PDA)

You will need:

Potato	200g
Dextrose	20 g
Agar	20 g
Tap water	1 l

1 Take 200g potatoes.

2 Scrub the potatoes clean - DO NOT PEEL.

3 Cut into 12 mm cubes.

4 Weigh out 200g potatoes.

5 Rinse rapidly in running water.

6 Place in one (1) litre water.

7 Boil until soft (1 hour).

8 Mash and squeeze as much of the pulp as possible through a fine sieve.

9 Add 20 g agar and boil till dissolved.

10 Add 20 g dextrose and stir till dissolved.

11 Make up to one (1) litre with water.

12 Sterilize at 15 psi for 20 minutes.

SABOURAUD DEXTROSE AGAR (SDA)

You will need:

D-glucose (Dextrose or Maltose)	200 g
Peptone	20 g
Agar	20 g
Tap water	1 l

1 Dissolve the D-glucose, peptone and agar in the tap water by boiling them together.

2 Autoclave at 115 psi for 15-20 minutes.

READY-MIXED POWDERED AGAR PRODUCTS

Follow the instructions given by the manufacturer.

POURING AGAR PLATES

1 Autoclave the agar (to melt and sterilize). Cool the agar until hand hot.

2 Lay out newly opened plastic Petri dishes OR sterile glass Petri dishes in a clean area.

3 Pour the agar into the dishes to a depth of 0.5 cm (approximately 15 ml in a 9 cm Petri plate)

4 Allow to cool uncovered in a sterile air cabinet, keeping the lids within the sterile air flow (do not touch the inside surface of the lids as this will cause contamination).

N.B. If you do not have a sterile air flow cabinet, replace the lid of each Petri plate immediately after adding the agar and allow the agar to set. Once cool, any condensation which has collected on the lids of the agar plates can be removed by taking the lid and giving it a short sharp shake. Replace the lids immediately.

4 Cover with lids.

5 If not needed immediately, store in the refrigerator (5°C) for several weeks.

PREPARING SLOPES IN BOTTLES

Use 25-30 ml Universal bottles; use SDA for *Metarhizium* and PDA, SDA or MEA for *Beauveria*

1 Prepare agar.

2 Put approximately 7 ml of agar in each Universal bottle.

3 Place the bottles in the autoclave with lids loose and autoclave as instructed in the agar recipe.

4 Tilt the bottles so that the agar forms a slope inside the bottle and let them cool.

5 Tighten the lids and store in a refrigerator until use.

For small scale production of spores use 300ml medical flats or 700 ml square sided whisky bottle.

1 Prepare agar.

2 Put 100 ml of agar in each 700 ml bottle and 40ml agar in each 300 ml bottle.

3 Autoclave the bottles (with lids loose) as instructed in the agar recipe.

4 Tilt the bottles and let them cool.

5 Tighten the lids and store in a refrigerator until use

4. SAFETY IN THE LABORATORY

NO SMOKING

NO EATING NO DRINKING

GOOD WASHING FACILITIES GOOD DISPOSAL FACILITIES

RESPONSIBILITIES

The laboratory supervisor/manager must ensure that:

- 1 The laboratory is a safe working environment.
- 2 Equipment is safe.
- 3 All technicians are aware of the dangers.

4 All technicians are properly trained to do their work.

5 Be responsible for chemicals.

Technicians are responsible for:

- 1 Keeping benches clean and uncluttered.
- 2 Keeping floors clean.
- 3 Cleaning and proper storage of glassware.

4 Storing chemicals in proper places (cupboards, stores).

5 Maintaining equipment.

6 Bringing any problems or potential problems to the attention of the supervisor.

CLOTHING

Technicians must always wear the right kind of protective clothing.

Laboratory coats should be worn (if the room temperature is high this may prove to be impractical).

Face masks must be worn when dealing with dusts.

Gloves must be worn when handling chemicals.

CHEMICALS

ALWAYS read the label on the container. 1 Orange background with black cross: harmful.

