Plant Disease Diagnosis Practical Laboratory Manual

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Plant Disease Diagnosis

Practical Laboratory Manual

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I. Introduction

The term *plant disease* refers to impairment in the structure or function of a plant that result in observable symptoms. Some plant diseases are easily recognizable based on specific symptoms expressed on host plants but others may not, hence a structured diagnostic process is recommended. Control measures depend on proper identification of causal agents of diseases. Therefore, disease diagnosis is one of the most important aspects of plant pathology. A definitive diagnosis is not always possible or even necessary. Most disease diagnosis procedures at the laboratory level aim to narrow the list of possibilities to a point where an informed management decision can be made.

Laboratory work is extremely important in plant pathology. Basic research is conducted in the lab, plant pathogens are isolated and identified, and inoculum is produced for doing artificial inoculations in both field and greenhouse. A well organized, equipped and accredited diagnostics laboratory that minimizes false positive results as much as possible not only gives reliable conclusions for disease management interventions but also saves a waste of time and money.

The ultimate goal of a Gene bank is to provide healthy PGR for utilization in breeding programs so that better and good quality crop varieties with desired traits can be developed for improving crop productivity. A fully-equipped Seed Health Testing laboratory is essential for testing the indigenous germplasm for the presence of associated pests (insects, nematodes, fungi, bacteria, viruses and weeds) by using standard seed health testing methods as well as greenhouse screening. Reduced germination is of particular importance for gene bank storage. Seed health analysis would generally focus on the precise identification of those seed-borne pathogens affecting seed germination and/or crop establishment. Conservation of well tested PGR minimizes the spread of important pests through collected germplasm within the country/ area or through domestic or international germplasm distribution.

This manual provides the basic diagnostic procedure in a plant pathology laboratory focusing on three major pathogens. It aims to serve as a guideline for easy diagnosis of plant diseases and also the identification of phytopathogenic seed-borne fungal or bacterial problems at least at the colony level.

Part I focuses on the isolation, purification and identification techniques as well as common general and selective growth media for fungi. Part II and III focus on the isolation/extraction and identification of plant pathogenic bacteria and nematodes, respectively. It also provides information about commonly followed procedure and basic instruments of a plant disease diagnostics laboratory.

II. Safety Precautions

The following are lists of common safety precautions that should be followed in a plant pathology laboratory to keep hazardous incidences to the minimum level as possible.

a. Check the safety aspects of all chemicals before use. Such information can be found on the product packaging or on the internet.
b. Use gloves where appropriate.
c. Chloramphenicol is a suspected carcinogen, and it and all other antibiotics must be handled with care.
d. Remember when making media to loosely screw on lids of bottles during autoclaving and tighten afterwards. This will prevent bottles from exploding under pressure and a lot of clean-up work.
e. Take care when opening the oven. High temperatures and steam can cause serious burns.
f. Do not open the autoclave until the internal air pressure reaches atmospheric pressure (reading 0 on the dial). Always use heavy duty material gloves when removing any material from the autoclave or oven.
g. Ethyl alcohol is highly flammable. Do not wipe benches near a flame.
h. Be careful not to place hot or flaming instruments in or near alcohol, since this is a fire hazard.
i. Keep a fire blanket in the laboratory to put out clothing fires.
j. Wear shoes in the laboratory to protect feet from sharp instruments dropped accidentally. Closed shoes also protect feet from broken glass and chemicals.
k. To avoid contamination problems, all plant materials entering the lab must be controlled. In addition, work tables should be routinely cleaned with alcohol or sodium hypochlorite.
l. Plant tissue samples brought into the lab should be kept to a minimum and above all should be handled as inoculum within the lab.
m. Work utensils (needles, forceps, etc.) should be sterilized after every use by cleaning them with alcohol and flaming.
n. Hands should be washed and cleaned with alcohol before working under sterile conditions. Care must be taken because some fungi present as saprophytes in many tissue samples or in the lab environment may grow and reproduce rapidly.

III. Basic equipment in a plant pathology laboratory

A. Autoclave

Autoclaves are used for sterilizing with moist heat. Water vapor inside an autoclave can reach a temperature of 120°C and 181b/in^3 (1.4 kg/cm^2) pressure. The heat source may be electric or, in the case of a pressure cooker, a gas burner. A pressure cooker works like a big autoclave but has the advantage of being very low-cost. Both devices are used primarily for sterilizing artificial media but can also handle soil, sand, vermiculite, or any other substrate used for growing plants and microorganisms.

B. Oven

An oven provides a wide temperature range by means of dry heat. It is used for drying plant or soil samples at constant temperatures and sterilizing glassware at high temperatures.

C. Isolation chamber

Laminar flow chambers (LFCs) have an air flow mechanism that filters outgoing air. This avoids contamination by microorganisms and provides sterile conditions. Microvoids work much the same way as LFCs but are smaller and cheaper. If LFCs and microvoids are not available, a small room with a gas or alcohol burner (to avoid contamination) and no air current may serve as an isolation chamber.

D. Incubator

An incubator can be calibrated to a wide range of light/ temperature combinations. Incubators are extremely useful for artificially growing many plant pathogens requiring specific light/temperature conditions. Partial incubators can be built by hand out of wood. Since temperature cannot be controlled in this type of incubator, it should be located in a place where temperature is 15-22°C, the range at which many plant pathogens develop.

Some incubators are provided with fluorescent light that can be used to encourage sporulation. Near-ultraviolet (NUV) light is supplied by fluorescent tubes, and a light control is used to regulate light/darkness cycles.

E. Refrigerator

It is a basic requirement in the microbiological laboratory and used for storing stock cultures of microorganism at 4°C to save sub-culturing every few days. The stored cultures at low temperature are fairly inactive and will not suffer damage due to evaporation of medium. It is

also used to store sterilized media to prevent dehydration and to serve as a repository for thermo-labile solutions, serums, antibiotics and biochemical reagents.

F. Water bath

This device consists of a container that maintains water at a constant temperature. Water baths have many uses. For example, Erlenmeyer flasks containing freshly sterilized culture medium can be placed in a water bath at 46°C for 30 minutes to cool it before pouring into Petri dishes. Water baths are essential for measuring growth of some organisms in liquid media at specific temperatures, and have other uses in bacteriology and virology.

Figure 1. Manually operated (A) and automatically controlled digital (B) autoclaves, C: Pressure cooker (Stovetop autoclave), D: Oven, E: Incubator (growth chamber), F: Isolation chamber (laminar flow hood), G: Water bath

IV. Common Procedure in the Laboratory

A. Maintaining a microbe-free environment

A microbe-free environment is essential for most of the work in a plant pathology laboratory. Contamination (growth of unwanted fungi, bacteria or any other microorganism) may come from various sources such as utensils, samples brought from a field and human interferences. All samples brought from the field must be considered as sources of inoculums and hence, must be treated with due care. In order to avoid false positive results, contaminants should be removed or maintained at its minimum level as possible. Common techniques used to maintain a microbe free environment are listed below.

1. Dry heat sterilization

Dry heat sterilization kills microbes by oxidation. It is used when the materials to be sterilized are not damaged by high temperature under dry conditions. This method is best for the sterilization of dry glassware such as test tubes, glass Petri dishes, flasks, pipettes, all glass syringes and instruments such as forceps, scalpels and scissors. An electric oven is a commonly used tool for heat sterilization. It can be adjusted to a wide range of temperature for specific duration of time.

