MANUAL ON LABORATORY HANDLING TECHNIQUES FOR PLANT QUARANTINE SERVICES

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1. INTRODUCTION

Plant quarantine service requires laboratory testing and diagnosis for the plant pests before issuing phytosanitary certificate, import permit and release order in case of export and import of plants and plant products. In order to accomplish any of the aforementioned phytosanitary processes,it is necessary to have a basic laboratory testing facility in each of the plant quarantine check posts and offices.

Each of the plant quarantine check posts of Nepal has a basic set of laboratory infrastructures. However, laboratory handling and testing is in weak situation due to the insufficient knowledge and skill of basic laboratory handling techniques of the working manpower. There has always been a lack of specific reference manual dealing with the laboratory handling techniques that would help to implement the laboratory testing activities by following the proper procedure.

This manual has provided the basic guidelines for handling the laboratory equipments and glass wares, media preparation, isolation techniques of plant pathogens, pure culture preparation and maintenance, collection and preservation of plant pests, along with basic facilities required in a diagnostic plant quarantine laboratory. This is the working manual for plant quarantine service laboratory, plant protection laboratories and students studying plant protection, plant pathology and agricultural entomology.

The laboratory is established to assist mainly with the laboratory diagnosis of plant diseases and insect pests. However, the facilities are also suitable for the isolation of common plant pathogens. Before working in any laboratory, potential safety issues and health risks must be considered. Safe handling of laboratory tools, equipments and chemical is important while working in any laboratories.

GENERAL REQUIREMENTS AND FACILITIES FOR A PLANT QUARANTINE LABORATORY

1. Infrastructure & Utilities

• Laboratory space with furnitures and immovable fittings for example: Working tables, laboratory chairs with adjustable height, book case, chemical rack, cupboards to store apparatus and supplies, specimen storing rack, microscopes etc

• Water (sink/basin with hot and cold water taps with mixing facilities with plastic tubing, good drainage system leading to concrete tank outside the laboratory)

• Electricity with several electrical wire outlets

• Information facilities (telephone, internet, email etc)

Wash room and lavatories

Guide keys, reference publications, databases

• Communication systems

• Transport (Standby Vehicle used for laboratory works)

• Storage/preservation facilities – Herbaria/culture collections

2. Human resources

• Trained diagnosticians/taxonomists

• Technical support staff

• Non-technical staff

• Service managers/co-ordinators

• Outreach/Public liaison staff

3. Location of the laboratory

4. Laboratory layout

When designing a laboratory there are many aspects to consider. It is important that work should be carried out in a logical order and, that particular parts of the diagnostic protocol are separated from one another. Figure 1 is the layout of a general diagnostic laboratory mainly of plant pathogens.

Preparation room

The preparation room is used for preparing media, including sterilizing items in the autoclave, sterilizing petri dishes in an oven, washing glassware and storing glassware, chemicals and other basic items. This room should have an exhaust fan to remove hot air produced by the autoclave and the oven.

Clean room

The clean room is used for isolating fungi and bacteria from cleaned subsamples of diseased plant tissue into pure cultures. It is also used for growing cultures under clean conditions. The microscopes are located in this room for examining cultures and fungal structures.

This room should be air-conditioned, if possible, to protect equipment and cultures. It should also be kept free from dust and insects. However, do not have an airtight clean room or humidity will be too high and fungus (mould) will develop on walls and equipment. A dehumidifier is useful in this room. No soil is allowed in the clean room as soil is a source of fungus-eating mites that can contaminate cultures.

Specific chambers within the rooms (for specific diagnostic laboratory):

Media preparation chamber : it is mainly to prepare media and should have a continuous and sufficient supply of single and double distilled water. It should also have a water still for storage, a double distillation unit, pH meter and balances.

Inoculation Chamber: This chamber should harbor inoculation cabined/ laminar flow cabinet (convenient for two persons to sit and work at the same time), the floor should be covered with linoleum or tiles to facilitate proper cleaning. The atmosphere in this chamber should be free from all types of contamination. This chamber should preferably be air conditioned for good air circulation and to maintain a constant temperature. A power back up system is required to protect incubated cultures.

Incubation chamber: Incubation of plant parts and tissues at a specified temperature (around 25 degree celcius) after the aseptic transfers needs a chamber. This has to be maintained with an air cooler or by window air conditioners. Controlled heating system may be needed in some check posts in the high mountains.

Washing and drying chamber: A sink has to be fitted in one corner of the chamber. racks to train and dry should be placed adjacent to the sink to facilitate the drying of cleaned glasswares.

Plugging Chamber: This chamber should have tables of sitting and standing height. Space to store the cleaned glassware to be provided. Glassware to be plugged with nonabsorbent cotton.

Media preparation chamber: Pressure cookers of ultradent type (30 cm *30 cm) to steam the media are provided in this chamber. Various instruments including pH meters, balances, centrifuge, refrigerators, deep freeze can be adjusted in this chamber. Vaccum lines and compressed air facility are necessary for filter sterilizing the solutions.

Media storage chamber: Storing of the media before its actual use is done in this chamber.

Sterilization chamber: Horizontal autoclaves of several dimensions may be kept in this chamber. An electric oven for drying the glassware may be accommodated. In one corner a sink may be fitted. Exhaust fans for proper ventilation should be provided.

Shaker chamber: It should be accommodate rotary shakers with variable speed controls, illuminated with fluorescent tube lights (40 w) may be fitted 60 cm above the shaker so that they can provide an intensity of 2000 lux to the cultures. A lux meter can be used to measure the light intensity in the inoculation and shaker chambers.

Other Chambers: Office, meeting hall and data collection/management chamber with communication facility as listed above should also be provided. An acclimatization chamber fitted with humidifier and temperature and air conditioning system should also be managed. The acclimatization chamber should also have the facility similar to the illuminated incubation chambers to regulate the variable light intensity.

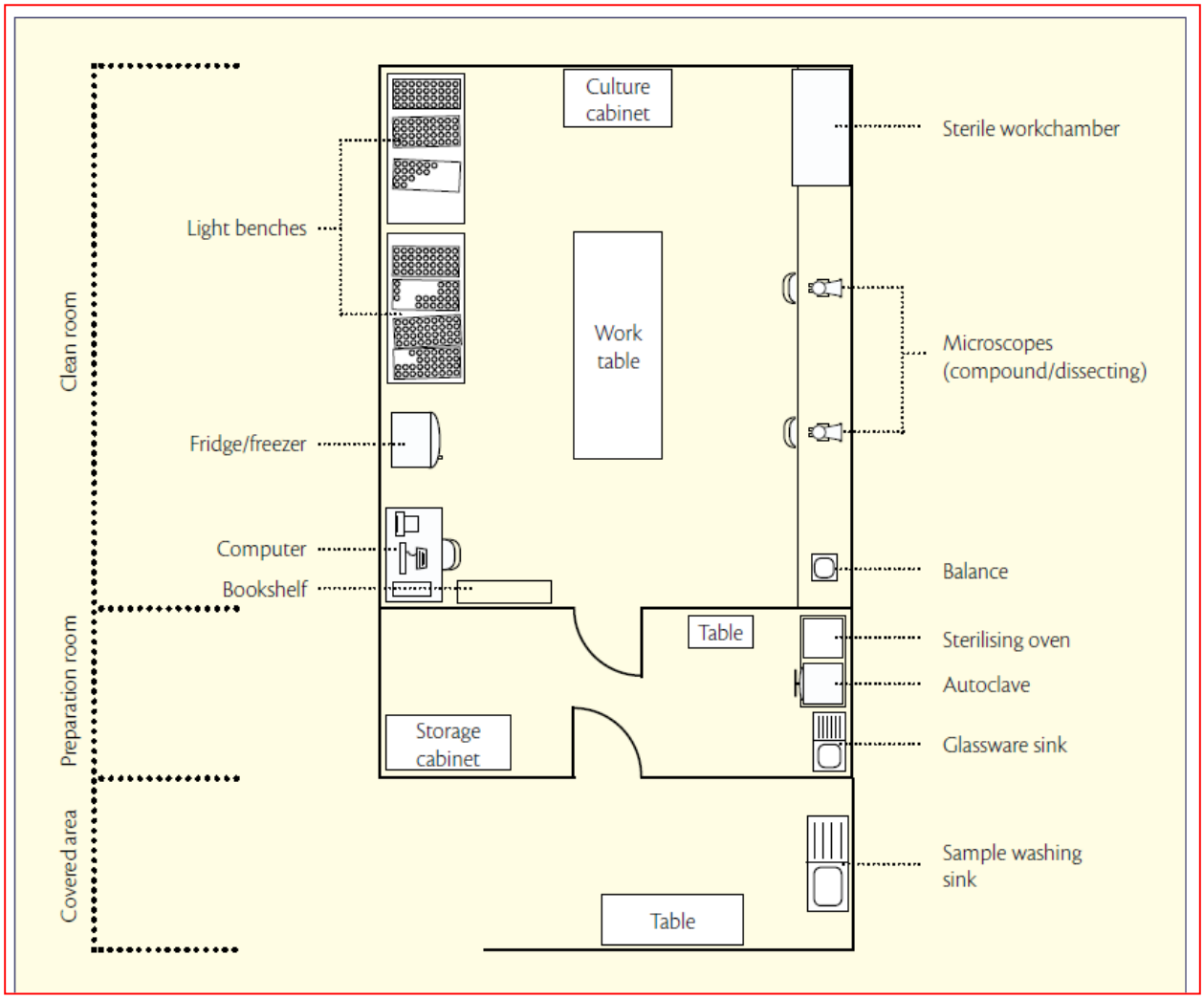


Figure: Typical floor plan and the layout of general plant quarantine/protection diagnostic laboratory [some modification can be made with floor availability and type of laboratory and others]

2. KNOWING LABORATORY EQUIPMENTS, APPLIANCES, GLASSWARE AND CHEMICALS WITH THEIR SAFE HANDLING TECHNIQUES [Plant Pathology and Entomology]

A LABORATORY EQUIPMENTS

1. Analytical Balances

An instrument used for accurate measurement /determination of weight of a sample in various operations. It has gone radical changes by the desire to produce an instrument which is more robust, less dependent upon the experience of the operator, less susceptible to operator and hasten the weighing operation

1.1 Conventional type : Free swinging balance, Equal arm balance, Two pan balances

1.2 Electric balance- no mechanical failure and greatly reduced sensitivity to vibration with it

Macro balance	150 g - 0.1mg
Semi microbalance	30 g - 0.01 mg
Microbalance	20 g - 1 µg
Ultra micro balance	5 g – 0.1 µg

Care and maintenance of analytical balance

→ Never exceed the stated maximum load of the balance
→ The balance must be kept clean
→ Objects to be weighed should never be handled with the fingers
→ Objects to weighed should be allowed to attain the temperature of balance
→ No objects which might injure the balance pan should ever be placed directly on it. Always use a watch glass or weighing bottle for weighing substance
→ Nothing must be left on the pan when the weighing has been completed

→ Exposure of the balance to corrosive atmosphere must be avoided

2. Heating Apparatus

Ovens

Ovens are the boxes designed to maintain a constant internal temperature; they are electrically heated and thermostatically controlled for drying the glass wares and other solids with very high melting points. Ovens are designed to mentain the differential internal temperature ranging from room temperature to about 250 - 300˚ C ± 1 – 2 ˚C. They are also useful for determining the moisture content of materials

Incubators

They are the boxes designed to maintain a constant internal temperature by the use of thermostat; and are used for rearing insects and prematurely born infants. An incubator comprises a transparent chamber and the equipment that regulates its temperature, humidity and ventilation and; is **u**sed in biological experiments to allow growth of organisms in optimal conditions of temperature and humidity.

Distillation Plant

It is an instrument used to purify the water through distillation. The working mechanism of the distillatioln plant allows the water to vapourized and condensed to form the distilled water.

Muffles furnaces

An electrically heated furnace of muffle

The maximum temperature of about 1200˚C

Hot Plates

The Electrically Heated Hot Plate, With Three Controls - Low – Medium – High

The Heating Elements and The Internal Wiring are Protected From Fumes or Spilled Liquids

Autoclave

A thick walled vessel with a tightly fitting lid, in which substances may be heated under pressure to above their boiling points

It is used for sterilizing instrument and in cooking

Heating Mantles

→ An equipment with a flexible knotted fiber glass sheath in it which fits snugly around a flask and contains an electrical heating element which operates at back heat

→ It is particularly designed for the heating flasks

→ It has wide application in distillation operations

Crucibles and Beaker Tongs

Apparatus such as crucibles evaporating basins and beakers which have been heated need to be handled with suitable tongs

Berlese Funnel

→ An apparatus comprising of funnel, lamp and collecting jar.

→ An apparatus for collecting and extracting small invertebrate animals from grains, soil and decomposing litter.

Berlese funnel extraction works in the following way:

- A 60-watt light bulb, housed in a reflective shade, is suspended above a funnel containing approximately 1,000 grams of suspect grain.
- A collecting jar is placed under the funnel.
- Water in the jar prevents insects from escaping.
- Over several hours, light and heat generated from the bulb and reflector cause stored product insect pests to move through the grain into the collecting jar where they can be identified

3. Cooling Apparatus

Refrigerators

→ Refrigeration is generally the cooling of a body by the transfer of a portion of its heat away from it

→ A refrigerator (often shortened to fridge) is an electrical or gas appliance that uses refrigeration to help preserve materials

→ They may consist of either a cooling compartment only (a larger refrigerator) or a freezing compartment only (a freezer) or contain both

4. Desiccators

→ A desiccators is a covered glass containers designed for the storage of objects in a dry atmospheres

→ It is charged with some drying agent such as silica gel, anhydrous calcium chloride

→ The exhausted silica gel (spent material) can be regenerated by heating in an electric oven at 150 180 ˚c

5. Magnifying Instruments

i. Hand lens
ii. Pocket lens
iii. Microscopes

Microscope is an important instrument, which enlarge the image of an object with the help of lenses so as to facilitate the morphological, anatomical and other characteristics of an object. It also facilitates measuring sizes of smaller objects.

Dissecting microscope, binocular microscope and electric microscope are common types of microscopes being used in entomological laboratory. The objects are measured by ocular and stage micrometers.

iv. Micrometer

→ An instrument for the accurate measurement of small distances or angles

→ Stage micrometer is a glass slide in which one millimeter scale is divided into 100 equal divisions and each division measures 0.01 mm (10 microns).

