ACP601 PLANT BACTERIOLOGY

PHYTOBACTERIOLOGY PRACTICALS

The practical sessions to be covered in this course are intended to acquaint students with basic methods and techniques for studying of plant pathogenic bacteria. The experiments will be continuous and progressive, and therefore, each student will be required to make continuous observation (sometimes at your own time) during every practical session. The students are thus advised to keep record of all the steps, methodology and results. Each student should ensure the cultures (either supplied or isolated) are adequately labeled and well maintained for use in subsequent experiments throughout the course. Each student is expected to hand results and answers to the questions at the end of each experiment. This will form part of the continuous assessment test marks.

PRACTICAL 1: SYMPTOMS AND ISOLATION OF PLANT PATHOGENIC BACTERIA

1. Objectives
   i. To familiarize with the common bacterial diseases affecting crops in eastern Africa
   ii. To learn the steps in preparation of culture media
   iii. To be able to recognize characteristic symptoms caused by bacteria on plants
   iv. To obtain skills in isolation of plant pathogenic bacteria

2. Symptoms of diseases caused by bacteria on plants

A study of symptoms invoked by plant pathogenic bacteria is an important step in characterization and identification of bacteria pathogens. The disease symptoms on plants due to bacteria include chlorosis, stunting, wilting, necrosis (either as leaf spots or blights), rots, cankers, scabs, and galls.

Requirements
- Infected plant materials
- Power point slides of common bacterial diseases

3. Preparation of media

This section of the practical will involve preparation of media which you will use in isolation of the bacterial pathogens. The success of the subsequent practicals will depend on how good your media is.

a) Nutrient Agar (NA)  g/l
   Beef extract  3.0
Peptone 5.0
Glucose 2.5
Agar 15.0

Dissolve the beef extracts and peptone in water. Adjust the pH to 7.0 by using 0.05 NaOH or 0.05 HCL

b) Yeast extract calcium carbonate agar g/l
Yeast extract 10.0
Dextrose (glucose) 20.0
Calcium carbonate (powder) 20.0
Agar 15.0

The autoclaved medium should be cooled to 50°C in water bath and CaCO₃ suspended by swirling before pouring into plates.

c) Kings medium B Agar
Proteose peptone 20g
K₂HPO₄.7HO 2.5g
Mg.SO₄.7H₂O 1.5g
Agar 20g
Glycerol 10ml
Distilled water 1000ml

Each group of three students will prepare 200ml of each of the above media. To make agar slants, fill 5 universal bottles with 10ml of media (a) and (b) and place the rest of the media separately in 250ml conical flasks and sterilize by autoclaving. After sterilization place the tubes containing the 10ml in an inclined position so that the slanted surface is formed and store at 10°C. Pour about 20 ml of the sterilized media in the conical flasks into sterile petri-dishes.

4. Isolation of plant pathogenic bacteria

Requirements:
- Infected plant materials - black rot of cabbage, halo blight of bean, bacterial canker of tomato
- Plates of nutrient agar medium
- 1-3% sodium hypochlorite for surface sterilization
- Scalpels or razor blades
• Spirit lamps
• Wire loops and innoculating needles
• Sterile distilled water
• 70% ethyl alcohol
• Sterile glass rods

Procedure
(i) Using a sterile scalpel or razor blade, cut out younger portions of lesions from recently collected plant materials.
(ii) Surface sterilize by dipping the cut portions in sodium hypochlorite solution for 1-3 minutes.
(iii) Immediately rinse in 3 changes of sterile distilled water.
(iv) Macerate or crush the tissues in a small amount (about ½ml) of sterile distilled water using a sterile glass rod and leave to stand for some time (about 10 minutes) to free bacterial cells.
(v) Using a flamed wire loop, streak the suspension on nutrient agar medium.
(vi) Incubate the plates in an inverted position at room temperature for 36 to 72 hours and make observations. (NB: It is essential that the surface of the medium is dry; wet media surface allows motile bacteria to swim in the surface moisture and a carpet of mixed growth results instead of discrete, well separated colonies).

Observations
Observe the plates for appearance of bacterial colonies. Well-isolated bacterial colonies should be sub cultured individually onto new medium to make pure cultures. Colonies of the pathogen will often predominate if isolations were made from young infections and freshly-collected material. Material with advanced symptoms and old samples are often invaded by saprophytic fungi and fast-growing bacteria.