2 Orange background with black skull and crossbones: toxic.

3 Orange background with black fire: flammable.

4 Orange background with black Bar or hand being eaten away: corrosive.

There may be written warnings, but they are often only in one language. Harmful solid! Target organ: Nerves! Possible teratogen*!

ALWAYS keep a list of chemicals and the dangers associated with them.

NEVER store chemicals in anything other than their original container, with the correct label.

ALWAYS keep flammable and toxic chemicals in a securely locked cupboard.

BIOLOGICAL HAZARDS

The entomopathogenic fungi used for locust control are very safe, BUT they may cause allergic reactions if they are inhaled.

Some common contaminants e.g. *Aspergillus* spp. are harmful.

Technicians must learn to recognise and dispose of contaminants safely.

ALWAYS keep benches, equipment and protective clothing clean.

FIRST AID

ALWAYS keep a first aid box in a secure place, in full view and check it regularly. Write all injuries in an accident book which should be kept next to the first aid box It is ESSENTIAL that at least on eperson in a laboratory is trained in first aid procedures.

KEEP AN ANTIDOTE FOR ANY ESPECIALLY DANGEROUS CHEMICALS

5. ISOLATING A FUNGAL PATHOGEN FROM AN INSECT

1 Find an area of the insect where fungi are growing free of visible contaminants.

2 Touch a sterile wire loop or needle to the fungus and tease a small amount off.

- 3 Streak across a PCA plate containing antibiotic.
- 4 Incubate at room temperature until growth occurs.

N.B. Any obvious fungal or bacterial contaminant can be removed by cutting it out with a sterile scalpel and throwing it away

6. MICROSCOPY



After sterile technique, learning to make good use of the microscope is the next technique to be mastered by any pathologist.

MICROSCOPES

A brief description of types of microscopes and their uses.

Dissecting:

taxonomy, surface examination of insects, dissecting to reveal internal organs, x10 - x50 magnification, reflected light source (i.e. light from above)

Compound:

pathogens, x100 - x1,000 magnification, transmitted light (from underneath)

Phase contrast:

some compound microscopes have a phase contrast condenser which gives greater clarity for examining pathogens at high magnification

Electron:

emits a beam of electrons instead of a light beam. This is for examining very fine structures. It is an extremely expensive piece of apparatus.

Scanning electron microscope (SEM):

x100 - x50,000 magnification gives three dimensional images for defining shapes in surface structure

Transmission electron microscope (TEM):

x10,000 - x1,000,000 magnification only for very thin sections or for isolated pathogens. Specialised equipment is required to prepare these sections.

USING A MICROSCOPE

1 Turn on the microscope and check that the light is working.

2 Place a piece of thin tissue on a slide with a coverslip on top.

3 Make sure the lowest power objective is in place.

4 Adjust the eyepieces to a comfortable viewing position.

5 Focus by lowering the objective towards the slide while watching from the side. Then look through the microscope and focus up slowly until the tissue comes into focus. Never focus down towards the slide.

6 Close the condenser so that the rim is just visible through the microscope and focus finely on it.

7 The condenser rim should be centered in the field

of view. If it is not, refer to the microscope manufacturer's handbook to find out how to centre it.

8 Now change to the next higher power objective and repeat the focusing of the objective and condenser.

9 You should always view specimens with the condenser just outside the field of view. Adjust light levels with the light power switch.

10 Take your time adjusting the microscope to get it just how you want it. You will probably need to readjust it whenever someone else uses it.

MAKING MICROSCOPE SMEARS

Some simple techniques for preparing specimens to be examined under the microscope.

External sporulating mycelium on an insect or fungus growing on a Petri dish.

1 Use sterile wire loop or forceps to transfer a small amount of mycelium and spores to a glass slide.

2 Add two (2) drops of lactophenol cotton blue on top.

- 3 Lower a cover slip gently over the slide.
- 4 Examine under a microscope.