Temperature (^{o}C)	Exposure time (Hrs)
180	1
170	2
150	4
120	12-16

2. Moist heat sterilization (**Autoclaving)**

Moist heat kills microorganisms, probably by coagulating and denaturing their enzymes and structural proteins, a process in which water participates. All culture media therefore are sterilized by moist heat. Autoclaving at temperatures greater than 100 °C is the most reliable method and widely used for the sterilization of culture media. Most autoclaves and pressure cookers operate at 121 °C, at which the minimum holding period for sterilization is 15 minutes. It is essential that all air is expelled from the autoclave; otherwise it will not reach the correct temperature.

3. Ultraviolet light radiation

Ultraviolet light is used for sterilization because it kills most microorganisms. While it is recommended for plastic materials, it cannot penetrate glass. Ultraviolet systems must be installed in a closed chamber to avoid exposure, since it is harmful to the eyes.

4. Sterilization of instruments

Forceps, inoculating needles and other instruments must be sterilized before contact with a culture to avoid cross-contamination. Inoculating needles are best sterilized by heating to red heat (incineration) in a flame. Needles must be allowed to cool to room temperature again before being used. Hot needles are the most common cause of failure of sub-culturing, hyphal tipping and single sporing. Forceps and scalpels are sterilized by dipping in alcohol. Before use, the alcohol is burnt off by passing the forceps through a flame to ignite it. Do not hold the instrument in the flame, since this will heat it up too much.

5. Sterilisation of work surfaces

Trays, benches and other surfaces may be sterilized with a liquid disinfectant. Alcohol is the most commonly used. Alcohol works best as a sterilant if it contains some water, and a solution of 70% ethyl alcohol is suitable.

B. Dilution

In a plant pathology laboratory, dilution techniques are often followed to lower the concentration of solutions.

Example: If one wants to dilute a 99.7% ethanol and make 500 ml of a 70% ethanol solution,

C_1= 99.7%

C_2= 70%

V_2= 500 ml

$$C_1V_1 = C_2V_2$$

$$V1 = \frac{C2V2}{C1}$$

$$V1 = \frac{(70\%) \text{ x } (500 \text{ ml})}{(99.7\%)}$$

V_1= 351 ml

Steps

1. Pour 351 ml from the 99.7 % labeled alcohol in to a jar
2. Fill the jar with dilute water to make the final volume of the solution 500 (add 149 ml water)
3. Shake the mixture.
4. Pour the new diluted solution into a new container.
5. Label it (70% Ethanol)

C. Microscopy

Microscopes and stereoscopes are essential for the identification, classification, and quantification of plant pathogens, as well as for observing symptoms and lesions and evaluating experiments.

1. Using a dissecting microscope (Stereoscopes)

A dissecting microscope is used to examine diseased materials for the presence of small fungal structures, such as pycnidia, acervuli, sporodochia and perithecia, under low magnification (up to approximately ×100). Using the dissecting microscope, such structures can be easily transferred to a slide preparation for examination under a compound microscope, at higher magnification (up to ×400).

A dissecting microscope is also used for fine work such as the transfer of germinated single spores or hyphal tips during purification of fungal cultures, and the examination of nematodes, which can also be transferred to a slide for examination under a compound microscope. Developing colonies are best examined under a dissecting microscope.

2. Adjusting the compound microscope

It is very important that objective lenses are not scratched or touched by agar, fungus or stained preparations. Cover slips are very thin and, if broken, can cut fingers.

1. Place a slide containing a specimen on the stage of the microscope.
2. Turn the lamp on and adjust the transformer to approximately 50% brightness.
3. Bring the specimen into focus with the ×10 objective.
4. Close the field iris diaphragm so that it becomes small.
5. Adjust the condenser height to bring the field iris diaphragm into focus.

6. Turn the two condenser centering knobs until the image of the field iris diaphragm is centered.

7. Open the field iris diaphragm until it just disappears from view (i.e. until it is slightly larger than the field of view).

8. Adjust the aperture iris diaphragm to clearly show the specimen. The setting number (numerical aperture) on the aperture iris diaphragm should be approximately 75% of the numerical aperture on the objective being used.

9. Examine the specimen.

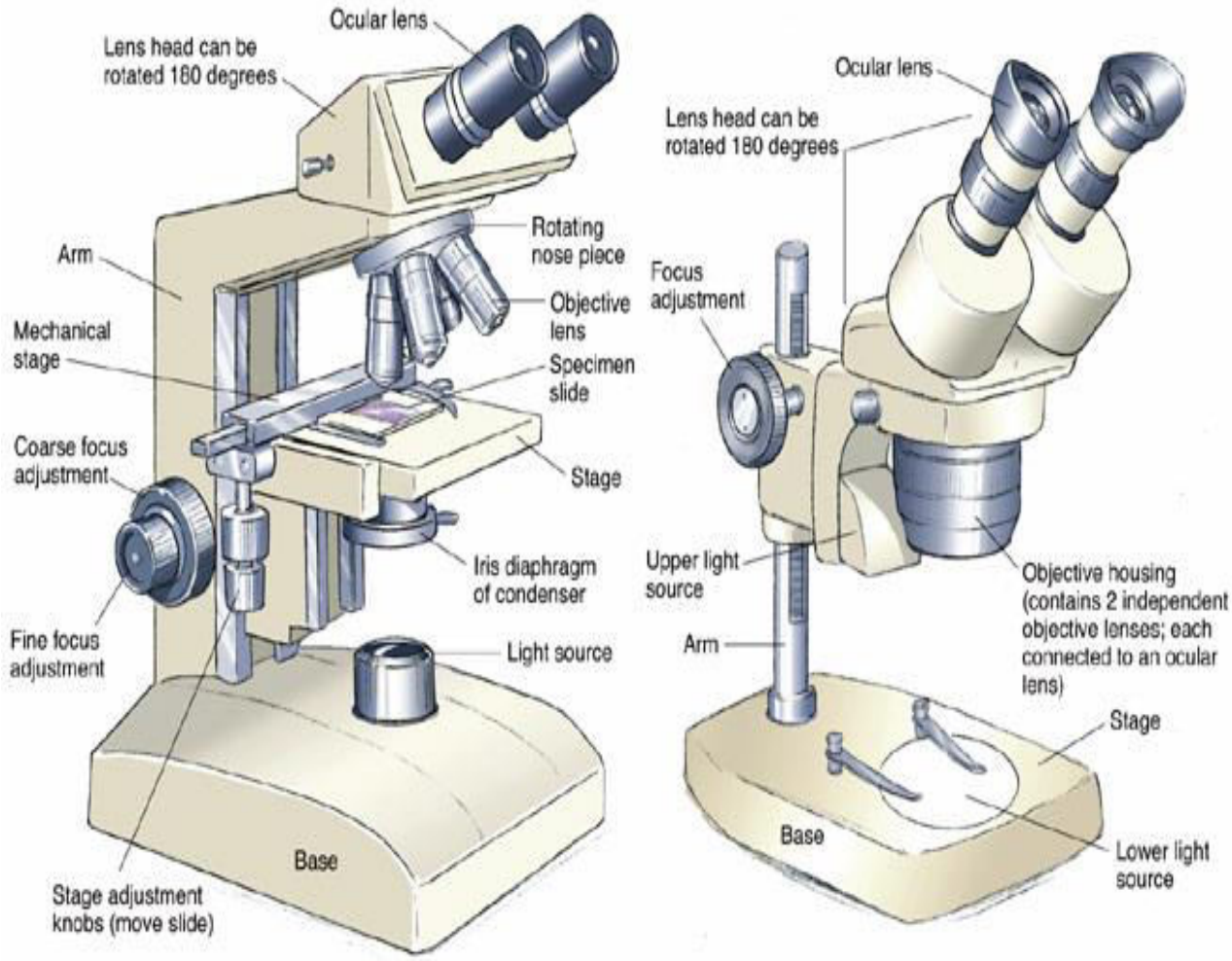

Figure 2. Compound and Stereo Microscopes

3. Mounting specimen

Specimen such as fungal spores or spore forming structures such as pycnidia, perithecia or cleistothecia can be mounted on slides in water or lacto phenol cotton blue solution. Spores from

a diseased plant or culture can be scraped off with a transfer needle and transferred to the water drop. Larger spore-forming structures should be examined under the dissecting microscope and then placed in the water drop and squashed (flattened) by pressing gently on the cover slip using a flat surface. On re-examination, pycnidia can be identified as containing only spores (conidia), while perithecia and cleistothecia will contain both asci and ascospores.

