Contents

A SAFRINET MANUAL FOR PHYTOBACTERIOLOGY

Introduction to Practical Phytobacteriology

Compiled by
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Bacterial Diseases Unit, ARC–PPRI, South Africa

Sponsored by SDC, Switzerland

Introduction to Practical Phytobacteriology

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by

SAFRINET, the Southern African (SADC) LOOP of
BioNET-INTERNATIONAL

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Preface

This manual is a guide to a course in practical phytobacteriology for technical assistants of SADC countries in the SAFRINET-LOOP of BioNET-INTERNATIONAL.

The course, presented by the staff of the Bacterial Diseases Unit of the ARC - Plant Protection Research Institute, comprises lectures, practical sessions and discussions aimed at teaching students to recognise and identify bacterial diseases of agricultural crops. Techniques to isolate and identify plant-pathogenic bacteria are presented, as well as information on how to preserve isolated pathogens for further study. The manual not only provides technical details but also lists the literature, including books and manuals, that should be available in laboratories specialising in phytobacteriology.

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Introduction

Although bacteria cause a rather small proportion of plant diseases, this does not mean that these diseases are unimportant. In North Carolina, USA, for instance, Granville or bacterial wilt of tobacco caused so much damage for 30 years after its appearance in 1880 that it forced banks to close, farms to be sold and towns to decline. A more recent example of a severe bacterial disease is watermelon fruit blotch, which appeared in watermelon-production areas of the USA. Pending lawsuits and the risk of future litigation forced major seed companies to suspend their watermelon seed sales in the autumn of 1994.

Other biotic agents implicated in plant diseases are fungi, viruses and nematodes; abiotic factors may also produce disease-like symptoms. A plant abnormality cannot always be diagnosed solely by symptoms as different agents can cause similar pathological symptoms (Fig. 1). Soft rot can be caused by fungi or bacteria; galls by

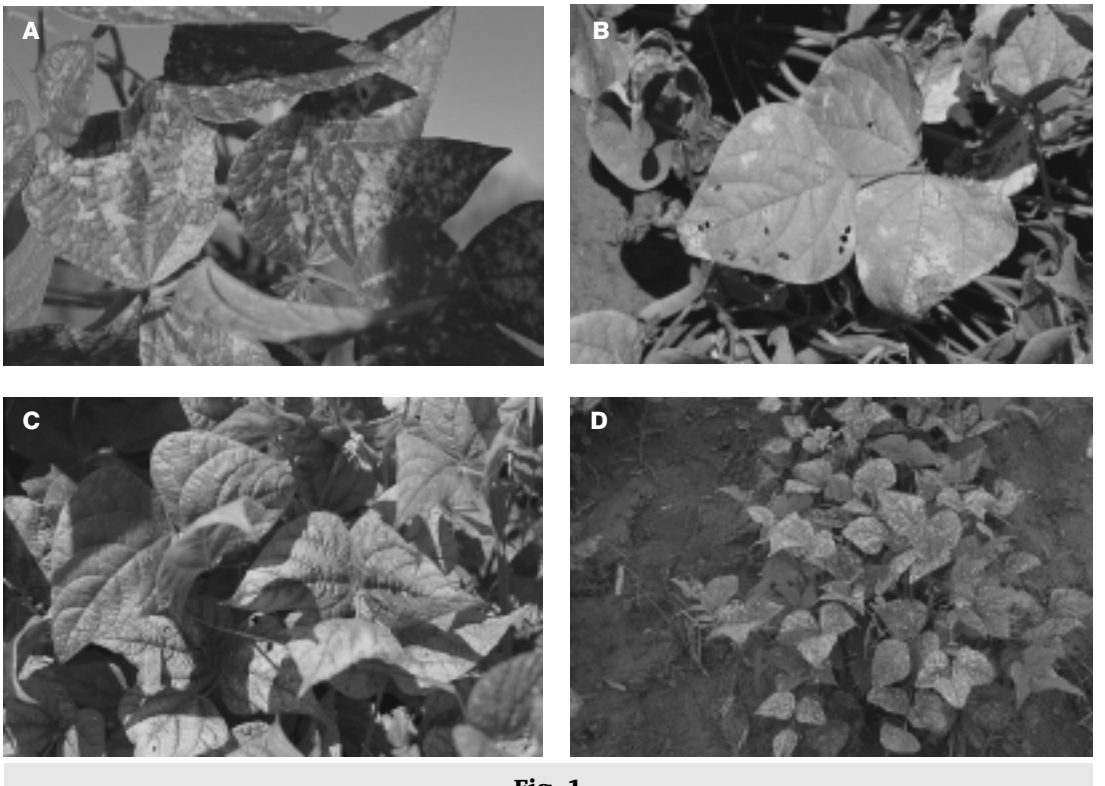

Fig. 1
Similar symptoms on bean plants caused by different agents: A – virus; B – bacterium; C – pesticide; D – fungus.

fungi, bacteria and insects; leaf spot by bacteria, viruses and fungi, and wilt diseases by fungi and bacteria. In a single plant species, symptoms caused by different bacteria may overlap, for example bacterial blight of bean and foliar bacterial diseases of tomato. Symptom expression of a particular disease can vary considerably, and may be influenced by crop cultivar, growth stage, environmental conditions and pathogen strain. Crop-production methods, such as production in controlled environments like greenhouses and in hydroponics, also play a role in symptom expression. Changes in production methods have also brought previously unknown and unimportant diseases to the foreground.

☞ *A preliminary diagnosis of the disease can be made on the basis of symptoms, microscopic examination and a few diagnostic tests. However, accurate diagnosis of the pathogen is always essential.*

Identification of bacterial plant diseases

In a diagnostic laboratory where plant material is analysed for the presence of a plant disease, a number of logical steps must be followed to identify the causal agent of the disease or plant abnormality.

Visual examination and gathering of information

- First step – be sure of the identity of the plant to be analysed.

  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, recent climatic information.

- Second step – familiarise yourself with the symptoms (Fig. 2).

  Gather information about the distribution of the disease in the crop. It is important to examine as many of the diseased plants as possible, from early to advanced stages of symptom development.

- Third step – obtain information about all the possible diseases reported on the crop in the country or subregion.

The Appendix to this manual and the *Index of Plant Pathogens and the Diseases that they Cause in Cultivated Plants in South Africa* (Gorter 1977), which contains the name of the crop, a list of pathogens reported on the crop, and the common names of the diseases, are good starting points in all SADC countries. Not all diseases are included in the *Index* and it is advisable to obtain as much information as possible from the literature. The *Disease Compendium Series* published by the American Phytopathological Society is very useful in this regard. It also contains photographs of diseases of particular crops.

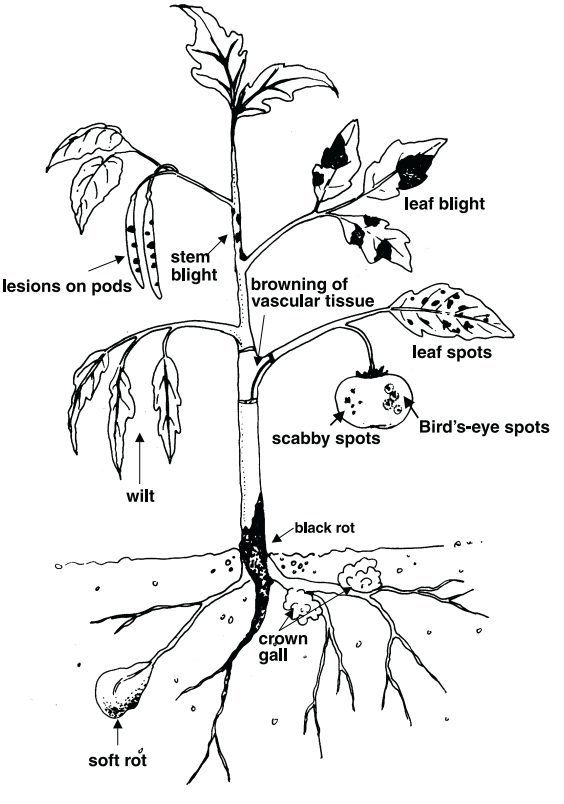

Fig. 2
Symptoms caused by bacteria on plants.

Testing for bacterial streaming

When wilt caused by *Ralstonia solanacearum* is suspected, a section from the lower part of the stem should be cut and placed in a glass of clear water to see whether bacterial streaming from the stem occurs (Fig. 3).

For leaf spot, thin sections from lesion margins should be made, mounted in a drop of water on a microscope slide, covered with a coverslip and examined microscopically for the presence of bacterial streaming (Fig. 4). Standard objectives can be used but phase contrast is better. Streaming of the bacteria from the material may only be evident after 10–15 minutes, especially if the material is not fresh.

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

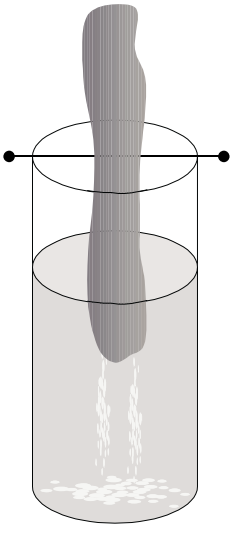

Fig. 3
Milky exudate from tomato stem infected by *Ralstonia solanacearum*.

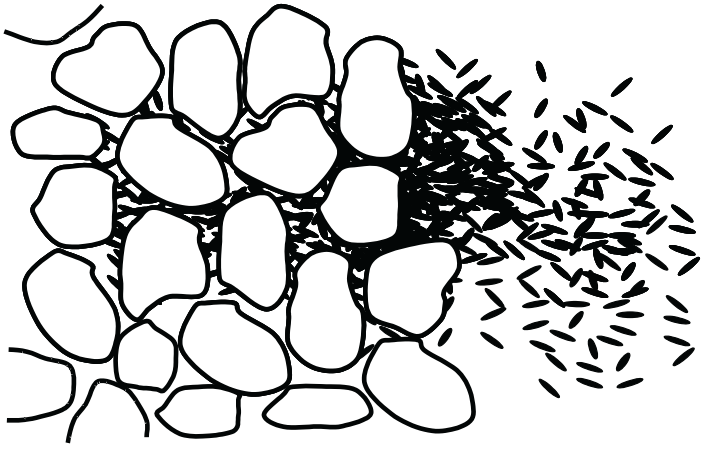

Fig. 4
Schematic drawing illustrating bacterial streaming from diseased tissue

may be obscured by or confused with high numbers of other particulate matter such as latex, plastids and starch granules.

Isolation

To identify a bacterium it must first be isolated (Fig. 5). The isolation medium or media chosen and the method of isolation or the isolation steps will be determined by the suspected disease.

> ☞ *Some disease symptoms caused by different bacteria are very similar and, in such cases, suitable isolation media for both or all suspected bacteria must be incorporated.*

General isolation media are suitable for the isolation of most phytopathogenic bacteria and should be used if the identity of the disease is unknown. Specific, semi-selective and diagnostic media are, however, available for most phytopathogenic bacteria (see chapter 'Media and Diagnostic Tests'). These media vary in complexity and usually contain antibiotics for the suppression of non-target organisms, and complex carbon sources, utilised by a small group of microorganisms on which the target bacterium displays diagnostic features. Media like these are used to isolate the bacteria from

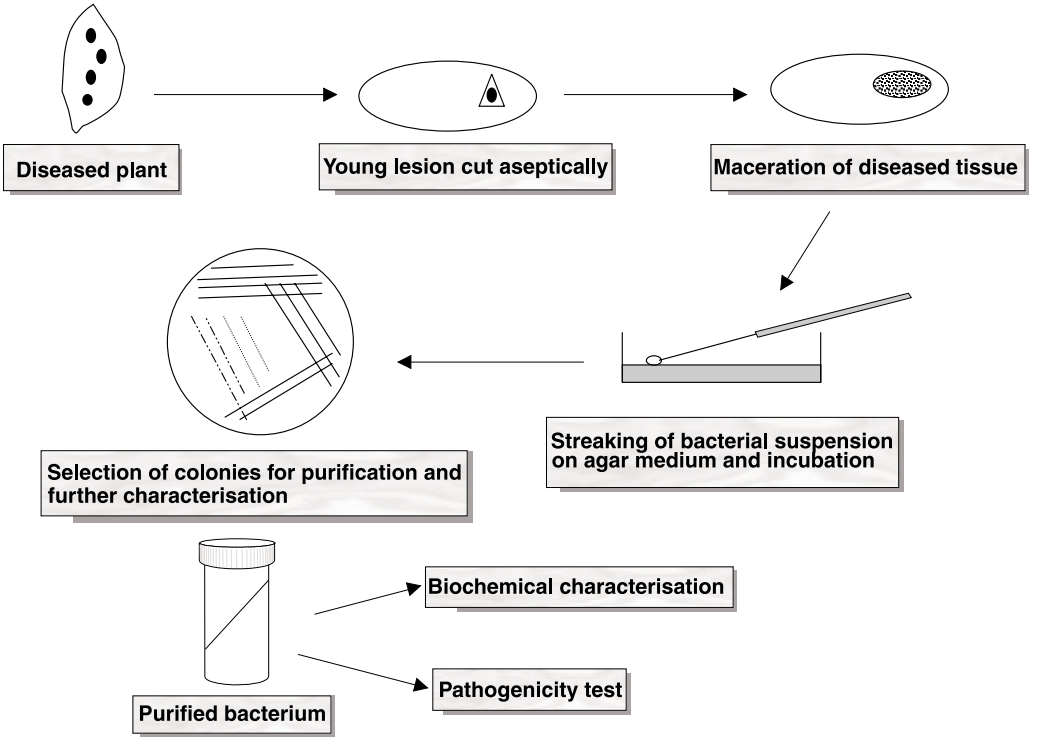

Fig. 5
Steps to isolate and identify phytopathogenic bacteria.

propagation material and from diseased plants. Preparation of these media is sometimes complicated and they are not used routinely in all diagnostic laboratories. However, for the isolation of some pathogens, such as soft rot *Erwinia* spp. and *Ralstonia solanacearum,* special media are recommended.

> ☞ Isolations should be made from the earliest symptoms of the disease; in the case of cankers and blights, from margins between diseased and healthy tissue. A high number of secondary invading bacteria and fungi are normally present in the advanced stages of a disease. These saprophytic invaders normally grow faster on agar media than bacterial pathogens, hampering the isolation of the target organism.

Plant material is rarely surface-sterilised but some phytobacteriologists do use a 0.5 % sodium hypochlorite solution to surface-sterilise plant material by dipping for 2–5 minutes. After surface-sterilisation, the material should be rinsed a few times in sterile distilled water to remove all traces of disinfectant. Washing the material in running tap water to remove soil may be necessary. The material must be left to dry completely before isolations are made.

Isolations 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.

Preparation of plant leachate/macerate

- Cut small sections 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 with a sterile glass rod.
- Set aside for at least 10 minutes.
- Plate on medium.
- Alternatively place plant tissue in a test tube containing 2–3 ml of one of the liquids mentioned above and allow to diffuse 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 (Fig. 6). If a bacterial wilt or soft rot is suspected, or when large numbers of saprophytes could be present, plating by dilution series is recommended. A series of 1:10 dilutions 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 as shown in Fig. 7. Separate, single colonies are more readily obtained in this way. The agar plates should be incubated at about 25 °C for at least 72 hours.

Colony appearance

Differentiation between phytopathogenic and saprophytic bacteria by colony appearance on an isolation medium is the first step in the identification of the pathogen. Colony morphology, growth rate, colour and appearance are typical for specific

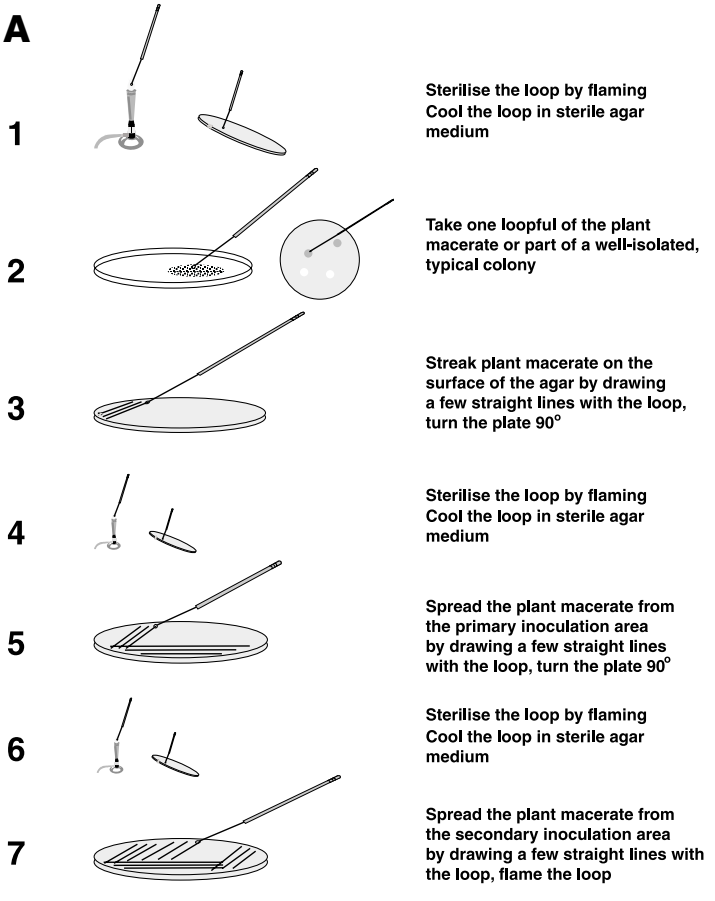

Fig. 6

Isolation of single colonies by streaking of bacteria on an agar medium using a wire loop.