→ Ocular micrometer is a small glass disc which can be fitted into an eyepiece of microscope. It has a scale of one centimeter long, divided into 100 equal parts and each division measures 0.1 mm (100 microns).

Care and maintenance of microscope

i. Carry the microscope in an upright position, by putting one hand on the handle and supporting the base with other hand.
ii. Avoid tarring suddenly

iii. Keep microscope free from dirt and dust
iv. Clean lenses with lens paper only
v. Always use clean glass slides with specimen on it.
vi. Reflect the mirror towards the light source
vii. Always start focusing with low power to bring the object in the objective field. Use higher magnification gradually.
viii. Clean the microscope after finishing your work.
ix. Store the microscope in a cabinet with a cover over it

6. **Rotary evaporator:** concentration and purification
7. **Suction pump:** useful to filter mixture with suction mechanisms through any filter media
8. **Centrifuge**

→ An apparatus for separating particles from a suspension
→ Tubes containing the suspension are attached to opposite ends of arms rotating rapidly about a central point
→ Suspended particles are forced outwards, and collect at the bottoms of the tubes

9. **Microtome**

→ Microtome is an apparatus for cutting thin sections of material for microscopic examination

10. **Vernier calipers:** A device for measuring sub-divisions of a scale. For a scale graduated in (say) centimeters and tenths, a vernier consists of a scale that slide along side the main scale, and on which a length of nine–tenth of a cm is subdivided into ten equal parts. Each vernier division is thus 0.09 cm. The vernier caliper is an instrument used to measure the length of a long or elongated object e.g. the internal diameter or external diameter of a cylinder tubes.
11. **Camera**
12. **Tally counter:** It is a device for counting individual events/objects/things to ensure the counting.
13. **Hygrometer**

It is an instrument designed to measure the relative humidity of the atmosphere. The variation of relative humidity of the air is shown by hygroscope.

14.Maximum and Minimum Thermometer

An instrument for the measurement of temperature is called thermometer. A thermometer that records the highest and lowest temperature reached during a period of time is known as maximum and minimum thermometer. It consists of bulb filled with alcohol, which, by expansion, pushes a mercury thread along a fine tube, graduated in degrees. At each end of the mercury thread is a small steel 'index' that is pushed by the mercury one is thus left at the farthest point reached by the mercury thread, corresponding to the maximum temperature and the other at the lowest point.

26. Grinder:

27. Micro Syringe:

B.GLASSWARE:

In order to avoid the introduction of impurities during analysis, apparatus of resistance glass should be employed. For most purposes Pyrex glass (a borosilicate glass) is to be preferred.

As a rule, glassware should not be heated with a naked flame, a wire gauze should be inter posed between the flame and glass vessel.

For special purposes, corning vycor glass (96% silica) may be used. It has a great resistance to heat and equally great resistance to thermal shock and is unusually stable to acids, water and various solutions.

Beakers

Pipette

Measuring cylinder

Conical flasks

Dessicator

Petri dishes

Specimen Vials

Reagent bottles

Funnel

Separatory funnel

Test tubes

Glass slides

Cover slips

Wash bottles

Droppers

Glass jars

Glass rod

C. CHEMICALS

1. Chemicals Used For Insect Killing

→ Ethyl Acetate
→ Carbon Tetra Chloride
→ Chloroform
→ Diethyl Ether
→ Amonia Water
→ Potassium Cyanide
→ Sodium Cyanide
→ Calcium Cyanide
→ Xylene + Ethyl Alohol

Ethyl Acetate is the Active Ingredient Commonly Used to Charge Collecting Jars

2. Chemicals Used As Preservatives

→ Ethyl Alcohol
→ Formaldehyde
→ Glacial Acetic Acid

3. Chemicals Used As Disinfectants

→ Formaldehyde
→ Hypochlorite Acid
→ Benzene- Used For Cleaning

4. Chemicals For Slide Preparation

→ Xylene- xylene bath will clear the specimen by removing fats and oils
→ Glycirine- for sealing the slides
→ Potassium Hydrooxide –remove clear the soft tissues
→ Canada Balsom- fixing the speciment (mounting cement)

5. Chemicals For Pesticide Residue Extraction

Acetone, Acetonytrile, Hexane, Petrolium Ether, Methylchloride (Pestide Will Be Dissolved On Organic Solvents, Particular Type Of Pesticides May Require Particular Type Of Chemical)

Sodium Chloride- Help to separate aqueous layer and solvent layer distinctly

Sodium Sulphate - Moisture absorption from solvents if any

Florosil – Absorb pigments used on purification process

Acetone – Washing (Purification & Concentration)

Teepol - Teepol is a relatively mild and inexpensive detergent which may be used for cleaning glassware

D. MISCELLANEOUS

Entomological Pins

Pinning Block

Dissecting Tray

Watch Glass Set

Setting Board

Glass Top Boxes

Insect Collection Box

Dissecting Needle

Wire gauze

Aluminum Foil

Camel Hair Brush

Galvanized Wire

Insect Rearing Cages

Insect Rearing Tray

Sieve

Dendrite

Quick Fix

What Man Filter Paper

Blotting Paper

microscopic cutting knives

glass wool

shovel/spade

camel hair brushes

teasing needles

pinning blocks

spreading boards

blotting papers

tissue papers

funnel rack

cavity slides

counting dishes/digital talley counter

wire meshes

slide labels

pinch cocks

gloves

droppers

wash bottles

Forceps: Insect Forceps are those instruments with two blades and a handle used for handling, grasping, or compressing. Insect Forceps have truncated tips instead of pointed ones, as is usual in common forceps, and the inside face of each tip is rough so as to have a better grip of the pins bearing the insects.

Soft-touch or "Feather-touch" forceps are best for the collection of entomological specimens without damage as they are constructed of thin and flexible metal that prevents the larval body from being crushed if too much force is applied by the collector

Aspirator : Used for collecting those very small insects which can not be collected through hand nets.

Nets: A net is made of a wire frame, preferably a folding bag, 30 – 37.5 cm in diameter and 45 – 75 cm deep, a stick or handle and a Y-shaped metal piece for fitting the two.

Vials: Vials of "Screw-cap" types are best.

For long term storage (for museum purposes), vials are available containing a neoprene cone insert in the lid, which reduces evaporation to negligible levels and periodic maintenance is not required as often.

Scalpels: A small straight knife with a thin sharp blade used in surgery and dissection. Can have a fixed blade, or a disposable blade

Scissors: A cutting instrument resembling shears, but smaller, consisting of two cutting blades with handles, movable on a pin in the center, by which they are held together

Relaxation bottle: An apparatus used to keep the insect soft for further handling

Aspirator: An apparatus used to trap the minute sized live insects.

SOME EQUIPMENTS FOR PLANT PROTECTION LABORATORY WITH GENERAL SPECIFICATION

S. N.	Products	Specification
1	Electronic analytical Balance	Weighing capacity 200g (incl. Tare Range). Readability. 0.0001g, Pan size 76mm diameter Reproducibility ± 0.0001g. Linearity ± 0.0003g. Calibration automatic with external weight. Complete with draft shield and calibration weight. Operating voltage 220V 50Hz
2	Micro Centrifuge	High speed 13000-15000 rpm with cool running. Rotor capacity 24x1.5ml or 2ml with precise digital speed indicator and 30-minute timer and continuous running. Powerful brushless drive motor. Electronically locked lid. Operating voltage 220V 50Hz
	low speed centrifuse	should be equipped with continuously variable electronic speed control, speed vibrator, Amp meter, timer, dynamic break, zero staring switch and fuse safety device for 230 V/50 Hz AC mains.
3	Colony Counter	Colony counter LED display 0 – 999 counts with reset to 0, three fold surface magnification, 100mm diameter. 20W illumination ring lamp, complete with plug-in flexible arm, switchable background (bright-dark) complete with pointer. Operating voltage 220V 50Hz
4	Hot Plate	Ceramic top, Infrared touch panel, 9 levels adjustable. Dimensions of ceramic area 280 x 280mm. spill proof ceramic glass top around hot plate area. Operating voltage 220V 50Hz
5	Incubator	Complete stainless steel interior and exterior. PID microprocessor controlled Electronic temperature controller and indicator with facility for setting up time from 0-99 hours. Temperature range from +30C to +70C. All four side Heating element for uniform heating and arrangement for natural air circulation No of shelves two all SS. Working chamber 480X320X250mm (W. H. D. approx.) adjustable overheats protection. Operating voltage 220V 50Hz
6	Magnifier	Magnifier with circular lamp for uniform illuminates and enlarges objects under the lens. Built-in adjustable handle on heavy duty base. Lens diameter 5 Inch, 3-diopter lens increases magnification up to 75% at a focal length of 13". Operating voltage 220V 50Hz
7	Biological Microscope Trinocular with SLR Camera	Universal Infinity-Corrected optical system. Built-in transmitted Koehler illuminator 6V/30W halogen lamp. Focusing stage height movement by roller guide (rack & pinion) Stroke per rotation 36.8mm. Full stroke range 25mm. Upper stopped by simplified-focusing dial. Tension adjustment on coarse focus adjustment knob. Revolving nosepiece – fixed quadruple with inward tilt. Trinocular observation tube 30; inclined. Mechanical stage with double slide holder. Abbe condenser. N.A. Achromatic objectives 4X, 10X, 40X & 100X with wide field 10X eye pieces, duct cover. SLR Camera with complete attachment for SLR camera. Operating voltage 220V 50Hz
8	Stereo Microscope	Zoom stereo microscope with greenough optical system for clear and sharp image reproduction with zoom magnification 0.8x to 4x (8 to 40 times using 10x eye-piece) and zoom ration 5:1, working distance 110mm 45º tube inclination angle. Optically components must be lead free. LED reflected/transmitted illumination stand with stage plate 100mm glass included. 10X comfort view eyepieces one pair. Operating voltage 220V 50Hz
9	Oven	Complete stainless steel interior and exterior. PID microprocessor controlled Electronic temperature controller and indicator with facility for setting up time from 0-99 hours. Temperature range from +ambient to 220ºC. All four side Heating element for uniform heating and arrangement for natural air circulation No of shelves two all SS. Working chamber 480X320X250mm (W. H. D. approx.) adjustable overheats protection. Operating voltage 220V 50Hz,
10	Shaker	Flask shaker with orbital motion and universal attachment set consisting of 2 fastening bars with 2 clamping rods. Dimensions of platform 330x330mm with 0-60minute timer or continuous operation. Shaking amplitude 10mm and frequency 20-500 min –1. A.C motor with over load protections electronic speed control stepless with gentle start-up. Operating voltage 220V 50Hz,
11	Thin Layer Chromatography set	Complete with chrome plated TLC spreading device, spreading template, 10 carrier plates 200 x 200mm, 2 carrier plates 200x 50 mm, 500g TLC silica Gel 60 GF 254, drying rack, spotting and evaluating template, capillary pipette 5ul, standard separating chamber with knob lid atomizer and rubber bellow.
12	Autoclave	Autoclave Complete stainless steel interior and exterior with radial locking lid, double jacketed, low water cut off device, timer, water level indicator and pressostate pressure control, safety valve, pressure gauge, steam release valve, Chamber size 410mm x 500mm Paddle lifting device for lid opening and lifting. Operating voltage 220V 50Hz,

13	Automatic Water Distillation Apparatus	Glass Double distillation set Tabletop horizontal model with two separate quartz boilers for first and second stage distillation, attached with condenser and low water cut-off device. Auto start for second stage boiler, vertical condenser on both boilers. Distilled water out put 1.5 Liter or more/Hr. Pyrogen free, Free of Heavy metal Conductivity of out put water not more then 1-2 us/cm Quartz heater. Power heater: 1.5KW each heater/220V/50Hz.
14	Digital camera	Zoom type with 5 mega pixels complete with carrying case
15	Waterbath	Compete stainless Steel interior and exterior Water Bath with Six openings and concentric rings. Electronic microprocessor PID controller cum indicator with continuous power adaptation. Solid state switching unit Auto diagnostic system for identification of faults. Integrated digital timer (pre-set time from 1 minutes to 999 hours) Digital Display (LED) of set and actual temperature as well as of (remaining) programe time. Overheat Safety Device Fixed temperature cut-out (TB) protection class I Textured Stainless Steel casing L x W x H: 350 x 290 x140mm, 14L Easy-to-clean interior made of stainless steel, reinforced by deep drawn ribbing, laser welded. Corrosion proof large-area heating on three sides. Temperature range at least 5ºC above ambient up to + 95ºC Voltage / Power Rating 230V(± 10%), 50/60 Hz /approx. 1800 W (during heating)
16	Thermo hygrograph	Sensor aged bimetallic strips for temperature, human hair bundle for relative humidity. Measuring range –4 to 40ºC for temperature & 5 to 100% R. H., Accuracy +/- 2ºC & +/- 5% for R. H. Chart graduation: 2ºC for temperature & 5% for R. H. Clock: Quartz clock. Drum duration 7 days with recording pen. Standard accessories: 7 days rotation chart pad with 55 sheets for one year use and spare cartridge violet & red pan.
17	Inoculation loop	Changeable nichrome loop holder made of SS rod with heat resistance handle, for 2mm diameter nichrome wire. With extra 10pcs nichrome loop.
18	Refrigerator	Refrigerator, CFC free compressor double door. Chamber Capacity 230Liter Net with Freezer 60Lit/170Liter refrigerator.
19	pH Meter	Analog Tabletop model easy to read mirror scale mV scale for ORP measurement and titrations. With combined glass electrodes with BNC connector. pH/mV/ORP with temperature setting for compensation. PH range 0.000 to 14.000, resolution 0.02 Two point buffer calibration. Electrode support stand Operating voltage 220V 50Hz
20	Grain Moisture Meter	Grain Moisture Meter portable hand held with digital reading for direct eight crops, automatic temperature compensation & easy calibration system. Measuring range 8 to 35%, accuracy +/-0.5% complete with 9V alkaline dry cell.
21	Incubation Chamber	All steel construction with interior of stainless steel capacity 38 liters temperature range from 5 to 50C, microprocessor based digital temperature controller with indicator, inner glass door for easy viewing of samples. Controller box must be separate for easy attachment with main unit. Operating voltage 220V 50Hz
22	Isolation chamber	All steel construction cabinet, working area 900 x 600 x 600mm complete with germicidal light, fluorescent light, gas cock, manometer for air pressure, front shield, table top model, Operating voltage 220V 50Hz
23	Desiccators	Desiccators with knobbed lid 150mm diameter made of Borosilicate glass complete with porcelain plate.
24	Deep Freeze:	CFC Free. Temperature –20'C chest type. Capacity 300 liter with spring loaded lid, 1 handled baskets, step divider, recessed adjustable, safety sentinel power on indicator, water drain outlet and detachable drain hose.