1. Place a small drop of sterilized water / lacto phenol cotton blue solution on a slide.
2. Place the material into the drop, under a dissecting microscope.
3. Place a cover slip with one side touching the slide near one edge of the mounting solution.
4. Gently lower the other side of the cover slip onto the specimen—this method excludes air bubbles from the preparation.
5. Use a strip of blotting or filter paper to blot excess water at the edge of the cover slip.

Spores from a diseased plant or culture can be scraped off with a transfer needle and transferred to the water drop. Larger spore-forming structures should be examined under the dissecting microscope and then placed in the water drop and squashed (flattened) by pressing gently on the cover slip using a flat surface. On re-examination, pycnidia can be identified as containing only spores (conidia), while perithecia and cleistothecia will contain both asci and ascospores.

D. How to prepare moist chambers

Moist chambers are a quick, direct way of stimulating sporulation and helping to identify the causal agents of some diseases. They are especially useful for identifying microorganisms that show rapid growth on the host and compete well with saprophytes in a moist chamber.

Preparation:

- Cut leaves or stems into small pieces and tape them onto a sterile slide.
- Sterilize pieces with 5% sodium hypochlorite for 30-60 seconds;
- Rinse samples in sterile water to remove hypochlorite and dry on paper towels.
- It's important to leave a few pieces untreated because some pathogens are very sensitive to sterilization.

- Do not select heavily damaged tissue to avoid saprophytic organisms.
- Place filter paper disk on the bottom of a Petri plate and moisten it thoroughly with sterile water.
- Place the slide on the paper disk.
- Close the plate and seal it with parafilm to hold in moisture.
- Incubate the plate at 18-22°C under alternating cycles of light and darkness (10 h light/14 h darkness) (many fungi will not develop to the reproductive stage without this alternating light/darkness regimen).
- Open the plate after 24, 48 and 72 h of incubation.
- Place the slide under the dissecting microscope, and observe the structures that have developed on the lesion.
- If necessary, take a tiny portion of the fungal growth with a dissecting needle and put it in a drop of water on a slide.
- Cover the slide, being careful not to form air bubbles (use a dissecting needle as an aid).
- Look for mycelium, conidia, conidiophores, and other fungal structures under the compound microscope.
- If no structure is visible, close and seal the plate again to make sure it doesn't lose moisture and continue observation.

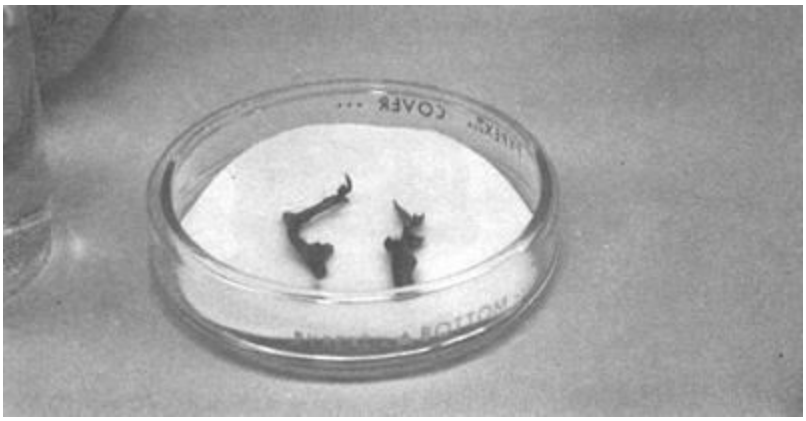

Figure 3. A moist chamber

V. Culture Media

There are many types of media which have been developed as general purpose media suitable for growing most fungi, but some are selective and are used for the isolation of particular fungi from

plants or soil. Growth media can also be classified as synthetic or natural based on their compositions. Synthetic media (those made entirely from defined chemical compounds) are by nature uniform as their chemical composition is standard whereas natural media (those made from natural material, usually plant extracts) are variable depending on the extract from the plant. If using natural media for distinguishing morphological characters or growth rate studies it is important that the same batch of media is used across all isolates.

1. Water agar (WA)

Water agar is the most useful general purpose isolation media often used to isolate and germinate spores, obtain monosporic culture, and verify inoculum viability.

Preparation:

WA (2%) consists of 20 g agar in 1 L of water and is recommended as the substrate for the germination of conidia used to initiate single spore cultures. Hyphal growth is sparse on this medium so it is suitable for cultures from which single hyphal tips are to be taken for the initiation of new colonies. Sparse growth on WA also facilitates the isolation of fungi from plant material, particularly roots.

For single sporing and hyphal tipping it is suggested that plates be poured when the medium is still quite hot so that thin plates can be produced—this restricts fungal growth and makes it easier to cut out the spores or hyphal tips.

WA (0.05%), 0.5 g agar in 1 L of water, is used in the preparation of soil dilution series. The small quantity of agar slightly retards sedimentation rates of fungal propagules. The agar is dissolved in water before being dispensed into McCartney bottles.

2. Potato-dextrose-agar (PDA)

Most widely used for isolating, multiplying, and storing fungi because it is suitable for a large number of species.

Components:

- Sliced potatoes 250 g
- Dextrose 10 g
- Agar 20g
- Distilled water 1000 ml

Preparation: Boil the potatoes in 500-700 ml of distilled water for 15-20 minutes. Filter through cheesecloth; pour into a flask and add dextrose, agar, and enough water to reach 1000 ml. Seal

flask with cotton or aluminum foil and sterilize. Let it cool and pour into Petri dishes (halfway). Stack dishes one on top of another to avoid condensation.

- If commercial powdered PDA is available, weigh 41g and add distilled water to make the volume of the mixture 1L. Then, follow the above steps.

3. V-8 agar

Useful for inducing sporulation in many fungi. V-8 juice is made by the Campbell Soup Co. and Herdez, a Mexican firm, among others; it contains tomato, celery, beet, parsley, lettuce, spinach, and watercress extracts. Since there are differences among brands, comparative testing on each species is recommended. If canned V-8 juice is not available, it can be replaced with a medium containing leaf extract (such as the following), especially for testing *Helminthosporium tritici-repentis*and H. *teres.*

Components:

- V-8 juice 200ml
- Calcium carbonate 3g
- Agar 15-20 g
- Distilled water 800ml

Preparation: Place agar and calcium carbonate in a flask; add the juice and mix with water to complete 1000 ml. Seal the flask and sterilize. Stir the medium before pouring to keep the juice from precipitating. This medium may be prepared at different concentrations by adjusting the amount of juice used.