Internal sporulating mycelium

- 1 Break open the insect body.
- 2 Proceed as for external sporulation.

N.B. Lactophenol cotton blue is hazardous and should be handled with care.

Insects with no sporulation

You need to use judgment and experience here. If the cadaver is hard, it may sporulate after incubation in a humid chamber for a few days. If it is soft (flaccid), proceed as below.

Large insects:

1 Use a syringe to extract haemocoel* from the insect.

- 2 Drop the haemocoel on to several slides.
- 3 Allow to dry.

4 Add two (2) drops of lactophenol blue, Giemsa or Naphthalene Black.

- 5 Lower a cover slip gently over the slide.
- 6 Examine under a microscope.

Small insects:

- 1 Cut open the abdomen.
- 2 Smear on to several slides.
- 3 Allow to dry.

4 Add two (2) drops of lactophenol blue, Giemsa or Naphthalene Black.

- 5 Lower a cover slip gently over the slide.
- 6 Examine under a microscope.

Some Useful Microscopy Solutions

<u>0.2 M Phosphate Buffer</u>

Solution A:

1 Take 28.39 grams of Na₂HPO₄.

2 Add distilled water to make one (1) litre (0.2M solution).

Solution B:

1 Take 31.21 grams of Na₂H₂PO₄.2H₂O.

2 Add distilled water to make one (1) litre (0.2M solution).

- 3 Add 55ml of solution A to 45 ml of solution B.
- 4 Make up to one (1) litre with distilled water.

The pH of the solution should be between pH 6.0 and pH 7.0.

<u>Cotton Blue Stain - Cotton blue (or trypan blue) in</u> <u>lactophenol</u>

1 67.0 ml anhydrous lactophenol

- 2 20.0 ml distilled water
- 3 0.1 g cotton blue or trypan blue
- 4~ On mixing, this will give a 0.1% stain (standard strength).

Simple Giemsa Staining

For fungal nuclei

1 Air dry a thin smear.

2 Soak for 1-2 minutes in Giemsa's fixative:

94% absolute alcohol

5% formalin

- 1% acetic acid (glacial)
- 3 Rinse under slow running tap water for 5-10 sec.

4 Use 10% Gurrs Improved R66 Giemsa in 0.02M Phosphate buffer pH 6.9 and stain for 45-60 minutes.

 $_{\rm 5}\,$ Rinse stain off under slow running tap water 5-10 sec.

6 If there is too much stain, stand smear in 0.02M buffer until the red colour on the glass slide disappears.

- 7 Blot on tissue.
- 8 Leave to dry.
- 9 Examine under microscope.

Naphthalene Black 12B (Acid Black 1) Stain

1 Mix 1.5 g naphthalene black 12B with 38-40 ml glacial acetic acid and 60ml distilled water.

- 2 Air dry smears.
- 3 Heat the solution to 40-44°C.

4 Put the slide into the hot solution for five (5) minutes.

- 5 Blot on tissue.
- 6 Dry and examine under microscope.

Proteinaceous bodies, NPV, CPV, GV and pox virus inclusion bodies stain black and the background is greyish green to light black.

7. MISCELLANEOUS TECHNIQUES

SPORE COUNTING USING AN IMPROVED NEUBAUER HAEMACYTOMETER

It is essential to know the spore concentration of all formulations to be used in the laboratory or in the field. The concentration of a spore suspension can be determined using a haemacytometer.

DILUTING FORMULATED SPORES IN OIL

For counting, the ideal concentration of a conidial suspension is between 1×10^6 and 1×10^7 conidia/ml. A conidial suspension of *Metarhizium* conidia will appear light green in colour when suspended in kerosene or water with 0.05% Tween 80. Conidial suspensions which are more concentrated than this, e.g. spores which are formulated ready for spraying in the field will need to be diluted before counting.

1 Shake the bottle containing the formulated spores to ensure that the spores are evenly distributed in the

solution.

2 Take 1 ml of the formulation and add this to 9 ml of kerosene or other oil (kerosene is better as the spores settle faster).