A – Steps to be followed during inoculation of an agar plate using a wire loop.

B – Bacterial colonies growing on an agar plate inoculated by streaking of plant macerate using a wire loop. The lines of growth show how the plate was inoculated.

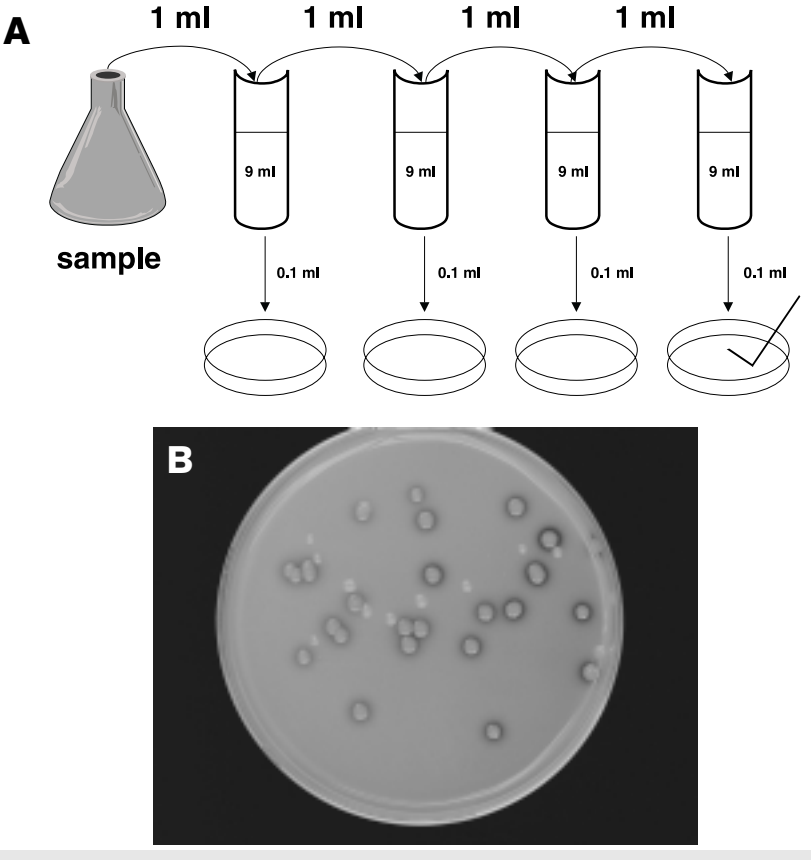

Fig. 7
Isolation of single colonies by spreading plant macerate or its dilutions on the surface of an agar using a sterile glass rod.
A – Tenfold serial dilutions are made from the plant macerate; 0.1 ml of each dilution is placed on the surface of an agar plate and distributed using a sterile, L-shaped glass rod.
B – Bacterial colonies growing on an agar plate inoculated by the spread method. Two types of well-separated colonies are visible on the plate.

phytopathogenic bacteria on different isolation media.

Phytopathogenic bacteria normally grow more slowly than common saprophytes and colonies are only visible after 36–72 hours. Some slow-growing organisms take 7 days or longer to form visual colonies on agar media. If selective media are used, growth of pathogens is also much slower than on general media and plates should be incubated and examined daily for at least 7–14 days. Antibiotics in selective media slow the growth rate of the pathogens.

☞ *Pure cultures of phytopathogenic bacteria are seldom obtained on isolation media (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. Examples are yellow-pigmented *Pantoea agglomorans* and fluorescent saprophytic pseudomonads that resemble pathogenic xanthomonads and pseudomonads. If large numbers of saprophytic bacteria are present, dilution of the leachate and the use of selective media must be considered.

A number of single colonies of the suspected pathogen should be purified by streaking on a non-selective agar medium using a wire loop (Fig. 6), incubated, examined for purity and restreaked.

If purity remains a problem, a single colony should be suspended in sterile water, shaken vigorously and plated for single colonies. Some phytopathogenic bacteria may lose their pathogenic ability during repeated culturing, which should therefore be limited. The pure culture can be restreaked on different agar media to determine colony appearance, including growth rate (colony size), colony morphology (Fig. 8) and pigmentation.

Colony morphology includes shape, size, texture, colony surface markings, elevation, margin type, consistency, colour, translucency or opaqueness.

☞ *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 pseudomonads (pathogenic as well as non-pathogenic) can be distinguished by the production of a blue to green, fluorescent pigment when the colonies are examined under short-wavelength UV light. Many other diagnostic media contain complex carbon sources, utilised by a small group of microorganisms, on which the target bacterium displays diagnostic features. Soft rot erwinias growing on a polypectate-based medium produce typical piths, from pectate degradation. Hydrolysis of starch (clear zones around target colonies) and lypolysis of Tween 80 (crystal precipitation around colonies) are used to distinguish xanthomonads. Proteolysis of casein in media containing skimmed milk, by some xanthomonads and pseudomonads, can be identified as clear zones around colonies.

Microscopic examination of isolated bacteria

☞ *Bacteria with cell walls are divided into two groups: Gram-positive and Gram-negative bacteria. The division is based on differences in cell wall composition and groups can be distinguished using the Gram staining procedure (page 10).*

After purification, Gram-staining is the first step in the identification of a bacterium.

The majority of phytopathogenic bacteria are Gram-negative and belong to either the Gram-negative aerobic rods or the facultatively anaerobic Gram-negative rods. Most Gram-positive phytopathogenic bacteria belong to the Actinomycetes and related

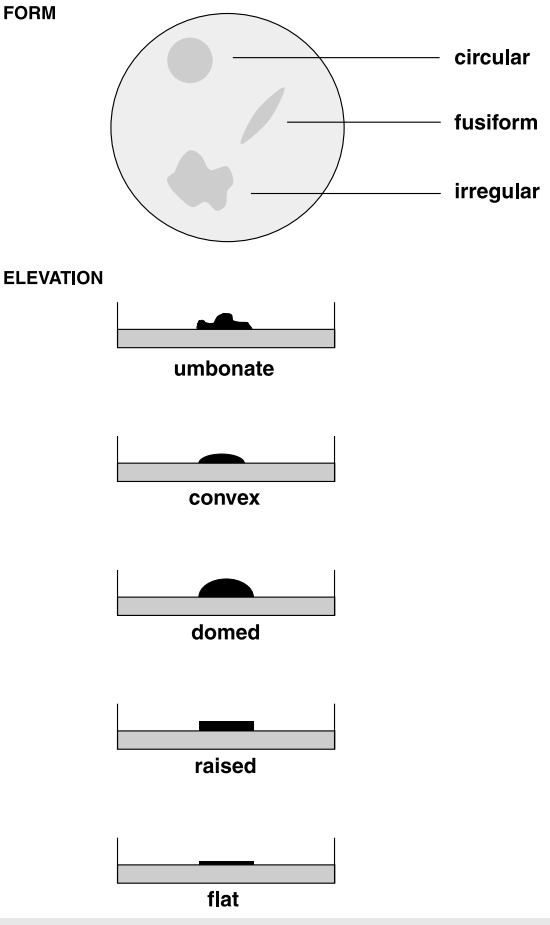

Fig. 8
The most common colony types of phytopathogenic bacteria.

organisms and can be divided into the Corynoform group and the Actinomycetales. Bacteria without cell walls cause relatively few plant diseases.

The Gram 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.

Gram-staining procedure

- Place a small drop of sterile water on a clean microscope slide.
- Remove 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 (recipe 1a in chapter 'Media and Diagnostic Tests') and set aside for 60 seconds.

- Rinse under running water.
- Drain off excess water.
- Flood with Lugol's iodine (1b) and set aside for 60 seconds.
- Wash with 95 % ethanol for 30 seconds.
- Rinse with water.
- Blot dry.
- Counter-stain with Safranine O (1c) for 10 seconds.
- Rinse with water and dry.
- Examine at ×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 mycelial-type growth. Gram-negative and Gram-positive cocci and spore-producing rods are not plant pathogens and should be discarded.*

Cells of Gram-positive corynoform bacteria are generally smaller than those of other Gram-positive bacteria (less than 0.8 μm wide) with typical configurations (L- and Y-shaped) often referred to as Chinese lettering.

☞ *A rapid method to distinguish between Gram-negative and Gram-positive bacteria is to test for solubility of the bacteria in 3 % potassium hydroxide.*

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 a Gram-negative bacterium (Fig. 9), if a watery suspension is produced, it is a Gram-positive bacterium.

Flagellation and motility

The number and orientation of flagella are major taxonomic criteria (Fig. 10). 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 0.5–1.0 ml sterile water.

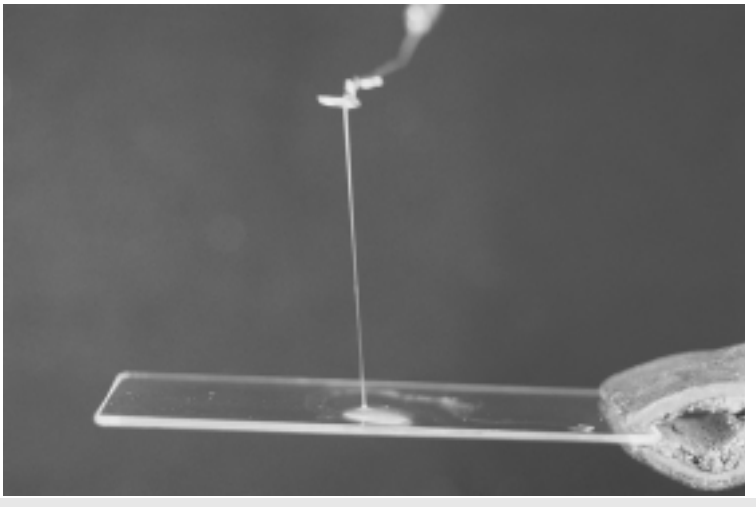

Fig. 9
A Gram-negative bacterium producing a mucoid thread in a KOH solubility test.

- Incubate overnight.
- Remove a loopfull of bacterial suspension from the bottom of the slant and place directly on a microscope slide.
- Cover with coverslip and examine under a ×40 objective.

  ➽ *or*

- 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.

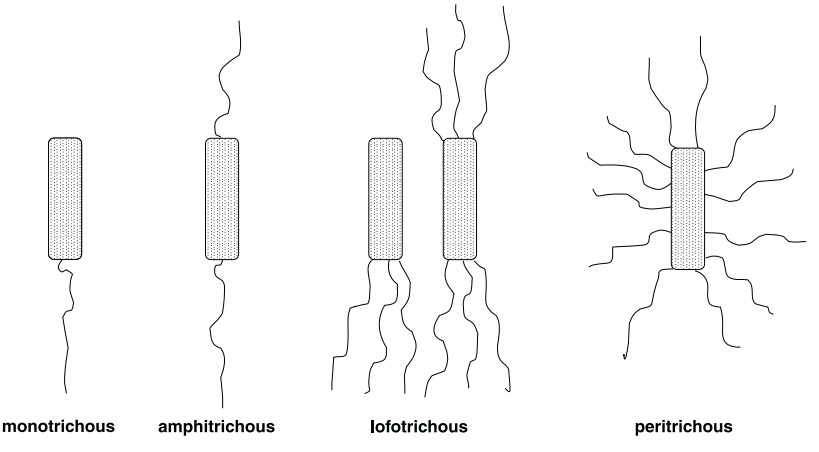

Fig. 10
Flagellation of bacterial cells.

Rapid, darting motility is characteristic of polar flagellate bacteria. Peritrichously flagellate bacteria show a period of relatively slow translational movement followed by chaotic tumbling.

Tests for characterisation of bacteria

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.

Recipes for media (4–42) are provided in the chapter 'Media and Diagnostic Tests'.

When carrying out tests always:

- Include positive and negative controls.
- Use young cultures (18–24 hours) for inoculation.
- Inoculate agar plates and slants by:
  - ✳ Streak-inoculation (Fig. 6).

    ➧ *or*

  - ✳ Spot-inoculation (spread single colony on the surface of agar over 1 cm^2 area using a wire loop or by placing 1 drop of water suspension containing 10^6–10^7 cfu/ml of test isolate).
- Stab-inoculate agar media in test tubes by stabbing (remove a part of colony to be tested using a straight wire and stab into the agar in a tube).
- Drop-inoculate liquid media by adding 1 drop of water suspension containing 10^7 cfu/ml of test isolate.

Utilisation and decomposition of carbon sources

Oxidative/fermentative use of carbohydrates

- Stab-inoculate 2 tubes containing basal mineral medium plus specific carbohydrate (22) with bacteria taken from a young colony.
- Add 1–2-cm sterile mineral oil to one tube.
- Incubate for up to 3 weeks.

In the **Hugh-Leifson** (23) **test** fermentative use of glucose by bacteria is examined. This is an important test for erwinias.

☛ Colour changes to yellow in only the 'open' tube: oxidative reaction.

☛ Colour changes to yellow in both tubes: fermentative reaction.

Utilisation of carbohydrates (growth)

The utilisation of a carbohydrate as a sole source of carbon energy is useful in the identification of bacteria, particularly pseudomonads.

- Spot-inoculate standard mineral base medium containing selected carbohydrate (24).
- Incubate for up to 14 days.

☛ Positive reaction – growth of bacterium.

Acid production from carbohydrates

- Streak-inoculate tubes containing Dye's medium C plus carbohydrate (26).
- Incubate.
- Examine after 2, 4, 6, 21 and 28 days.

☛ Yellow colour: acid production.

Levan production

Levan is a substance produced through the action of the enzyme levan sucrase. Most fluorescent pseudomonads that utilise sucrose as a sole carbon source, produce this enzyme.

- Streak-inoculate nutrient agar with 5 % sucrose (27).
- Incubate for 3–5 days.

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

3-Ketolactose production

Agrobacterium tumefaciens biovar1 oxidises lactose to 3-ketolactose.

- Spot-inoculate (1 cm^2) lactose agar (28).
- Incubate for 2 days.
- Flood with Benedict's reagent (28).
- Incubate for 1 hour at room temperature.

☛ If 3-ketolactose is produced, a yellow ring of Cu_2O precipitate is visible.

Decomposition of nitrogenous compounds

Arginine dihydrolase

Ammonia is produced from arginine under anaerobic conditions. The test is used to differentiate pseudomonads.

- Stab-inoculate tubes containing arginine medium (29).
- Cover with sterile mineral oil.
- Incubate for 24–48 hours.

☛ Positive reaction – colour changes from yellow to red/pink.

H_2S production

H_2S production from organic sulphur compounds is an important test for differentiating *Xanthomonas* spp. and *Erwinia* spp.

- Drop-inoculate test tube containing medium 30.
- Suspend a lead acetate strip (30) over the medium (strip is held by a lid).
- Incubate for up to 14 days.

☛ Positive reaction – black discoloration of strips.

Indole production

- Drop-inoculate 2 tubes containing tryptone/tryptophane medium (31).
- Incubate for 5 days.
- Add 0.5 ml Kovacs' indole reagent (31) after 2 and 5 days.

☛ Positive reaction – cherry red colour that fades after 15 min.

Urease production

The enzyme urease hydrolyses urea to ammonia. Alkaline reaction in the medium is detected by a change in the pH indicator.

- Drop-inoculate tube containing urea medium (32).
- Incubate for up to 7 days.

☛ Positive reaction – colour changes to dark pink.

Decomposition of macromolecules

Gelatine liquefaction

- Stab-inoculate tubes containing 12 % (w/v) gelatine (Difco) (33).
- Incubate for up to 15 days at 20 °C.
- Keep at 5 °C for 15 minutes before determining liquefaction.

☛ Positive reaction – liquefaction of gelatine seen when tubes are tilted.

Aesculin hydrolysis

- Streak-inoculate slants or plates containing aesculin medium (34).
- Incubate for 2–5 days.

☛ Dark colour develops if β-glycosidase activity is present.