4. Leaf extract medium

Used primarily for stimulating growth and formation of asexual structures in some fungi. Leaves of different crops may be used, depending on the pathogen.

Components:

- Agar 15-20 g
- Distilled water 1000 ml
- Fresh leaves (wheat, oats, barley, etc.) 100 g
- Sucrose 10 g

Preparation: Boil leaves for 20-30 min and filter the water. Add filtrate to agar and mix with water to 1000 ml. Sterilize and pour into Petri dishes or test tubes.

5. 4-4-4 agar-malt-yeast medium

Frequently used for isolating and multiplying *Septoria tritici.*

Components:

- Malt extract 4g
- Yeast extract 4g
- Sucrose 4g
- Agar 18 g
- Streptomycin 0.1 g
- Distilled water 1000 ml

Preparation: Place all ingredients except the antibiotic in distilled water. Mix well and sterilize for 20 min. Add the antibiotic while the medium is still warm, then pour into Petri dishes or test tubes.

6. Medium for *Septoria nodorum*

Recommended for the development and growth of *S. nodarum* pycnidia.

Components:

- Malt extract 3g
- Yeast extract 2g
- MgS04·7H20 0.5 g
- Agar 20g
- Streptomycin 0.1 g
- Distilled water 1000 ml

Preparation: Mix all the ingredients and sterilize for 20 min; add the antibiotic while the medium is still warm, then pour into Petri dishes or test tubes.

7. Chinese bean (*Vigna radiate*) broth

Medium used to increase *Fusarium graminearum.* If Chinese bean is available, this medium is inexpensive and very efficient for producing large amounts of inoculum.

Components:

- Chinese beans 20 g
- Distilled water 1000 ml

Preparation: Boil the beans in water for 20 min; strain the solution and sterilize the filtrate for 20 min in 500 ml flasks (just half full).

Inoculum increase: Inoculated flasks are incubated for five days on rotary shaker (100 rpm).

8. Lima bean agar

This medium is utilized for *Rynchosparium secalis* isolation and inoculum increase, as well as for fungi that attack the roots and crown. It can be prepared from lima beans or commercial preparations can be purchased.

Components:

- Lima beans *(Phaseolus lunatus)* 8g
- Agar 15-18 g
- Distilled water 1000 ml

Preparation: Boil the lima beans in water for 20 min; filter the infusion, add the agar and sterilize for 20 min.

9. Sacarose-yeast liquid medium

Used only for increasing *Septaria tritici,* and only if a shaker is available and existing conditions favor daily inoculum applications, since viability is lost after five days.

Components:

- Sacarose 10 g
- Yeast extract 10 g
- Distilled water 1000 g

Preparation: Mix the components and sterilize for 20 min in 250-ml flasks. Let the medium cool, then inoculate and shake continuously during the entire incubation period. Use the inoculum after five days.

10. Leaf segment medium

Appropriate for stimulating the formation of sexual structures in some fungi.

Components:

- Agar 15-20 g
- Distilled water 1000 ml
- Small pieces of fresh leaves (try different species)

Preparation: Dissolve the agar in water. Place the leaf pieces in Petri dishes and sterilize. Pour the sterilized water-agar into the plates. When the agar has nearly solidified, place 5-10 leaf pieces on each plate so they will stay in the top part of the agar. The pieces are inoculated with the fungus under study and periodically observed.

11. Antibiotics

Antibiotics may be added to fungal isolation media to prevent the growth of bacteria or unwanted fungi. Most antibiotics (except chloramphenicol) are unstable if heated and need to be added to the medium after autoclaving. These antibiotics are dissolved in a small quantity of sterile distilled water, according to the recipe. For most purposes this may be added directly to the medium but, for critical work the antibiotic solution should be filter-sterilised before use.

Commonly used antibiotics

Antibiotic	Active against	Solubility
Penicillins	Gram-positive bacteria	Water soluble
Streptomycin	Gram-negative bacteria	Water soluble
Neomycin	Gram-positive bacteria	Water soluble
Chloramphenicol	Gram-positive and negative bacteria	Ethanol soluble

VI. Fungal diseases identification

For a correct identification of the causal agent of plant diseases and make reliable conclusions, appropriate isolation procedure must be followed. The successful isolation of fungi from diseased plants depends on several factors:

- type of diseased tissue (seeds, leaves, stems, roots)
- method of surface sterilization
- plating procedure
- isolation medium
- incubation conditions

A. Isolation from leaves and stems

Avoid using potato dextrose agar (PDA) or other carbohydrate-rich media for isolation from diseased plant tissues, especially if isolating from roots. Saprophytic fungi and bacteria grow quickly on carbohydrate-rich media and suppress the growth of slower growing fungal pathogens.

Isolation from stems is often improved by removing the bark or outer stem tissues before surface sterilization.

1. Wipe the work area with 70% ethyl alcohol.
2. Dip instruments (forceps and knife or scalpel) in 70% ethyl alcohol and flame dry.
3. Rinse leaf or stem tissue in water to remove soil and other debris.
4. Surface sterilize leaf or stem tissue by wiping the surface with soft paper (paper tissue) dipped in 70% ethyl alcohol or by briefly dipping thick leaves in 70% ethyl alcohol for 5 seconds, rinsing in sterile water and damp-drying on sterile paper tissue.
5. Aseptically cut small pieces (approximately 2 × 2 mm) from the margin of the healthy and diseased tissue, and transfer them to a low-nutrient medium (e.g. water agar [WA]) or a selective isolation medium, placing the pieces near the side of the plate.
6. Incubate the plates at approximately 25°C, ideally under lights.
7. Check plates each day, and when fungal colonies develop from the pieces of plant tissue, transfer material from the margins to a medium such as PDA or WA that contains sterile pieces of plant tissue, for example, pieces of green rice stem, carnation leaf or bean pod.

(Sterile pieces of plant tissue encourage sporulation, which aids in identification of the pathogen.)

8. Make a final identification using pure cultures grown from a single germinated spore or a hyphal tip.

B. Alternative method for isolating from leaf spots

1. Place the leaf or leaf piece on moist paper in a Petri dish in a humid chamber.
2. Incubate at approximately 25 °C under lights to promote sporulation.
3. Examine after 1–2 days under the dissecting microscope to locate spores or spore-forming structures such as pycnidia, acervuli or sporodochia.
4. Pour isolation plates containing WA with a drop of lactic acid (which reduces the pH and suppresses bacterial growth) or with added antibiotics.
5. Using a sterile transfer needle, transfer the spores to the plates.

C. Isolation from seeds

1. Soak seeds in 1% NaOCl for 1-5 minutes (based on the texture of the seed coat)
2. Soak seeds for 2 min in 70% ethanol and rinse in 3 changes of sterile distilled water.
3. Dry the seeds on sterile paper tissues before plating on moistened blotter or agar.

The blotter technique

1. Place sterile blotting paper (3-layers) in sterilized Petri dishes.
2. Moisten the papers with sterilized distilled water so that little amount of surplus water will be left on the surface of the papers
3. Place surface disinfested seeds on the water soaked blotters and keep them in a controlled room at a temperature of 20±2ºC under a pair of tube light mounted at about 40cm above the seeds with alternating cycles of 12hr near ultraviolet light and darkness.
4. Examine the growing fungi on the seed after 8days of inoculation and record the data.

D. Isolation from small, thin roots

Do not use severe surface sterilization of small rootlets as the sterilant may kill all the fungi in the rootlet, including the pathogen.