3 If the solution looks too concentrated, shake the suspension well and redilute 1 ml of this solution with a further 9 ml of kerosene.

N.B. Be as accurate and exact as possible when preparing the 9ml of kerosene and when taking the sample of concentrated spore suspension. If these volumes are not measured accurately, your results will be meaningless.

N.B. If the spores are suspended in aqueous suspension e.g. water and Tween 80, you must use water and Tween for the dilution.

PREPARING THE HAEMACYTOMETER

The haemacytometer must be dry and free of grease such as finger prints etc. Grease should be removed using a tissue and alcohol.

1 Using a clean finger wipe some saliva over the two edges of the cover slip.

2 Place it on the haemacytometer and apply a little pressure so that you can see a rainbow (Newton's rings) at both edges of the slide.

³ Use a Pasteur pipette to take a small amount of the diluted solution.

4 Drop the solution at the edge of the slip; it will be drawn up under the cover slip. Do not overfill the chamber, the channels (grooves) at the sides of the chamber are for taking up any excess solution only and should not be filled with liquid.

5 Leave for a few minutes until the spores have settled (15 minutes for kerosene, 30-60 minutes for viscous oil, 5 minutes for aqueous suspensions).

COUNTING THE SPORES

1 Under the microscope you can see a grid as shown in Figure 3.1.

2 If the concentration is very weak count all 25 squares.

3 If the concentration is higher, count 5 squares on the diagonal.

One large square is made up of 16 smaller squares and is bordered by three (3) lines. There are 25 large squares on each grid an improved Neubauer haemacytometer.

You must count between 30 and 300 spores on each haemacytometer grid. If more or less are present, use a different dilution. If your haemacytometer has two grids marked on it, you should count both grids. If you only have one grid, you should wash and prepare the haemacytometer again.

ALWAYS make two counts to get an accurate estimate of the spore concentration.

CALCULATING THE CONCENTRATION

If 5 large squares were counted

1 Count the number of spores in each of 5 large squares.

2 Add up the total number of spores counted in 5 squares.

3 Repeat this count on a second grid as indicated above.

4 To calculate the mean spore count:

$$X = \frac{a+b}{2}$$

Let a and b =totals for each grid.

Let X = mean count of 5 squares.

 $_5\,$ You used 0.1 μl of spore suspension over 25 squares.

Let c = concentration of spores per ml.

$X.5.10^{4}$

6 Let n be the number of dilution's and

C = the concentration of spores in the original solution.

 $C = cx10^n$

CALCULATING THE CONCENTRATION

If 25 large squares were counted

1 Count the squares on each grid.

2 Add up the total number of spores counted in 25 squares.

3 To calculate the mean spore count:

Let a and b = totals for each grid.

Let X = mean count of 25 squares

$$X = \frac{a+b}{2}$$

 $4\;$ You used 0.1 μl of spore suspension over 25 squares.

 $X.10^{4}$

Let c = concentration of spores per ml.

5 Let n be the number of dilution's.

$$C = c.10^{n}$$

6 Let C = the concentration of spores in the original solution

OBTAINING THE REQUIRED CONCENTRATION

$$x = \frac{\text{Required Concentration x Final Volume Needed}}{\text{Counted Concentration}}$$

where

x = number of ml of spores suspension to be added to make 10ml)

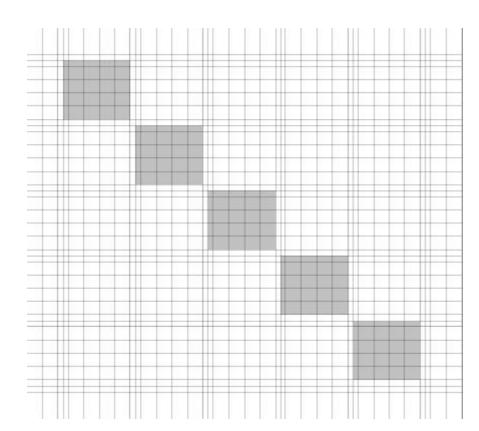
example.