	Laminar flow cabinet	

Figure Typical arrangement of equipment in a diagnostic laboratory: a) and (b) two views of clean room, (c) and (d) two views of preparation room.

3. Killing, Preservation and transportation techniques for insect pests

3.1.1 Killing Methods

The killing of insect has important effect on its permanent preservation. The insect to be preserved have to be well handled throughout the procedures. Each and every insect part is important for its identification, so care should be taken not to damage them. The collected insects have to be effectively killed. For this purpose killing jars are used, with some sort of killing agent in it. The time for killing the insects differs according to the size of the specimens. The most widely used method for killing the collected specimens is the killing jar (bottle). In the field work, it is always not possible to take/get killing bottles. For this purpose, a heavy scale plastic can also be used as it is easier to transport and handle. Take some amount of liquid killing agents in a tight vial along with forceps and some cotton pad. Place the collected insects inside the plastic and then put small piece of cotton pad soaked with liquid killing agent inside. Then tie up the plastic and carry to the laboratory.

One must be careful at the time of keeping insects in the killing jar/plastic after collection. Put insects according to their behaviour. Try to put the insects according to their respective taxonomic orders. Do not put Orthopteran insects with other group of insects. Generally after putting the liquid killing agents inside the killing jar/plastic, the orthopteran insects jump and kick damaging body parts of other insects. So insects are placed differently as per their behaviour: butterfly – moth – dragon flies - beetle and bug – bees – dipteran flies accordingly.

Killing agents: Various liquid or solid chemicals may be used as killing agent. Liquid killing agents are generally slower acting but safer to use than solids. All killing agents are to some extent hazardous to human health, so all killing jars or bottles should be clearly labeled "POISON".

Liquid killing agents: The frequently used liquid killing agents are ethyl acetate, Carbon tetrachloride, chloroform, benzene, ether (diethyl ether, trichloro-ethylene) and ammonia water solution. Among these ethyl acetate and carbon tetrachloride are commonly used. These chemicals can be used in lower quantity

but the killing agents like benzene has to be used in more quantity though it is regarded bit safer.

Solid killing agents: The most often used solid killing agents in killing jars are the cyanides – potassium cyanide, sodium cyanide, or calcium cyanide. All the cyanides have to be handled with extreme care because they are dangerous, rapid-acting poisons. But nowadays, the cyanide is very difficult to find at regular market, but it can be used making special request for scientific use.

All killing agents are to some extent hazardous to human health. So all killing jars or bottles should be clearly labeled "POISON" and should be kept away from children or persons who may be unaware of their potential danger.

3.1.2 Relaxing

It is always not possible to pin the specimens collected at the same day. So before pinning these specimens, left in temporary storage, it is necessary to restore their flexibility. This is called as “relaxing” the specimens. Relaxing can be done by placing the insect specimen in a relaxing jar for certain time. Generally the freshly killed specimens can also be kept at relaxing jar for some time. The relaxing jar is simple equipment and can be made by any glass material. Some moist sand are placed at the bottom and covered with blotting paper where the insect specimens will be placed. The growth of mold is also to be avoided, since it will ruin specimens left too long in relaxing chambers unless a chemical mold inhibitor has been added.

3.1.3. Cleaning specimens

At most time, cleaning the specimens is important for further preservation process. The soil insects and also other insects and their immatures might be unclean at the time of collection. Cleaning can be done in different ways as per quantity of dirt, size and type of specimen. A fine camel-hair brush can work for dirt, pollen dust type materials. Similar dirt can be removed in water to which synthetic detergents has been added. Specimens can be picked by fine forceps and drop at the dish containing clean water or mixed with detergents for some time. Greasy materials in the specimen have to be cleaned with the help of organic solvent. Specimen covered with mold or fungus can be removed by fine brush dipped in carbolic acid.

Beetles and other hard insects can be made clean by gently scrubbing with water or weak alcohol. After cleaning specimen it can be returned to the relaxing jar.

Papering: This is the storage method, known as papering has been used since long time for larger specimens of Lepidoptera, Trichoptera, Neuroptera, Odonata and some other groups. This is a traditional way of storing un-mounted insects and still been used. At first envelope are made using some glassine papers, where the specimens are placed with the wings folded together dorsally. These paper triangles are stapled and piled up in dry condition. Care should be taken to avoid mold develop.

3.1.4. Insect labeling

A collection has little value unless each specimen is properly and accurately labeled. Their precise collection locations, habitats and other related information are important documentation. Labeling must be done as soon as possible after collecting, pinning and mounting otherwise the information may be lost.

Labeling paper: The paper used for making labels should be heavy enough so that the labels remain flat and do not rotate loosely on the pin.

Writing ink: The ink should be a good grade of ink (India ink or permanent ink), which will be permanent. Be sure the ink is completely dry before placing the label in the liquid. Generally HB pencils are used if the label is to be put in the alcohol for short term storage.

Printing labels: Though computer printed labels is easy to use, hand written labels using technical pens with very fine points are still widely used at many institutions. If typewritten or computer generated labels are to be used, take care of the proper size. The letter size must be very small (about 6 point on most fonts) but readable.

Label Data: The information on label must answer the questions of where, when, and who in that order. This kind of data should be given as follows:

Locality: The collection locality should be given in such a manner that it could be found on any good map.

Date: Write day, month, and year in that order

Collector: Write the last name of the collector or collectors, using initials for given names if space permits.

Other data: It is often useful to have additional information, like host plant, found under rock, trapping methods etc. in separate label called as ecological label.

More than one label can be placed according to the information collected. The top label should have the locality from which the insect was collected, the collection date and the name of the collector. The lower label should show the taxonomic information of the insect. Many entomologists place another label beneath the collection label which gives the host plant, habitat or other pertinent information called as ecological label.

For labeling the vials, special care should be taken with labels placed in alcohol. The paper should be of high quality. Always place labels inside the vial because if it is fixed at the outer surface of the vial, it will be difficult to observe the specimens inside. A single label large enough to include all data should accompany material in fluid.

3.2Preservation

The process of preservation starts at the time of insect collection. There are several insect collection methods. Thereafter the insects are killed using some type of insect killing chemicals. Proper care should be followed during insect collection and killing process, because each and every part of the insect is very important for the process during insect identification. Once the specimens are preserved they will be valuable material. When carefully collected and preserved, insects can last indefinitely. Several different types of preservation methods are used, each appropriate for different class and type of insects. In general the insects can be preserved in two ways – Although there is not any fixed method, but a general thumb rule is that – insect larger than common housefly is kept as dry preservation (by drying the insect) whereas smaller than housefly and soft bodied are kept as wet (kept in some sort of liquid) preservation

- **Dry preservation and**
- **Wet preservation**.

3.2.1. Dry preservation of insects

Specimens to be prepared as permanent dead or dry collection must be fresh or flexible, by which it is easy to show different body parts. Usually it is advisable to use the fresh specimens immediately after collection, but if left for some period, they must be well relaxed for some time.

Relaxing of insects can be done by placing the specimen in a relaxing jar for certain time. It is also advisable to keep freshly collected insects for sometime in the relaxing jar. The relaxing jar can be made using any type of materials especially made of glass. Some moist sand or newspaper is placed at the bottom of the jar. This sand/paper is then covered with blotting paper where the insect specimens will be placed. Care should be taken for the growth of mold, since it will ruin specimens, if left too long in relaxing chambers. A small amount of carbolic acid is placed

Equipment typically needed to mount specimens includes:

Insect pins, Minuten pins, Pinning block, Spreading board, Stiff good quality paper, Scissor, Forceps, Glue, Specimen box, Polyporous pith / fine cork / soft wood/fine textured plastic, camel hair brush, insect rearing cages, insect tray, narrow paper stripes, etc

Hand lens; microscopes; petri dishes, vials, reagent bottles, test tubes, glass slides, cover slips, wash bottles, droppers

Ethyl acetate, carbon tetra chloride, ethyl alcohol, formaldehyde, benzene,

The main goal of proper pinning procedures is to display the taxonomic characters to be used for insect identification. The pinning procedure for large insects to be kept as dry preservation are described below:

Large insects: Insects larger than medium size (house-fly) are generally pinned –

The killing jar is emptied onto a piece of clean white paper – if the specimen is not clean, it has to be cleaned – then take the individual insects to be pinned between forefinger and thumb. The pin is first inserted through the right side of the thorax (the pinning position are different in different insect orders). Place the pinned specimen in pinning block to maintain the appropriate pinning height. After the pin is inserted and before the specimen is dry – the legs, wings, and antennae should be arranged so that all parts are visible for study (the spreading insect body parts are different in different insect orders). The pinned specimens may be arranged closely on the setting board in series applied with paper strips for winged insect. Specimens either on spreading boards or blocks should be placed in a warm, well-ventilated place for drying. Always keep temporary data labels with specimens on spreading boards so as the important information may not be lost.

Although the insects smaller than common house fly are kept as wet preservation, but sometime certain condition may come, so these insect have to be kept as dry preservation. The pinning procedure for small insects to be kept as dry preservation are described below,

Small insects: Insects smaller than a house-fly if preserved as dry preservation should be either pointed, carded, double mounted or mounted on microscope slides. In working with small insects, a large magnifying lens may be very helpful.

Pointing: Card points used for pointing are slender little triangles made by cutting the stiff paper. Card points may be cut with scissors from a strip of paper. They should be made only from good quality paper. Now cut the paper in triangle form and push a regular insect pin through the end of the triangle. Level the triangle by pushing the pin through the hole in pinning block. For most insects, the card point is attached to the right side of the specimen. Pick up the small insect and mount it by touching it on its thorax to the small drop of glue. If specimens are in good condition and are well prepared, they may reasonably be kept in museum collections for a long time.

Carding: Rectangular pieces of card, little larger than used for pointing are mounted on insect pin and a small strip of glue is applied at the middle of the card using the

minimum amount of glue for the size of the insect to be mounted. This might be preferable then pointing, as the specimen is protected by the card.

Double Mounts: Generally pointing or carding of small insects is sometimes not recommended because the insect is often obscured by the glue. So for the small insects, it is wise to use double mount. This method is little tired and time consuming. This term refers to the insects being mounted on a minuten (small insect pin), which in turn is mounted or attached to a standard insect pin. Minutens are finely pointed at one end, headless on the other, and generally of stainless steel. Double mount is the similar procedure as that of pinning larger insects. It is done by inserting the minuten at the standard pinning position of the specimens – which then is placed into a small cube of soft material for spreading and drying.

3.2.2. Wet preservation of specimens

Many types of small insects, including aphids, springtails, thrips, mayflies or silverfish, are soft-bodied and cannot be pinned successfully. Such insects must be preserved in liquids in air tight glass vials. For the long term storage, insects are preserved in ethyl alcohol, usually of about and above 70 percent concentration (70 percentage alcohol and 30 percentage distilled water). Isopropyl alcohol and formaldehyde solution can also be used in different concentration, but alcohol is common and easily available.

Take a neatly cleaned conical flask or any type of glass jar. The jar must be tightly fitted with lid for long time storage of chemical. Then pour alcohol in measuring cylinder into 70 ml mark and add water to make it 100 ml.

Special care should be taken with labels placed in alcohol. Paper should be high quality. Similarly the simple lead pencil is also widely used. For the preservation of specimens in liquid, do not use necked vials, use shell vials with screw top. Also it is advisable to use glass vials instead of plastic vials. The vials are kept in wide mouthed jars with straight sides. Generally it is recommended that each jar contain between 10 and 40 vials. Avoid glass-glass contact by placing folded paper towel in the bottom of each jar. Keep vials upright within jars.

3.3 Transportation of insect pest specimens

The collected specimens have to be well identified for different purposes including management of insect pests. The specimens, after applying appropriate preservation techniques have to be stored at local level laboratory. Identification is not only possible at field or local level, so it has to be sent at national museum or similar museums or sometimes abroad also.

Before sending the specimen prior approval from the concerned institution is very necessary. The concerned personnel must be notified, so that, he can make necessary arrangements for taking the specimens at his laboratory. Prior information is necessary also because, the concerned official have enough time and space to place the specimens. Also, all the specimens cannot be identified at same laboratory. It should be noted that there are many procedures to be completed before arranging the specimens for identification.

The insects to be sent have to be categorized and packed accordingly. The small, soft bodied insects have to be sent in liquid preserved materials, while large insects have to be sent in dry pinned condition.

Procedures for packing dried pinned specimen:

- Generally medium to large insects are sent in dry pinned process.
- The insects are pinned in standard pinning positions and well dried
- Few to large quantity can be sent by this method
- Small postal boxes are preferred for pinned specimens; however various type of boxes made by wood, cardboard paper, metal etc can be used
- At first cork, thermocol or similar material has to be cut to the size of the postal boxes and placed tight at its bottom
- The dried pinned specimens have to be kept accordingly
- The pins must be pushed deep into the cork
- The insects should be prevented from rotating on their pins by extra pins just touching each side of the body

- The postal boxes should be well packed by sticking packaging tape at all sides
- Each and every specimens kept inside the boxes should be numbered and well labeled with all the information.

Procedures for packing liquid preserved specimen:

- Generally small, soft bodied insects are sent in liquid preserved materials.
- At first make the liquid preservatives ready
- Normally 70 % alcohol (70 parts alcohol and 30 parts distilled water) are used
- Then get ready some vials
- Screw-capped tubes or vials are preferred for specimens in liquid
- Place the specimens in vials and cover the vials with liquid preservatives
- Tubes/vials should be wrapped individually in cotton-wool, tissue paper, or polystyrene sheet to avoid glass surface touching each other
- The vials can be placed in postal boxes with extra care
- Special boxes are also available at scientific equipment stores

3.4 Culture of insects

- Culturing of insects involve **keeping, rearing and breeding insects**.
- Most insects can be kept alive for some time after capture
- Larva or pupae can usually be induced to complete their development.
- However, breeding means maintaining them through a complete life cycle, mating, oviposition, successive larval moults, pupation and emergence of adults.

- Insects that can readily be bred continuously in the laboratory are those whose immature stages are passed in a uniform and stable medium.