1. Select diseased rootlets with both healthy (symptomless) and diseased parts, and wash them in three changes of sterile water in a small bottle. Add a small drop of detergent to the first wash.

2. Wipe the work area with 70% ethyl alcohol.
3. Dip instruments (forceps and knife or scalpel) in 70% ethyl alcohol and flame dry.
4. Dip the rootlets briefly in 70% ethyl alcohol, rinse quickly in sterile water and then damp-dry on sterile paper tissue. Alternatively, surface sterilize the rootlets in 1% sodium hypochlorite or in 10% ethyl alcohol for 10–15 seconds only, immediately rinse in sterile water and allow to air-dry on sterile paper tissue in a sterile work chamber.
5. Aseptically cut root pieces 1–2 mm in size at the margin of healthy and diseased tissue and transfer onto WA or a selective medium.
6. Press the pieces gently into the surface of the agar to ensure good contact between the entire root segment and the antibiotics in the agar.
7. Incubate at approximately 25 °C and check each day under the dissecting microscope for fungal growth from the root pieces.
8. Subculture each colony onto PDA or WA containing sterile pieces of plant tissue, such as green rice stem pieces.
9. Purify by hyphal tipping or by the single germinated spore technique before final identification.

E. Sub-culturing from isolation plates

Sub-culturing is the stage between isolation from plant material and the creation of pure cultures. This stage helps to determine which organism has been isolated.

1. Examine the plates under the dissecting microscope each day and assess the growth of fungal hyphae from the segments of plant tissue.
2. Determine if there is more than one fungal species growing.
3. Subculture when there is approximately 5 mm of hyphal growth from the plant tissue.
4. Cut out a small block of agar (2 × 2 mm) from the margin of each colony and transfer it to PDA or a natural substrate medium.

F. Purification of cultures

The final stage in identifying fungal pathogens is the creation of pure cultures. Only a single spore or hyphal tip is transferred to ensure a pure culture is produced.

1. Single sporing

Single sporing involves the transfer of a single germinated conidium to obtain a pure culture. This method is suitable for species of fungal genera that produce spores in culture, for example, *Fusarium*, *Colletotrichum*, *Alternaria*, *Stemphylium*, *Bipolaris*, *Verticillium*, *Phoma, etc*.

1. Sterilise transfer needle.
2. Create a spore suspension by removing a small amount of surface mycelium with conidia or a small scraping of sporodochia from *Fusarium* spp. and place in 10 mL of sterile water in a test tube.
3. Shake the suspension to disperse the spores and check the spore concentration by holding the tube against the light or by examining a drop of the suspension under a dissecting microscope. Avoid high concentrations of spores. Dilute with sterile water if needed.
4. Pour the spore suspension onto a Petri dish containing a thin layer of water agar.
5. Pour out excess water. This leaves some spores on the agar.
6. Store the plate on its side for 18 hours until the spores germinate.
7. Examine the Petri dish under a dissecting microscope with a light source underneath. (Adjust the mirror on the light source carefully to obtain a good contrast between the agar and the conidia and germ tubes.)
8. Remove a single germinating spore using a flat transfer needle and transfer it to a new medium.

2. Hyphal tip transfer

Hyphal tipping involves the transfer of a single hyphal tip to obtain a pure culture. This method is suitable for species of fungal genera such as *Phytophthora*, *Pythium*, *Rhizoctonia*, *Sclerotium* and *Sclerotinia.*

1. Pour a plate of water agar so that it is shallow on one side of the plate.
2. Inoculate on the side of the plate where the agar is deeper with a small agar block taken from a subculture isolation plate.
3. Place the plate under a dissecting microscope and focus on the hyphae at the growing margin of the colony. (The hyphae will grow sparsely across the shallow region of the agar.)
4. Adjust the light source (mirror) to obtain a good contrast between the medium and the hyphae.

5. Aseptically transfer a small block of agar containing a single hyphal tip to a plate of suitable agar medium, using a flat transfer needle.

G. Final Identification of fungal pathogens

Microscopic examination is generally required to enable identification of the pathogen and diagnosis of the disease. The identification of fungal pathogens is based initially on morphological features, such as spores and spore-forming structures. For example, most fungal pathogens that cause leaf diseases produce spore forming structures; perithecia, pycnidia, acervuli, sporangiophores or conidiophores that can be readily examined microscopically and, to some extent, the characteristics of the fungus body (mycelium).

The shape, size, color, and manner of arrangement of spores on the sporophores or in the fruiting bodies, as well as the shape and color of the sporophores or fruiting bodies, are sufficient characteristics to suggest, to one somewhat experienced in the taxonomy of fungi, the class, order, family, and genus to which the particular fungus belongs.

Once the genus of the fungus has been determined, descriptions of the known species are found in monographs of genera or in specific publications in research journals. Because there are usually lists of the pathogens affecting a particular host plant, one may use such host indexes as short cuts in quickly finding names of fungus species that might apply to the fungus at hand. Host indexes, however, merely offer suggestions in determining identities, which must ultimately be determined by reference to monographs and other more specific publications.

Books, keys and manuals should be kept as resources in diagnostic laboratories, and fortunately many scientific journal publications on taxonomy and identification can be accessed via the internet.

VII. Bacterial diseases identification

A. Introduction

Bacteria are microscopic organisms typically composed of single cells. About 200 types of bacteria are known to cause plant diseases. Due to their small size, a high-magnification microscope is required to observe them. Occasionally, when a large number of cells are present, plants may be observed 'oozing' bacteria and other organic byproducts. Bacteria are capable of rapid reproduction through a process known as binary fission. In this process, one cell divides to become two, then two divide to become four cells, and so on. Within a few hours one bacterial cell can become thousands, and under ideal conditions, populations can double in as little as 20 minutes.

B. Visual examination and Information Gathering

a. **First step -** be sure of the identity of the plant to be analyzed. Besides the plant's identity, as much information as possible on the crop must be gathered, for example location of crop, method of cultivation, irrigation methods, chemicals applied and recent climatic condition.

b. **Second step** - familiarizes your-self with symptoms. Gather information about the distribution of the disease in the crop.

c. **Third steps** - Obtain information about all the possible diseases reported on the crop in the country or sub-region.

Testing for bacterial streaming

Most Phytopathogenic bacteria are motile by flagella and the motility can be readily observed if the material is fresh. It is important to note, however, that absence of bacterial ooze does not mean that the lesion is not caused by a bacterium. It is also sometimes difficult to observe the bacteria in some plant species, because it may be obscured by or confused with high numbers of other particulate matter such as latex, plastids and starch granules.

C. Preparation of Plant Leachate / Macerate

- Cut small section of lesion or canker margins with a sterile scalpel.
- Place in a drop of sterile water, buffered saline or quarter-strength ringer solution in a sterile petri dish.

- Chop tissue with a sterile scalpel or grind a sterile glass rod.
- Set aside for at least 10 minutes.
- Plate on medium

D. Isolation of Phytopathogenic Bacteria

Isolation from leaf spots should be made from small, water soaked lesions rather than from larger brown or necrotic spots. In the case of canker or wilt diseases, the plant should be torn open by hand to expose internal tissues and prevent contamination by epiphytic bacteria.

Steps

- ✓ Diseased plant must be present
- ✓ Cut young lesion aseptically
- ✓ Maceration of Diseased tissue
- ✓ Streaking of bacterial suspension on agar medium and incubation
- ✓ Selection of colonies for purification and further characterization
- ✓ Purified bacterium made for biochemical characterization and pathogenicity test.