Milk proteolysis

Milk proteolysis is a diagnostic characteristic for most xanthomonads.

- Spot-inoculate medium containing milk (35).
- Observe for clear zone around growth after 3, 5 and 7 days.

☛ Clear zone around growth indicates milk proteolysis.

Starch hydrolysis

- Streak-inoculate starch plates (36).
- Incubate for 2–7 days.

- Flood with iodine solution (1b).

  ☛ Starch stains blue-black; a clear zone around growth indicates starch hydrolysis (amylase activity).

Tween 80 lypolysis

On nutrient media containing Tween 80 and $CaCl_2$, opaque zones develop around colonies of bacteria that produce the enzyme esterase.

- Streak-inoculate Tween 80 medium (37).
- Incubate for up to 7 days.

  ☛ Positive reaction – opaque zone around growth.

Lecithinase production

The test is used to differentiate *Erwinia* spp.

- Spot-inoculate egg-yolk agar (38).
- Incubate for 7 days.

  ☛ Positive reaction – opaque zone around growth after 7 days.

Other tests

Poly-β-hydroxyburate granules (PHB)

Some phytopathogenic bacteria produce organic reserve materials in the form of polyesters of β-hydroxy butyric acid, which can be microscopically observed after PHB staining. PHB staining is used to distinguish between species in the non-fluorescent pseudomonad group (Table 1).

- Heat-fix bacteria on a glass slide as for Gram staining.
- Flood slide with Sudan Black (3) for 5–15 minutes.
- Drain off and blot dry.
- Cover slide with xylene for 10 seconds, blot dry.
- Counter-stain for 5 seconds with 0.5 % aqueous safranine (1c), wash and dry.
- Observe under oil immersion.

  ☛ Positive reaction – blue-black or blue-grey granules in a pink cell.

Oxidase test

- Prepare 1 ml 1 % aq., w/v solution of NNN'N'-tetramethyl-p-phenylene-diamine-dihydrochloride solution (40).
- Place a few drops of solution on a new piece of Whatman No. 1 filter paper with a clean Pasteur pipette.
- Remove part of a colony with a sterile toothpick (**do not use a metal loop**).
- Smear onto the moistened paper.

  ☛ Colour changes to dark purple within 30 seconds is positive; if it takes longer, up to 60 seconds, it is weakly positive.

Salt tolerance

- Prepare nutrient broth (6) with a range of NaCl concentrations (1–5 %).
- Drop-inoculate 2 tubes of each NaCl concentration.
- Incubate for 14 days at 25 °C.

☛ Positive reaction – growth visible as a turbidity in the tube.

Growth at minimum or maximum temperature

- Drop-inoculate nutrient broth (6) tubes.
- Incubate at required temperatures for 7 days.

☛ Positive reaction – growth visible as a turbidity in the tube.

Potato soft rot

- Cut 7–8-mm-thick slices from washed, alcohol-flamed, peeled potatoes.
- Place each slice in a Petri dish.
- Add sterile distilled water to a depth of 3–4 mm.
- Make a nick in the centre of each slice.
- Spot-inoculate with a loopful of a nutrient agar culture.
- Incubate for at least 24 hours.
- Draw inoculating loop across inoculated part to determine whether the slice has decayed beyond the point of inoculation.

☛ Positive reaction – decaying of potato beyond the point of inoculation.

Tobacco hypersensitive reaction (HR)

If some plant species are inoculated with pathogenic bacteria that are not a pathogen of the plant, a rapid defence mechanism in the plant is triggered. Plant cells in the invaded area die off, restricting the invading pathogen by preventing further spread to the rest of the plant. The triggering of HR in plants by phytopathogenic bacteria is used as a diagnostic tool, especially for fluorescent pseudomonads.

- Prepare an opaque suspension (10^8–10^9 cells per ml) of the isolate in sterile distilled water.
- Infiltrate the lower surface of a mature tobacco leaf by pressing a syringe containing the suspension against the leaf, forcing the suspension into the leaf.
- Use distilled water as a negative control.
- Best results are obtained if inoculation is carried out before 10:00 or after 15:00.

☛ Positive reaction – the infiltrated area becomes dry and necrotic within 24 hours.

Tyrosinase activity

- Streak-inoculate a test strain on agar containing L-tyrosine (39).
- Incubate for 2–5 days.

☛ Red to reddish-brown, diffusible pigment indicates tyrosinase activity.

Acetoin production

- Drop-inoculate yeast salts broth + glucose (41).
- Incubate on a rotary shaker for 5 days.
- Transfer 1 ml to test tube after 2 and 5 days.
- Add 0.6 ml 5 % α-naphtol (41 A), and shake for 5 seconds.
- Add 0.2 ml 40 % KOH (41 B), shake vigorously.
- Set aside.
- Observe after 30 minutes, and 2 and 4 hours.

☛ Positive reaction – appearance of a crimson to ruby colour at the top or throughout the mixture within 2 hours.

☛ Negative reaction – appearance of colour after 4 hours.

Catalase

- Add a few drops of 3 % H_2O_2 to a 24-hour-old colony.

☛ Positive reaction – gas bubbles.

Determination of pathogenicity

Determination of pathogenicity and fulfilment of Koch's postulates is a very important step in the identification of phytopathogenic bacteria. Pathogenicity tests are not standardised and depend on host–pathogen combination. Inoculation methods for foliar, root and vascular pathogens are different. The general steps in pathogenicity tests are (Fig. 11):

- Inoculum preparation.
- Inoculation of greenhouse-grown host plant.
- Incubation of plants under conditions favourable to disease development.
- Interpretation of disease symptoms.
- Reisolation and identification of the bacterium.

Pathogenicity tests are normally performed on greenhouse-grown plants, but detached plant parts like fruit and pods are also sometimes used. For foliar diseases, plants should be in the optimal stage of development (immature, rapidly developing tissue) and it is important to incubate them at high relative humidity for 18–48 hours after inoculation (in a humidity chamber or cover plants with a wet plastic bag). Inoculum is prepared by suspending 24–48-hour growth from a non-selective agar medium in sterile distilled water and adjusting the concentration to ~10^7 cfu/ml. It is advisable to use different concentrations – from 10^6 cfu/ml to 10^8 cfu/ml. A reference strain should be included if available. Plant pathogens, especially pseudomonads, can cause a variety of reactions (necrotic spots, toxin reactions and even watersoaking) on non-host plants if the inoculum level is too high. It is essential to include a negative control.

Inoculation can be carried out as follows:

- Spray both surfaces of a leaf or an entire plant with a hand-held atomiser, set to a fine mist, till run-off without infiltrating the leaf. 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.
- Stems are inoculated by placing a drop of inoculum at the base of a leaf petiole and stabbing it into the stem. Leaves or twigs can also be removed with a razor blade and a drop of inoculum placed on the wound.
- Immature fruit are inoculated by placing a drop of inoculum on the fruit and then pricking it.
- Tomato plants can be inoculated with *Ralstonia solanacearum* either by placing a drop of inoculum on the stem and pushing a needle through the stem or by adding 10^8 cfu/ml inoculum to soil after roots have been cut in several places with a scalpel.
- For *Agrobacterium tumefaciens,* young tomato, sunflower and datura plants are used as indicators. The stems of the plants are wounded by making a slit with a sharp needle on the stem, applying a drop of inoculum and making a number of small wounds with the needle at an angle of 90° across the initial wound.
- Inoculum can also be introduced into plants by using toothpicks, scalpels, needles, injection or knives.

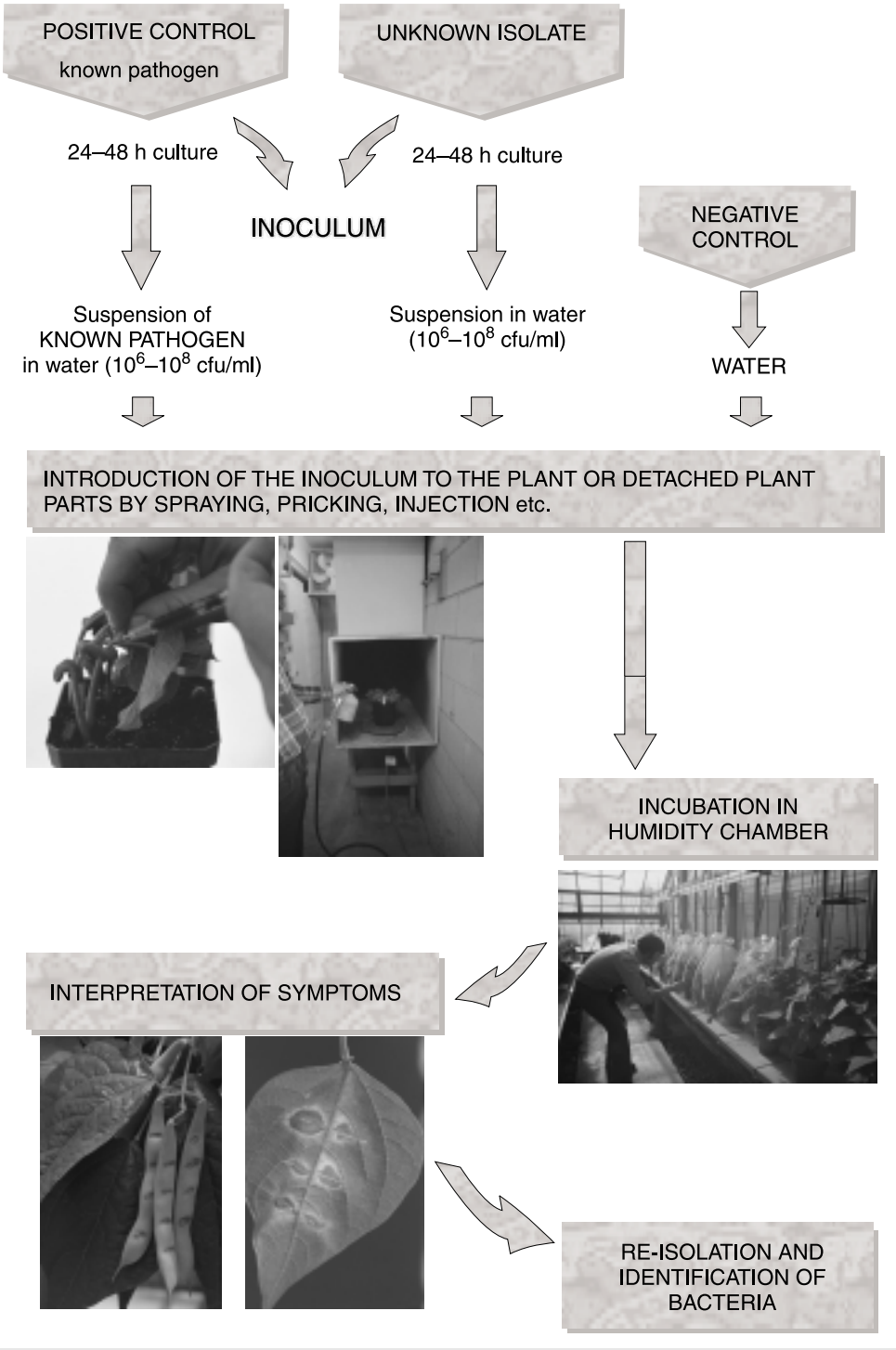

Fig. 11
Pathogenicity test (Koch's postulates).

Classification of bacteria

Bacteria belong to the kingdom *Procaryotae* (or *Monera*). The prokaryotes differ from other forms of life in the structure and organisation of the cells, genetic material and method of genetic exchange. They all have cell membranes and a non-membrane-bound nucleoid, whereas eukaryotes have a membrane-bound nucleus and cell organelles like mitochondria and chloroplasts (Fig. 12). Most bacteria, except mycoplasmas, have a cell wall that consists of peptidoglycan.

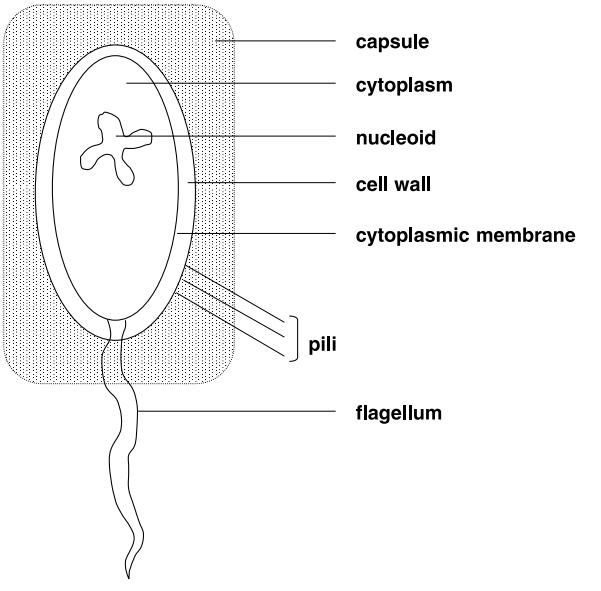

Fig. 12
Schematic diagram of a typical rod-shaped bacterium.

Bacterial cells come in different shapes and sizes (Fig. 13), but most phytopathogenic bacteria are rod-shaped, 1–3 μm in length and about 1 μm wide.

It is difficult to verify all characteristics of a bacterium, and differentiation between species is not always apparent. Intermediate strains occur. Some groups of phytopathogenic bacteria can only be differentiated by their pathogenicity on plants; an infraspecific division called pathovar (pv.) was established to accommodate these groups. An example is *Pseudomonas syringae* pv. *tomato,* the causal organism of bacterial speck of tomato. Some phytopathogenic bacteria attack only specific plant species; some have a narrow host range while others have a wide host range. Some genera and species of phytopathogenic bacteria have in recent years been reclassified.

☞ *Bergey's Manual of Systematic Bacteriology* is the basic manual for the classification of bacteria. New names and taxonomic changes in bacterial classification are published in the *International Journal of Systematic Bacteriology.*

☛ In bacteria, morphological features alone are of little taxonomic value, because they are too simple to provide enough taxonomic information. Bacteria are mainly characterised by their physiological, biochemical and molecular characteristics.

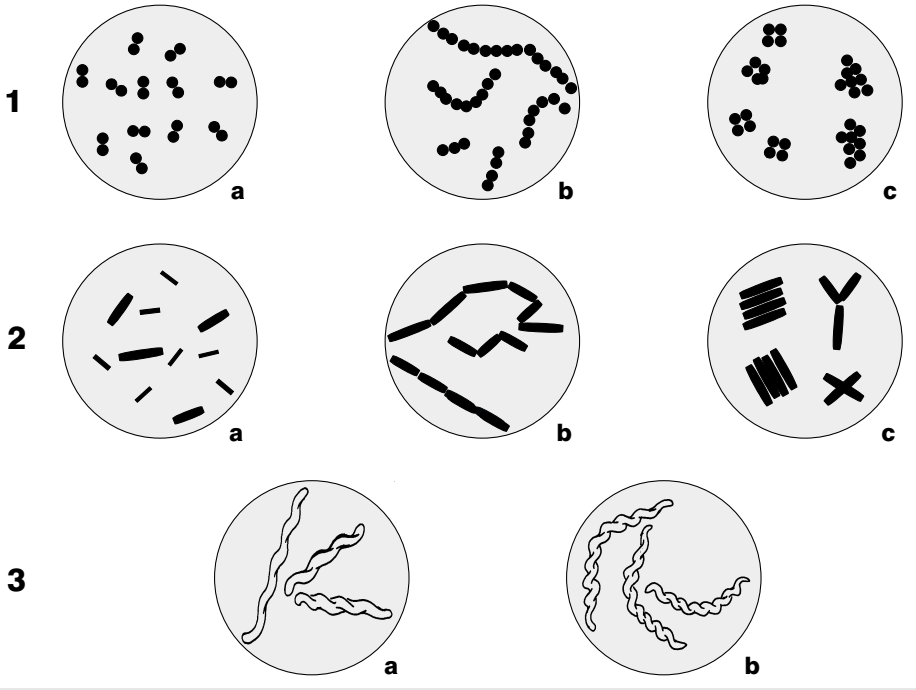

Fig. 13

Bacterial cells usually aggregate in the shape of cocci, rods or spirals.

1 – cocci: a) diplococci; b) chains of cocci; c) tetrads and clusters.
2 – rods: a) single rods; b) chains of rods c) palisades, X and Y formations.
3 – spirals: a) spirochetes b) spirilla.

1. Gram-negative bacteria

☞ Gram-negative rod-shaped, motile bacteria; do not produce yellow colonies on YDC agar and are fluorescent on KB medium – *Pseudomonas* sp.
Fluorescent pseudomonads are divided into 5 groups according to the LOPAT tests (page 26).