The concentration that you counted was 5.35×10^7 but you require a concentration of $3.75 \times 10^7 \cdot 10$ ml of this concentration will be sufficient for your needs so:

$$3.75 \times 10^7 = \frac{3.75 \times 10^7 \times 10 \text{ml}}{5.35 \times 10^7} = \frac{37.5}{5.35} = 7.00 \text{ concetration} =$$

10ml of 3.75×10^7 sp/ml + 3.00 pure oil/water

i.e. 7 ml of your initial spore suspension plus 3 ml of diluent will make 10 ml of the required spore concentration (3.75×10^7) .



GERMINATION TEST

Figure 3.1.

Fungal spores are living organisms. The viability of the spores (proportion of live spores) diminishes with time depending in the conditions in which they are kept. It is therefore essential to know what percentage of the spores in a given sample are viable before using them. If the percentage of viability has diminished, the concentration of spores must be increased accordingly. However, if the percentage is lower than approximately 70%, the spores cannot be used.

1 Use Sabouraud dextrose agar (SDA) in small Petri dishes.

2 Take a sample of spores and suspend them in either kerosene or water plus 0.05% Tween 80 at a low concentration $(10^4-10^5/ \text{ ml})$.

3 Spread 0.1-0.2 ml of the spore suspension (1-2 drops from a pasteur pipette) on the agar and put the lid on the Petri dish. Using a microscope check that the spores are well distributed. i.e. that individual spores can be easily distinguished from each other.

4 Incubate the plates at 25-30°C for 20-24 hours, then use a microscope to count germinating and non germinating spores. Record the time exactly. Any budding spore should be counted as germinating.

⁵ Count at least 300 germinating and non germinating spores by moving the field of view of the microscope so as to cover a large area of the Petri dish.

6 Calculate the percentage of germination as follows:

 $[a/(a+b)] \ge 100 = \%$ germination

where

- a = germinating
- b = non-germinating

7 Depending on the degree of precision required and the objective of the experiment, these observations can be repeated three (3) times on the same Petri dish or three (3) Petri dishes may be set up at the same time

8. BIOASSAY OF FUNGAL ENTOMOPATHOGENS AGAINST GRASSHOPPERS OR LOCUSTS

WHY DO BIOASSAYS?

1 To find new isolates of fungal pathogens which could be used in biological control of insect pests.

2 To compare new isolates with the ones we already know about.

³ To assess the virulence* of new isolates against other insect pests.

4 To check the virulence of spores from mass production.

INSECT BIOASSAY

See Section 6 on maintenance of an insect colony.

Before you begin make sure that the insect colony is free of protozoan infections such as *Malamoeba* and *Nosema*. Some insects, in particular *Schistocerca gregaria* survive well in individual plastic boxes with no food. Other species may need to be kept in cages and provided with food.

Schistocerca gregaria - in boxes



1 You will need equal numbers of adults of both sexes, 9-12 days post fledgling (not sexually mature).

2 Take plastic food boxes (not airtight).

3 Put a piece of dry tissue paper in the bottom of each box - nothing else.

4 Put one insect in each box.

5 Finish handling the insects before you touch the fungus.

6 If you are using an uninoculated control, set up the control before you touch the fungus.

7 After handling fungus DO NOT enter the insect room again on the same day.

SPORE PREPARATION

(Metarhizium and Beauveria spp.)

1 Use SDA slopes in 25 ml Universal bottles.

2 To inoculate, streak the agar liberally and evenly with conidia of the fungus to be tested.

3 Incubate at 22-25°C in 12 hours artificial light and 12 hours darkness.

4 Harvest conidia 10-30 days after inoculation.

N.B. It is a good idea to choose a standard age e.g. 15 days.

8 To harvest conidia: add 10 ml good quality oil (groundnut or soya oil, without added antioxidants or fungicides) to each bottle.