4. PREPARATION OF CULTURE MEDIA FOR FUNGI AND BACTERIA

Medium (media pl.) is the substance which provides nutrients for the growth of microorganisms. The nutrient preparation on which culture is grown in the laboratory is called culture medium. Microbes require different nutrients for their growth. There is no single medium which can support the growth of majority of microbes.

Thus, different types of media and environmental condition are to be used for a given group of microbes. Many special purpose media are needed to facilitate, recognition, enumeration and isolation of certain microbes.

Based on chemical composition, media can be classified into.

1) Natural 2) Semi-synthetic 3) Synthetic.

1. Natural medium: The exact chemical composition of this media isn't known properly. It includes ingredients of natural origin like yeast extract, beef, milk, tomato juice, blood etc.

Sometimes this medium is also referred to as complex medium or non-synthetic medium because medium is of complex type and contain various ingredients of unknown chemical composition. This type of media is useful for cultivation of microbes whose specific growth factor requirement is not known.

Eg. Carrot slices, potato plugs, twigs, milk, meat extract, peptone etc.

2. Semi-synthetic: The chemical composition of media is only partially known. Media, which contains Agar, is semi-synthetic medium.

Eg. Potato Dextrose Agar medium, Nutrient Agar media.

3. Synthetic medium: The chemical composition of the medium is completely known.

These media are very useful in studying the physiology, metabolic nature and nutritional requirements of microbes. Both autotrophs and heterotrophs can be grown in these media.

Eg. Mineral glucose medium, Richard's solution, Raulins medium etc.

Based on consistency the media are of three types as 1**) Liquid 2) Semisolid 3) Solid medium**

1. Liquid medium: Nutrient broth is the common liquid medium used in a microbiological laboratory. Its drawback is that the morphology of bacterial colony cannot be studied. But it supports a high microbial population.

2. Semi-solid medium: A semisolid medium is prepared with agar of concentration of 0.5% and is useful in the cultivation of micro-aerophilic or studying bacterial motility.

3. Solid medium: If agar is added to a nutrient broth, it becomes solid medium. It is used for isolating microbes and to determine characteristics of colonies. It remains solid on incubation and not destroyed by proteolytic bacteria. The addition of 15-20 gm (2%) of agar in 1Ltr of liquid culture will produce a gel that liquefy at 95°C and solidifies at 40-45°C into gel.

Based on application or function, media can be classified as follows:

1. Selective media: Provide nutrients that enhance the growth and predominance of particular microbe and don't enhance or may inhibit other types of organisms that may be present. For instance, isolation of bacterium *Neisseria gonorrhoeae* from a clinical specimen is facilitated by the use of media containing certain antibiotic. These antibiotics don't affect pathogenic but inhibit the growth of contaminating bacteria.

2. Differential media: Certain reagents or supplements when incorporated into culture media may allow differentiation of kinds of bacteria. If a mixture of bacteria is inoculated on to blood agar media, some of bacteria destroy the RBC and others don't. Thus one can distinguish between haemolytic and non- haemolytic bacteria on the same medium.

3. Assay media: Media of prescribed composition are used for the assay of vitamins, amino acids, antibiotics etc.

4. Enumeration media: Specific kinds of media are used for determining the bacterial population in milk, water, soil and food etc.

5. Maintenance media: It is used for satisfactory maintenance of viability and physiological characteristics of culture.

GENERAL PROCEDURE FOR THE PREPARATION OF BASIC MEDIUM

Media containing nutrients are usually solidified by the addition of agar for the preparation of basic media.

Eg. Potato Dextrose agar medium, Nutrient agar medium.

A) Preparation of Potato Dextrose Agar Medium:

It is commonly used in isolation and maintenance of common fungi.

Materials Required:

Peeled potatoes - 200g,

Dextrose - 20 gm

Agar - 20 gm

Distilled water -1 Ltr,

Beaker 1L, 250 ml conical flasks, knife, muslin cloth, measuring cylinder, cotton nonabsorbent, Autoclave.

Procedure

1. Take 500 ml of distilled water in 1L beaker and add 200g of peeled and sliced potato boil the potatoes till they become soft.

2. Filter the contents of the beaker through muslin cloth and squeeze out all liquid

3. Add the dextrose dissolved in water to this extract.

4. Adjust the pH of medium to 6 to 6.5 using 0.1 N HCl or 0.1N NaOH as per need

5. Add the dissolved agar to dextrose-potato extract and make the volume to 1Ltr and dispense 200ml each to 5 conical flask and plug with non absorbent cotton.

Sterilise the flasks at 15 Ibs pressure for 15 minutes in a autoclave.

6. Allow the autoclave to cool, "Remove the conical flask and store at room temperature. Allow the flask to cool until the flask can be held by hand.

7. Prepare agar plate by pouring the media into Petri-dish quickly. Using aseptic condition, allow the media in Petri-dish to solidify to produce the agar plate.

Nowadays, readymade PDA can be purchased from market, which can be prepared directly @40gm/L of distilled water. Which is dissolved in water, boiled and autoclaved.

B. Preparation of Nutrient Agar Medium:

It is commonly used for the maintenance and isolation of bacteria.

Materials:

Peptone - 5gm,

Beef extract - 3gm,

Agar - 20g,

Distilled water -1Ltr,

Petri-dish, 1Ltr Beaker, 250 ml Conical flasks, Measuring cylinder, Non absorbent cotton, Autoclave and hot plate.

Procedure

1. Dissolve the weighed amounts of peptone and beef extract into 500 ml of water.

2. Heat and dissolve the chemicals and adjust the pH of medium to 7 by adding 0.1N HCl or 0.IN NaOH.

3. Weigh 20g agar and dissolve in 500 ml of distilled water in another beaker

4. Mix the dissolved agar with chemical solution and make up the volume to 1 Ltr.

5. Dispense 200 ml each into 5 conical flasks.

6. Plug the flask with non absorbent cotton and sterilize at 15 Ibs pressure for 15 minutes in a autoclave.

7. Allow the autoclave to cool, remove the conical flask and store at room temperature, or

8. Allow the flask to sufficiently cool and prepare agar plates by pouring media into Petri-dish under aseptic condition; allow the media with Petri-dish to solidify.

Precautions

1. Don't pour the media over 2/3 of flask capacity.

2. Cotton plug must be loose while autoclaving.

3. Don't pour media to Petri-plate when the medium is too hot since it produce condensation of water on underside of Petri plate lid and thus can fall on to agar surface and may lead to contamination and spreading of colonies.

4. Pour medium quickly to avoid contamination by air-pores and close lid down as soon as possible.

5. Perform the pouring of medium in inoculation/laminar flow chamber fitted with U. V. lamp with filtered air.

6. Pouring should be performed near the flame.

5. ISOLATION OF PLANT PATHOGENIC FUNGI FROM DISEASED PLANT MATERIAL

Isolation of the fungal pathogens from diseased material is made by surface sterilizing the diseased area with surface sterilizing agents, removing a small portion of the infected tissue (leaves, stems, fruits etc.) with a sterile scalpel, and plating it in a plate containing a nutrient medium. The most common method, for isolating fungal pathogens from infected leaves as well as other plant parts involves cutting several small sections 5-10 mm-square from the margin of the infected lesion to contain both diseased and healthy looking tissue. These are placed in surface sterilizing agents solutions for about 15-30 seconds the sections are taken out aseptically and blotted dry on clean, sterile paper towels or washed in three changes of sterile water and are finally placed on the nutrient medium, usually three to five per dish. The pathogen will grow from the sections and the colonies of the pathogen are sub cultured aseptically for further study.

Materials required

Infected specimens, sterile Petri -dishes, PDA slants, sodium hypochlorite solution (1%), sterile water, razor blade, forceps, inoculation needle, burner/spirit lamp, spirit, incubator, PDA medium.

Procedure

1. Select infected host tissue from the advancing margin of the lesions.

2. Cut into small pieces (2-5 mm) containing both the diseased and healthy tissue and keep in sterile Petri dishes

3. Dip the pieces into 1 % sodium hypochlorite solution for about one minute.

4. Transfer the pieces to Petri - dishes containing sterile distilled water and wash thoroughly in two changes of sterile water to free them from the chemicals if any.

5. Wash hands with rectified spirit and wipe the table top of inoculation chamber with rectified spirit.

6. Lit the burner

7. Hold the flask containing sterile Luke warm PDA in the right hand and remove plug near the flame. Lift the lid of Petri dish gently with left hand and pour about 20 ml of medium. Close the mouth of the flask with plug near the flame

8. After solidification of the medium, place four sterilized pieces at different distance in a single PDA plate.

9. Incubate the Petri dishes in an inverted position at 25° C and examine for 3-5 days.

Observations

Observe the incubated plates from the second day onwards for the growth of the fungus. Aseptically transfer the bits of mycelia from the margin of the colonies on fresh PDA slants for further study, sporulation and identification. Mycelia growth on the medium from the infected tissues indicates that the disease may be due to a fungus.

Isolation of fungus from soil (rakhne ki narakhne?)

6. ISOLATION OF PHYTO-PATHOGENIC BACTERIA FROM DISEASED PLANTS

Isolation and identification of bacteria associated with diseased plant is important to determine whether bacteria are involved in plant disease. The method normally used to isolate phyto-pathogenic bacteria differs from that used for fungi. A suspension of bacterium is prepared from the infected material and loopfuls of this are streaked onto nutrient agar plates. The aim is to produce single colony that can be sub-cultured pure.

Pure cultures are absolutely essential for pathogenicity assays and characterizing the pathogen for identification. The serial dilution method is used for isolating bacteria from diseased tissues contaminated with other bacteria. After surface sterilization of sections of diseased tissues, the sections are ground in small volumes of sterile water and then part of this homogenate is diluted serially. Finally, plates containing nutrient agar are streaked with a loop dipped in each of the different serial dilutions and single colonies of the pathogenic bacterium are obtained from the higher dilutions that still contain bacteria.

Choice of material: Selection of the diseased tissue is important because pathogenic bacteria may occupy different locations in the plant. In isolation of bacteria, it is generally better to use newly collected material. The earliest stages of symptom development should be used. Old lesions and dead areas usually contain few pathogens and many saprophytes. Necrotic diseases usually start with tiny, dark greenish, spots, which are excellent for isolations. Cankers and soft rots should either be at an early stage, or if, older lesions only are available, the advancing edge must be used, where the disease is spreading into healthy tissue. When crown gall is suspected in a woody plant a search must be made for young galls on young green stems. With wilts and other vascular infections small pieces of infected stem are usually good for isolation.

Preparation of material: Clean leaves and stems, carefully chosen and "handled aseptically, can often be used without surface sterilization. Roots and parts contaminated with soil should be gently washed with clean water as soon as possible after collection.

Medium: Nutrient agar is suitable for the isolation of most plant pathogens. The medium used for isolations must have a dry surface. If water is present the bacteria move around and a carpet of mixed growth results instead of the required single colony.

Materials

Disease specimens, growing media (petri-plates) surface sterilizing agents (1 % sodium hypochlorite), sterile razor blade, glass rod, sterile water, sterile test tubes and Petri-dishes, sterile pipettes (1 ml), inoculation loop.

Procedure

Put on the U.V lamp of inoculation chamber for 5 minutes. Wipe the table top with rectified spirit, and wash hands with rectified spirit and air dry. Lit the burner or spirit lamp, arrange sterile Petri dishes near the burner.

1. Select a diseased specimen and cut out a small portion of the diseased tissue from the advancing lesion using sterile razor blade in a drop of sterile water and after several minutes, examine under microscope. If bacterial ooze is seen, proceed for isolation

2. Surface disinfests the cut portions by dipping in sodium hypochlorite solution for 60 sec. and then immediately rinse three times with sterile water.

3. Immerse the disinfested cut portions in I ml of sterile water taken in a clean sterilised test tube.

4. Crush the cut portions of the leaf with a sterile glass rod. Allow it to stand for 5 minutes to allow the bacteria to diffuse out of the cut tissue and into the water.

5. Gently lift the lid of a Petri dish with left hand and using inoculation loop transfer several loopfuls of the bacterial suspension to sterile Petri-dishes (three) containing 1 ml of sterile water and mix thoroughly.

6. Hold flask filled with sterile Luke warm nutrient agar medium in the right hand and remove cotton plug near the flame and pour about 20 ml of medium into each dish and mix thoroughly by gentle rotation. Allow time for solidification of medium.

7. Incubate the dishes in an inverted position at 25°C for 36 to 72 hours.

8. Observation: Observe the dishes for appearance of desired bacterial colonies. If colonies appear, select consistently found and well isolated colonies of the pathogen, for sub-culturing and further studies.

9. Select the isolated colonies and streak on the surface of a solidified medium in a zigzag manner and incubate the dishes at 25^{o}C. Bacteria isolated from nature may be contaminated with saprophytic species; hence, re-streaking for isolation ensures a pure culture. Transfer some of the purified colonies to NA slants and grow them for further use.

Specifically following method is followed for bacterial isolation

The Plant Pathogenic Bacteria isolation from disease samples or diseased plants depends upon the nature of source from which the suspension has bee prepared. Some of them are explained as follows:

1. Leaf: - Take fresh leaf showing lesion of disease causing organism, surface sterilize the infected weak surface sterilizing agent i.e. (Sodium hypochloride/oxychloride) Chlorox. Mercuric Chloride should not be used because it may kill the pathogen. The concentration of sterilizing agent Chlorox is 0.5 to 1%, chemically known as Sodium Hypochloride a weak sterilizing agent, also known as Sodium Oxychloride.

The disease specimen was taken on Petri plates chopped with blade, before the sample or medium then confirm the bacterial infection by using ooze test.

Now take a loop full of suspension. Isolate the plant pathogens bacteria using following methods:

Serial dilution method: Streak plate method (only for isolation of bacteria) as pour plate method (counting individual colony).