Alternative Method:

- Place the plant tissue in a test tube containing 2-3 ml of a liquid (sterile water, buffered saline or quarter-strength ringer solution) and allow diffusion at room temperature for 30-60 minutes.
- The leachate/ macerate should be streaked onto the appropriate agar media with a wire loop to obtain single colonies.
- If a bacterial wilt or soft rot is suspected, when large numbers of saprophytes could be present, plating by dilution series is recommended.
- A series of 1:10 dilution of the leachate are made in sterile water/ buffered saline/ringer solution and plated by spreading 0.1 ml on the surface of dried agar plates with a sterile L-shaped glass rod.
- Separate, single colonies are more readily obtained in this way. The agar plate should be incubated at about 25^0C for at least 72 hours.

E. Purification of Phytopathogenic bacteria

Steps of Purification

- Sterilize the loop by flaming and cool the loop in sterile agar medium.
- Take one loopful of the plant macerate or part of a well-isolated, typical colony
- Streak plant macerate on the surface of the agar by drawing a few straight lines with the loop, turn the plate 90^0c
- Sterile the loop by flaming cool the loop in sterile agar medium
- Spread the plant macerate from the primary inoculation area by drawing a few straight lines with the loop, turn the plate 90^0c
- Sterile the loop by flaming cool the loop in sterile agar medium.
- Spread the plant from the secondary inoculation area by drawing a few straight lines with the loop, flame the loop.

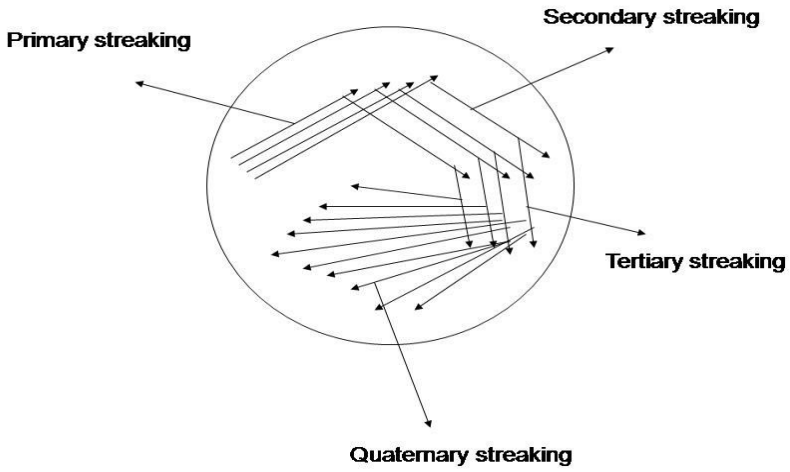

Figure 4. Pattern of streaking for obtaining pure cultures

F. Examination of isolated bacteria

1. Colony appearance

Differentiation of phytopathogenic and saprophytic bacteria by colony appearance on isolation are the first step in the identification of the pathogen. Colony morphology, growth rate, colour and appearance are typical for specific phytopathogenic bacteria on different isolation media

2. Gram Staining

Bacteria with cell walls are divided into two groups; Gram-positive and Gram-negative bacteria. The division is based on the differences in cell wall composition and groups can be distinguished using the gram stain procedures.

After purification, gram –staining is the first in the identification of a bacterium. The majorities of the phytopathogenic bacteria are gram-negative and belong to either the gram-negative aerobic rods or the facultatively anaerobic gram-negative rods. Gram-positve phytopathogenic bacteria belong to the Actinomycetes and related.

Gram-staining Procedures

- Place a small drop of sterile water on a clean microscope slide.
- Remove a part of a young colony, with a cold, sterile loop, from the agar medium.
- Smear the bacteria onto the slide. The smear should be just discernible.
- Air –dry and heat-fix the bacteria on the slide by passing the slide four times through a Bunsen flame, but do not overheat it.
- Flood the slide with crystal-violet and set aside for 60 seconds.
- Rinse under running water
- Drain off excess water
- Flood with lugol's iodine and set aside for 60 seconds.
- Wash with 95% ethanol for 30 seconds.
- Rinse with water.
- Blot dry
- Counter-stain with safranine for 10 seconds.
- Rinse with water and dry.
- Examine at X 100 magnification using oil immersion.
- Gram-positive=dark purplish
- Gram-negative = red.

Gram-positive corynoform and all Gram-negative phytopathogenic bacteria are rod-shaped.

Streptomyces have a mycelia-type growth. Gram-negative and Gram-positive cocci and spore producing rods are not plant pathogens and should be discarded.

3. KOH Solubility test

- Place a drop of potassium hydroxide (KOH) (3% aq., w/v), using a Pasteur pipette, on a microscope slide.
- Remove part of a single colony, using a cooled sterile loop, from agar medium.
- Mix bacteria into KOH solution until an even suspension is obtained.
- Lift the loop from the slide.
- If a mucoid thread can be lifted with the loop it is Gram-negative bacterium, if a watery suspension is produced, it is a Gram-positive bacterium.

4. Flagellation and Motility

The number and orientation of flagella are major taxonomic criteria. Flagella staining methods for light-microscopy are available but consistent results are not readily obtained. Electron-microscopy is often used to study flagellation.

The hanging-drop method is used to provide information on flagellation and motility.

Testing for motility

- ✓ Inoculate actively-growing bacteria on nutrient agar (NA) slants containing 0.5-1 ml sterile water.
- ✓ Incubate overnight
- ✓ Remove a loopfull of bacterial suspension from the bottom of the slant and place suspension from the bottom of the slant and place directly on a microscope slide.
- ✓ Cover with coverslip and examine under the 40 X objective.

Alternative method

- ✓ Place a drop of the bacterial suspension on the underside of a coverslip (hanging drop).
- ✓ Suspend the coverslip between 2 matchsticks mounted on a microscope slide.
- ✓ Examine the drop as described above.
    - Rapid, darting motility is characteristic of polar flagellate bacteria.

G. Identification of phytopathogenic bacteria

Pure cultures of phytopathogenic bacteria area seldom obtained on isolation (even on semi-selective media). Colonies of the pathogen will often predominate if the isolations were made

from young infections and freshly-collected material; material with advanced symptoms and old samples are often invaded by a succession of saprophytic fungi and fast growing bacteria. Some non-target saprophytes are often present and can easily be confused with pathogens. Colony features of some common saprophytes may easily be confused with those of phytopathogenic bacteria and identification on the basis of colony morphology alone should never be carried out, even if selective and diagnostic media are used.

Selective or semi-selective media used in phytobacteriology render preliminary identification of suspected colonies easier. King's medium B is the most widely used diagnostic medium. The fluorescent pseudomonas (pathogenic as well as non-pathogenic) can be distinguished by the production of blue to green, fluorescent pigment when the colonies are examined under short-wavelength UV light. Many other diagnostic media contain complex carbon sources, utilized by a small group of microorganisms, on which the target bacterium displays diagnostic features.

In bacteria, morphological features alone are of little taxonomic value, because they are too simple to provide enough taxonomic information. Bacteria are mainly distinguished by their physiological and biochemical characteristics.

VIII. Nematode Extraction Techniques

A. Introduction

Nematodes are generally minute, colourless, unsegmented and worm-lke animals that lack eyes, appendages, circulatory and respiratory systems but have well-developed excratory, digestive, reproductive and nervous systems. They live virtually in every environment that support life and are found in fresh water, in salt water, in all kinds of soil and as internal parasites of animals and plants.