Review questions
1. State the functions of the various ingredients of the nutrient agar media used - 2 marks
2. Explain why different bacteria will grow differently in different culture media – 2 marks
3. Why is the length of time for surface sterilization important for successful isolation of bacteria from plant tissues? - 2 marks
4. With a diagram, illustrate the pattern you used to streak suspension containing bacteria on agar medium - 2 marks
5. For each type of media and for each bacteria, describe the following characteristics of the bacterial colonies you isolated: colour, shape, size, elevation, surface, production of pigment (use a table to present your results) - 4 marks
PRACTICAL 2: TESTS FOR CHARACTERIZATION OF PLANT PATHOGENIC BACTERIA
CULTURAL CHARACTERISTICS AND GRAM STAIN REACTION

Objectives
i. To study the characteristics of bacterial colonies growing on culture media
ii. To differentiate bacteria into Gram positive or Gram negative by performing simple
    and fast potassium hydroxide test
iii. To perform Gram stain test

1. Colony appearance
Colony morphology, growth rate, colour and appearance are typical for specific
phytopathogenic bacteria when grown on different isolation media. Phytopathogenic
bacteria grow more slowly than common saprophytes and colonies are only visible after 36-
72 hours. Some slow growing bacteria take 7 days or longer to form visible colonies on agar
media.

Observe the cultures of different bacteria you isolated on solid medium during the last
practical and note the following:
- Colony colour
- Colony shape (circular, irregular, filamentous)
- Colony elevation (flat, raised, convex)
- Colony margins (curved, smooth, filamentous)
- Pigment production
- Colony relative size (pin point, small, large)

2. Pigmentation production
Plant pathogenic bacteria produce a variety of pigments some of which only manifest
themselves on special media. Pseudomonas spp produce several kinds of pigments which
are of taxonomic and diagnostic value. Fluorescent pseudomonads produce yellowish-green
diffusible pigments on King’s medium B medium. The fluorescence can readily be seen
under ultraviolet light. Yellow carotenoid pigments are produced by Xanthomonas spp.

3. Gram Stain
Gram staining is the first step in the identification of a bacterium. Majority of plant
pathogenic bacteria are Gram-negative rods and are either aerobic or facultatively
anaerobic. Gram stain reaction will determine which criteria will be used for further
identification, and cell shape and size will determine whether or not the bacterium is a
possible plant pathogen. Young, actively growing cultures (24 hours) should be used for
Gram staining.
**Procedure**

1. Place a small drop of sterile water on a clean microscope slide.
2. Remove part of young colony, with a cold, sterile wire loop, from agar medium.
3. Smear the bacteria onto the slide. The smear should be just discernible.
4. Air dry and heat fix the bacteria on the slide by passing the slide rapidly four times through a bunsen flame, but do not overheat it.
5. Flood the slide with crystal violet solution for 1 min.
6. Wash in a gentle stream of running tap water until no more stain can be removed from the smear.
7. Flood with Lugol’s iodine solution for a minute.
8. Wash as in 5 and blot dry.
9. Decolorize by washing in gentle stream of ethanol (95%) for no more than 30 seconds to remove stain that will easily wash away and blot dry.
10. Counterstain by flooding with safranin for 20 seconds.
11. Wash with tap water, blot-dry and examine by adding a drop of immersion oil and examine at x100 magnification under the light compound microscope.

**3. Potassium Hydroxide (KOH) solubility test**

This is a rapid method to distinguish between Gram-negative and Gram-positive bacteria and it is an alternative to Gram stain reaction.

1. Place a drop of 3% (w/v) aqueous sodium hydroxide (KOH) solution using a Pasteur pipette, on a microscope glass slide.
2. Take a loopful bacteria from a well grown single colony and mix in KOH drop for not more than 10 seconds. A toothpick can be used instead of a loop.
3. Raise the loop or the toothpick a few centimeters from the glass slide.

If a mucoid thread can be lifted with the loop it is a gram-negative bacterium; if watery suspension is produced, it is a gram-positive bacterium. Gram-positive bacteria do not produce such strands even on repeated strokes of the loop/or toothpick.

**Review questions**

1. For each type of media and for each bacteria isolated, describe the following characteristics of the bacterial colonies you isolated: colour, shape, size, elevation, surface, production of pigment (use a table to present your results) - 4 marks
2. Why is it necessary to use young cultures for Gram stain reaction? - 2 marks
3. Explain the purpose of following in Gram staining:
   a. Making a very thin bacterial smear - 2 marks
   b. Fixing of the smear - 2 marks
   c. Dissolving cells in KOH solution in the KOH solubility rapid test - 2 marks
Objective
To familiarize with procedures in carrying out physiological tests used to differentiate bacterial genera

1. Catalyse test
- 24-hr old bacteria cultures on nutrient agar
- 3% hydrogen peroxide

Method
Using a dropper add a few drops of 3% hydrogen peroxide to the bacterial growth. Look for gas bubbles immediately and if absent look again after 5 minutes. Record as catalyze-positive if bubbles of gas form.