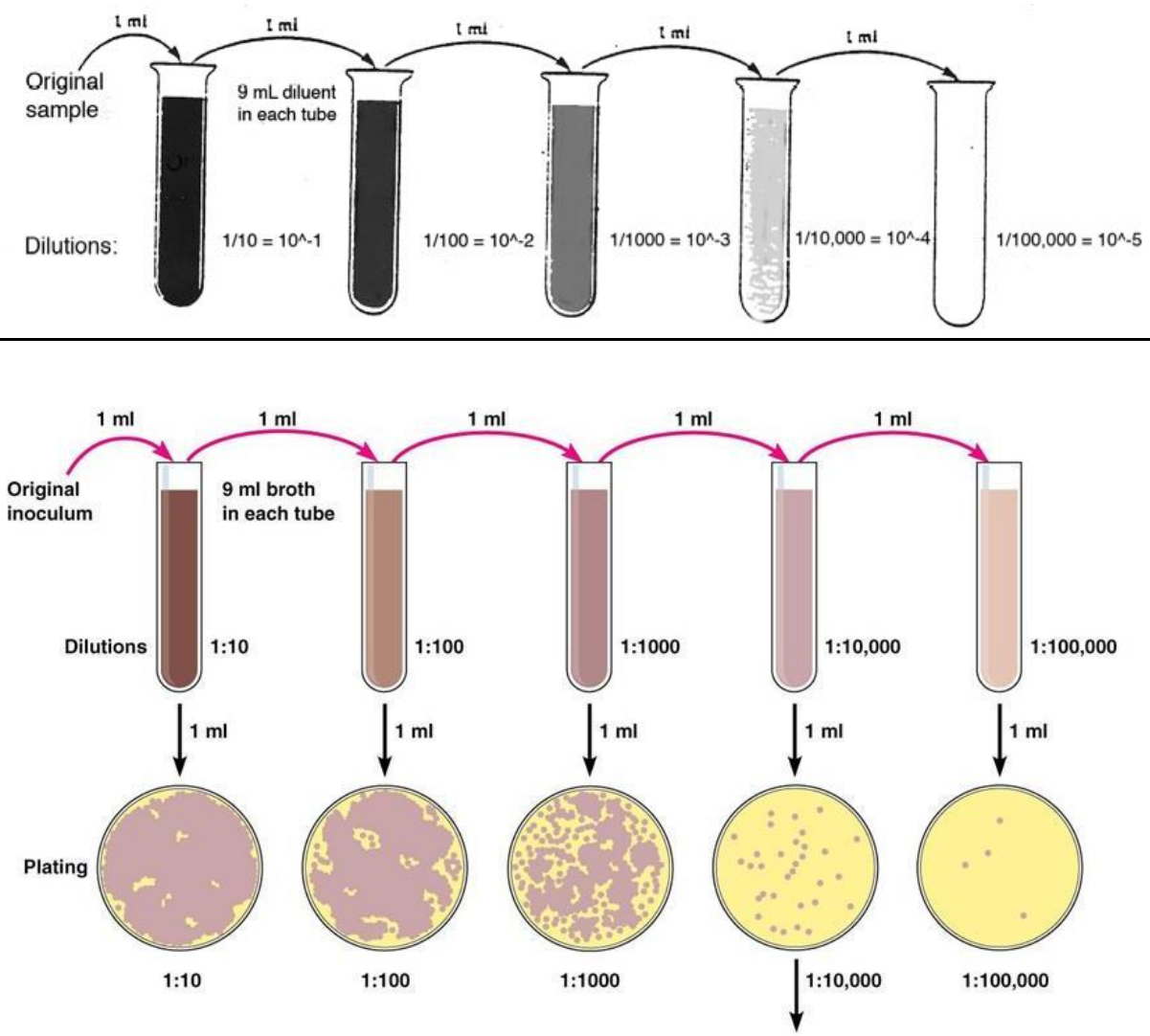


Fig. Serial dilution method

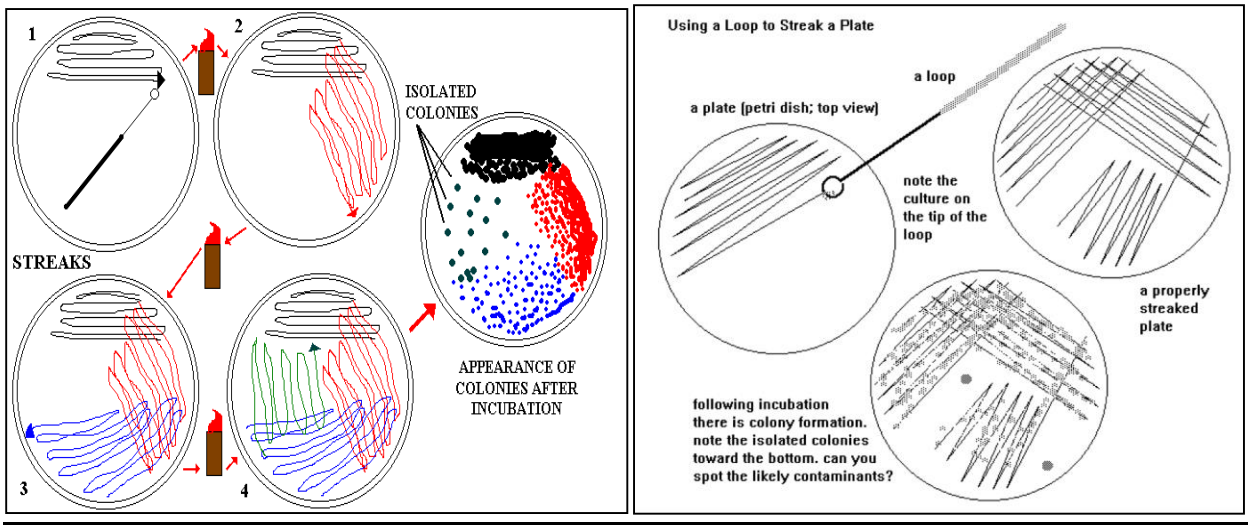


Figure: Streaking method

Plate count method: By pour plate method, Take 1 ml of the stock solution of the bacterial cell suspension aseptically, add it 9 ml of sterilized distilled water blank. After preparing cell suspension of obtained from the difference sources, isolate the bacteria by Agar plate (fungus conidia), Streak plate and Pour plate (both for bacteria).

In case of pour plate method,

First pour the bacterial cell suspension in sterilized Petri plate containing medium ranging from 0.1 ml to 1 ml depending upon population of plant pathogenic bacteria.

Take sterilized medium in 40-50 ^{0} C (Chick test), 10-15 ml per sterilized plate media is poured to each plate. Incubate the material in BOD (Biological Oxygen Demand) incubator 25 ±2 ^{0}c.Observe the development of colony and pick up the desired colony, then purify the culture using subsequent transfer and streak method. Maintain the pure strand and keep them in refrigerator.

2. Vascular tissue: - In this case, the infected tissue may be uniform. The sample must be taken from initial stage of infection. The vascular tissue containing infected parts is cut into pieces. Generally 1-2 cm in size- the

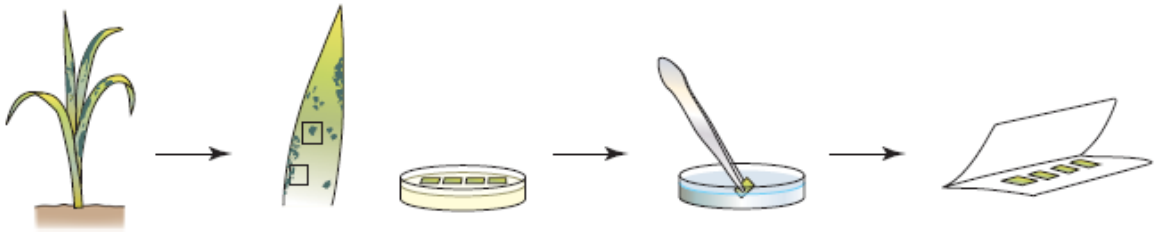
tissues are either surface sterilized or put directly and aseptically after washing with sterilized distill water.

Add 1-2 drops of sterilized distill water in surface sterilized tissue taken in Petri plates. Macerate with the help of sharp blade to get cell suspension. The maceration should also be done aseptically in such a way that the temperature of surrounding should not so high.

Then proceed for isolation of pathogen as discussed early.

In case of fleshy roots, leaves etc., the cell suspension of bacterium can be directly obtained after surface sterilization, later on by squeezing.

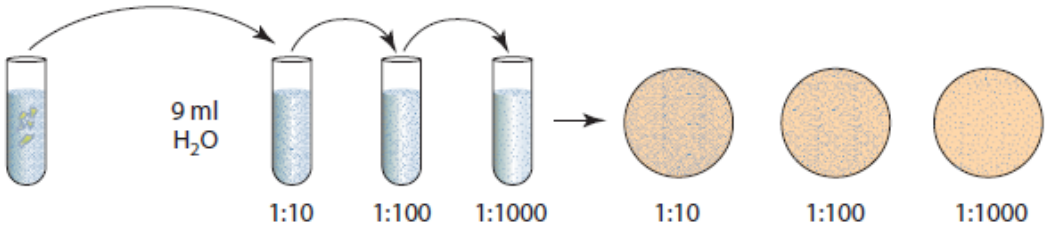
3. Galls/woody tissues: - They are surface sterilized by using Chlorox 1% for 1-2 minutes. Then small pieces of samples are taken in sterilized Petri plates. The infected tissue is macerated to prepare the bacterial cell suspension. This macerated portion is used for isolation of PPB as discussed earlier.
4. Seeds: - Incase of seed infection, the bacterial cell suspension is obtained by taking soaked seed for 4-6 hours in distilled water. The soaked seeds are macerated and the extract is prepared. The surface sterilization of the seed is followed only in those cases, in which the bacterial cells are present inside the seed coat. The seeds on which the bacteria adhere to the surface should not be surface sterilized because the bacteria will be killed during surface sterilization.



Infected plant

Cut out small infected areas or at margin of large one. Place in 10% Clorox for different durations

With sterile forceps rinse tissue sections in distilled water and blot on sterile paper towel



Place tissue pieces in tube of sterile water and macerate

Make serial dilution by transferring 1ml of bacterial suspensions from one tube to the next

Place 0.5ml of each dilution into separate petri dishes. Add melted but cool agar, stir gently and let solidify

In a few days single colonies appear at one or more of the plates

Single colonies are subcultured and the properties of their bacteria compared

7. ISOLATION OR EXTRACTION METHODS FOR NEMATODES FROM PLANT AND SOIL SAMPLE

There are several methods of extraction of nematodes from soil and plant samples, such as Baermann funnel, Bearmann tray combination, Cobb's decanting-sieving method, centrifugal flotation with heavy sugar, Elutriation, Fennick can method etc. One or more methods are often combined for the extraction of plant pathogenic nematodes. Selection of methods depends upon facilities available, type of targeted nematode, size of sample, time of collection etc. Nematode populations may consist of relatively motile to quiescent forms such as cysts, individual eggs, eggs in masses or various combinations of these. Ectoparasitic forms occur mainly in the soil, whereas high percentages of endoparasitic nematodes are found in roots, root fragment or other plant parts during certain period of the year. Efficiency of extraction depends on the nematode type as well as extraction methods with their respective nematodes, which are as follows:

1. Baermann funnels	Trichodorus, Helicotylenchus
2. Baermann trays	Trichodorus, Helicotylenchus
3. Centrifugal floating	Mostly all types of nematodes
4. Elutriator	Criconemoides, Criconema, Hemicreconemoides

EXTRACTION FROM SOIL SAMPLE

- Take about handful (250g) from different field depth 5-25cm 20 sasmple per ropani.
- Mix all sample and weight 100g put in a beaker.
- Now put 100g sample in a sieve 35mesh-500µ pore size.
- Put in metallic sieve, disperse soil particle with water and collect in a bucket, and let the suspension stand for 1minute until this soil sediment (but nematodes are still swimming in the sample).
- Now filter the suspension from the 50 micron and repeat the process again. In case 100 micron, repeat the process for the 4 times.

- Purify the sample from the Baremann fennel method and observe the sample after 24 hours whether nematodes are present of not.

Baermann funnel method

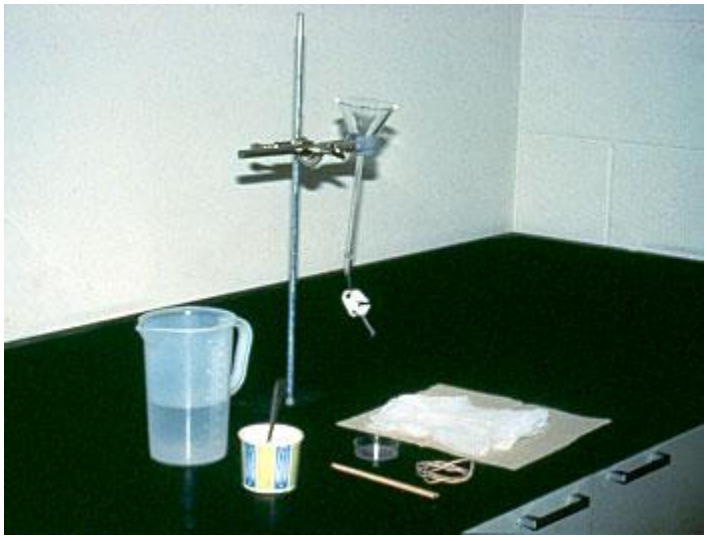
This is the useful method for collecting smaller nematodes from the soil or endoparasitic nematodes from infected plant tissue. It is based on the principle that nematodes have a tendency to move downward where the soil or plant material is placed in funnel. Only active and motile nematodes are collected by this method.

