**Lesson 25**

**Title of the Experiment:** Identification of pathogens by culturing on artificial media
(Activity number of the GCE Advanced Level practical Guide - 38)

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**Introduction:**

Plant disease diagnosis is important in developing or selecting a method for disease management. Numerous methods are available for disease diagnosis of which some are fairly simple, quick and cheap. Examples of such conventional simple methods include: use of symptoms, isolation and identification of the pathogenic organism(s) using culture methods and microscopy, studies on the biochemical and physiological nature of the pathogenic organism(s).

The focus of this laboratory exercise is pathogen identification/disease diagnosis by culture based methods.

Isolation of the pathogen from infected tissues into synthetic culture media is the first step in identifying the causal agents of a particular disease. Different methods of isolating pathogens from infected plant tissues are used depending upon the plant part infected and the type of pathogen. Direct isolation of fungi from infected plant tissues involves placing pieces of the infected plant on a growth medium. Grinding the infected plant parts and then dilution-streaking the macerated tissue onto a growth medium is used in isolating culturable bacterial pathogens. When using infected plant material for isolation of causative agents, it is important that the surface contaminants (organisms on the surface) are removed. Surface sterilization of the material by immersing in an appropriate agent/chemical for a required time period will ensure the removal of organisms on the surface.

The disadvantages of this method are that only pathogens that can be cultured on artificial media can be identified. Therefore, biotrophs (organisms that needs living host tissue/material to survive) such as some fungi, phytoplasma, MLOs, and viruses cannot be identified by this method. Also, these culture methods are not highly accurate or sensitive and the results would be evident only after a considerably long period of time.

Therefore, when quick diagnosis with a high level of accuracy and sensitivity is required and when the causative agent is suspected to be a biotroph, immunological methods such as ELISA, nucleic acid based methods and other more sophisticated methods have to be carried out.

The use of immunological and nucleic acid based methods for pathogen identification will be discussed in the next experiment in this manual.

**Preparation of culture media**

Different types of artificial media can be used to grow different groups of pathogens *in-vitro*. Potato Dextrose Agar (PDA) is frequently used to culture fungal plant pathogens whereas Nutrient Agar (NA) medium is commonly used to culture bacterial plant pathogens. After adding all the components, culture media is autoclaved at $121^{o}$ C, 15 lb/inch$^2$ for 15 minutes. If a fungal pathogen is to be isolated or grown, bacterial contaminants can be eliminated/reduced by adding antibiotics to the medium. To further purify a fungal isolate into pure culture, subsequent sub culturing into

water agar is often practiced. When a fungus grows in water agar, it will grow sparsely searching for nutrients due to lack of available nutrients. Therefore, the mycelia will be well separated from each other enabling the isolation of the tip of a single hypha easily into pure cultures for identification.

Although it is not entirely correct, for obtaining pure fungal cultures, sub culture of isolated fungal spp. into fresh PDA plates instead of into water agar is practiced in some instances.

**Learning outcomes:**
At the end of this laboratory, students should be able to,

1. Prepare artificial culture media
2. Apply aseptic techniques
3. Isolate fungi and bacteria into pure cultures for identification
4. Prepare microscopic slides to observe fungal mycelia and conidia
5. Differentiate Gram –ve and +ve bacterial strains by KOH test

**Preparation of Potato Dextrose Agar (PDA) plates**

**Materials:**

Blades/scalpel
Potatoes 200 g
Glucose 20 g
Agar 20 g
Water 1 L
Sterilized Petri dishes
Autoclave
Bunsen burner/hot plate
Materials for surface sterilization of the work bench

**Procedure:**

Boil 200 g of peeled and sliced potatoes in 1 L of water until the potatoes are soft. Strain through cheesecloth into a conical flask/beaker. Add glucose and agar and mix well. Adjust the volume to 1 L with distilled water. Autoclave for 15 min at 121$^{o}$ C, 15 lb/inch$^{2}$. Allow to cool to about 50-60$^{o}$ C. Pour autoclaved, cooled medium into sterile Petri dishes under aseptic conditions.

**Isolation of plant pathogenic fungi by culturing on the prepared PDA plates**

**Materials:**

Infected plant tissue
PDA plates
Sterilized distilled water
Sterilized Petri dishes for serial washings
70% ethanol
Cotton wool
Scalpel /blades and forceps
Sterilized filter papers
Bunsen burners

**Procedure:**

Each group will receive one diseased plant tissue and two PDA plates to isolate the suspected fungal plant pathogen.

a) Carefully examine the symptoms and signs on the plant tissue provided**.**

b) Carryout the following steps aseptically to isolate fungal pathogen into PDA media:

**Select the appropriate tissue:**
Pathogens are usually isolated from the boundary between healthy and infected tissue. Remove small pieces of tissue from the area between healthy and infected tissue with a flame sterilized razor blade.