- Oxidase reaction negative = *P. syringae* pathovar or *P. viridiflava.*
- Potato soft-rot positive = *P. viridiflava.*
- Potato soft-rot negative = *P. syringae* pathovar.
- Most *P. syringae* pathovars = levan positive.
- *P. viridiflava* = levan negative.
- Most *P. syringae* pathovars utilise sucrose as sole carbon source.
- *P. viridiflava* does not utilise sucrose as sole carbon source.
- Oxidase reaction positive = *P. marginalis, P. tolaasii, P. agarici, P. cichorii* or non-pathogenic *Pseudomonas* (mostly *P. fluorescence* or *P. putida*).

- Levan, potato rot and arginine dihydrolase positive but tobacco hypersensitivity (HR) negative = *P. marginalis.*
- Isolated from mushroom; levan, potato rot and tobacco HR negative but arginine dihydrolase positive = probably *P. tolaasii.*
- Isolated from mushroom: levan, potato rot and arginine dihydrolase negative but tobacco HR positive = probably *P. agarici.*

If isolated from other plants but gives same reaction = probably *P. cichorii.*

☞ Gram-negative rod-shaped, motile bacteria; do not produce yellow colonies on YDC agar and are non-fluorescent on KB – *Pseudomonas* spp., *Burkholderia* spp., *Acidovorax* spp., *Ralstonia* spp., *Agrobacterium* spp., soft rot *Erwinia* spp. and some non-soft rot *Erwinia* spp.

- Pit formation on CVP medium and fermentative utilisation of glucose in Hugh-Leifson test = soft rot *Erwinia* spp.
- No pit formation on CVP medium and fermentative utilisation of glucose (Hugh-Leifson test) = non-soft rot *Erwinia* spp. and *Pantoea* spp.
- Pit formation on CVP medium and oxidative utilisation of glucose (Hugh-Leifson test) = *Pseudomonas* spp. and *Xanthomonas* spp.
- No pit formation on CVP medium and oxidative utilisation of glucose (Hugh-Leifson test) =*Pseudomonas* spp., *Burkholderia* spp., *Acidovorax* spp., *Ralstonia* spp. and *Agrobacterium* spp.

Tests to distinguish *Pseudomonas* spp., *Burkholderia* spp., *Acidovorax* spp. and *Ralstonia* spp. are presented in Table 1. Most species are oxidase positive (except *P. amygdali, B. andropogonis, P. fiscuserectae* and *B. glumae*) and have PHB inclusions.

Table 1. Tests to differentiate between non-fluorescent *Pseudomonas* spp., *Acidovorax* spp., *Burkholderia* spp. and *Ralstonia solanacearum*.

Test	A	B	C	D	E	F	G	H	I
PHB	–	+	+	+	+	+	ND	V	+
Number of flagella	1–6	–	>1	>1	>1	>1	1–3	1	>1
Levan	+	ND	ND	ND	–	+	ND	–	–
NO_3 reduction to NO_2	–	–	+	+	+	V	+	–	+
Denitrification	–	–	–	–	–	–	–	–	V
Kovac's oxidase	–	–	+	+	+	+	–	+	+
Gelatine hydrolysis	–	–	V	V	+	+	+	–	+
Starch hydrolysis	–	–	–	–	–	–	V	–	–

Test	A	B	C	D	E	F	G	H	I
Arginine dihydrolase	–	–	–	–	–	–	–	–	–
Growth at 4 °C	+	ND	–	–	ND	–	ND	–	ND
Growth at 41 °C	–	–	+	V	–	+	+	+	–
Acid from sucrose	+	–	+	V	+	+	–	–	+
Diffusible pigments	–	–	y,g	V(y)	y,g	y,g	–	–	V(b)
Non-diffusible pigments	–	–	–	y,g(pl)	–	–	–	–	–

A, *Pseudomonas amygdali;* **B**, *Burkholderia andropogonis;* **C**, *B. caryophylii;* **D**, *B. cepacia;* **E**, *P. corrugata;* **F**, *B. gladioli;* **G**, *B. glumae;* **H**, *Acidovorax avenae* subsp. *citrulli;* **I**, *Ralstonia solanacearum.*

ND, not determined; **V**, variable reaction.

Pigments: **b**, brown; **y**, yellow; **g**, greenish; (**pl**) purple (variable reaction).

☞ *Gram-negative rod-shaped, motile bacteria; produce yellow colonies on YDC agar and are non-fluorescent on KB – Erwinia* spp., *Pantoea* spp. and *Xanthomonas* spp.

- Fermentative utilisation of glucose (Hugh-Leifson test) = *Erwinia* spp., *Pantoea* spp. or another member of the Enterobacteria.
- Oxidative utilisation of glucose (Hugh-Leifson test) = *Xanthomonas* spp., *Pseudomonas* spp. (see Table 1for pigmented pseudomonads) or non-pathogenic bacterium.

  Bacteria with gliding motility, spreading growth on agar media, and short or long flexuous rods that are oxidase positive may be *Cytophaga* or *Flexibacter,* and non-motile short rods with small, smooth colonies that are also oxidase positive may be *Flavobacterium.* These bacteria are not pathogens.
- Oxidase reaction positive = non-pathogen or pigment-producing pseudomonad (see Table 1).
- Oxidase reaction negative; slow-growing (2 days or more); typical colony type on YDC agar is circular, smooth, domed and mucoid = *Xanthomonas* spp.

Gram-negative aerobic rods and cocci

This group contains a large variety of phytopathogenic bacterial species. The main genera include *Pseudomonas, Xanthomonas* and *Agrobacterium.*

Genus *Acidovorax*

☞ *Bacteria in this genus are non-fluorescent, motile by a single polar flagellum, oxidase and catalase positive and accumulate poly-β-hydroxyburate. Colonies are white or colourless but some strains may produce a yellow or brown diffusible pigment.*

Some of the plant pathogens in this genus are:

- *A. avenae* subsp. *citrulli*: bacterial fruit blotch of watermelon (Fig 14). Major outbreaks of this disease recently occurred in the USA and other parts of the world.
- *A. avenae* subsp. *avenae*: bacterial leaf blight of oats, bacterial leaf blight and stalk rot of maize and bacterial brown stripe of foxtail (*Alopecurus* spp.) and other grasses.
- *A. avenae* subsp. *cattleyae*: brown spot disease on orchids.

Fig. 14
Bacterial fruit blotch of watermelon, *Acidovorax avenae* subsp. *citrulli*.

Genus *Burkholderia*

☞ Bacteria in this genus are non-fluorescent, oxidase variable, catalase positive, accumulate poly-β-hydroxyburate and are motile by a single polar flagellum or polar tuft. All strains can grow on glucose, glycerol, inositol, galactose, sorbitol and mannitol.

Some of the plant pathogens in this genus are:

- *B. andrapogonis*: bacterial leaf spot of clover and velvet bean, bacterial stripe of sorghum and leaf spot on carnation, tulips and other ornamentals.
- *B. caryophylli*: bacterial wilt and bacterial stem crack of carnation.
- *B. cepacia*: sour skin of onion.
- *B. gladioli*: leaf spot and corm scab of *Gladiolus* spp. as well as soft rot of onion.
- *B. glumae*: bacterial grain rot of rice.

Genus *Pseudomonas*

☞ This is a very heterogeneous genus that can be conveniently divided into 2 groups:
- fluorescent.
- non-fluorescent.

The fluorescent *Pseudomonas* produce a yellow-green to blue fluorescent pigment on iron-deficient media (KB medium) and have been further divided into 5 groups using 5 key tests:

- **L**evan production
- **O**xidase reaction
- **P**otato rot
- **A**rginine dihydrolase production
- **T**obacco hypersensitivity

These are generally known as the **LOPAT** tests.

Group I (LOPAT + – – – +) includes over 50 *P. syringae* pathovars and causes a wide range of diseases on various plant species.

P. syringae subsp. *savastanoi, P. syringae* pv. *glycinea* and *P. syringae* pv. *phaseolicola* are distinct from *P. syringae* pv. *syringae* according to DNA hybridisation data. A new species, *P. savastanoi,* was described that includes these 3 pathogens recently recognised as *P. savastanoi* pv. *savastanoi, P. savastanoi* pv. *glycinea* and *P. savastanoi* pv. *phaseolicola.*

P. syringae pv. *syringae* has the widest host range in the group, having been reported on over 180 plant species. Some of the major plant diseases caused by *P. syringae* pathovars are:

- *P. syringae* pv. *syringae*: bacterial canker of stone-fruit trees; bacterial blossom blight or blast of pear; brown spot of bean; citrus blast and black pith.
- *P. syringae* pv. *lachrymans*: angular leaf spot of cucurbits.
- *P. syringae* pv. *morspronorum*: bacterial canker of stone-fruit trees.
- *P. syringae* pv. *pisi*: bacterial blight of pea.
- *P. savastanoi* pv. *phaseolicola* (previously known as *P. syringae* pv. *phaseolicola)*: halo blight of *Phaseolus* spp. (Fig. 15).

Fig. 15
Halo blight of bean, *Pseudomonas savastanoi* pv. *phaseolicola*.

- *P. syringae* pv. *tabaci*: wildfire of tobacco, soybean and sometimes bean.
- *P. syringae* pv. *tomato*: bacterial speck of tomato.
- *P. savastanoi* pv. *savastanoi* (previously *P. syringae* pv. *savastanoi*): galling and excrescence of Oleaceae and *Nerium oleander.*

P. syringae pathovars can also be differentiated by biochemical tests. As some plant species are attacked by two or even more *P. syringae* pathovars, biochemical differentiation between pathovars is important. Beans are attacked by *P. syringae* pv. *syringae, P. syringae* pv. *tabaci* and *P. savastanoi* pv. *phaseolicola.* Bacterial canker on stone-fruit is caused by *P. syringae* pv. *syringae* and *P. syringae* pv. *morsprunorum.* *P. syringae* pv. *syringae* and *P. syringae* pv. *pisi* cause bacterial blight of pea. Differential tests are given in Table 2.

Table 2. Tests to differentiate between some *Pseudomonas syringae* and *P. savastanoi* pathovars.

Test	*P. s. syringae*	*P. s. tabaci*	*P. s. tomato*	*P. s. pisi*	*P. s. phaseolicola*	*P. s. glycinea*
Ice nucleation	+	–	–	+	–	+
Utilisation of:						
Sucrose	+	+	+	+	+	+
Sorbitol	+	+	+	+	–	–
Mannitol	+	+	+	+	–	V
Erythitol	+	V	–	V	–	–
L-Tartrate	–	+	–	–	–	–
D-Tartrate	V	–	+	–	–	–
L-Lactate	+	–	–	V	–	–
Homoserine	–	–	–	+	–	–
Inositol	+	+	+	+	–	+

V = variable reaction.

P. syringae pv. *syringae* (**PSS**) and *P. syringae* pv. *morsprunorum* (**PSM**) can be differentiated by the GATTa test. GATTa stands for:

- **G**elatine liquefaction
- **A**esculin hydrolysis
- **T**yrosinase activity
- **Ta**rtrate utilisation

Test results: **PSS** = G+, A+, T–, Ta–**; PSM** = G–, A–, T+, Ta+

Group II (LOPAT – – + – +) includes *P. viridiflava,* a common epiphyte but sometimes a potential pathogen on a wide variety of plants. It is often a secondary invader of plants infected with a *P. syringae* or *Xanthomonas campestris* pathovars, hampering the isolation of the primary pathogen. It can be a rapid, invasive primary pathogen under certain conditions such as after frost damage, causing a rapid watery rot of succulent material. Hosts include pea, bean, crucifers, tomato, chrysanthemum, grape and peach.

Group III (LOPAT – + – – +) includes 2 major pathogens, namely *P. cichorii* and *P. agarici.*

- *P. cichorii* is common in soil and as an epiphyte on plants. It may occur on decaying lettuce and, together with a wide variety of other bacteria, can cause large necrotic spots on the outer leaf layers of cabbage heads. Other reported hosts are chicory, clover, chrysanthemum, celery and cauliflower.
- *P. agarici* causes drippy gill of mushroom (*Agaricus bisporus*).

Group IV (LOPAT + + + + –) includes *P. marginalis* that has a tuft of polar flagella, does not accumulate PHB, is nitrate reductase and denitrification positive; gelatine is hydrolysed but not starch and it can grow at 4 °C but not at 41 °C. It includes 3 pathovars, namely:

- *P. marginalis* pv. *alfalfae*: browning of roots and stunting of alfalfa or lucerne plants.
- *P. marginalis* pv. *marginalis*: marginal leaf spot of lettuce as well as a range of other diseases.
- *P. marginalis* pv. *pastinacae*: bacterial rot of parsnip roots.

Group V (LOPAT – + – + –) includes *P. tolaasii,* the causal organism of bacterial blotch of mushroom.

☛ Apart from LOPAT tests, ability to grow on agar media containing a single carbon source is an important classification criterion for fluorescent pseudomonads (Table 3).

Many previous groups of non-fluorescent *Pseudomonas* have recently been recognised as new genera: *Acidovorax, Burholderia* and *Ralstonia.* Species remaining in *Pseudomonas* are:

- *P. amygdali*: bacterial canker on *Prunus amygdalus.* Similar to *P. syringae* but lacks fluorescent pigment.
- *P. corrugata*: tomato pith necrosis. It is oxidase positive, levan negative and tobacco HR variable.
- *P. ficuserectae*: leaf spot and shoot blight on *Ficus erecta.* It is oxidase negative, arginine dihydrolase negative, tobacco HR positive and potato rot negative.
- *P. meliae*: bacterial gall on *Melia azadarach.* It is oxidase positive, tobacco HR and potato rot negative, arginine dihydrolase positive and produces levan.
- *P. rubrisubalbicans*: mottled stripe of sugarcane. It is oxidase positive and arginine dihydrolase negative.

Table 3. Carbon source utilisation by fluorescent phytopathogenic *Pseudomonas* spp.

Utilisation of	*P. marginalis*	*P. tolaasii*	*P. agarici*	*P. cichorii*	*P. viridiflava*	*P. syringae* *P. savastanoi* pathovars
Mannitol	+	+	+	+	+	V
Benzoate	–	–	+	–	–	–
Cellobiose	–	–	–	–	–	–
Sorbitol	+	+	–	–	+	V
Trehalose	+	V	–	–	–	–
Sucrose	+	–	–	–	–	V
Meso-tatrate	V	+	–	+	+	V
D-tatrate	V	–	–	–	+	V

V = variable reaction.

Genus *Ralstonia*

☞ The bacterium accumulates PHB, does not form levan from sucrose, does not hydrolyse starch and aesculin, reduces nitrate, is oxidase positive, arginine dihydrolase negative and does not grow at 40 °C. The species can be divided into 5 biovars according to acid production from 3 disaccharides and 3 sugar alcohols (Table 4).

One of the most destructive bacterial pathogens of plants, *Ralstonia solanacearum*, belongs to this genus. Previously this pathogen was considered a non-fluorescent *Pseudomonas* but was later assigned to the genus *Burholderia* and then *Ralstonia.*

The species is very heterogeneous and has been divided into 3 races on the basis of pathogenicity. Race 1 affects tobacco, tomato, many weeds, particularly solanaceous; Race 2 causes bacterial wilt of triploid bananas (Moko disease). Race 3 affects potato and tomato but is not highly virulent on other solanaceous crops.

Genus *Xanthomonas*

☞ Most species in this genus produce smooth, circular and butyrous or viscid colonies with a typical yellow pigment. A few members lack pigment and produce white colonies. The bacteria are motile by a single polar flagellum and are catalase positive, oxidase negative or weakly positive; nitrate is not reduced; extra-cellular polysaccharides (EPS) are commonly produced.

Most species are plant pathogens which were formerly considered members of the *Xanthomonas campestris* group that included more than 130 pathovars. They are now recognised as new species with new pathovars, with *X. axonopodis* including most of the pathovars. Some pathovars have been divided into separate groups, for instance

Table 4. Tests to differentiate between biovars of *Ralstonia solanacearum*.