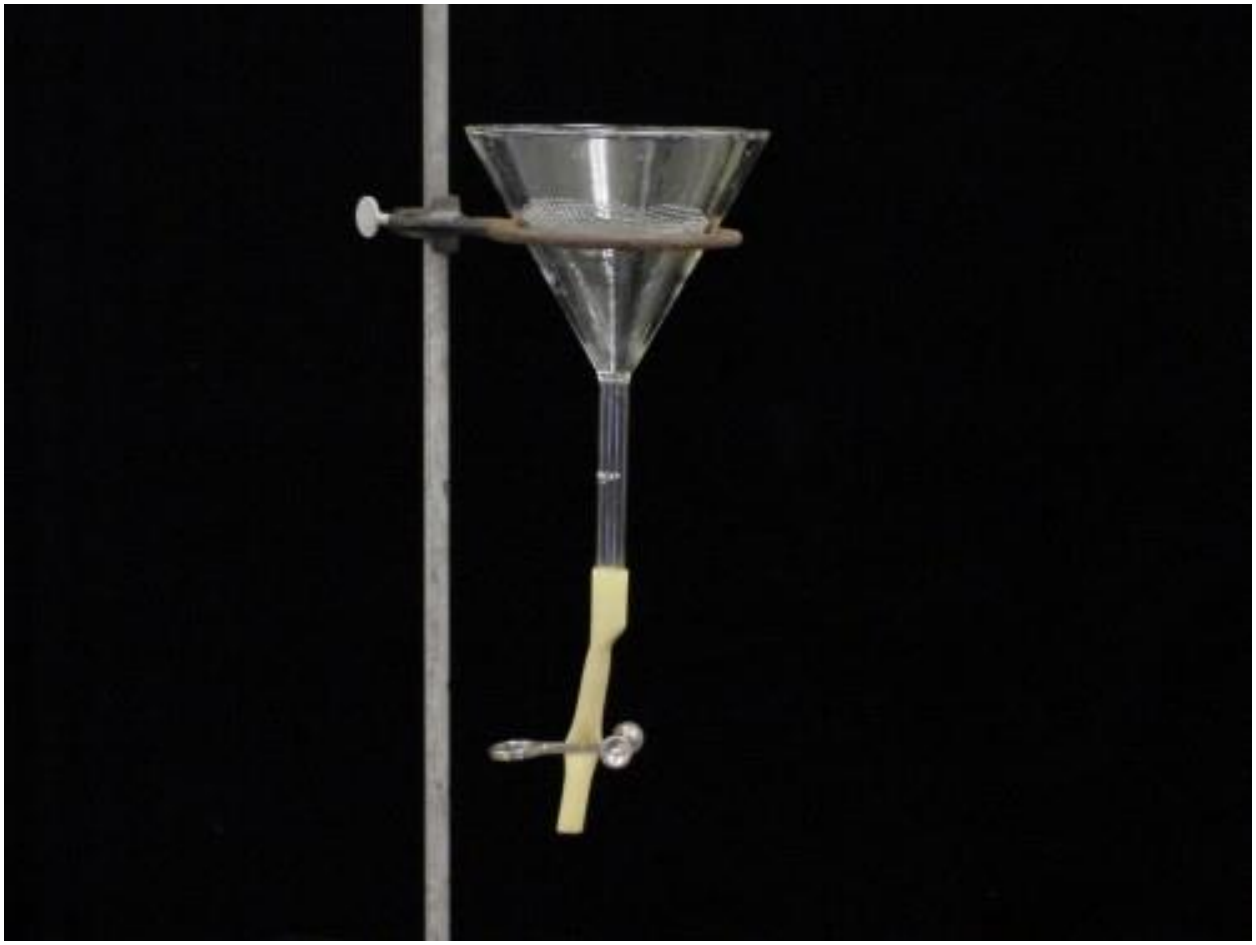
Procedure

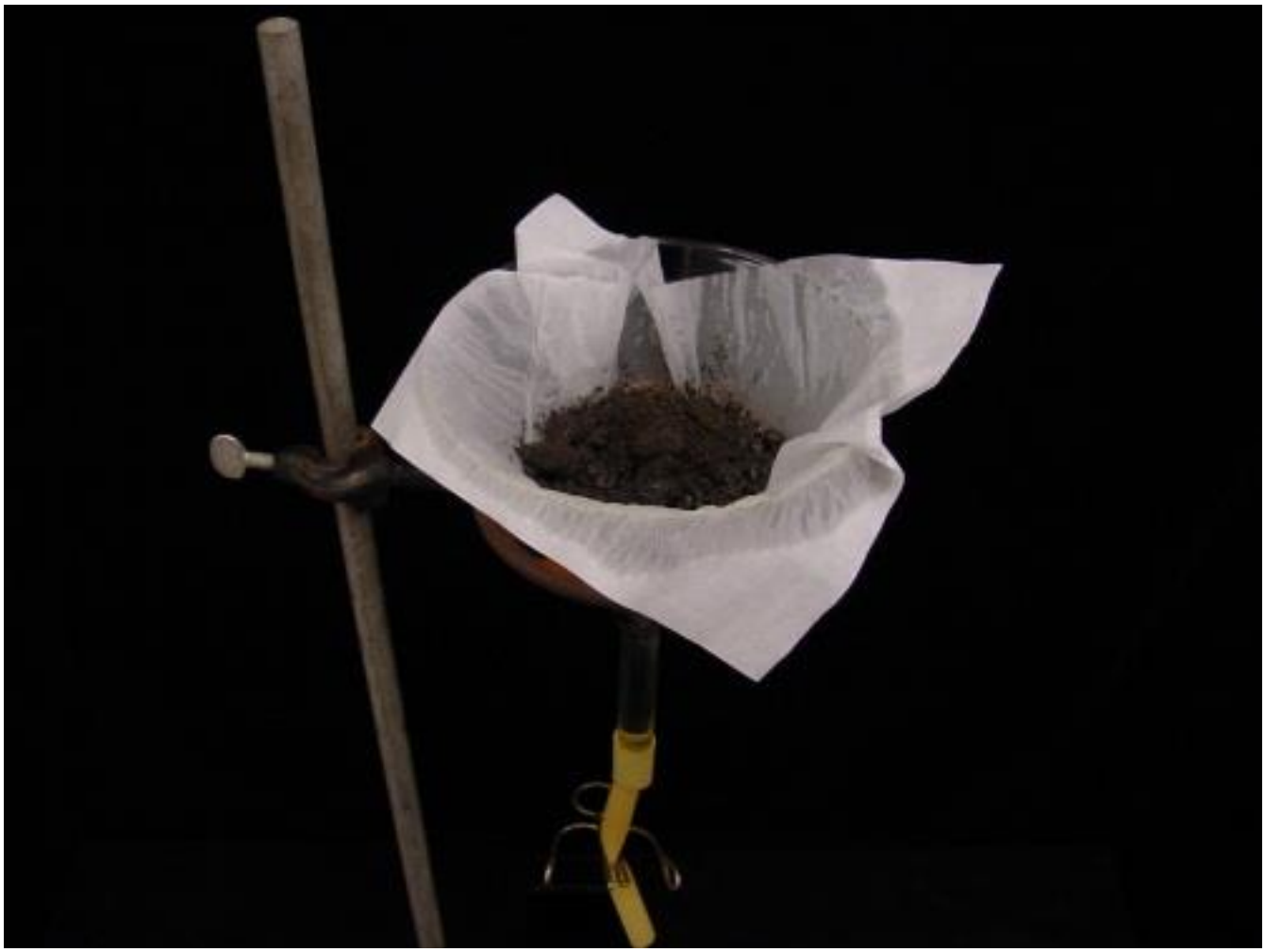
1. Take 200 gm of soil sample and place it on fine tissue paper in a wire-gauge and place it in a funnel filled with water. The funnel has rubber tubing clipped. Care is taken that there should not be air bubble inside the tubing. (Please see the figure)
2. Leave the sample in the funnel for 48 hours for nematode movement due to gravitational forces.
3. After 48 hours, collect nematode suspension in a beaker.
4. Let it settle for 30 minutes and reduce the suspension and observe under dissecting microscope.

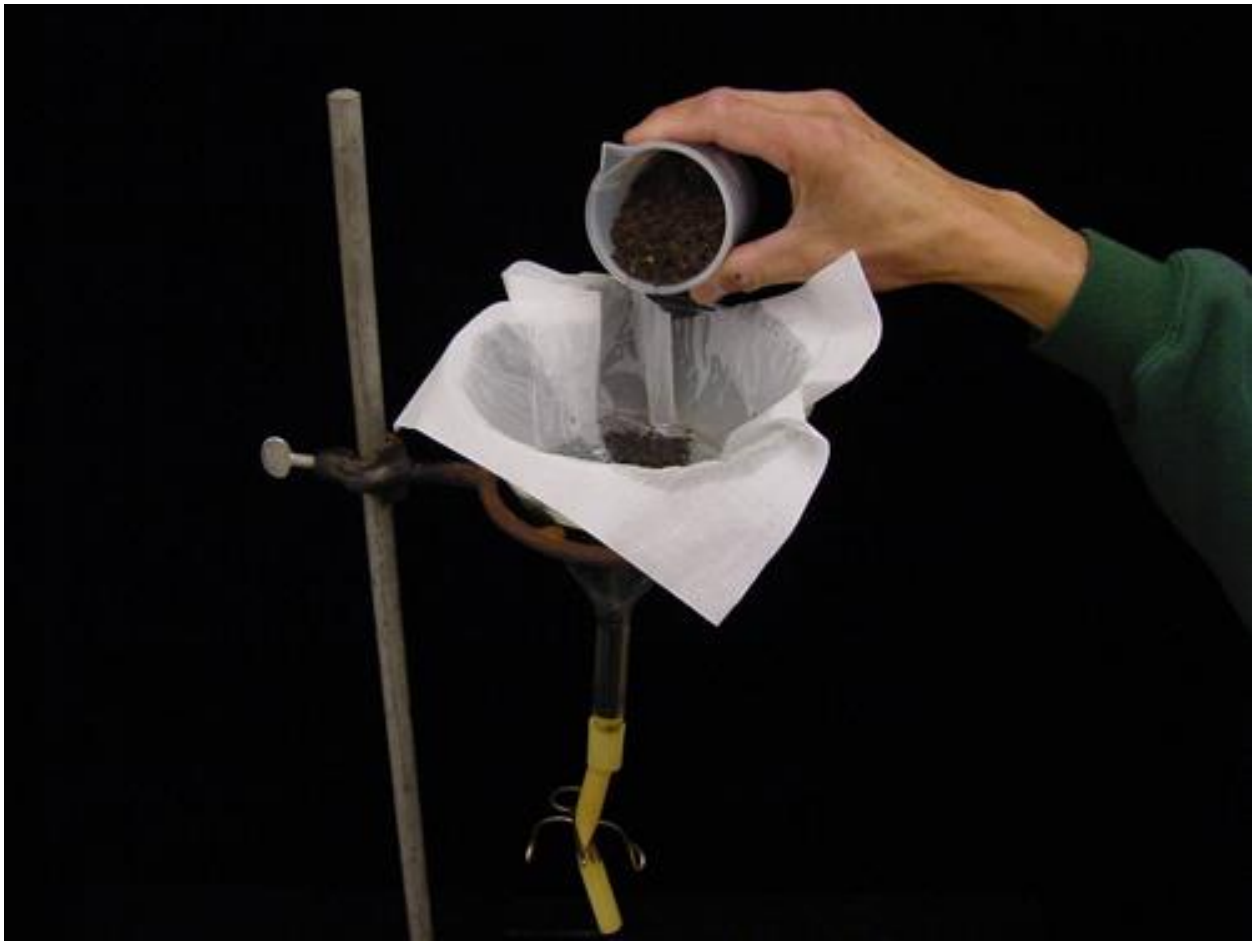
Observation:

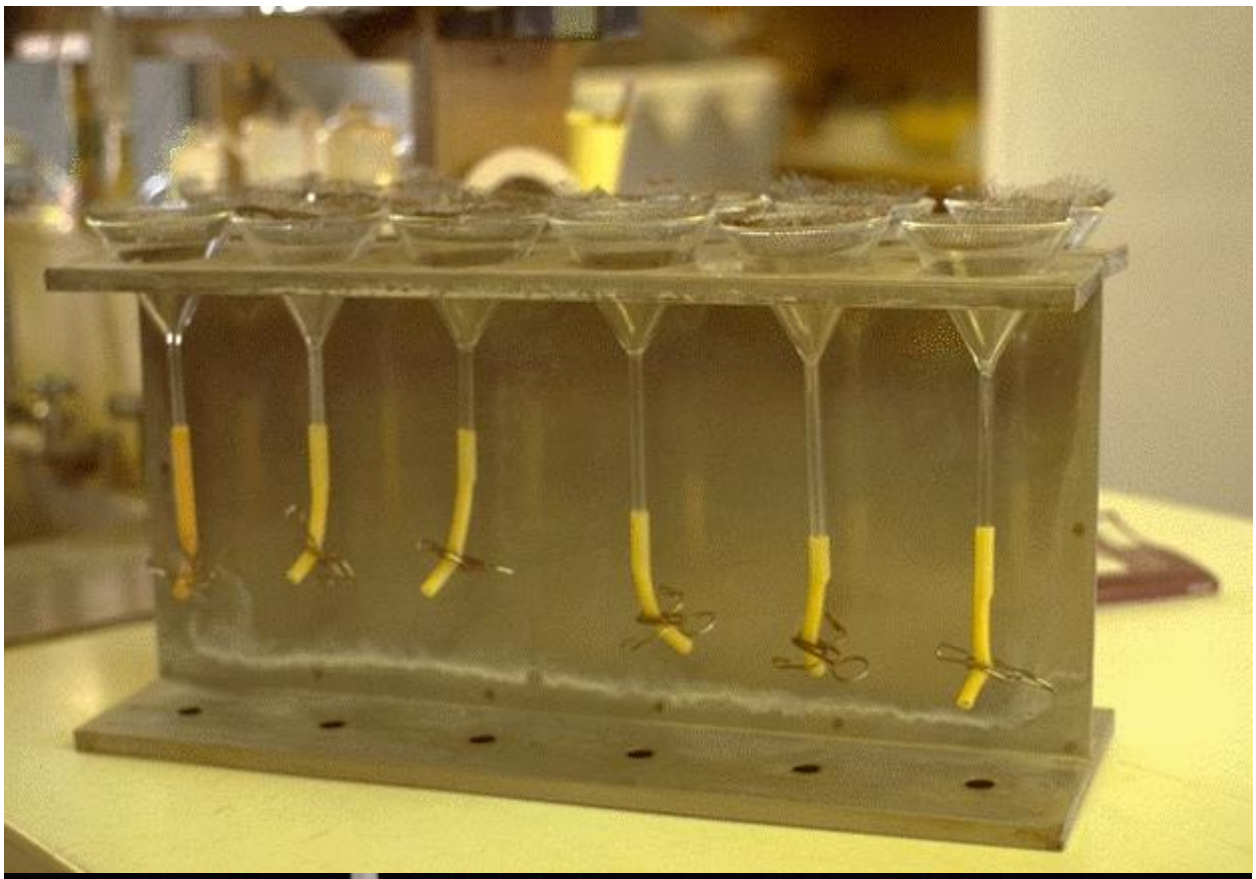
Different plant parasitic and free-living nematodes will be observed by these isolation methods. See the presence of stylet in plant pathogenic nematodes.