The easiest way to isolate nematodes from their host material is by submerging the sample in water and select the nematodes under a microscope. However, this is a tedious and laborious job and it can only be done with very small samples. For that reason most of the extraction methods are indirect, making use of a number of properties to separate nematodes from the surrounding medium. Considering material and technical resources in our laboratory, only the Baermann funnel technique can be applied. The Seinhorst mist extraction is included just as an option.

B. The Seinhorst Mist Extraction/Mistifier Technique

This technique was originally described by Seinhorst (1950) and is used to extract motile nematodes. It consists in principle of a Baermann funnel or Oostenbrink dish placed in a mist or fog of water to avoid oxygen depletion. The mist is produced by spray nozzles over the plant material or by nozzles spraying upwards so that the droplets fall in a soft curve onto the plant material. Sap and toxic decomposition products of the plant material are washed off with the funnel overflows allowing extraction times of possibly up to 6 weeks.

Area of application: It is used for the extraction of nematodes from soil, river silt, manure, litter and other substrates which may harbor nematodes and can be washed into a suspension.

Principle: It makes use of nematode mobility and extracts suspension decantation.

Materials required

- Knife, pair of scissors or blender;
- Mistifier spray apparatus;
- Baermann funnel or Oostenbrink dish;
- Supports (sieves, wire baskets) with legs;

- 20 or 25 µm aperture sieve;
- Glass beaker;
- Dissecting, inverse or compound microscope;
- Counting slide;
- Microscope slide.

Procedures

1. Place 100 g of seeds on a layer of cheesecloth, in a 60 mesh sieve (Fig.1).
2. Place the sieve inside a funnel, the stem of which reaches the bottom of a 250 ml beaker.
3. Spray the seeds with a continuous fine mist of tap water at a rate of 4L/hr. Heating the water to 20^{O}C improves extraction especially for those larvae underneath the seed testa.
4. After 48 hours of spraying, remove the beaker and allow the leachates to settle for 5 hours, then decant carefully, leaving 40-50 ml of extract in the bottom of the beaker.
5. Pour this extract (containing larvae) into a counting dish and examine under a microscope at about 25 X magnification.
6. Measure the length of at least 15 males and 15 females for identification

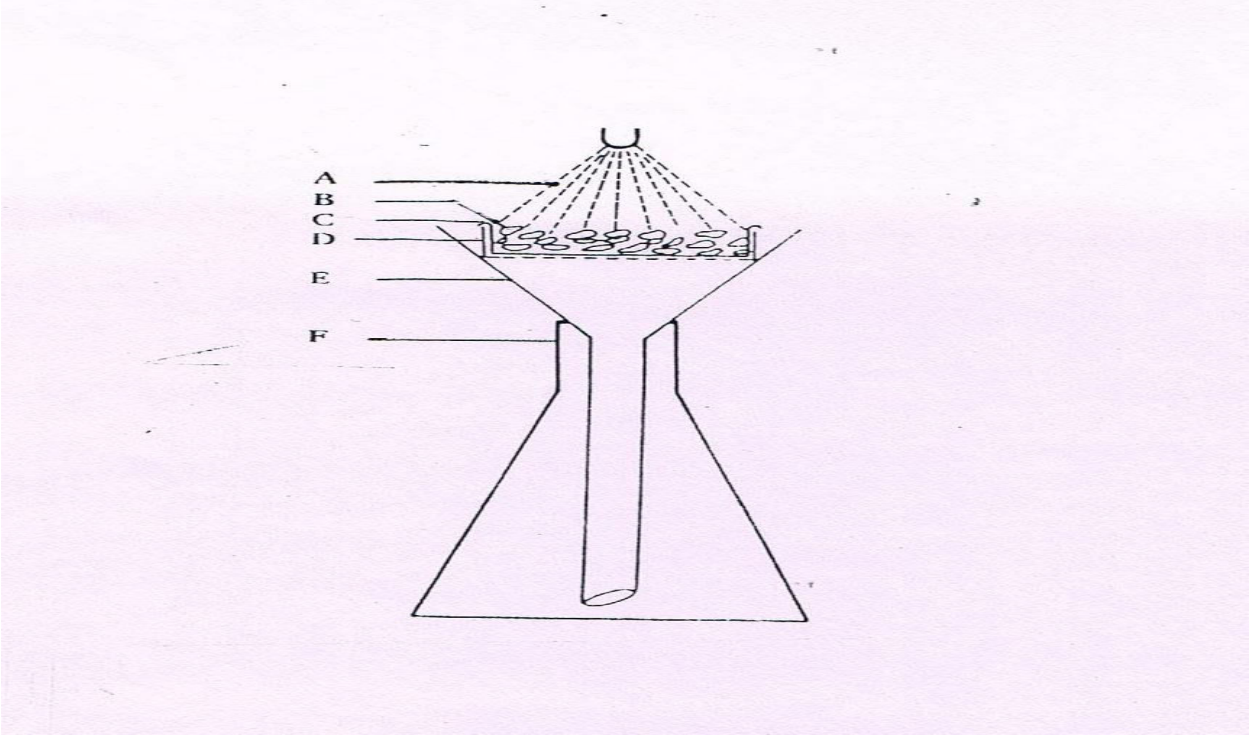

Figure 5. Seinhorst mist extraction apparatus for nematode assay in plant parts and soil. A: water mist, B: plant part or soil, C: cheesecloth, D: sieve, E: funnel and F: beaker

C. The Baermann Technique

This method for the extraction of motile nematodes was introduced by Baermann (1917) using a funnel.

Area of application: The Baermann funnel is used for extraction of active nematodes from plant material and soil. The sample size depends on the funnel diameter and the type of material. If extraction is from soil, the final suspension is dirty.

Principle: The method makes use of nematode mobility. If (infected) plant material is placed in water, nematodes crawl out of the material and sink.

Materials required

- Knife, pair of scissors or blender;
- Cotton-wool milk filter or equivalent (e.g. cheesecloth, filter paper, paper towel);
- Funnel made of glass with a piece of soft polyethylene tube attached to the stem and closed with a spring or screw clip. Recommended slope of funnel is approx. 30°;
- Stand to hold the funnel;
- Support, such as plastic sieve or wire basket of large enough aperture to allow nematode passage (i.e. 250 µm);
- 20 or 25 µm aperture sieve;
- 100 mL glass beaker;
- Dissecting, inverse or compound microscope;
- Counting slide;
- Clean water
- Microscope slide.

Procedures

1. Place a thin layer (1-2 cm) of soil, plant tissue cut into small pieces, or seeds on a cheesecloth in a 40 or 60 mesh sieve (Fig. 2).
2. Submerge the sieve in tap water in a shallow dish. The sieve should be supported so that it stays 1 cm above the bottom of the dish.

3. After 24 hours, remove the sieve, pour the water from the dish into a glass cylinder and allow settling for 5 hours. Then decant carefully leaving 50 ml of concentrated nematode suspension at the bottom of the cylinder.
4. Pour this suspension into a counting dish and examine under a microscope at about 25 X magnification.