2. Levan (poly-fructose) formation test
Levan is a substance produced through the action of the enzyme levan sucrose. It is produced by most fluorescent pseudomonads that utilize sucrose as a sole source of carbon. Therefore, this reaction is useful in the identification of pseudomonads. *Pseudomonads syringae* produces white, domed, shining, mucoid, levan type colonies on a 5% sucrose nutrient agar. Isolated colonies are usually 3-5 mm after 3 days at 27°C.

Recipe for SNA (sucrose nutrient agar/nutrient agar + 5% sucrose)

- Peptone 5g
- Beef extract 3g
- Agar 20g
- Sucrose 50 g
- Distilled water 1000ml

Inoculate plates by streaking interspersed by surface sterilization of loop in order to obtain separate colonies when testing pure cultures. Serial dilutions from infected samples or seed extracts are similarly used for the inoculation of the plates.

Levan is produced when colonies are convex, white, domed and mucoid.

3. Gelatin Hydrolysis Test:

Recipe for gelatin medium

- Yeast extract 3g
- Peptone 5g
- Gelatin 120g
- Distilled water 100ml
Allow the solids to stand in the water for 15 minutes and dissolve by heating. Adjust pH to approx. 7 if necessary. Dispense into test tubes to a depth of approximately 5cm. Sterilize by autoclaving at 121°C for 15 min.

**Procedure**

i. Stab inoculate test tubes containing molten gelatin medium. Include controls. Incubate at 27°C.

ii. Every 2-3 days record liquefaction of the medium. If needed: Cool the tubes together with the controls at 5°C for 30 minutes before recording the observations.

**Results**

Gelatin hydrolysed: If the test culture fails to solidify.

Weekly positive reaction: The culture liquefies before the positive control tube.

Negative reaction: No liquefaction

4. **Starch Hydrolysis**

**Recipe for starch Agar Medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble starch</td>
<td>2g</td>
</tr>
<tr>
<td>Peptone</td>
<td>5g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3g</td>
</tr>
<tr>
<td>Agar</td>
<td>20g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
</tbody>
</table>

Dissolve the nutrient agar powder in the water by heating. Dissolve the starch in 10ml distilled water and add to molten agar.

**Lugols iodine**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodine</td>
<td>5g</td>
</tr>
<tr>
<td>KI</td>
<td>10g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>500ml</td>
</tr>
</tbody>
</table>

Dissolve by stirring in a closed container for several hours to complete dissolution.

**Procedure**

i. Inoculate starch agar plates by making a single streak in the middle and incubate for 2 to 7 days at 27°C.

ii. Flood with Lugol’s iodine.
Results

Positive: appearance of yellowish, clear zones around or under the bacterial growth indicate starch hydrolysis (amylase activity).

Negative: the medium will turn blue-black when starch has not been hydrolyzed.

Note: reddish-coloured zones indicate that starch has been partly hydrolyzed to dextrine and is usually considered negative reaction.

5. Oxidative/Fermentative metabolism of carbohydrates (Oxidation and fermentation of glucose)

Many bacteria are capable of utilizing simple sugars like glucose and sucrose as a carbon and energy source. Utilization is demonstrated either by the presence of growth or by a biological change brought about in the medium (acid or alkali shift demonstrated by the use of a suitable indicator). The tests can also be used to determine whether a bacterium has a oxidative or fermentative metabolism. All known plant pathogenic bacteria except Clostridium spp are aerobic although some like Erwinia spp are also facultative anaerobes and have a fermentative metabolism. Utilization of the sugars results in production of acid or a gas but sometimes only acid is produced. Some bacteria fail to metabolize any of these carbohydrates, hence neither acid nor gas is produced.

Medium recipe

Peptone 1g
NH₄H₂PO₄ 1g
KCL 0.2g
MgSO₄ 7H₂O 0.2g
Bromothymol blue powder (2.5ml of 1.6% alcoholic solution) 0.08g
Distilled water 1000ml

Adjust pH to 7 - 7.1 (an olivaceous green colour) by drop-wise addition of 20% NaOH solution and dispense 9ml in each universal bottle. Place small tubes in inverted position in each universal bottle and autoclave at 121°C for 15 minutes. Autoclave separately 10% (w/v) glucose solution and add 1ml to each of the test tubes.

