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.

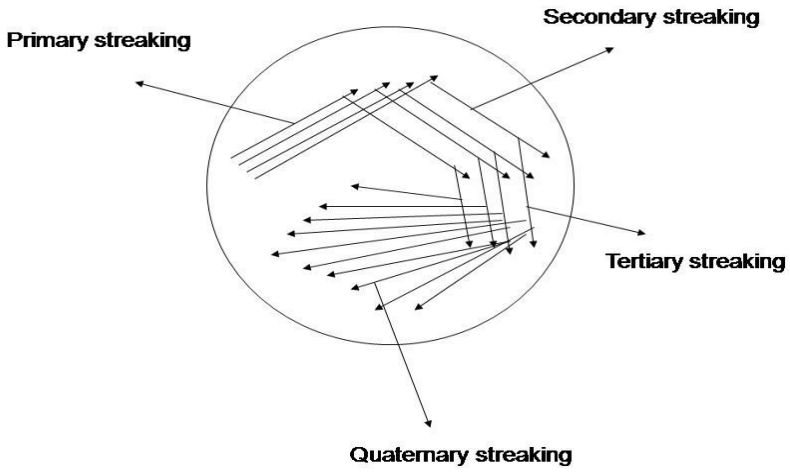

Figure 4. Pattern of streaking for obtaining pure cultures

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.