Utilisation of	Biovar				
	I	II	III	IV	V
Maltose	–	+	+	–	+
Lactose	–	+	+	–	+
Cellobiose	–	+	+	–	+
Mannitol	–	–	+	+	+
Sorbitol	–	–	+	+	–
Dulcitol	–	–	+	+	–

X. campestris pv. *vesicatoria* has been split into *X. axonopodis* pv. *vesicatoria* and *X. vesicatoria.*

Twenty *Xanthomonas* species are recognised:

1. *X. fragariae*: leaf spot of strawberries.
2. *X. hortorum,* 3 pathovars, previously *X. campestris* pathovars.
3. *X. populi*: bacterial canker of poplar.
4. *X. arboricola,* 5 pathovars, previously *X. campestris* pathovars. *X. arboricola* pv. *pruni*: leaf spot on *Prunis* spp.
5. *X. cassavae,* previously *X. campestris* pv. *cassavae*: necrotic leaf spot on cassava.
6. *X. codiaei,* previously *X. campestris* pv. *poinsettiicola*: leaf spot on *Euphorbia.*
7. *X. bromi,* previously *X. campestris* pv. *graminis:* bacterial wilt of grasses.
8. *X. cucurbitae,* previously *X. campestris* pv. *cucurbitae*: leaf spot on pumpkin, squash and watermelon.
9. *X. axonopodis,* previously *X. axonopodis* and 38 former X. *campestris* pathovars. *X. axonopodis* pv. *manihotis* (non-pigmented pathovar): cassava bacterial blight. *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* (dark pigmented strain): common blight of bean (Fig. 16). *X. axonopodis* pv. *vesicatoria*: bacterial spot of tomato and pepper.
10. *X. oryzae* pv. *oryzae, X. oryzae* pv. *oryzicola*: leaf streak on rice.
11. *X. vasicola,* 2 former *X. campestris* pathovars.
12. *X. pisi,* previously *X. campestris* pv. *pisi*: water-soaked lesions on stems of pea, necrosis of pea leaves.
13. *X. melonis,* previously *X. campestris* pv. *melonis:* fruit soft rot of melon.
14. *X. vesicatoria,* previously *X. campestris* pv. *vesicatoria*: leaf spot, spots or scabs on fruit – tomato and capsicum.
15. *X. campestris,* 6 pathovars, all pathogens of crucifers. *X. campestris* pv. *campestris*: black rot of Cruciferae (Fig. 17).

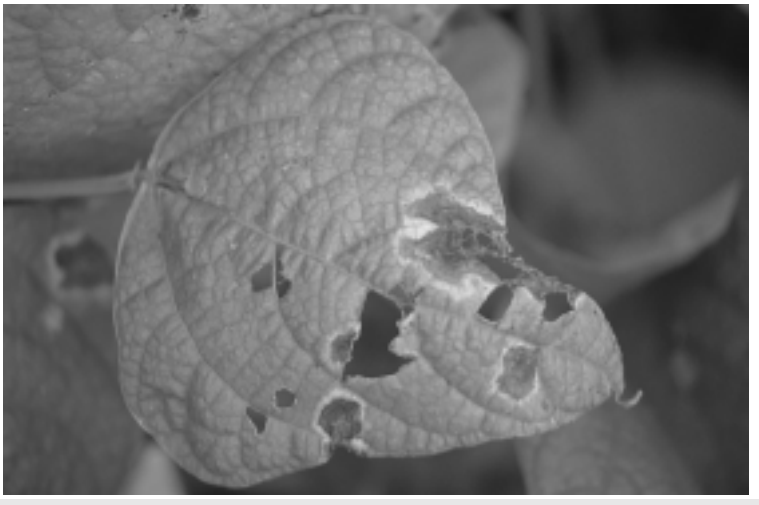

Fig. 16
Common blight of bean, *Xanthomonas axonopodis* pv. *phaseoli*.

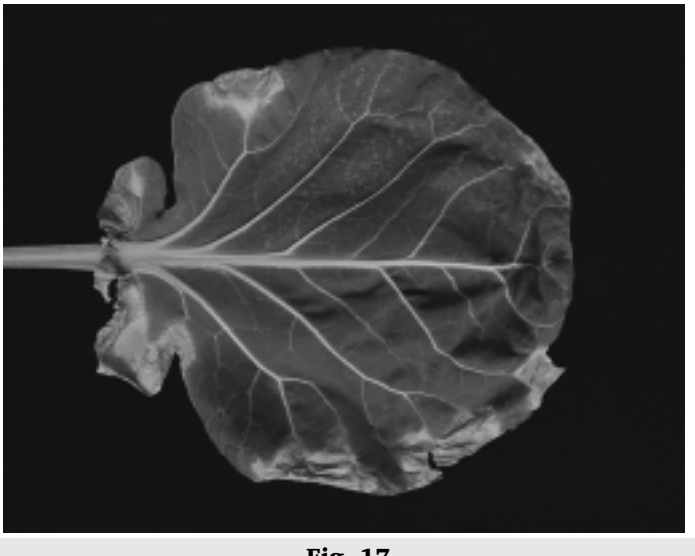

Fig. 17
Black rot of cabbage, *Xanthomonas campestris* pv. *campestris*.

16. *X. translucens,* 10 former *X. campestris* pathovars: bacterial streak on barley.
17. *X. hyacinthi,* previously *X. campestris* pv. *hyacinthi*: yellow disease of hyacinth.
18. *X. theicola,* previously *X. campestris* pv. *theicola*: bacterial canker on tea.
19. *X. sacchari,* previously *'X. albilineans'*.
20. *X. albilineans*: leaf scald and white stripe of sugarcane.

☛ Differential tests for *Xanthomonas* spp. are given in Table 5.

Table 5. Tests to differentiate between some *Xanthomonas* spp.

Test	*Xanthomonas* spp.	*X. fragariae*	*X. albilineans*
Mucoid growth on SPA	+	+	–
Hydrolysis of:			
Gelatine	V	+	V
Aesculin	+	–	+
Starch	V	+	–
Milk proteolysis	+	–	–
H_2S from peptone	+	–	–
Growth at 36 °C	+	–	+
Growth on 2 % NaCl	+	–	–
Acid from:			
Arabinose	+	–	–
Mannose	+	+	+
Galactose	+	–	V
Trehalose	+	–	–
Cellobiose	+	–	–
Fructose	+	+	–

V = variable reaction.

Genus *Xylophilus*

☞ Growth is very slow; on nutrient agar, yellow-pigmented colonies are visible after 6 days, and are about 1 mm in diameter. Acid is produced from arabinose and galactose in Dye's medium C, but not from glucose, maltose or cellobiose. Urease is produced. Tolerance of NaCl is 1 % and the maximum temperature for growth is 30 °C.

Xylophilus ampelinus, the causal organism of bacterial blight of grapevine (Fig. 18), is the only member of this genus.

This destructive disease of grapevine occurs in the Mediterranean region of Europe and in the Western Cape Province in South Africa.

Fig. 18
Bacterial blight of grapevine, *Xylophilus ampelinus*.

Genus *Agrobacterium*

☞ This non-pigmented phytopathogen is motile by 1–6 peritrichous flagella, produces white to cream-coloured, convex, circular, smooth colonies on nutrient agar. It is both catalase and urease positive and the strains can be oxidase positive or negative.

The nomenclature has been based on pathogenicity, and the genes of pathogenicity are located on large plasmids, the Ti (tumour-inducing) or Ri (root-inducing) plasmids, which can be transferred from one bacterial cell to another. If the Ti plasmid is present, the bacterium is *A. tumefaciens* (Fig. 19) and if the Ri plasmid is present, it is *A. rhizogenes.* If the plasmid is absent, it is *A. radiobacter,* which is a saprophyte. This species is further subdivided into 3 biovars or biotypes on the basis of physiological tests. Recently, Biovar 3 has been renamed *A. vitis,* which only infects grapevines. Biovars 1 and 2 of *A. tumefaciens* can cause symptoms on nearly all dicotyledonous and some gymnospermous plants. The host range of different isolates can vary, depending on the specific Ti or Ri plasmid present.

Differential characteristics of *Agrobacterium* spp. are given in Table 6.

Fig. 19
Crown gall of roses, *Agrobacterium tumefaciens*.

Table 6. Tests to differentiate between *Agrobacterium tumefaciens* biovars and *A. vitis*.

Test	Biovar 1	Biovar 2	*A. vitis*
Oxidase reaction	+	–	+
3-Ketolactose production	+	–	–
Growth on 2 % NaCl	+	–	+
H_2S production	+	–	ND
Zone production on PDA + $CaCo_3$	–	+	–
Acid from:			
Erythitol	–	+	–
Ethanol	+	–	–
Melezitose	+	–	–
Dulcitol	+	+	–
Alkali from:			
L-tatrate	V	+	+
Propionate	V	–	–
Malonate	–	+	+

V = variable, ND = not determined.

Other Gram-negative pathogens

- *Acetobacter aceti* and *Gluconobacter oxydans*: pink disease of pineapple fruit.
- *Rhizobacter daucus*: bacterial gall and corky root of carrot.
- *Serratia marcescens*: crown rot of lucerne.
- *Xylella fastidiosa* (xylem-restricted): Pierce's disease of grape, phony disease of peach and some other diseases.

Gram-negative facultatively anaerobic rods

All Gram-negative, facultatively anaerobic rods are plant pathogens in the family Enterobacteriaceae. Most bacteria in this family are motile by peritrichous flagella (except *Pantoea stewartii* subsp. *stewartii*). Bacteria in this group can grow in the presence or absence of oxygen (fermentative) although anaerobic growth by some species is weak. The family contains genera like *Salmonella, Shigella* and *Escherichia* that are of medical importance. The plant pathogens are in 2 genera, namely *Erwinia* and *Pantoea.*

Genus *Erwinia*

☞ The colony morphology of this genus is variable. White, cream-coloured and yellow pigmentation has been noted and colonies are generally round and convex. The bacteria are oxidase negative, catalase negative and Hugh-Leifson test fermentative.

The genus can be broadly divided into 2 groups, a soft-rot group and a group that causes wilt or blight diseases. The soft-rot group produces pectolytic and other macerating enzymes resulting in a characteristic soft rot, especially of vegetables, although a wide range of non-woody plants like ornamentals and field crops are also infected.

Pathogens in the soft-rot group include:

- *E. carotovora* subsp. *atroseptica*: potato is the main host where the bacterium causes soft rot and black leg, but numerous other crops are also infected.
- *E. carotovora* subsp. *carotovora* attacks a wide variety of plant species, under certain conditions causing soft rot of especially large fleshy organs. It can also cause black leg of potato.
- *E. chrysanthemi* is heterogeneous and has a wide range of hosts and some pathogenic specialisations. The pathogen has been subdivided into 7 pathovars. It causes stunting, yellowing, wilting or soft-rot of a wide range of hosts.

☛ Tests to differentiate between soft rot erwinias are given in Table 7.

The non-soft rot erwinias include:

- *E. amylovora*: fireblight on members of the family Rosaceae, apples and pears

Table 7. Tests to differentiate between soft rot *Erwinia* spp.

Test	*E. carotovora* subsp. *atroseptica*	*E. carotovora* subsp. *carotovora*	*E. chrysanthemi*
Indole	–	–	+
Phosphatase	–	–	+
Lecitinase	–	–	+
Growth in 5 % NaCl	+	+	–
Erythromycin sensitivity	–	–	+
Acid from:			
Lactose	+	+	–
Maltose	+	V	–
α-methyl-D-glycoside	+	–	–
Trehalose	+	+	–

V = variable reaction

being the most susceptible. Fireblight is a destructive disease but does not occur in southern Africa.

- *E. tracheiphila*: bacterial wilt of cucurbits. Insects, particularly cucumber beetles, transmit this vascular disease.

Genus *Pantoea*

☞ Members of this genus were previously assigned to the so-called 'Herbicola' group of *Erwinia*. The yellow-colony-forming bacterium *P. agglomerans* (formerly *E. herbicola*) is a widely distributed plant-associated bacterium that is often confused with phytopathogenic bacteria, as it may be a secondary invader.

Important plant pathogens in the genus are:

- *P. stewartii* subsp. *stewartii*: Stewart's bacterial wilt of maize, which is especially important on sweetcorn in the USA. The bacterium is non-motile, overwinters, and is transmitted by an insect vector, the corn flea beetle. The disease has not been recorded in southern Africa.
- *P. stewartii* subsp. *indologenes*: leaf spot on foxtail and pearl millet.

Gram-positive bacteria

Actinomycetes and related organisms

The corynoform group of bacteria as well as the family Streptomycetaceae of the order Actinomycetales contain phytopathogens.

Corynoform group

This group contains some important plant pathogens. The bacteria are non-acid-fast, non-spore forming, pleomorphic rods and are all strict aerobes, catalase positive and oxidase negative. A variety of pigments are produced by the different species (Table 8) Most of the *Clavibacter* spp. are non-motile whereas most of the *Curtobacterium* spp. are motile.

Genus *Clavibacter*

☞ Non-motile pleomorphic rods that are often in angular arrangements. Growth factors are required, nitrate and nitrite are not reduced, casein is not hydrolysed, and they are catalase positive and oxidase, lipase, tyrosinase and urease negative.

Important phytopathogens in the genus are:

- *C. michiganense* subsp. *michiganense*: bacterial canker of tomato (Fig. 20) is a cosmopolitan, economically important, seed-borne disease of tomato.
- *C. michiganense* subsp. *nebraskensis*: Goss's bacterial wilt and blight on maize.
- *C. michiganense* subsp. *insidiosum*: bacterial wilt of lucerne. The disease requires a cool climate and can be a serious factor in stand decline.
- *C. michiganense* subsp. *sepedonicum*: ring rot of potato, an important disease in the northern hemisphere.
- *C. xyli* subsp. *xyli*: ratoon stunting disease (RSD) of sugarcane, a very slow-growing bacterium that does not grow on general media.

Table 8. Tests to differentiate between some phytopathogenic corynoform bacteria.

	A	B	C	D	E	F	G	H	I	J
Characteristic:										
Motility	–	–	–	–	–	–	–	V	V	+
Pigment	y/b	y/v	o/v	w	y	y	w	y/o/p	Y	y
Acid produced:										
Ribose	–	–	–	–	–	–	–	+	+	+
Sorbitol	–	–	+	+	–	–	–	–	+	–
Inulin	–	–	–	–	–	+	–	–	–	–
Utilisation of:										
Acetate	–	–	+	+	–	+	–	+	+	–
Formate	–	–	–	–	–	–	–	–	–	–
Hydrolysis of:										
Casein	–	–	–	–	–	–	–	+	–	+
Aesculin	+	+	+	+	+	+	–	+	+	+

A, *Clavibacter michiganense* subsp. *insidiosus;* **B**, *C. m. michiganense;* **C**; *C. m. nebraskense;* **D**, *C. m. sepedonicum;* **E**, *Rathayibacter rathayi;* **F**, *R. tritici;* **G**, *C. xyli* subsp. *xyli;* **H**, *Curtobacterium flaccumfaciens* subsp. *flaccumfaciens;* **I**, *C.f. betae;* **J**, *C. f. oortii.*

V = variable reaction; **ND** = not determined.

Pigment production on NA agar: **y**, yellow; **b**, blue; **o**, orange; **w**, white or colourless; **p**, purple; **v**, various pigments.

Genus *Curtobacterium*

☞ Usually motile by lateral flagella. Rods also pleomorphic (straight to slightly curved or wedge-shaped), showing bending-type cell division (V, Y and palisade arrangements). Produces small (2–4 mm) colonies after 4 days that are smooth, entire, convex and semi-fluid. Yellow, orange or pink pigments may be produced.

Diseases caused by members af this genus are:

- *C. flaccumfaciens* pv. *betae*: silvering disease of red beetroot.
- *C. flaccumfaciens* pv. *flaccumfaciens*: bacterial wilt of bean and other legumes.
- *C. flaccumfaciens* pv. *oortii*: yellow pustule and hell-fire of tulips.

Genus *Rathayibacter*

Members of this genus were formerly assigned to the genus *Clavibacter.* Pathogens

Fig. 20
'Bird's eye' spots on tomato fruit, *Clavibacter michiganense* subsp. *michiganense*, causal agent of bacterial canker of tomato.

include:

- *R. irinicus*: gumming disease of wheat.
- *R. rathayi*: gumming disease or cocksfoot on *Dactylis glomerata.*
- *R. tritic*: gumming or slime disease of wheat.

Actinomycetes

Genus *Streptomyces*

☞ The genus produces well-developed, branched, coenocytic and rarely fragmented mycelium. Aerial mycelium produces chains of 3 to many non-motile, round to oval spores. Colonies are lichenoid, leathery or velvety.

Most species are saprophytic and pathogens include:

- *S. ipomoeae*: scab of sweet potato.
- *S. scabies*: scab of potato in most potato-producing areas of the world.

Cell-wall-free procaryotes

Spiroplasmas and mycoplasma-like organisms (MLOs) lack a true cell wall and occur in the phloem tissue of plants. The spiroplasmas are helical, spherical to ovoid in shape and the MLOs are pleomorphic. Disease symptoms caused are yellowing, little leaf, phyllody, stunting and proliferation disorders. The organisms are transmitted by phloem-feeding leafhoppers. Spiroplasmas can be cultivated on special media but not the MLOs.

Basic keys for the identification of phytopathogenic bacteria

In the laboratory several selective and general agar media should always be in stock. Tubes and plates with diagnostic media for performing biochemical tests and reagents for the Gram test should be prepared on a regular basis. Tobacco, bean, tomato and other plants should always be maintained in the greenhouse for pathogenicity tests.