**Surface sterilize the tissue:**
Immerse the small pieces of the tissue cut in the above step in 70% ethanol solution (note: usage of mercuric chloride is banned in many countries and 5% commercial bleach, chlorox, is also effective in most instances). When using a chemical for surface sterilization, it is important that the plant material and the pathogen(s) within are not killed in the process. Most materials can be surface sterilized for 30 to 60 seconds without killing the pathogen. The length of immersion depends on the type of plant tissue: woody tissues or other dense materials can be immersed much longer than delicate roots or leaves. The length of immersion can be increased or, sterilization with commercial bleach followed by 70% ethanol can be used when contamination persists. Remove the disinfectant by three serial washings with sterilized distilled water, blot dry it on a dry sterilized paper towel before plating.

**Place the tissue on an appropriate growth medium:**
PDA plates can be used for the isolation of fungi. Trim the four edges of the sterilized plant tissue. Cut about 5 mm pieces and place several (about 4) in a single PDA plate. Be careful not to open the lid of the petri dish more than necessary when transferring disinfected tissue pieces. Lift the lid on one side only and place the tissue on the surface of the medium while covering the dish with the lid.
Incubate the plates for 4-7 days at room temperature and then examine for the growth of fungal mycelia out of the tissue.

**Prepare pure cultures:**
Sub culture each fungal spp**.** that grows out of cultured leaf pieces onto fresh PDA plates separately.

Further purification steps can be conducted as described below to obtain a pure culture of the pathogenic fungal species. (However, these steps will not be carried out in the laboratory and are stated here only for your information.)

Inoculate each sub cultured fungus onto Water agar (WA) plates and incubate for about one week. Cut out the growing tip of a hypha while observing under the microscope and place on a fresh PDA plate. This is known as the Hyphal tip method and it ensures that the ensuing colony is a pure culture. Repeat this for all fungal spp. isolated from the diseased leaf pieces.

The pure cultures obtained by either method can be used for identification of fungal pathogen(s).

**Identification of isolated fungal pathogens:**

**Materials:**

PDA with fungal pure cultures
Inoculation needle
Cotton blue in lactophenol
Slides and cover slips
Microscope

**Procedure:**

Each group is provided with the previously isolated pure cultures of a fungal pathogen grown on a PDA plate. Remove a portion of the culture using a needle and mount on a glass slide. Add a drop of cotton blue in lactophenol. Place a cover slip carefully and observe under the microscope.

Observe the different shapes of conidia, spores and sporulating structures of the fungal spp.
Observe the permanent slides of different pathogens provided. Make appropriate drawings.

**Isolation of plant pathogenic bacteria by culturing on artificial media**

Streaking is one of the techniques used to isolate a pure strain/a single species of bacteria. The isolated pure strain taken from the resulting colonies can be grown on a new plate so that the organism can be identified, studied, or tested.

**Materials:**

Infected plant tissue
Scalpel /blades and forceps
Sterilized distilled water (SDW)
Sterilized Petri dishes for serial washings
70% ethanol
Cotton wool
Clean watch glass
Inoculating loops
Sterilized filter papers
Nutrient Agar (NA) plates
Bunsen burners

**Procedure:**
You are given soft rot infected carrot roots and nutrient agar plates for the isolation of a bacterial pathogen by dilution streaking.
Follow the procedure described below for isolation of bacteria using streak plate method:

**Surface sterilize the tissue:**
Cut small pieces of carrot root provided using a scalpel. Surface sterilize using 70% ethanol. Wash with sterile distilled water (3 serial washings).

**Macerate the tissue:**
Place three or four surface disinfected tissues in a clean watch glass. Add 1 ml of sterilized distilled water. Cut the infected tissue into tiny pieces using a sterile scalpel to release the bacterial cells from inside the tissues to water.

**Streak the macerated tissue:**
Sterilize the provided inoculating loop by holding it in the flame until red-hot, allow it to cool, and then immerse it in the macerate. Make one streak along the edge of a Petri dish containing NA as indicated in the accompanying Figure 1 below, which shows one of the streaking methods. Sterilize the loop again and draw it across one end of the first streak along the edge of the dish. Sterilize the loop again and draw it across the end of the second streak. Sterilize the loop again and draw it across the end of third streak towards the center of the dish in an S-shaped" pattern. Incubate the dish at room temperature for about 48 hrs and observe the isolated colonies.