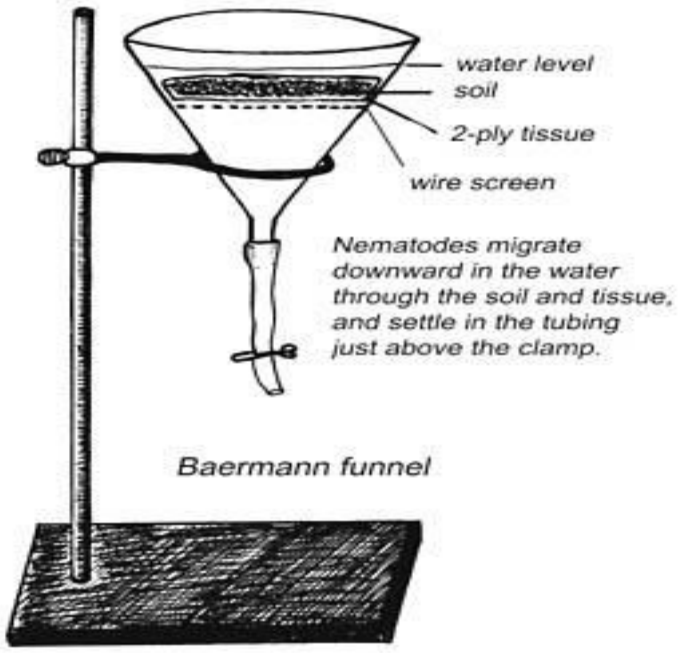

Figure 6. Baermann funnel nematode extraction apparatus

IX. Pathogenicity testing

To test pathogenicity, susceptible plant species are grown under controlled conditions and inoculated with a suspected pathogenic organism. Pathogenicity tests can provide information to:

a. Confirm an isolated organism as a plant pathogen using Koch's postulates
b. Determine the host range of a pathogen
c. Measure the virulence of different isolates of a pathogen.

Since cultivars can differ significantly in susceptibility, it is important to use the same cultivar (variety) from which the pathogen was isolated when choosing healthy plants to inoculate for a pathogenicity test to confirm Koch's postulates. High levels of moisture facilitate the infection and spread of many diseases. Mist sprays or humid chambers (made from plastic bags covering pots) can create a moist environment and significantly increase the success rate of pathogenicity tests. Pots in moist chambers or with plastic bag covers should not be placed in direct sunlight.

Steps to perform Koch's postulates

a. Describe the symptoms expressed by the diseased crop plants.
b. Isolate the suspected pathogen—the same cultures should be isolated from plants with similar symptoms.
c. Obtain a pure culture and use it to inoculate healthy plant material.
d. Observe the symptoms expressed by the inoculated plants—symptoms should be the same as those observed originally in the crop plants.
e. Re-isolate the pathogen from the newly diseased material—the culture should be the same as the original purified culture.

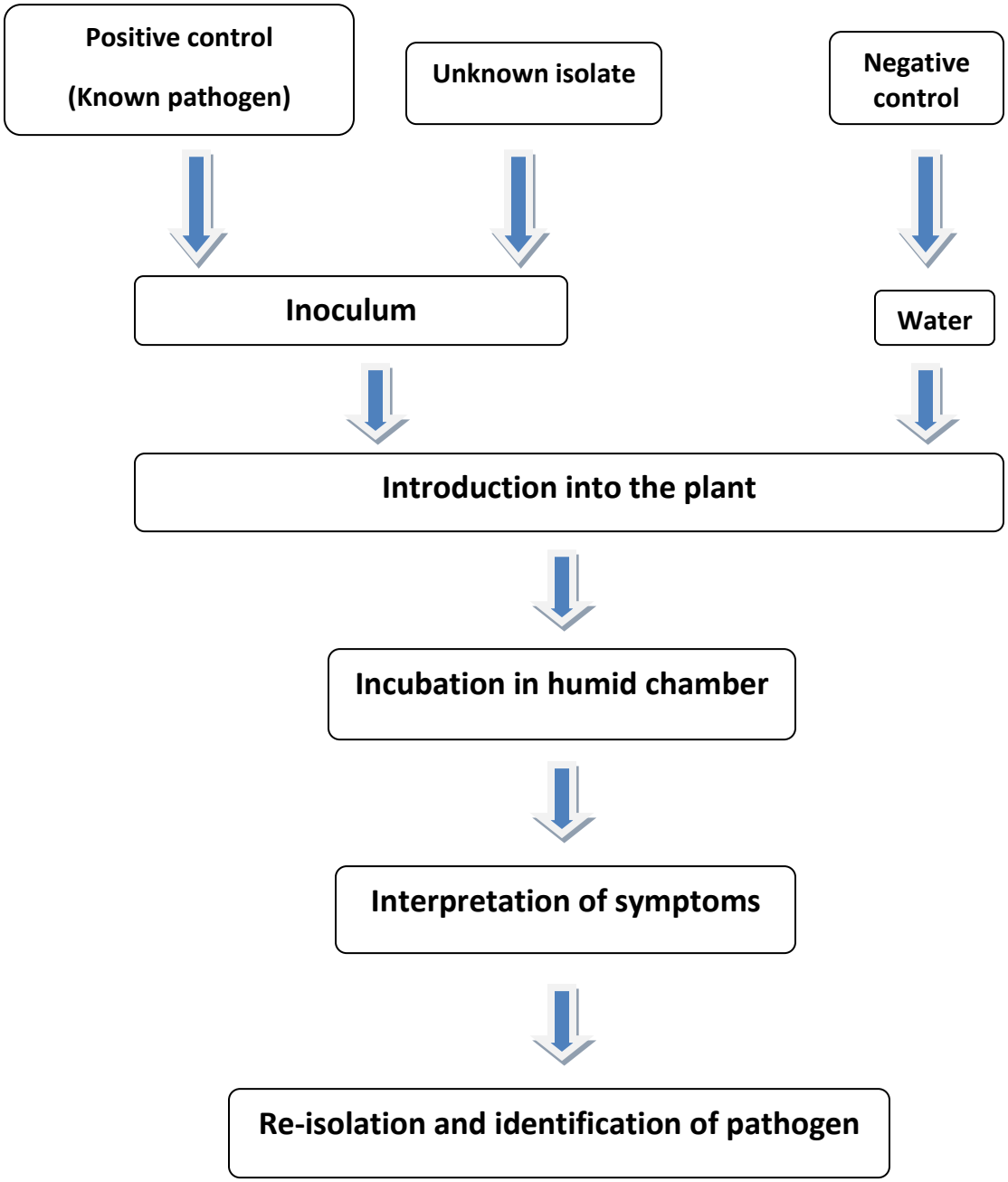

Figure 7. General steps in pathogenicity tests (Koch's postulate)

X. References

- Agrios G. N. 1936. Plant Pathology. 5th ed.
- ARC-Plant Protection Research Institute. 1999. Collecting and Preserving Nematodes: A Manual for Nematology. SAFRINET, the Southern African (SADC) Loop of BioNET-INTERNATIONAL.
- Bezooijen J.V. 2006. Methods and Techniques for Nematology. Revised version. Wageningen.
- Burgess L.W., Knight T.E., Tesoriero L. and Phan H.T. 2008. Diagnostic manual for plant diseases in Vietnam. ACIAR. Canberra.
- Gilchrist-Saavedra L., Fuentes-Davila G. and Martinez-Cano C. 1997. Practical Guide to the Identification of Selected Diseases of Wheat and Barley. CIMMYT. Mexico
- Goszczynska T., Serfontein J.J and Serfontein S. 2000. Introduction to practical phytobacteriology: A manual for phytobacteriology. SAFRINET, the Southern African (SADC) LOOP of BioNET-INTERNATIONAL.
- Hanounik S.B. 1986. Screening Techniques for Disease Resistance in Faba Beans. ICARDA. Syria.
- Johnson D.R.M. and Chandra S.S.R.I.V. 2012. Principles of Plant Pathology: Practical Manual. Acharya N G Ranga Agricultural University. India.