Keys for the identification of phytopathogenic bacteria presented below are based on practical experience in the Plant Protection Research Institute's diagnostic laboratory. The following media and tests are routinely used:

Agar media for plating plant extracts:

(See chapter 'Media and Diagnostics Tests')

- TGA
- KBC
- Tween A
- SCM
- TZC
- NA
- CVP
- KB
- NASA
- YMA+C
- MT

Diagnostic tests

- Gram reaction and KOH solubility test
- Utilisation of carbon sources: sucrose, inositol, mannitol, erythitol, sorbitol, homoserine
- Hugh-Leifson test
- LOPAT tests
- Urease production
- Fluorescence under UV light on KB, KBC, MT media
- Gelatine liquefaction
- Aesculin hydrolysis
- Starch hydrolysis
- Pathogenicity tests

☛ Special agar and diagnostic media are prepared when necessary.

☞ *Recommended steps for identifying a disease:*

- Identify the diseased plant (bean, pea, tomato etc.).
- Examine symptoms both macro- and microscopically.
- Undertake literature search for pathogens occurring in a particular country.
- Compare disease symptoms with symptoms described in the literature.
- Select isolation media.
- Isolate.
- Incubate.
- Examine agar plates with bacterial growth.
- Select suspect colonies.
- Purify selected colonies.
- Perform required tests.
- Pathogenicity test.

☛ If you cannot identify the pathogen, consult an expert plant pathologist in your area.

Key No. 1 — Bean, pea

Pod spot, leaf spot and blight

No.	Description	Go to
1	Plate on KBC	2
	MT	8
2	Colonies fluorescent under UV light	3
	Colonies non-fluorescent	Discard
3	Off-white, semi-transparent colonies, Gr– rods	4
	Yellow or other colour colonies	Discard
4	OX–	5
	OX+	Discard
5	LOPAT (+ – – – +)	6
	Other pattern	Discard
6	Carbohydrate utilisation: Sucrose + Inositol + Mannitol + Erythitol + Sorbitol + Homoserine –	7
	Other pattern	Discard
7	Pod test – green, sunken lesions after 3 days	*Pseudomonas syringae* pv. *syringae*
	No lesions or small, brown lesions within 24 hours	Discard

8	Colonies fluorescent under UV light, Gr– rods	9
	Non-fluorescent, yellow colonies, Gr– rods	16
9	Clear zone around colonies	11
	No zone	10
10	LOPAT (– + – – +)	*Pseudomonas chicorii*
	Other pattern	11
11	Potato-rot positive, no sucrose utilisation	*Pseudomonas viridiflava*
	Potato-rot negative, sucrose utilised	12
12	LOPAT (+ – – – +)	13
	Other pattern	Discard
13	Pathogenic reaction on tobacco	*Pseudomonas syringae* pv. *tabaci*
	Hypersensitive reaction on tobacco	14
14	Carbohydrate utilisation Sucrose Inositol Mannitol Erythitol Sorbitol Homoserine	
	+ + + + + –	7
	+ – – – – –	15
	+ + + V + +	Isolated from pea: *Pseudomonas syringae* pv. *pisi*

15	Pod test – water-soaked lesions after 3 days	*Pseudomonas savastanoi* pv. *phaseolicola*
	No lesions or small, brown lesions within 24 hours	Discard
16	Two zones around colonies: bigger: clear; smaller: opaque	17
	No zones around colonies	Discard
17	Brown diffusible pigment present	*Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans,* but go to 19
	No brown pigment	18
18	CVP Starch Tween 80 – + +	19
	+ + +	Probably non-pathogenic, but go to 19
19	Pathogenicity test – positive	*Xanthomonas axonopodis* pv. *phaseoli*
	Pathogenicity test – negative	Discard

V = variable reaction.

Key No. 2 — Cowpea

Leaf spot or leaf blight

No.	Description	Go to
1	Plate on KBC	2
	MT	3

2	Fluorescent, off-white, semi-transparent colonies, Gr– rods	Key No. 1, point 4
	Yellow or differently coloured colonies, non-fluorescent	Discard
3	Fluorescent, off-white, semi-transparent colonies, Gr– rods	Key No. 1, point 9
	Yellow colonies, Gr– rods	4
4	Two zones around colonies, bigger: clear; smaller: opaque	5
	No zones	Discard
5	Pathogenicity test – positive	*Xanthomonas axonopodis* pv. *vignicola*
	Pathogenicity test – negative	Discard

Key No. 3 — Tomato

No.	Description	Go to
1	Wilt	Key No. 5
	Galls	Key No. 7
	Canker, browning of vascular tissue	Key No. 4
	Soft rot of fruit and stems	Key No. 6
	Leaf, fruit and stem spots	2
	Pith necrosis, plate on MT, KBC, TGA	12

2	KBC	Key No. 1, point 2
	SCM	Key No. 4, point 4
	MT	3
3	Off-white, semi-transparent colonies, Gr– rods	4
	Yellow colonies, Gr– rods	9
4	Colonies fluorescent under UV light	5
	Colonies non-fluorescent	Discard
5	Clear zone around colonies	6
	No zone	6
6	LOPAT (– – + – +)	*Pseudomonas viridiflava*
	LOPAT (– + – – +)	*Pseudomonas chicorii*
	LOPAT (+ – – – +)	7
7	Carbohydrate utilisation Sucrose Inositol Mannitol Erythitol Sorbitol Homoserine	
	+ + + – + –	8
	+ + + + + –	*Pseudomonas syringae* pv. *syringae*
8	Pathogenicity test – leaf spots	*Pseudomonas syringae* pv. *tomato*
	Pathogenicity test – negative	Discard

9	Two zones around colonies: bigger: clear; smaller: opaque	10 and 11
	No zones	Discard
10	Go to Table 5 (p. 32) for more tests	11
11	Pathogenicity test – leaf spots	*Xanthomonas vesicatoria*
	Pathogenicity test – negative	Discard
12	**MT**: non-fluorescent, circular beige colonies with raised centre, ~3 mm in diameter, 2 zones around colonies: bigger: clear; smaller: opaque	13
	KBC: non-fluorescent, circular greenish colonies, ~1 mm in diameter	
	TGA: smooth, circular, slightly raised colonies with darker centre, 3 mm in diameter, orange-brown	
13	Gr– rods: possibly of *Pseudomonas corrugata*	14
	Gr+	Discard
14	See Table 1 (p. 23) for more tests	15
15	Pathogenicity test – pith necrosis	*Pseudomonas corrugata*
	Pathogenicity test – negative	Discard

Key No. 4 — Tomato

Canker and wilt

No.	Description	Go to
1	Plate on TZC by dilution plating, SCM and TGA	2

2	**TZC**: mucoid, drop-shaped, white colonies with pink, half-moon-shaped centres	3
	TGA: mucoid, white colonies	
	SCM: fluidal, mucoid, yellow colonies with grey flecks	4
	TGA: yellow, mucoid, circular colonies	
3	Gr– rods	Key No. 5, point 6
	Gr+	Discard
4	Gr+	5
	Gr–	Discard
5	Conduct pathogenicity test by inoculating tomato plants with 10^7 cfu/ml suspension of pathogen. Symptoms develop after 14 days at 25 °C	6
6	Pathogenicity test – positive	*Clavibacter michiganense* subsp. *michiganense*
	Pathogenicity test – negative	Discard

Key No. 5 — Potato wilt

No.	Description	Go to
1	Milky exudate from stems after 30 minutes	2
	No milky exudate from stems	3
2	Isolate on TZC and TGA by dilution series and spread-plating	4

3	Isolate on CVP, TGA and TZC as in 2	5
4	**TZC**: mucoid, drop-shaped white colonies with pink, half-moon-shaped centres	6
	TGA: mucoid, white colonies	
5	**CVP**: pith formation, Gr– rods	6
	TGA: circular, white colonies, Gr– rods	
	TZC: mucoid, drop-shaped white colonies with pink, half-moon-shaped centres, Gr– rods	6
	TGA: mucoid, white colonies, Gr– rods	
6	Hugh-Leifson test – oxidative	7
	Hugh-Leifson test – fermentative	11
7	OX +	8
	OX –	Discard
8	Colonies non-fluorescent on KB	9
	Colonies fluorescent on KB	Discard
9	PHB inclusions present	10
	No PHB inclusions	Discard
10	Pathogenicity test at 28 °C – wilting	*Ralstonia solanacearum* see Table 4 (p. 30) for biovar classification
	Pathogenicity test – negative	Discard

11	Potato soft rot positive	*Erwinia,* see Table 7 (p. 35) for further classification
	Potato soft rot negative	Discard

Key No. 6 — Soft rots

Fruits, tubers, bulbs and leaves

No.	Description	Go to
1	Plate on CVP, TGA and in the case of potatoes include TZC	2
2	**CVP**: pith formation	3
	TGA: circular, white or cream-coloured colonies	
	TZC: mucoid, drop-shaped, white colonies with pink half-moon-shaped centres	Key No. 5, point 6
	TGA: mucoid, white, fluidal colonies	6
3	Gr– rods	4
	Gr+, Gr– cocci	Discard
4	Potato soft-rot positive	5
	Potato soft-rot negative	Discard
5	Hugh-Leifson test – fermentative	*Erwinia,* see Table 7 (p. 35) for further classification
	Hugh-Leifson test – oxidative	6
6	Colonies fluorescent on KB	7
	Colonies non-fluorescent on KB	Discard

7	Utilisation of sucrose and trehalose	8
	No utilisation of sucrose and trehalose	9
8	LOPAT (+ + + + –)	*Pseudomonas marginalis*
9	LOPAT (– – + – +)	*Pseudomonas viridiflava*

Key No. 7 — Galls

☛ include at least 6 isolates from each gall.

No.	Description	Go to
1	Isolate on NASA, YMA+C and NA	2
2	Gr– rods, Hugh-Leifson oxidative	3
	Gr+, Gr– cocci, Hugh-Leifson fermentative	Discard
3	Catalase +, urease +	4
	Other pattern	Discard
4	**NA**: white to cream-coloured, circular, convex and smooth colonies	5
	YMA+C: pink, circular, transparent, convex to dome-shaped colonies	
	NASA: shiny, convex, circular colonies with bright pink centre and white margin after 5 days	
5	Pathogenicity test on tomato, tobacco or datura plants – galls	*Agrobacterium,* see Table 6 (p. 34) for biovar classification
	Pathogenicity test – negative	Probably *A. radiobacter*

Key No. 8 — Crucifers

Leaf spot, black rot and soft rot

No.	Description	Go to
1	Plate on MT, SX and CVP	2
2	**SX**: clear zones around colonies	3
	MT: yellow colonies with 2 zones: bigger: clear; smaller: opaque, or	
	Off-white, semi-transparent, fluorescent colonies	5
	CVP: pith formation	Key No. 6, point 3
3	Gr– rods	4
	Gr+, Gr– cocci	Discard
4	Pathogenicity test – leaf spot, black rot	*Xanthomonas campestris* pv. *campestris*
	Pathogenicity test – negative	Discard
5	OX+	6
	OX–	7
6	LOPAT (– + – – +), no zone on MT	*Pseudomonas chicorii*
	LOPAT (+ + + + –)	*Pseudomonas marginalis*
7	LOPAT (– – + – +)	*Pseudomonas viridiflava*
	LOPAT (+ – – –+)	*Pseudomonas syringae* pv. *maculicola*

Other methods to detect and identify phytopathogenic bacteria

Besides conventional isolation and identification methods used in phytobacteriology, other methods are also used to detect and identify phytopathogenic bacteria. These methods include phage-typing, sero-typing and detection, and molecular methods.

Phage-typing

The use of bacteriophages specific to the target organism. A bacteriophage is a virus that infects bacteria.

Serological methods

Antisera are used to identify as well as to detect bacteria in plant material. Polyclonal antisera are raised in animals against bacteria or specific components of bacteria. Monoclonal antibodies are produced against specific epitopes in myeloma cells and are more specific than polyclonal antibodies. Serological methods used to detect and identify bacteria are immunofluorescence microscopy, immunofluorescent colony staining, ELISA, agglutination and others.

Polymerase chain reaction

PCR is used in the detection and identification of phytopathogenic bacteria. Specific target nucleic acid is artificially multiplied by repeated cycles.

Fatty acid analysis, protein electrophoresis, restriction fragment-length polymorphism (RFLP) analysis, random amplified polymorphic DNA (RAPD) analysis and rRNA sequencing are all used to identify pure cultures of bacteria for comparison with databases of profiles.

Preservation of bacterial cultures

Culture collections

Isolated and identified bacterial strains should be preserved for future studies and as reference strains. Preserved bacteria are kept in culture collections. There are 3 main types of culture collections:

- ☛ Private – for personal research purposes.
- ☛ Institutional – for internal use in the institution concerned.
- ☛ Commercial – for supply to clients on demand.

The main function of a culture collection is to preserve living cultures and make them available to scientists who wish to repeat, compare or extend their work.

Some institutions that supply reference strains of plant pathogenic bacteria are:

- American Type Culture Collection, 12301 Parklawn Drive, Rockvile, Maryland 20852, USA.
- International Collection of Phytopathogenic Bacteria, Department of Bacteriology, University of California, Davis, California 95616, USA.
- Collection Française de Bactéries Phytopathogénes, Station de Phytobactériologie, Angers, France.
- National Collection of Plant Pathogenic Bacteria, Plant Pathology Laboratory, Ministry of Agriculture, Fisheries and Food, Harpenden, Hertfordshire, England.

Preservation of bacteria

There are many ways of preserving bacteria. The choice of method depends on the nature of the microorganism, the preservation objectives and available facilities.

☞ *The main aims of preservation are to:*

- ✳ Reduce the metabolic rate of the organism.
- ✳ Eliminate damage and changes in pathogenicity.
- ✳ Maintain high recovery and survival rate.

Short-term storage

Periodic subculture

This method is traditionally used to maintain frequently used isolates. Bacteria can be stored on sealed agar plates or slants at room temperature or in a refrigerator (Fig. 21). The humidity of the storage area is important because slow dehydration leads to a loss of

Fig. 21
Bacterial cultures preserved on agar slants.

viability. Several agar media can be used: glucose yeast extract $CaCO_3$ agar, tryptone glucose extract agar, nutrient agar etc. (see chapter 'Media and Diagnostic Tests').

Periodic subculturing is not recommended for long-term preservation. The likelihood of contamination, loss in pathogenicity, mislabelling and risk of culture loss are great. Some bacteria like *Pseudomonas* spp. generally survive for long periods without subculturing.

Storage under mineral oil

Sterile paraffin oil must cover the slants completely. Cultures are stored at room temperature or at 4 °C.

Long-term storage

Storage in distilled water

A dense suspension of culture is prepared in sterile distilled water and stored at room temperature. This method is suitable for *Ralstonia solanacearum, A. tumefaciens* and *X. albilineans.* Some isolates stored in this way can survive for up to 20 years.

Deep-freezing

Most bacteria can survive in the frozen state for a long time. The metabolic rate is greatly reduced and the lower the temperature the less is the loss of viability of most micro-organisms.

Before deep-freezing, prepare a dense suspension of bacteria, from a 3–4-day-old growth on agar, in milk-glycerol (10 % skimmed milk, 15 % glycerol in distilled water) or in NGY (0.8 g nutrient broth, 15 ml glycerol, 0.2 g yeast extract, 0.5 g glucose in 100 ml distilled water). Place 0.5 ml of the suspension in a small, labelled tube or bottle, seal it

with parafilm and store at –20 °C, but preferably at –70 °C.

The use of ultra-low temperatures obtained by freezing in liquid nitrogen (–196 °C) reduces metabolism to almost nil and genetic stability of cultures is largely preserved. When genetic stability of cultures must be preserved, freezing in liquid nitrogen at –196 °C is the recommended method.

Freeze-drying

Freeze-drying (lyophilisation) combines 2 long-term preservation methods, **freezing** and **drying**. First the cell suspension is frozen in ampoules and then water vapour is removed under vacuum sublimation from the frozen state.

Procedure

1. Preparation of ampoules
   - Prepare labels on filter paper by typing onto them the number of the isolate and the date of freeze-drying.
   - Cut the labels to size.
   - Insert a label into each ampoule.
   - Plug each ampoule with cotton wool.
   - Pack the ampoules in an autoclave bag and sterilise by autoclaving for 20 minutes.
2. Preparation of the bacterial culture
   - Plate the isolate on a suitable agar medium.
   - Incubate for the desired period at the optimum growth temperature. The culture should reach the **early stationary phase** of growth (cultures harvested in the actively growing stage do not survive well).
   - Examine the plate culture for purity.
   - Open the bag of sterilised ampoules.
   - Prepare a very dense suspension (~10^{10} cells/ml) of the culture in a suitable suspending fluid such as:
     - 7.5 % glucose horse serum.
     - 5 % peptone.
     - 10 % sucrose in water.
     - 5 % inositol serum.
     - 10 % skimmed milk.
   - Place 1 drop of the bacterial suspension at the bottom of an ampoule using a sterile Pasteur pipette, replace the cotton-wool plug and place the prepared ampoule in a rack.
3. Operation of the freeze-dryer

Procedures depend on available facilities (consult manual for operating a freeze-dryer).