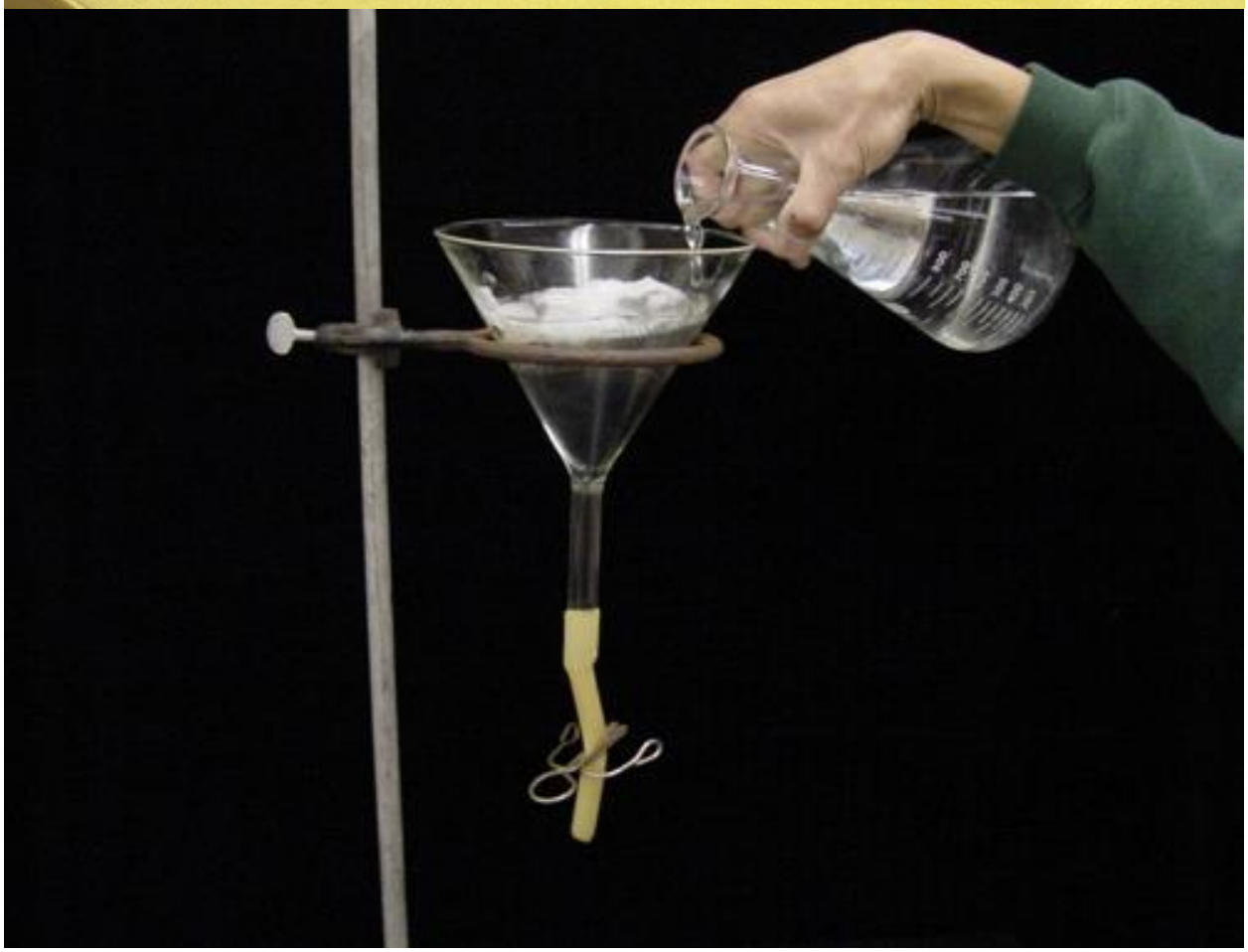












Extraction of nematodes from the plant sample

Isolation of nematodes in from plant tissue viz. roots seeds and stems can be done in a number of ways. The most common method is teasing. Simple technique of teasing method can be conducted as follows

1. Remove the suspected part and place in a glass slide containing water
2. With the use of dissecting needles tease out the tissue apart.
3. Examine the material under the microscopes for the presence or absence of the nematodes.
4. Record the color, movement and stage of devolvement of the nematodes

- Please consider, for the collection and isolation of nematode; different infected or suspected plant part may have different types of nematodes i.e. Dichylenchus-stem, Aphelenchus-leaf nematode, Hetereopdera/Globodera cyst

-Also after cutting/tearing the infected tissue, we can isolate nematodes by Baermann funnel method

- Cut or serrate of the sample
- Tear in small piece. Then extract by Baermann funnel method. Now they come out and swim freely in water and settle down as they have higher specific gravity of nematode. Root knot nematode lays eggs in mass (400-500) with in a jelly. Female, juvenile can also be extracted from gall for this collect root with fresh gall, Record symptoms, Observe gall under microscope where we can see male egg, female can be observed or egg can also collect and observed under microscope

Different genera of nematodes can be identified on the basis of :

- Body length and thickness

- Size and shape of the stylet, it may be straight, curved, thick, thin or curved.
- Size of lips, location of the vulva from the mouth and tail

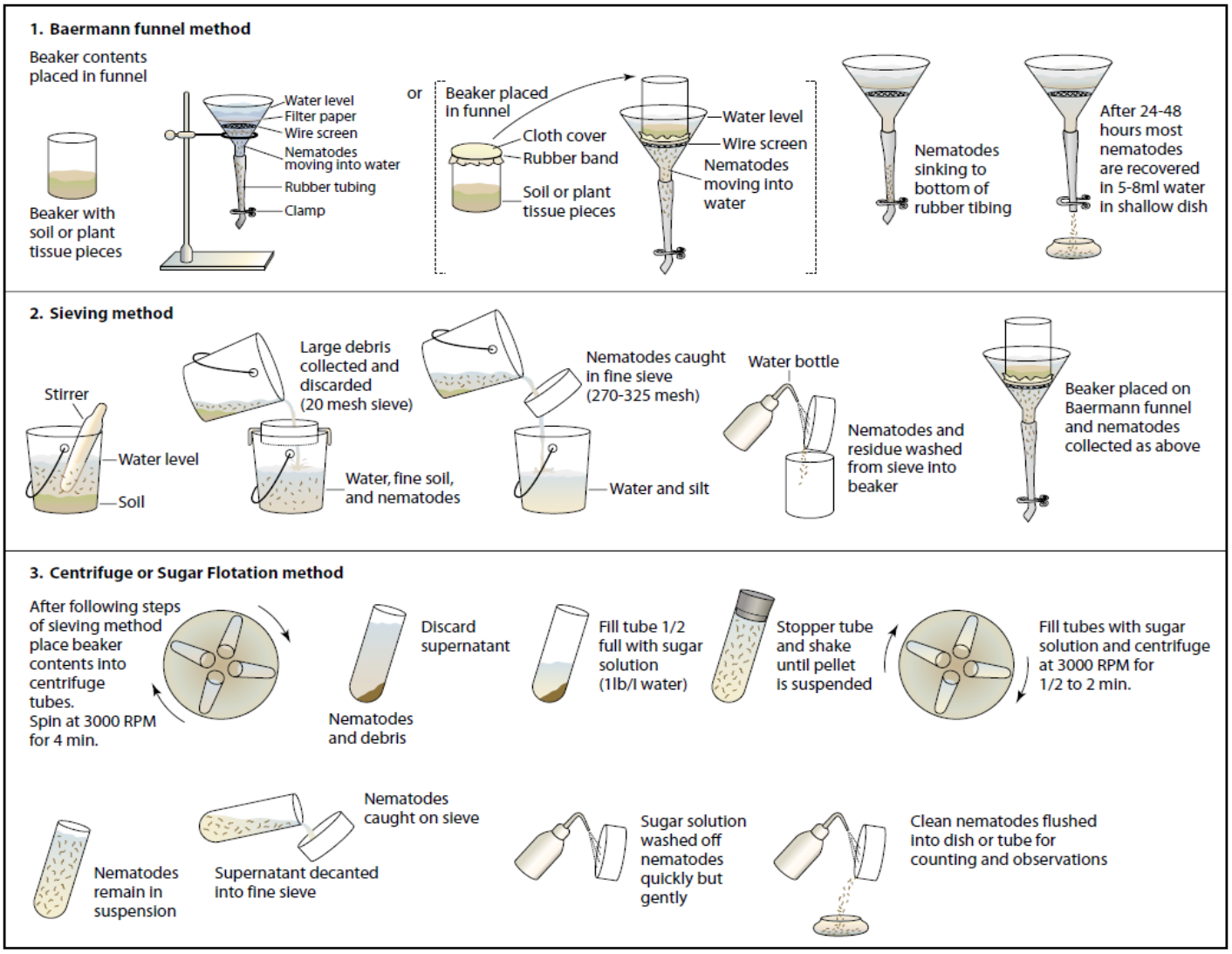


Figure: Isolation of plant pathogenic nematodes from soil, plant parts (Source: Agrios G.N.,2005)

8. MAINTENANCE AND STORAGE OF VIRUS ISOLATES

Introduction

Virus isolates can be maintained for long periods by sub culturing on their respective hosts, but may become contaminated or attenuated during repeated sub culturing. It is advisable, therefore to store samples of the original isolates as soon as possible after they have been acquired. When a fresh isolate is obtained, its concentration should be built up by sub-culturing once or twice in a suitable host plant. Then it should be preserved by any one of the following methods.

1. As dried powder

i. Place infected leaves over anhydrous calcium chloride in an airtight, screw topped bottle for several months.

ii. Grind the dry leaves to a powder with a pestle and mortar, and store in a tightly sealed capsule.

2. In liquid nitrogen

i. Grinding infected leaves in K_2HPO_4/Na_2SO_3 solution as if preparing inoculum for sap transmission.

ii. Place 0.5 ml aliquots of sap in sealed capsules and store under liquid nitrogen in a commercial storage flask.

3. By lyophilization

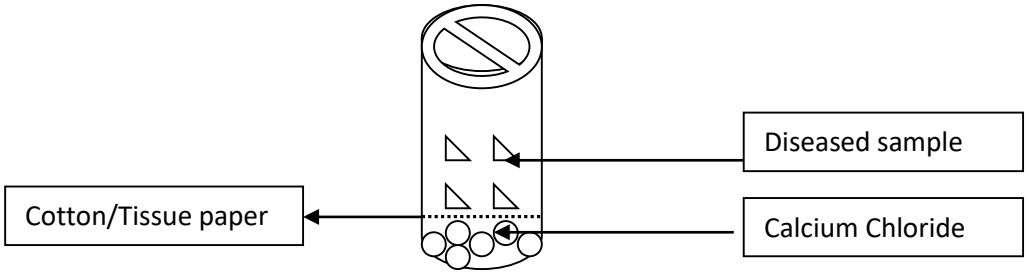
I. Take a small piece of infected leaf and place in a glass ampoule suitable for the freeze-drying machine to used. Add a label to identify and date the isolate. Constrict the tube and evacuate the air. Seal the tube and store at room temperature.

II. Alternatively, grind infected leaves in a minimal volume of K_2HPO_4/Na_2SO_3 solution. Take 10 ml of the filtered sap and add o.7 gm D-glucose + 0.7gm peptone. Shake the mixture to dissolve the additives. Pipette 0.25 ml aliquots of the mixture

in to the freeze drying ampoule. Add a small plug of non adsorbant cotton wool, followed by a suitable paper label. Proceed the lyophilization as described above.

Procedure for dry preservation

First collect and place viral infected in laboratory by pressing in newspaper and in open air to dry these samples. After a week after complete drying, grind sample to a powder form with mortar and pestle. Then place sample in airtight bottle with



calcium chloride ($CaCl_2$).

The following is the purification method of plant virus (Source: Agrios G.N., 2005)

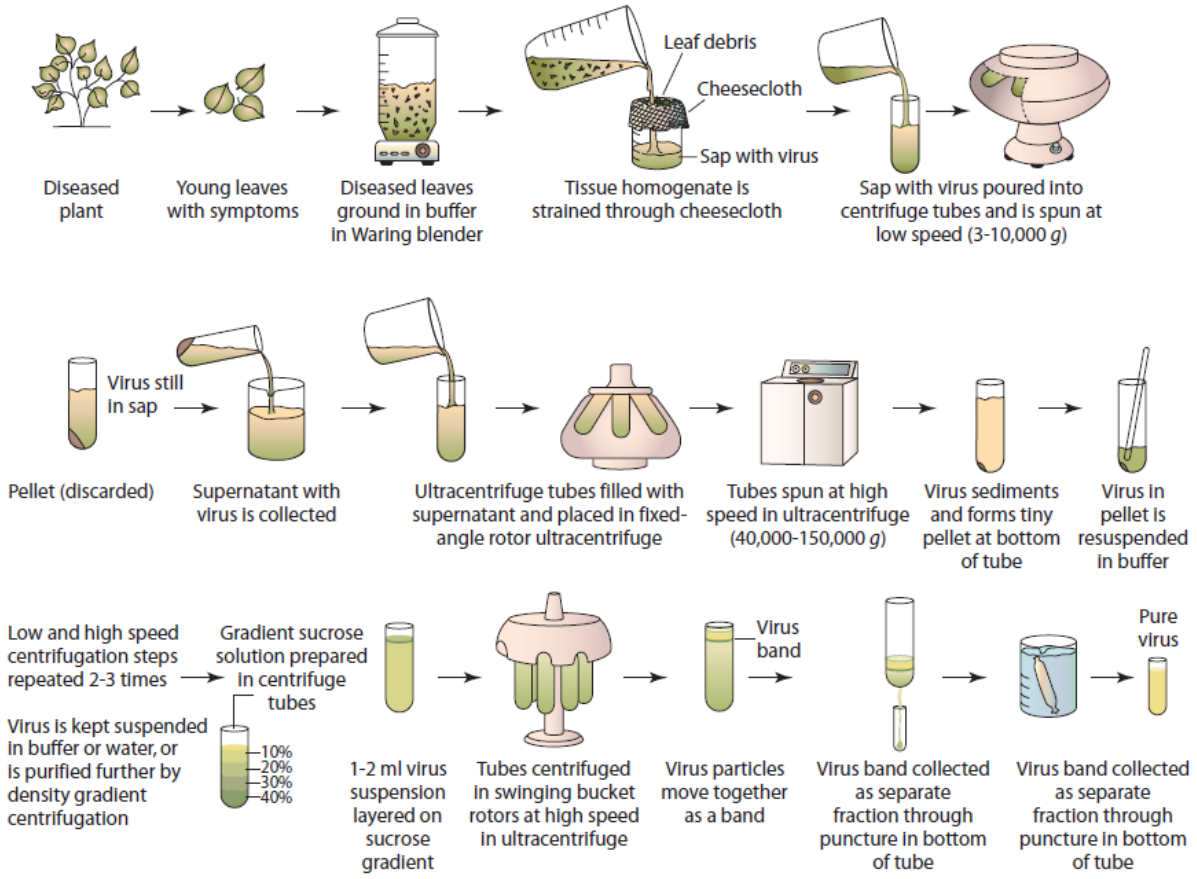


Figure: Purification and maintenance of plant virus (Agrious, G.N., 2005)

8. PRESERVATION OF DISEASE SAMPLES

Preservation of diseased specimens is of much importance in the study of plant diseases. Such preserved specimens can be used as reference materials for comparisons. Dry and wet specimens of fungal and bacterial diseases can be preserved.

a. Collection and dry preservation of plant disease specimen

The main objective in preservation of plant specimens for herbarium use and storage is to dry the material quickly to prevent moulding and minimize discolouration.