Method

i. Prepare nutrient broth with glucose or sucrose plus pH indicator and dispense 10 ml into universal bottles.

ii. Sterilize at 121°C for 15 minutes. Allow the media to cool.

iii. Label each tube with name of bacteria to be inoculated (2 tubes per bacteria)

iv. Take a loopful of bacteria and inoculate into each of the tubes. Leave 2 tubes uninoculated as controls.
v. Seal one of the tubes with sterile mineral oil to form a layer about 1cm depth into one of the tubes of each pair
vi. Incubate at room temperature for up to 14 days.
vii. Examine daily for up to 14 days. A change to yellow indicates acid production; the change in colour may be accompanied by gas production in the small tubes

Organisms with an oxidative metabolism for glucose produce acid only at the top, 1-2 cm of the open tube when conditions are aerobic; in a fermentative reaction acid is produced under aerobic and anaerobic conditions – both the unsealed and the sealed tube change to yellow.

6. Nitrate Reduction Test
Plant pathogens differ in their effect on nitrate, some produce nitrite from nitrate, others such as Xanthomonads do not reduce nitrate, others produce gas from nitrate: they denitrify.

The reaction from the denitrifiers is not strong and may not be evident on all media and may require long incubation periods, up to seven days. Therefore, the media must not be shaken as dissolved oxygen inhibits the reactions. Always check for good growth in the medium employed.

Recipe for nitrate-semi-solid medium
Peptone 10g
NaCl 5g
KNO$_3$ 2g
Distilled water 1000ml
Agar 3g

7. Salt tolerance
Prepare nutrient broth with a range of sodium chloride (NaCl) concentrations (1 – 5%). Dispense in to universal bottles and sterilize by autoclaving. Inoculate each salt concentration with the test bacteria (2 tubes for each) and incubate for 14 days at 25°C.

Positive reaction is indicated by growth visible as turbidity in the tube.
**Review questions**

1. Record the results of the other tests in a table as follows:  
   (You may use a different table format)  
   - 4 marks

<table>
<thead>
<tr>
<th>Test</th>
<th>Bacteria A</th>
<th>Bacteria B</th>
<th>Bacteria C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalyse test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levan formation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatine hydrolysis</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Starch hydrolysis</td>
<td></td>
<td></td>
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<tr>
<td>Oxidative/fermentative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salt tolerance</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. Based on the results of the table above, compare the bacteria tested. What is the explanation for the observed differences/similarities?  
   - 4 marks

3. Explain the production of bubbles in the catalyse test  
   - 2 marks

4. What is the explanation for formation of mucoid colonies in the levan test?  
   - 2 marks

5. Briefly describe the basis of formation of clear zones around bacterial growth in positive starch hydrolysis and gelatine liquefaction tests  
   - 2 marks

6. Explain the source of gas in positive oxidative tests  
   - 2 marks

7. What is the ecological significance of the differences in salt tolerance/intolerance?  
   - 2 marks
PRACTICAL 4: PATHOGENICITY TESTS FOR PLANT PATHOGENIC BACTERIA

Objective
The aim of the host test is to determine whether or not a suspected pathogen can cause disease symptoms in the host from which it was isolated.

Pathogenicity tests are usually done on greenhouse-grown plants, but detached plant parts like fruits, leaves, stems, tubers and pods may be used. For foliar diseases, plants should be in the immature, rapidly developing stages of development and it is important to incubate the inoculated plants at high relative humidity for 18-48 hours for infection to occur. This can be ensured by incubation in a humidity chamber or covering plants with plastic bag.

The general steps in pathogenicity tests are:
1. Inoculum preparation
2. Inoculation and incubation under conditions favourable for disease development
3. Assessment of disease severity.
4. Re-isolation and identification of the bacterium.

Preparation of inoculum
Inoculum is prepared by suspending 24-48-hour old cultures grown on non-selective agar medium in sterile distilled water and adjusting the concentration to about $10^7 \text{cfu/ml}$ ($10^5 - 10^9 \text{CFU/ml}$ depending on the inoculation method). It is essential to include a negative control (inoculation with sterile distilled water only).

Inoculation
1. **Leaf and stem spot pathogens** - spraying both surfaces of leaves or the entire plant with a hand-held atomizer, set to a fine mist, till run off. Some diseases require wounds and damage to the leaf by needle-pricking, by rubbing the leaf with carborundum or by infiltrating the leaf with inoculum.