The freeze-dryer consists of a freezing block and a high-vacuum pump.

- The ampoule with suspension is frozen in a mixture of dry ice and alcohol (–78 °C).
- The water vapour is removed under vacuum.

4. Sealing ampoules under vacuum

- Trim the cotton-wool plugs at the rim of the ampoules.
- Push the cotton-wool plug down the ampoule. The bottom of the plug should be 5 mm above the label.
- Seal the tubes by heating with a Bunsen flame while they are under vacuum.
- Check ampoules for leaks with a high-frequency tester.

Ampoules (Fig. 22) are stored at room temperature or refrigerated. Cultures preserved by freeze-drying can survive for 20–30 years.

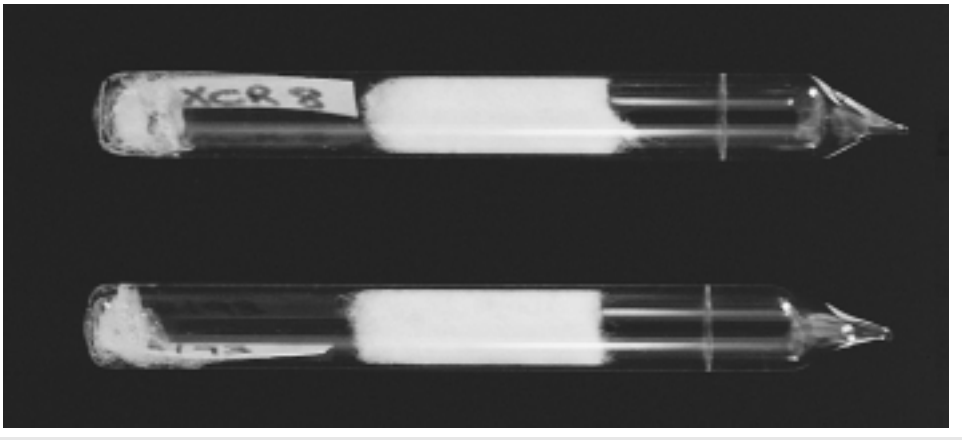

Fig. 22
Ampoules with freeze-dried bacterial cultures.

Epidemiology and control of bacterial diseases

Although each bacterial plant disease has its own disease cycle, some aspects in the development of a bacterial plant disease epidemic can be generalised. The first requirement is a primary inoculum source; i.e. the pathogen must be present. The second requirement is the presence of the host and the last requirement is the presence of favourable environmental conditions for the development of the disease. Environmental conditions may have an influence on the host, the pathogen or both. Without any one of these three basic factors, the development of a plant disease is not possible. The level of disease development (disease severity) and spread in a planting (disease incidence) will depend on these three factors. Some cultivars of a crop are more tolerant than others and, in these, disease severity will be lower. Diseases will spread more rapidly from diseased plants under certain environmental conditions; for example, wind-driven rain induces higher disease incidence. In seed-borne diseases both disease severity and incidence are often high.

Inoculum sources

Both primary sources of inoculum (introduction of disease or source of disease at the beginning of the season) and secondary sources (spread of disease within or between plantings) are important.

Primary sources

Seed/propagation material

Many bacterial plant diseases are disseminated via seed or other propagation material. Mostly the seed/propagation material does not show symptoms of the disease (i.e. latent infections). Very low levels of seed infection can result in an epidemic; one bean seed in 10 000 infected with *P. savastanoi* pv. *phaseolicola* is enough for the development of a halo-blight epidemic in beans. Control strategies for many bacterial diseases are based on the use of clean propagation material. This includes the production of propagation material under conditions not suitable to the development and spread of the disease, as well as routine inspection of plantings and laboratory seed health testing. Hot-water treatment of seed is effective in controlling some seed-borne pathogens in small-seed crops.

Water and soil

Some bacterial plant pathogens (*R. solanacearum, Agrobacterium* spp. and soft rot *Erwinia* spp.) can survive for extended periods in soil and water. Most of the other pathogens are poor competitors in soil and do not survive for extended periods, although some do survive in the soil between growing seasons.

☛ Control strategies include:

- Crop rotation with non-susceptible crops.
- Use of clean water or chemical treatment of irrigation water.
- Use of resistant varieties.
- Biological control of *Agrobacterium* spp.

Crop debris and volunteer plants

Although many pathogens do not survive in the soil, they do survive between seasons in crop debris left on the soil surface. These can be a source of inoculum. Volunteer plants can also be a primary source.

☛ Control strategies include:

- Crop rotation with non-susceptible crops.
- Deep-ploughing, removal or burning of crop debris.
- Control of volunteer plants.

Weeds and other crops

Some weeds and even other crops may serve as an inoculum source of some pathogens. The bacteria do not necessarily infect these non-host plants, but grow epiphytically. An epiphytic population is one that can survive, grow and spread on host and non-host plants without infecting the plants and causing disease. Epiphytic populations may be important inoculum sources of some diseases.

☛ Control strategies include:

- Weed control.
- Control of epiphytic populations on the crop by a preventative copper bactericide spray programme.

Insects

In the USA, *P. stewartii* subsp. *stewartii,* the causal agent of Stewart's wilt in sweet corn, overwinters in the corn flee beetle. The harshness of the winter determines the survival rate of corn flee beetles and the severity of Stewart's wilt during the next season.

Secondary sources

The rate of secondary spread of bacterial diseases depends on environmental conditions as well as on human activities. In foliar diseases, the bacteria infect plants though natural openings (hydatodes and stomata) and wounds (wind, hail and insect damage) in the presence of free water. Wind-driven rain and hail cause wounds to plants and facilitate the spread of bacteria between plants and plantings. Aerosols produced during rain showers can contain bacteria and spread them over considerable distances. Bacteria can also spread from infected plantings on dry, hot days by upward

drafts and wind. Human activity can also result in the spread of bacteria between plants, especially when plants are wet. Pruning shears can spread bacteria in perennial crops and any movement in and between stands can spread it as well.

Dissemination of soil-borne diseases is mainly by runoff water and soil particles attached to implements and shoes. Insects are usually important in the distribution of specific diseases, and can distribute pathogens like the soft rot *Erwinia* spp. Flies attracted to decaying vegetables spread the bacteria to healthy plants and fruit.

☛ Control strategies include:

- Sanitation: removal of decaying fruit and diseased plants; disinfection of implements and pruning shears.
- Planting at times and in areas that will not allow disease development.
- Preventative spraying programmes with copper bactericides.
- Avoidance of plant damage.
- Use of resistant varieties, if available.
- Not working among plants when they are wet.
- Planting wind breaks at windy sites.

Conclusion

Much can be done to minimise damage caused to crops by bacterial diseases. If a susceptible host crop is present, inoculum levels of a virulent pathogen are high and environmental conditions for disease development are favourable, no disease control strategy will prevent epidemics of a bacterial disease. Most strategies for the control of bacterial diseases in plants are based on the use of clean propagation material, and for long-term control, the introduction of resistant varieties. Resistance breeding is based on single-gene resistance, but pathogens overcome the resistance by the formation of new races. Control of crown gall is achieved by treating propagation material of some crops with a specific non-pathogenic *Agrobacterium* strain that prevents infection of the plants by pathogenic *Agrobacterium* strains. This is one of the best known success stories in the biological control of plant diseases.

Media and diagnostic tests

Essential laboratory equipment

- Autoclave
- Balances
- Laminar-flow bench
- Pipettes, preferably a 100 µl, 1000 µl and 5000 µl micropipette
- Wire loop for plating
- Straight wire for stab cultures
- Bunsen flame
- Glass rods
- Microscope, slides and cover slips
- Incubator
- Cold room or fridge
- Magnetic stirrer
- Sterile filters and syringes
- Scalpels
- Distilled water
- pH meter
- Glassware:
  - Sterile Petri dishes (glass or plastic)
  - Test tubes
  - Schott or other autoclavable bottles for media preparation
  - Erlenmeyer flasks
  - Measuring cylinders

☛ All glassware must be clean.

Numerous advanced bacteriology manuals and a few comprehensive books containing materials and methods used in phytobacteriology are available (see Recommended reading). At least one of these manuals or books should be available in the laboratory.

Some key tests are given in full below, with reference to more specialised tests where necessary.

Staining of bacteria and KOH solubility test

1. Gram stain

Solutions

a) Crystal violet (0.5 % aq., w/v)

Dissolve in distilled water and filter through Whatman No. 1 paper.

b) Lugol's iodine

Dissolve 2 g potassium iodine in 25 ml distilled water, add 1 g iodine; make up to 100 ml.

c) Safranine O (0.5 % aq., w/v)

☞ Gram-positive = dark purplish

☞ Gram-negative = red

2. KOH solubility test

Prepare 3 % KOH solution in distilled water.

3. Poly-β-hydroxyburate granules (PHB)

Preparation of Sudan Black B

Sudan Black B	0.3 g
Ethanol (70 %)	100 ml

☞ Shake vigorously and allow to stand overnight.

Preparation of culture media

Microbiological media are:

- Solid – e.g. for separating bacteria and allowing them to grow into isolated colonies.
- Semi-solid – e.g. for motility test.
- Liquid (broth) – e.g. to cultivate large quantities of bacteria; for performing biochemical tests.

Media are also classified by their function:

- General isolation media – support the growth of most bacteria.
- Selective media – inhibit the growth of some microbes; colonies of target bacteria may differ in appearance from others and can be easily distinguish (e.g. appearance of zones around colonies, pigmentation).

How to prepare a medium

- Weigh all chemicals.
- Place chemicals in a flask.
- Add distilled or deionised water.
- Dissolve chemicals in water by using magnetic spinbar and plate stirrer.

Agar media for isolating bacteria

- Warm mixture in a microwave oven or on hot-plate stirrer (when heating the medium, stir it constantly to prevent scorching).
- Autoclave medium.

- Remove medium from autoclave and cool to about 50 °C.
- Add antibiotics or other filter-sterilised chemicals if required (under sterile conditions).
- Mix medium well using magnetic stirrer.
- Pour the medium into sterile Petri dishes.

☞ After medium has solidified, label the plates, leave at room temperature for 24 hours, place plates inverted in plastic bags and store at 4 °C.

Agar media for storage of bacteria

- Warm mixture in a microwave oven or on hot-plate stirrer (when heating the medium, stir it constantly to prevent scorching).
- Distribute medium in 9 ml aliquots to small screw-cap bottles or test tubes.
- Close the bottles.
- Autoclave.
- Form slants by placing tubes at 45° angle to solidify.

☞ Store at 4°C.

Agar media for biochemical tests

- Sterilise test tubes or screw-cap bottles by autoclaving.
- Warm mixture in a microwave oven or on hot-plate stirrer (when heating the medium, stir it constantly to prevent scorching).
- Autoclave.
- Cool to 50 °C.
- Add filter-sterilised chemicals.
- Mix using magnetic stirrer.
- Dispense into sterile test tubes under sterile conditions.
- Close the tubes using sterile cotton plugs.

☞ Cool and store at 4 °C.

Broth media for growing bacteria

- Distribute medium in aliquots to flasks.
- Close flasks by using cotton plugs, cover plugs with aluminium foil.
- Autoclave.

☞ Cool and store at 4 °C.

Liquid media for biochemical tests

- Sterilise test tubes or screw-cap bottles by autoclaving.
- Autoclave medium.
- Cool to 50 °C.
- Add filter-sterilised chemicals.
- Mix using magnetic stirrer.
- Dispense into sterile test tubes under sterile conditions.
- Close the tubes using sterile cotton plugs.

General isolation media

4. KB (King's medium B)

	Per 1000 ml distilled H_2O
Proteose peptone No. 3 (Difco)	20.0 g
Glycerol	15.0 ml
K_2HPO_4 (anhydrous)	1.5 g
$MgSO_4 \times 7\ H_2O$	1.5 g
Agar	15.0 g

5. NA (nutrient agar)

(Available commercially)

	Per 1000 ml distilled H_2O
Beef extract (Difco)	3.0 g
Peptone (Difco)	5.0 g
Agar	15.0 g

6. NB (nutrient broth)

Prepare as for nutrient agar but without the agar.

7. NDA (nutrient dextrose agar)

For isolation of *Agrobacterium* spp., some *Clavibacter* spp. and some non-soft rot. *Erwinia* spp.

	Per 1000 ml distilled H_2O
Nutrient agar (Oxoid, CM3)	28.0 g
D-glucose	10.0 g

8. YDC agar (glucose yeast extract calcium carbonate agar)

Suitable for maintenance of *Xanthomonas* spp. and isolation of most pathogens.

	Per 1000 ml distilled H_2O
Yeast extract	10.0 g
D-glucose	20.0 g
$CaCO_3$	20.0 g
Agar	15.0 g

- Mix and heat all ingredients except $CaCO_3$.
- Add $CaCO_3$.
- Autoclave.
- Cool, mix, pour.

9. NYA (nutrient yeast agar)

Nutrient agar (5) supplemented with 0.2 % yeast extract (Difco).

10. TZCA (tetrazolium chloride agar)

General isolation and growth medium for *Ralstonia solanacearum.* White colonies with pink centres.

	Per 1000 ml distilled H_2O
Peptone	10.0 g
Casein hydrolysate (Difco)	1.0 g
Glycerol	5.0 ml
Agar	15.0 g
• Autoclave, cool to 50 °C and add:	
2,3,5-triphenyl tetrazolium chloride (TZC)	0.05 g

☞ TZC is added as 1 ml of filter-sterilised 0.5 % aqueous solution per 100 ml molten (60 °C) sterilised medium before pouring.

11. TGA (tryptone glucose extract agar)

Useful for growth and storage of most pathogens.

(Available commercially)

	Per 1000 ml distilled H_2O
Beef extract	3.0 g
Tryptone	5.0 g
Dextrose	1.0 g
Agar	15.0 g

Selective media

Selective media have advantages (suppress non-target organisms) and disadvantages (complex, expensive and may suppress target organisms).

12. MCVP (modified crystal violet pectate medium)

For selective isolation of soft rot *Erwinia* spp.

Crystal violet (0.075 % aq.)	1.0 ml
NaOH (1M)	4.5 ml
$CaCl_2 \times 2\ H_2O$ (10 % aq. fresh solution)	6.0 ml
Difco agar	2.0 g (4.0 g for erwinias)
$NaNO_3$	1.0 g

- Add all chemicals to 500 ml boiling distilled water while blending or stirring at high speed.
- Slowly add 9 g sodium pectate (HP Bulmer Pectin) while blending or stirring at high speed.
- Place in 2 l flask.
- Add 0.5 ml 10 % sodium lauryl sulphate (SDS).
- Cap with aluminium foil.
- Autoclave for 25 minutes.
- Pour plates as soon as possible.
- Dry plates at least for 48 hours, surface must be dry.

13. Tween A medium (modified)

For isolation of *Xanthomonas* spp.

	Per 1000 ml distilled H_2O
Peptone	10.0 g
KBr	10.0 g
$CaCl_2$	0.25 g
Agar	15.0 g
• Autoclave.	
• Cool to 50 °C and add:	
Tween 80 (autoclaved)	10.0 ml
Cephalexin (10 mg/ml water)	3.5 ml
5-Fluorouracil (6 mg/ml water)	2.0 ml
Cyclohexamide (100 mg/ml 75 % ethanol)	0.5 ml

14. YSSM-XP medium

For isolation of *X. axonopodis* pv. *phaseoli* from seed and plants.

Stock salt solution (SSS)

	Per 1000 ml distilled H_2O
$NH_4H_2PO_4$	2.5 g
K_2HPO_4	2.5 g
$MgSO_4 \times 7\ H_2O$	1.0 g
NaCl	25.0 g

Medium

SSS	200 ml
Yeast extract	5 g
H_2O	800 ml
• Adjust pH to 7.4.	
Soluble potato starch	10.0 g
Agar	15.0 g
• Autoclave, cool to 50 °C and add:	
Bravo 500F (15 mg/ml water)	1 ml
Cephalexin (10 mg/ml water)	2 ml
Brilliant cresyl blue (1 mg/ml water)	1 ml
Kasugamycin (10 mg/ml water)	2 ml
Methanol	20 ml

15. NSCAA (nutrient starch cycloheximide antibiotic agar)

For isolation of *X. campestris* pv. *campestris* from seed.

