

EXERCISE # 4**ISOLATION OF BACTERIA FROM PLANT TISSUE**

A critical step for working with plant pathogenic bacteria is obtaining a pure culture of the organism. Pure cultures are absolutely essential for pathogenicity assays and characterizing the pathogen for identification. General methods for isolation and purification are the subject of this protocol.

MATERIALS

Each student or team of students will require the following items:

- ❖ Infected plant material we suggest the following bacteria and diseases for this exercise, although other materials will also be appropriate: *Ralstonia solanacearum* (Granville wilt of tobacco), *Pseudomonas syringae* (angular leaf spot) and/or *Pectobacterium (Erwinia) carotovorum* (hand rot of tobacco/soft rot of potato).
- ❖ 10% commercial bleach solution (90 mL of water and 10 mL of bleach; equal to 0.5 to 0.6% sodium hypochlorite)
- ❖ Distilled or sterile distilled water
- ❖ Several 12- x 75-mm snap cap sterile test tubes
- ❖ Glass rod
- ❖ Several sterile transfer pipettes
- ❖ 1.5-mL eppendorf tubes and micro centrifuge
- ❖ Nutrient agar [8 g powdered medium (Difco, Detroit, MI), 20 g agar and 1 l of water]
- ❖ Bacterial loop
- ❖ Incubator set for 25°C (optional)

ISOLATION OF PATHOGENIC BACTERIA

1. Select tissue to be sampled. Tissue selected for sampling can be any plant part with symptoms. Remove any unnecessary material. For example, do not use the whole leaf, but a portion of the leaf with the suspect lesion surrounded by some apparently healthy tissue. Select the portion to fit the container used in Step 3.
2. Surface-disinfect plant organs with 0.5 to 0.6% sodium hypochlorite for 30 to 60 sec, and then rinse three times with freshly deionized, distilled, or autoclaved water.
3. Immerse the tissue in 1 to 2 mL of distilled water in a clean or sterile 12- x 75-mm snap cap tube. Crush the tissue with a clean glass rod or other convenient implement.

Let the mixture stand for 5 to 10 minutes to allow any bacteria to diffuse of the tissue and into the water.

4. Agitate the mixture briefly to suspend any bacteria evenly in the water. Allow the mixture to stand until the larger portions of remaining tissue settle to the bottom of the tube. Using a transfer pipette, remove as much of the supernatant as possible to one or more sterile, and 1.5-mL eppendorf tubes.
5. Briefly centrifuge at 14 to 17 K for 30 to 60 sec in a micro centrifuge to pellet bacteria. Gently pour or pipette the supernatant off allowing only the pellet.
6. Streak directly from the remaining volume onto either a general bacteriological medium such as nutrient agar. Incubate the medium for 72 to 96 h at 25°C.
7. Select colonies that are well isolated and represent the most numerous colony morphology. Generally, pathogens are in high populations in lesions.
8. Restreak the selected colony at least once onto fresh isolation medium. Each time, select isolated colonies representing the most numerous colony morphology. Bacteria isolated from nature may be contaminated with saprophytic species; restreaking for isolation ensures a pure culture.
9. Bacterial cells from isolated colonies may be suspended in 15% sterile aqueous glycerol (w/v) and stored at -20 to -80°C in snap cap, 1.5-mL conical tubes. It is wise to make several glycerol tubes so that you have a fresh tube for each experiment. The bacterial strain isolated from plant tissue is ready for pathological studies and identification.

ANTEDATED RESULTS

Growth of bacteria on the initial dish of nutrient agar may be heavy and individual colonies may be difficult to select. Restreaking a small portion of an individual colony onto the second dish should result in growth of a number of individual colonies. Pure cultures should be obtained with as few as one or two restreaking.

QUESTIONS

1. Ideally during isolation, how many bacteria form an individual colony on a nutrient agar dish?
2. Why is it important to have pure cultures of plant pathogenic bacteria?