The preserved specimen should remain in readily recognizable condition. The plant press is commonly used in the dry method. Plant or plant parts are arranged inside the folder newspaper, sheet with care that the materials remains flat and not

variously folded. The news paper is then placed between the blotters and the press is tightened.

For drying it is placed in the sun or in a drying oven. When the plant is dry, it is placed in paper packets or mounted on herbarium sheets. The fleshy specimens can be dried in drier fitted with an electrical bulb.

Materials Required: Paper envelopes, plant press with blotters and newspapers, herbarium sheets.

Procedure

1. Collect infected plant parts and place them in paper envelops. Use separate envelops for different kinds of diseased specimens and bring to the laboratory.

2. Arrange (spread) the diseases specimens on the sheets of newspaper.

3. Place the newspaper between the blotters and tighten the bolts of the press.

4. Change the blotters at frequent intervals till the specimens become dry.

5. Keep the press under sun for complete drying.

6. Remove dried specimens from the press and keep them in non absorbent tissue papers.

7. Examine a few bits of specimens as early as possible after collection and identify the pathogen and "label all the specimens giving the following information.

a) Name of the collector:

b) Specimen Number:

c) Name of the Host:

d) Name of the causal organism (binomial):

Record

Label all the specimens properly and prepare a list of specimens preserved

Make sketch of plant press, if possible, along with the specimen.

b. Wet preservation of diseased specimens

Formalin-acetic alcohol (FAA) is a good killing and fixing agent. After treatment with FAA, specimens can be stored in dilute (5 % commercial) formalin. Virus affected green tissues, rust affected plant parts and leaf spots can be preserved in copper acetate solution.

Materials: Fresh diseased leaves, formaldehyde/formalin, ethyl alcohol, glacial acetic acid, copper acetate crystals, zinc chloride, glycerol, vessel for heating, glass containers with screwed tops.

Procedure

For general wet preservation of diseased specimens use the following preservatives.

5% formaldehyde (40%) in water : 40 ml

Ethyl alcohol : 150ml

Water : 1 litre

For colour preservation of green plant parts, follow the following steps

a) Thoroughly wash the specimen in running water

b) Place leaves in the boiling mixture containing 1 part of glacial acetic acid saturated with copper acetate crystals and 4 parts of water

c) Continue boiling until copper acetate replaces the natural green color of cells, (approx. 15 min.)

d) Take out the material; wash thoroughly in running water again to remove excess copper.

e) Preserve the specimen in 5% commercial formalin in glass containers/specimen jars with screwed caps.

For preservation of colored fruits or stems of woody tissues, the prepare the following solution

Zinc chloride: 50 g

Formaldehyde (40%): 25 ml

Glycerol: 25 ml

Water: 1 litre

Wash and preserve the specimens as such in the above solution without boiling.

9. PURE CULTURE MAINTENANCE OF PLANT PATHOGENS

Collection of the diseased material and isolation of the pathogens

- Sample showing clear symptoms of the disease should be used for isolation of the pathogen.
- To avoid saprophytic growth on the diseased samples, paper bags should be used for collection, storage and transportation of diseased samples.
- A dried material is always more suitable for this purpose than wet samples.
- The tissue should be disinfected/surface sterilized in 10% Clorex (0.5%) solution for 2 minutes.
- Thereafter, wash the material thoroughly using sterilized distilled water.
- The fresh leaf spot together with healthy tissue are cut out in small sections with a sterilized knife and put on to the surface of the sterile pates (9mm diameter) of nutrient medium under aseptic condition preferably under laminar flow.
- These plates are incubated at 25-30^{0}Cfor 7 days.
- The pathogens grow out of the host tissue.
- A small segment o this culture is further transferred to a fresh sterilized culture pate of nutrient medium as state above for sub culturing and incubated for 7 days.
- For diurnal fungal pathogens, The culture plates are incubated at an alternate period of 12h ADL (Artificial Day Light) or NUV (Near Ultra Violet light) Light and 12h darkness.

(i) Streaking technique

A loopful of spore suspension or the fungus of a culture is streaked onto agar. The spore suspension is streaked on the agar on one side of the petri dish and streaks it up and sown that side until the spores are evenly spread along a line. The

streaking is done with an inoculating needle having its tip bent into a loop so that it will not cut the surface of the medium, sterilize the loop and do a second steal at right angle to the first along another side of the dish. This results in a second line bearing far fewer spores than the first. Repeat this a third time to yield another line. If it seems necessary, do it a fourth time to yield the fourth line but be careful not to streak into the first line. After two or more days one of the streaks will be seen to contain several separate colonies that can be used for transferring or else the single spore can be located on the fourth line in the centre of low power microscope field and the area illuminated in a darkened room by closing the diaphragm. The area is cut around as disc with a suitable tool usually with a flattened appear-shaper tip of platinum needle and the spore is transferred to a fresh agar plate or tube. A number of microscope attachments have also been derived to facilitate spore location. One such devise is a glass tube drawn out to a cone and fixed around the lens of a low –power objective. The smaller end (1.8mm) is racked sown into agar to cut a circular plug on which a single spore has been located. A similar method uses a biscuit cutter mounted below an objective and centered. This is swung into a place where he single spore is located and lowered to cut a disc of agar. The discs of agar thus cut and are transferred by use of needle to fresh agar plates or tubes.

(ii) Spore suspension dilution technique

A similar result can be obtained by using dilution of spore suspension as described under streaking technique. It will, however, prove more useful than the streaking technique when spores are mixed and outnumbered in a sample of spore suspension. For locating the single spore of the desire type, just take a small loopful of mixture of spore suspension and stir it up in above 10ml of sterile water and salute down several times and plate out each dilution separately. The ideal dilution will produce plate containing about 60 spores scatted all slants as described earlier or the spores may be allowed to produce the colonies fo culture of different isolate/races of speculating pathogens as *Alternaria* and *Helmithosporium* species. Alternatively, the spore suspension is prepared and sufficiently diluted till1 ml of suspension contains not more than 5-10 spores. The spore suspension (0.5-1 ml) is poured aseptically over a molten bu still warm plain agar 92%) plate. The plate is

then observed under the microscope and the area having spores are marked with a glass pencil on the back of the petri dish. Now the spore along with some medium is scooped out and transferred to a slant to obhtain a single – spore culture.

(iii) Single – hyphal tip culture

In this method, the growth of spore is allowed on a plain agar for 24-48 hr as described above and hyphal tip coming out from the single spore cell of multiseptate spore are marked and transferred.

10. STERILIZATION TECHNIQUES IN LABORATORY

- **Sterilization**

A physical or chemical process that completely destroys or removes all microbial life, including spores.

- **Disinfection**

It is killing or removing of harmful microorganisms

- **Disinfectant**

Products used to kill microorganisms on inanimate objects or surfaces.

- **Antiseptic**

A product that destroys or inhibits the growth of microorganisms in or on living tissue.

- **Aseptic**

Characterized by the absence of pathogenic microbes.

1. Physical methods
    - Heat
        - Dry
        - Moist
    - Radiation
        - U.V. light
        - Ionizing radiation
    - Filtration

2. Chemical Methods

Methods of Sterilization

Radiation:

- U.V. light- Has limited sterilizing power because of poor penetration into most materials. Generally used in irradiation of air in certain areas eg. Operating Rooms and T.B. laboratories.

Ionizing radiation- *e.g.* Gamma radiation: Source Cobalt^{60} has greater energy than U.V. light, therefore more effective. Used mainly in industrial facilities e.g. sterilization of disposable plastic syringes, gloves, specimens containers and Petri Dishes.

- Filtration
- May be done under either negative or positive pressure. Best known example is the membrane filter made from cellulose acetate. Generally removes most bacteria but viruses and some small bacteria *e.g.* Chlamydias & Mycoplasmas may pass through. Thus filtration does not technically sterilize items but it is adequate for circumstances under which it is used.
- Main use: for heat labile substances e.g. sera, antibiotics.

- **The recommended size filter that will exclude the smallest bacterial cells is 0.22 micron**
- Sterilization by Heat: Most common method
- Dry Heat
  - Simplest method is exposing the item to be sterilized to the naked flame e.g. Bunsen burner- for sterilizing bacteriological loops, knives, blades.
  - Commonly used time per temperature regimes are 1 hrs at 180 0c, 2 hrs at 170 0c.Glassware should be completely dry before placing in a hot air oven

- Used for Metals, Glassware

Moist Heat: Uses hot water. Moist heat kills microorganisms.

Boiling – quite common especially in domestic circumstances.

Tyndallization named after John Tyndall

- Lengthy process designed to reduce the level of activity of sporulating bacteria that are left by a simple boiling water method.

Moist heat:

Tyndallization

- The process involves boiling for a period (typically 20 minutes) at atmospheric pressure, cooling, incubating for a day, boiling, cooling, incubating for a day, boiling, cooling, incubating for a day, and finally boiling again.
- The three incubation periods are to allow heat-resistant spores surviving the previous boiling period to germinate to form the heat-sensitive vegetative (growing) stage, which can be killed by the next boiling step.
- The procedure only works for media that can support bacterial growth - it will not sterilize plain water.

Moist heat:

Pasteurization

- It aims to reduce the number of viable pathogens in liquids so they are unlikely to cause disease
- It uses heat at temperatures sufficient to inactivate harmful organism in milk. Does not achieve sterilization.
- Temperature may be 138°C for a fraction of a second (flash method), 71.7°C for 15-20 seconds or 62°C for 30 minutes.

Autoclaving – Standard sterilization method in hospitals.

- The Autoclave works under the same principle as the pressure cooker where water boils at increased atmospheric pressure *i.e.* because of increased pressure the boiling point of water is >100°C.

The autoclave is a tough double walled chamber in which air is replaced by pure saturated steam under pressure.

Sterilization by Chemical Methods

- Useful for heat sensitive materials e.g. plastics and lensed instruments endoscopes).
- **Ethylene Oxide Chamber:**
    - Ethylene oxide alkylates DNA molecules and thereby inactivates microorganisms.
    - Ethylene oxide may cause explosion if used pure so it is mixed with an inert gas e.g. Neon, Freon at a ratio of 10:90
    - It requires high humidity and is used at relative humidity 50-60% Temperature : 55-60°C and exposure period 4-6 hours.
- **Activated alkaline Glutaraldehyde 2%:**
    - Immerse item in solution for about 20 minutes if organism is TB. In case of spores, the immersion period is extended to 2-3 hours.

Types of Disinfectants

Phenol and phenolics

- Phenol (carbolic acid) is seldom used today. Derivatives of the phenol molecule, however, are widely used.

- Phenolics injure plasma membrane, inactivate enzymes, or denature proteins. They are stable, persistent, and are not sensitive to organic matter.

O-Phenylphenol

- It is the main ingredient in most formulations of Lysol.

Hexachlorophene

- It is main ingredient of a prescription lotion, pHisoHex, used in nurseries and for surgical and hospital microbial control procedures to control gram positive skin bacteria such as staphylococci and streptococci.
- Excessive use can cause neurological damage.

Triclosan

- It is a widely used found in many household products. It has broad spectrum of activity, especially against gram positive bacteria. It is also effective against gram negative bacteria and fungi.

Biguanides

- Chlorhexidine, a member of the biguanide group, is not a phenol, but its structure and applications resemble hexachlorophene. It is frequently used for surgical skin preparation and surgical hand scrubs.

Halogens

- **Iodine** is effective against all kinds of bacteria, many endospores, fungi, and some viruses. Its mechanism of activity may be its combination with the amino acid tyrosine in enzyme and cellular proteins.
- An iodophore is a combination of iodine and an organic molecule. Iodophores do not stain and are less irritating than iodine. Examples are Isodine and Betadine.

- **Chlorine** is used as a gas or in combination with other chemicals. Chlorine gas is used for disinfecting municipal water supplies, swimming pools, and sewage. Sodium hypochlorite – ordinary household bleach- is good disinfectant.
- Chloramines consist of chlorine and ammonia. They are more stable than most chlorine. The U.S. military uses tablets for field disinfection of water.
- Chlorine dioxide in gaseous form is used for area disinfection, most notably to kill endospores of anthrax bacteria.

Alcohols

- Both ethanol and isopropanol (rubbing alcohol) are widely used, normally at a concentration of about 70%.
- Concentrations of 60% to 95% are effective.
- They are bactericidal and fungicidal but are not effective against endospores or non-enveloped viruses.
- Alcohols enhance the effectiveness of other chemical agents.

Heavy metals and their compounds

- Tiny amount of heavy metals (e.g. silver and copper) are effective antimicrobials. A silver coin on an inoculated nutrient medium will inhibit growth for some distance.
- 1% silver nitrate solution has been used to prevent gonorrheal ophthalmia neonatorum, which the infants might have contracted as they passed through the birth canal (recently been replaced by antibiotics).
- Silver-sulfadiazine is used in wound dressings. Available as topical cream for use on burns.

- Mercuric chloride is highly bactericidal, but is toxic and corrosive and is inactivated by organic matter. Organic mercury compounds such as Mercurochrome are less irritating and less toxic than inorganic mercury.
- Copper sulfate is often used to destroy green algae in reservoirs or other water.

Zinc chloride is used in mouthwashes, and zinc oxide is used in paints as antifungal.