Test pathogens
   a. *Xanthomonas campestris pv campestris* – black rot on cabbage seedlings (about 2 to 3 weeks after transplanting)
   b. *Pseudomonas savastanoi pv phaseolicola* – halo blight on bean seedlings (4 leaf stage)
   c. *Xanthomonas axonopodis pv phaseoli* – common bacterial blight on bean (4 leaf stage)

Inoculate by spraying and leaf infiltration.
Leaf infiltration – i) select the young actively expanding upper leaves and make small prick the underside with needle, ii) fill a 2 ml syringe with bacterial suspension and without a needle, place tip on injured (prick); place a fore finger on opposite side of leave directly against the tip of syringe nozzle, iii) gently apply pressure to force liquid into tissues – indicated by water soaking appearance.
2. **Detached fruits** – swab the fruit with 70% ethanol and wash in sterile distilled water. Place a drop of inoculum on the fruit surface and puncture the fruit by pricking through the drop with a sterile needle. Incubate at 25°C in moist chamber.

   Test pathogens
   a. *Pseudomonas savastanoi pv phaseolicola* – halo blight on bean pods (young actively expanding, succulent pods)
   b. *Xanthomonas axonopodis pv phaseoli* – common bacterial blight on bean pods (young actively expanding, succulent pods)
      i. Surface sterilize pods in 70% ethanol and rinse in sterile distilled water
      ii. Place 4 to 6 pods in moist chamber (sandwich box lined with wet, sterile paper towel)
      iii. Place drops of bacterial suspension at 1 cm points on surface of the pod.
      iv. Using a sterile needle or tooth pick, pierce the pod surface through the drop of suspension
      v. Incubate for 5 to 10 days and observe water soaking lesions

3. **Soft rotting pathogens** – May use potato tubers, carrot root, cucumber fruits or onion bulbs.

   Test pathogens
   Erwinia carotovora pv carotovora – soft rot on potato tuber or carrot root
   a. Wash the tubers or roots under tap water and surface sterilize in 0.5% sodium hypochloride.
   b. Make a wound either by inserting a sterile cork borer to a depth of 0.5-1cm into the tissue or by removing a small piece of tissue.
   c. Apply bacterial suspension to the cut surface and seal with paraffin wax.
   d. Incubate the tubers in the dark in moist chamber

   Alternatively, prick the tuber or root surface with a toothpick dipped into a dense bacterial suspension.

4. **Root inoculation (vascular wilt-inducing pathogens)** –

   Test pathogens
   a. *Clavibacter michiganense subsp michiganense* – bacterial canker on tomato seedlings (about 15 cm tall hardened seedlings ready for transplanting)
   b. *Ralstonia solanacearum* – bacterial wilt on tomato seedlings (about 15 cm tall hardened seedlings ready for transplanting)
      i. Lift young plants from pots and wash the root system clean of soil.
ii. Trim the roots with scissors or knife and dip into a bacterial suspension for 5-10 minutes. The plants may be placed in a strong air current during this period to facilitate transpiration and uptake of the bacterial cells.

iii. Transplant the seedlings in a suitable sterile growth medium.

5. **Inoculations of bean cotyledons**

Test pathogens

a. *Pseudomonas savastanoi pv phaseolicola* – halo blight on bean cotyledons (seeds pre-germinated on wet paper towel)

b. *Xanthomonas axonopodis pv phaseoli* – common bacterial blight on bean cotyledons (seeds pre-germinated on wet paper towel)

i. Surface sterilize seeds of a susceptible bean variety in 2.5% sodium hypochlorite and rinse in sterile water

ii. Place between sterile, wet paper towel or rolled paper towel in moist chamber and allow 3 – 4 days to germinate (crook-neck stage)

iii. Dip a sterile toothpick or needle in the bacterial culture growing on nutrient agar

iv. Stab the needle through the cotyledon. Turn the toothpick or needle slightly while withdrawing to release bacteria. Re-infesting the toothpick or needle between inoculations

v. Inoculated seedlings are carefully placed in damp soil or potting medium in pots and allowed to grow under humid conditions (70–80%RH) for 4–5 d at 20–25 °C. Record symptoms after 4–8 days. Inspect the flat inner sides of the cotyledons for typical ‘greasy’ spots at the point of inoculation

**Review questions**

1. Explain the need to standardize the bacterial inoculum in testing for pathogenicity

2. Briefly explain how the bacterial suspension is standized to a given number of colony forming units

3. Why is it necessary to injure the plant tissues before inoculation

4. Explain the importance of surface sterilizing the detached plant organs and seeds before inoculation

5. In pathogenicity test using soft rotting bacteria, why is it necessary to seal the inoculated tissues with paraffin wax?

6. Describe the observations for each of the pathogenicity tests carried out