

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 25⁰C 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°c
- 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°c
- 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.

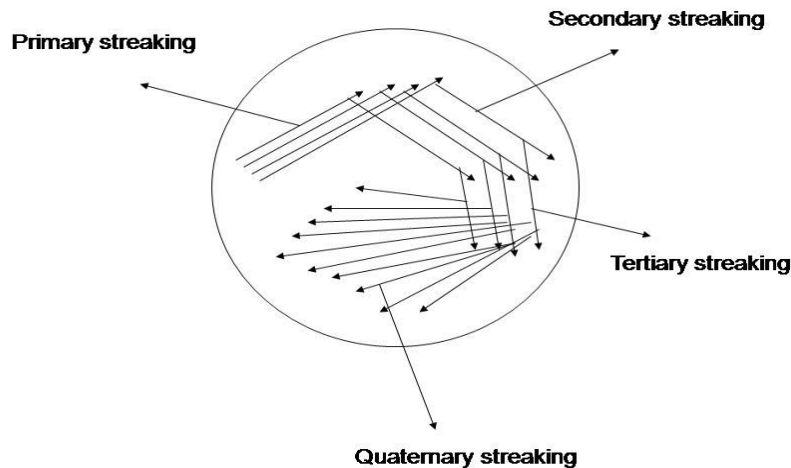


Figure 4. Pattern of streaking for obtaining pure cultures

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-positive 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 safranin 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 are 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.