9 Use a paint brush or spatula to brush the conidia into the oil.

10Sieve the suspension through a 75 micron sieve (this removes hyphal fragments).

11 Agitate the suspension in a bath sonicator for three (3) minutes or a probe sonicator for 1 min. If the conidia are still clumped agitate for a further two (2) minutes.

N.B. If you do not have a sonicator, add glass beads to the spore suspension and use a Vortex/Whirlimixer or shake the bottle vigorously.

12 The water in the suspension will settle to the bottom. Remove as much of the water as you can by decanting.

13 Use the formulation the same day.

14 If you cannot use the formulation the same day, you must add dry, non-indicating silica gel to absorb water.

N.B. You must remove all surplus water to prevent conidia germinating

15 Check conidial concentration using a haemacytometer.

16 Calculate the dilution needed to get $5x10^{\prime}$ spores/ml.

17 Use two (2) microlitres 5×10^7 spores/ml

suspension, this is equivalent to 10⁵ spores for each adult locust (approximate weight 2 g). Reduce the number of spores for smaller insects.

INOCULATION

1 For each strain use 25 insects.

If you are screening new isolates you do not need to replicate the assays. For replicate assays, use 3x20 insects.

2 <u>Large grasshoppers</u>: to inoculate use a micropipette to place 2 μ l of suspension behind the pronotum^{*}. You can hold the pronotal shield forward with a blunt needle or small spatula but be careful not to damage the cuticle.

N.B. Inoculating with 2µl of 5x107 conidia/ml gives a dose of 100,000 conidia per insect (105 spores/insect).

3 Put the insect back in the box.

4 Store at 30°C in 12 hours light and 12 hours dark.

5 Finally, check the viability of spores in each spore suspension used by spreading 0.1 ml of the spore suspension onto an SDA plate as described in the section on Germination testing in this section.

RECORDING

1 Assess mortality every day.

2 If you find dead insects, look for a red colouration, this often is indicative of death due to infection by *Metarhizium* and *Beauveria* spp. Death caused by these fungi also results in the insect cadaver becoming rigid. Insects killed by other causes such as bacterial infection normally appear limp and the limbs will appear loose. A black colouration under the thorax or abdomen of the dead insect is also indicative of bacterial infection.

3 Check your diagnosis, by saturating the tissue paper in the box with water and incubate the dead insect for a few days in the box to see the growth of fungus on the outside of the insect.

4 Even if a fungus does not develop on incubation, it may still have been the cause of death e.g. by toxins produced by the fungus. Compare with a blank formulation control to see whether the pathogen did cause death or whether there was some other cause. If the controls are healthy, then record all mortality as caused by the fungus. Record assays for 12 days. For the final result calculate each day as cumulative percentage mortality over the assay period.

GRASSHOPPERS - SPECIAL POINTS

1 Separate males and females at first since they may react differently to infection.

2 Record average weights for both sexes before beginning the bioassay.

³ For each new species check that it is susceptible to the standard strain.

4 Check each new species to verify that it is not affected by a control inoculation with blank oil

ANALYSIS

1 Plotting cumulative mortality curves gives a basic comparison between strains and you can read LT50s from these lines.

2 Calculate the average and the variance in survival times so that you can make quantitative comparisons between strains.

³ For a more detailed comparison, use a range of doses and calculate the LD50 by probit analysis. Specialised software such as the MELTIMOR programme, or the Kaplan-Meier survival analysis in SPSS should be used to analyse the data.

BIOASSAYS AGAINST OTHER INSECTS

1 It is necessary to develop a bioassay protocol for any new insect species; normally an oil drop bioassay gives the most reliable results, but it is necessary to find the best place for the oil droplet.

2 It is also important to ensure that the insects are kept under ideal conditions, with very low control mortality.

3 Always select a standard strain which can be included in all bioassays for comparison.

4 Check each new species to verify that it is not affected by a control inoculation with blank oil.