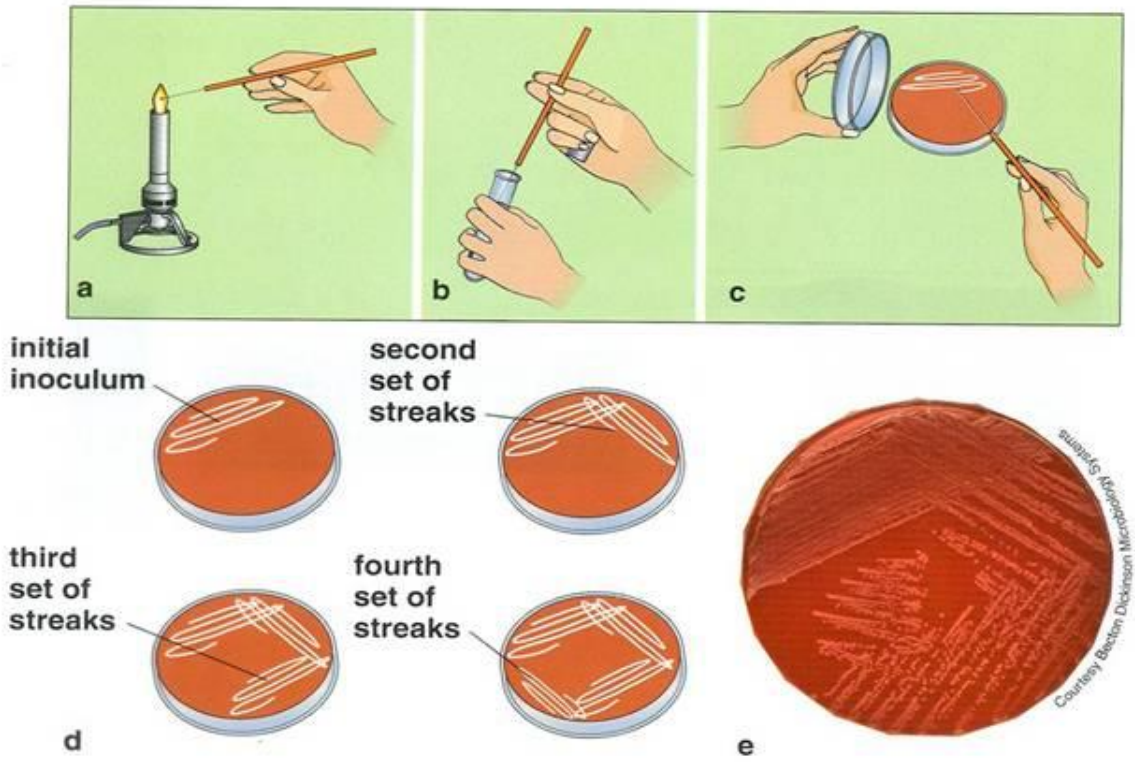


**Figure 1:** Steps involved in dilution streaking of bacteria
(Figure Source: http://intranet.tdmu.edu.ua/data/kafedra/internal/micbio/classes)

**Identification of bacteria**

One of the first steps in identification of bacterial isolates is to differentiate them as Gram +ve or Gram –ve strains. Based on their cell wall properties, several methods are available for this differentiation.

i. **Gram stain:**
The performance of the Gram Stain on any sample requires four basic steps that include applying a primary stain (crystal violet) to a heat-fixed bacterial smear, followed by the addition of a mordant (Gram's Iodine), rapid decolorization with alcohol and counterstaining with safranin. Organisms of the Domain Bacteria are differentiated based on the peptidoglycan content of their cell walls. **Gram +ve** bacteria have cell walls that contain thick layers of peptidoglycan. Therefore, they will retain the crystal violet-iodine complex and **stain purple** whereas **Gram –ve** bacteria have cell walls with thin layers of peptidoglycan and thus will not retain the above complex. These will **stain pink** by the counter-stain safranin.

**ii. Potassium hydroxide (KOH) String test:**

The KOH String test is carried out by placing a drop of 3% KOH on a glass slide and mixing a loopful of cells from a single, well-isolated bacterial colony of the test bacterium with the KOH drop. If the mixture becomes viscous within 60 seconds of mixing (KOH-positive) then the colony is considered to be Gram –ve. The reaction occurs due to the lysis of the Gram –ve cell walls in the dilute alkali solution releasing cellular DNA to turn the suspension viscous. The cell wall integrity of Gram –ve bacteria is easily damaged as their cell wall contains a thin peptidoglycan layer. The cell walls of Gram +ve bacterial cells will remain intact as they contain a thick peptidoglycan layer. Thus they are considered to be KOH negative.

**Materials:**

3% KOH
Clean glass slides
Isolated pure cultures of different bacterial strains

**Procedure:**

Place a drop of 3% KOH on a slide and place a clump of bacterial cells from the NA plates provided. Mix them together. Allow a minute for the reaction to occur. Record your observations

(By using E. *coli* as the test bacterium a KOH positive reaction can be observed.)

**iii. Identification of bacterial diseases by bacterial ooze and bacterial streaming in water**

A common diagnostic test for bacterial wilt involves cutting an infected plant stem, rejoining the cut surfaces for few seconds, and then slowly drawing the cut ends apart. The presence of bacterial slime (masses of bacteria streaming from xylem tissues) extending from one cut surface to the other is a positive indication of bacterial wilt. A variation of this test involves placing the cut stem or crown in water. Bacteria will ooze from the exposed vascular elements of infected plants, forming milky strands flowing from the cut surface into the water (Figure 2).

**Figure 2:** Sticky strand test on cut stems, with bacterial slime streaming from xylem tissues (left).Bacterial ooze from cut tomato stem infected with *Ralstonia solanacearum* (right) (Figure Source- http://www.apsnet.org/edcenter/intropp/topics/Pages/PlantDiseaseDiagnosis.aspx)

**Reference:**

Agrios, G.N. (2005). Plant Pathology. 5th Edition. Academic Press