	Per 1000 ml distilled H_2O
Nutrient agar (Difco)	23.0 g
Soluble starch	15.0 g

• Autoclave, cool to 50 °C and add:

Cycloheximide (100 mg/ml 75 % ethanol)	2 ml
Vancomycin (10 mg/ml water)	50 µl

16. KBC medium

For selective isolation of *P. syringae* pv. *syringae* from bean seed and plants.

I. Difco proteose peptone No. 3	20.0 g
Glycerol	15.0 ml
K_2HPO_4 (anhydrous)	1.5 g
$MgSO_4 \times 7\ H_2O$	1.5 g
Agar	15.0 g
Distilled water	900 ml
II. Boric acid	1.5 g
Water	100 ml
• Autoclave **I** and **II** separately, cool to 50 °C, mix and add:	
Cephalexin (10 mg/ml water)	8 ml
Cyclohexamide (100 mg/ml 75 % ethanol)	2 ml

17. SCM medium

For selective isolation of *C. michiganense* subsp. *michiganense.*

	Per 1000 ml distilled H_2O
Sucrose	10.0 g
Yeast extract	0.1 g
K_2HPO_4	2.0 g
KH_2PO_4	0.5 g
$MgSO_4 \times 7\ H_2O$	0.5 g
Boric acid	1.5 g
Agar	15.0 g
• Autoclave, cool and add:	
Cyclohexamide (100 mg/ml 75 % ethanol)	2 ml
Naladixic acid (salt) 10 mg/ml 2 % 0.1N NaOH)	1 ml
Difco Chapman tellurite (1 % solution)	1 ml
Nicotinic acid (free acid) (10 mg/ml aq.)	10 ml

18. MT medium (milk–tween)

Semi-selective medium for isolation and differentiation of *Pseudomonas syringae* and *P. savastanoi* pathovars, and *Xanthomonas* spp.

I. Proteose peptone No. 3 (Difco)	10.00 g
$CaCl_2 \times 2\ H_2O$	0.33 g
Tyrosine	0.50 g
Agar	15.00 g
Distilled water	500 ml

II. Skimmed milk	10.0 g
Distilled water	500 ml
III. Tween 80	10 ml
• Autoclave **I**, **II** and **III** separately and mix when still hot.	
• Cool to 50 °C and add:	
Cephalexin (10 mg/ml water)	8 ml
Cycloheximide (100 mg/ml 75 % ethanol)	2 ml
Vancomycin (10 mg/ml water)	1 ml

19. NASA medium

For isolation of *Agrobacterium* spp.

Add to 1000 ml of autoclaved nutrient agar (5):

Cellenite (0.5 g/100 ml water)	10 ml
Cycloheximide (actidione) (0.5 g/100 ml water)	10 ml

20. YMA+C medium

For isolaton of *Agrobacterium* spp.

	Per 1000 ml distilled H_2O
Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4 \times 7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	0.4 g
Congo Red (1 g/400 ml w/v)	10.0 ml
• Adjust pH to 7.0.	
Agar	12.0 g

21. SX agar

For isolation of *Xanthomonas campestris* pv. *campestris*

	Per 1000 ml distilled H_2O
Potato starch (soluble)	10.0 g
Beef extract (Difco)	1.0 g
NH_4Cl	5.0 g
K_2HPO_4	2.0 g
Methyl violet 2B (1 % in 20 % ethanol)	0.4 ml
Methyl green (1 % in water)	2.0 ml
Agar	15.0 g
• Autoclave, cool to 50 °C and add:	
Cycloheximide (100 mg/ml in ethanol)	2.0 ml
• Mix and pour	

Media for characterisation of phytopathogenic bacteria

Utilisation and decomposition of carbon sources

22. Oxidative/fermentative use of carbohydrates

Basal medium

	Per 1000 ml distilled H_2O
$NH_4H_2PO_4$	1.0 g
KCl	0.2 g
$MgSO_4 \times 7\ H_2O$	0.2 g
Peptone Bacteriological	1.0 g

- Adjust pH to 7.2 with 40 % NaOH.
- Add:

Bromothymol blue	0.08 g
Agar	3.0 g

- Heat to dissolve, autoclave, cool to 50 °C.
- Add 5 ml 10 % solution (w/v) filter-sterilised carbohydrate to basal medium just before dispensing in sterile, cotton-plugged test tubes (10 ml).

23. Hugh-Leifson medium

Basal medium

	Per 1000 ml distilled H_2O
Peptone	2.0 g
NaCl	5.0 g
KH_2HPO_4	0.3 g
Bromothymol blue (1 % aq.)	3.0 ml

- Dissolve all chemicals.
- Adjust pH to 7.1.

Agar	3.0 g

- Mix, heat to dissolve.
- Transfer 5 ml of basal medium to test tubes.
- Close using cotton plugs.
- Autoclave.
- Cool to 50 °C.
- Add 0.5 ml of 10 % aqueous filter-sterilised glucose to each tube.

24. Utilisation of carbohydrates

Basal medium

	Per 1000 ml distilled H_2O
$NH_4H_2PO_4$	1.0 g
KCl	0.2 g
$MgSO_4 \times 7\ H_2O$	0.2 g

Bromothymol blue (1.6 % aq.)	1.0 ml

- adjust pH to 7.2.

Agar	12.0 g

- Mix, heat to dissolve.
- Autoclave, cool to 50 °C.
- Add 1.0 g of carbohydrate (dissolved in water and filter-sterilised).
- Mix and pour.

25. Tartrate utilisation

	Per 1000 ml distilled H_2O
Sodium tartrate	2.0 g
$MgSO_4 \times 7\ H_2O$	0.2 g
NaCl	5.0 g
$NH_4H_2PO_4$	1.0 g
K_2HPO_4	1.0 g
Bromothymol blue (1.5 % in ethanol)	10 ml

- Adjust pH to 7.2 with 40 % NaOH.

Agar	15.00 g

- Heat to dissolve, autoclave and pour.

26. Acid production from carbohydrates

Dye's medium C

	Per 1000 ml distilled H_2O
$NH_4H_2PO_4$	0.5 g
K_2HPO_4	0.5 g
$MgSO_4 \times 7\ H_2O$	0.2 g
Yeast extract (Difco)	1.0 g
Bromocresol purple (1.5 % in ethanol)	0.7 ml
Agar	12 g

- Adjust pH to 6.8.
- Autoclave.
- Cool to 50 °C.
- Add 5 g of carbon source (dissolved in water and filter-sterilised).
- Mix.
- Pour into sterile test tubes.
- Solidify to form slants.

27. Levan production

Nutrient agar (5) with 5 % sucrose.

28. 3–Ketolactose production

Medium

	Per 1000 ml distilled H_2O
α-lactose	10.0 g
Yeast extract	1.0 g
Agar	20.0 g

Benedict's reagent

- Dissolve 173 g sodium citrate and 100 g anhydrous sodium carbonate in 600 ml distilled water while heating (solution A).
- Dissolve 17.3 g cupric sulphate in 150 ml distilled water (solution B).
- Slowly add B to A in a large beaker while stirring.
- Adjust volume to 1000 ml with distilled water.

Decomposition of nitrogenous compounds

29. Arginine dihydrolase

	Per 1000 ml distilled H_2O
Peptone	1.0 g
NaCl	5.0 g
K_2HPO_4	0.3 g
L-(+)arginine HCl	10.0 g
Phenol red	0.01 g
Agar	15.0 g

- Adjust pH to 7.2.
- Dissolve by heating.
- Dispense 3 ml in Bijoux bottles.
- Autoclave.

30. H_2S production

Medium

	Per 1000 ml distilled H_2O
$NH_4H_2PO_4$	0.5 g
K_2HPO_4	0.5 g
$MgSO_4 \times 7\ H_2O$	0.2 g
NaCl	5.0 g
Yeast extract (Difco)	5.0 g
Peptone	0.5 g

- Dissolve.
- Dispense in 5 ml lots in tubes.
- Autoclave.

Lead acetate strips

- Cut filter paper to strips, 1 × 10 cm.
- Immerse strips in 5 % lead acetate solution.
- Air-dry.
- Autoclave.
- Store at room temperature.

31. Indole production

Medium

	Per 1000 ml distilled H_2O
Tryptone	10.0 g
L-tryptophan	1.0 g

- Dispense medium into test tubes (10 ml per tube).
- Autoclave.

Kovacs' indole reagent

p-Dimethylaminobenzaldehyde	5.0 g
Amyl alcohol	75.0 ml
HCl (concentrated)	25.0 ml

32. Urease production

$NH_4H_2PO_4$	0.5 g
K_2HPO_4	0.5 g
$MgSO_4 \times 7\ H_2O$	0.2 g
NaCl	5.0 g
Yeast extract (Difco)	1.0 g
Cresol red	0.016 g
Water	800 ml

- Dissolve.
- Autoclave.
- Cool and add 200 ml 10 % filter-sterilised aqueous urea solution.
- Dispense 5 ml into sterile test tubes.

Decomposition of macromolecules

33. Gelatine liquefaction

	Per 1000 ml distilled H_2O
Beef extract	3.0 g
Peptone	5.0 g
Gelatine	120 g

- Dissolve while heating.
- Dispense into 10 ml per tube.
- Autoclave.

34. Aesculin hydrolysis

	Per 1000 ml distilled H_2O
Peptone	10.0 g
Aesculin	1.0 g
Ferric citrate	0.5 g
Agar	15.0 g

35. Milk proteolysis

- Sterilise reconstituted powdered milk containing 0.0004 % (v/w) bromocresol purple by steaming for 30 minutes on 3 successive days.
- Mix at 48 °C with sterile, melted yeast extract nutrient agar (NA containing 5 g/l yeast extract) to obtain a 10 % v/v.
- Pour over plates containing thin layer of NA.

36. Starch hydrolysis

Medium

- Add 0.2 % soluble starch to nutrient agar (5).
- Adjust pH to 6.8.
- Autoclave.

Iodine solution

See Gram stain (1b).

37. Tween 80 lypolysis

	Per 1000 ml distilled H_2O
Peptone (Difco)	10.0 g
NaCl	5.0 g
$CaCl_2 \times 2\ H_2O$	0.1 g
Agar	15.0 g

- Mix, heat to dissolve.
- Autoclave.
- Add 10 ml Tween 80 (autoclave separately).
- Mix and pour.

38. Lecithinase activity

(Available commercially.)

For differentiation of erwinias

- Wash fresh chicken egg in soap solution.
- Rinse with water.
- Surface sterilise in 70 % ethanol for 5 min.
- Flame the egg.
- Break aseptically.
- Separate the yolk into a sterile measuring cyllinder.
- Dilute to 40 % v/v with sterile distilled water.
- Incorporate the egg yolk into molten nutrient agar (5) cooled to 55 °C just before plates are poured at the rate of 10 ml/100 ml of medium.

39. Tyrosinase activity

	Per 1000 ml distilled H_2O
Glycerol	5 ml
Casein hydrolyzate (Oxoid)	10.0 g

K_2HPO_4	0.5 g
$MgSO_4 \times 7\ H_2O$	0.25 g
L-tyrosine	1.0 g
Agar	15.0 g

- Adjust pH to 7.2.
- Autoclave.
- Pour.

Other tests

40. Oxidase test

- Prepare 1 ml of a 1 % aq., w/v solution of NNN'N'-tetramethyl-p-phenylene-diamine dihydrochloride solution.
- Solution must be prepared just before use.

41. Acetoin production

Yeast salts broth + glucose

	Per 1000 ml distilled H_2O
$NH_4\ H_2PO_4$	0.5 g
K_2HPO_4	0.5 g
$MgSO_4 \times 7H_2O$	0.2 g
NaCl	5.0 g
Yeast extract	5.0 g
Glucose	5.0 g

- Dispense medium into 125 ml Erlenmeyer flasks (20 ml per flask).
- Autoclave.

Reagents

A . α-naphthol 5 % (w/v) in absolute ethanol.

B. KOH 40 % (w/v).

42. Catalase

Mix young culture with 3 % H_2O_2.

Tests used to characterise some genera of bacteria

PSEUDOMONADS	LOPAT	Levan production
		Oxidase
		Potato soft rot
		Arginine dihydrolase
		Tobacco hypersensitive reaction
	GATTa	Gelatine liquefaction
		Aesculin hydrolysis
		Tyrosinase activity
		Tartrate utilisation
	Other	Carbon source utilisation
		Pectolytic enzyme
		Toxin production
		Maximum growth temperature
		Ice nucleation activity
AGROBACTERIUM		3-Ketolactose test
		Growth and pigmentation in ferric ammonium broth
		NaCl tolerance
		Maximum growth temperature
		Acid production from carbohydrates
		Utilisation of L-tyrosine
		Alcali from malonic acid
SOFT ROT ERWINIAS		Hugh–Leifson test
		Acetoin production
		Catalase
		Indole production
		Lecithinase production
		Growth at 36 °C
		Potato soft rot
		Acid from organic compounds
		H_2S from cysteine
		Urease production
XANTHOMONAS		Milk proteolysis
		Acid production from carbohydrydates
		Starch hydrolysis
		Tween 80 lypolysis
		Aesculin hydrolysis
		Gelatine liquefaction
		Urease production
		Growth at 35 °C

Recommended reading

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The Disease Compendium Series of the American Phytopathological Society

Alfalfa Diseases

Apple and Pear Diseases

Barley Diseases

Bean Diseases

Beet Diseases and Insects

Citrus Diseases

Corn Diseases

Cotton Diseases

Elm Diseases

Grape Diseases

Ornamental Foliage Plant Diseases

Pea Diseases

Peanut Diseases

Potato Diseases

Raspberry and Blackberry Diseases and Insects

Rose Diseases

Sorghum Diseases

Soybean Diseases

Strawberry Diseases

Sweet Potato Diseases

Tobacco Diseases

Tomato Diseases

Wheat Diseases

Useful Internet sites

The Internet offers a large variety of useful web sites for the benefit of farmers, extension officers, researchers and lecturers in the field of plant pathology. Some sites have links to other useful sites and various search engines should be used.

☛ Some useful sites for the plant pathologist include:

//www.ippc.orst.edu/cicp/gateway/bacteriology.htm (Internet Resources on Bacterial Diseases of Plants)

//www.ifgb.uni-hannover.de/extern/ppigb/ppigb.htm (Plant Pathology Internet Guidebook)

//www.studyweb.com/agriculture/ (a link to various sites)

//pppis.fao.org/ (the Global Plant Protection Information System)

☛ Useful sites for plant disease diagnosis are:

//cygnus.tamu.edu/texlab/table.html

//www.ipm.iastate.edu/ipm/hortnews/

//www.ipm.ucdavis.edu/default.html

//hammock.ifas.ufl.edu/txt/fairs/17503

//www.crop-net.com/disease.html

//plants.okstate.edu/pddl/diseases.htm

//www.ces.ncsu.edu/depts/ent/clinic/

//www.ksu.edu/plantpath/extension/facts/

//ndsuext.nodac.edu/extpubs/plantsci/crops/pp533w-3.htm

Index to isolation media and diagnostic tests

Glossary

abiotic: non-living.

aerobe: microorganism living and active only in the presence of free oxygen.

anaerobe (anaerobic): organism able to live in the absence of oxygen.

antagonism: microorganism produces toxic metabolic products that kill, injure or inhibit the growth of some other microorganisms.

autoclave: chamber used to sterilise with pressurised steam.

bacteriophage (phage): virus that infects and causes lysis of bacterial cells.

biotype: infraspecific group of organisms that differ in one or more biochemical or physiological properties.

causal agent or organism: bacterium that induces disease.

cfu/ml (colony-forming units per millilitre): the number of live bacterial cells per millilitre of suspension fluid.

colony: macroscopically visible group of bacteria on a solid culture medium.

control of plant diseases: prevention of disease development, disease management.

dehydrate: to reduce water content.

diagnosis: identification of the nature and cause of disease.

dilution, serial: dilution of a specimen (a 1:10 dilution = 1 ml of specimen plus 9 ml sterile water).

disinfectant: chemical or physical agent that kills or inactivates microorganisms.

ELISA: enzyme-linked immunosorbent assay, a serological method for detecting bacteria.

epiphyte: bacteria living on a plant but not causing a disease.

fluorescence: visible emission of light when organism is placed in ultraviolet (UV) light.

freeze-drying (lyophilisation): preservation of living bacteria by removing water under high vaccum while tissue remains in a frozen state.

Gram-positive or Gram-negative: bacteria that stain violet or red, respectively, after treatment with Gram's stain.

halo: discoloured or water-soaked area of diseased tissue surrounding a lesion.

host range: range of plants that may be attacked by a given pathogen.

incubate: allow bacteria to grow under certain conditions.

inoculation: artificial introduction of a pathogen at a site of infection of a host to induce disease; introduction of bacteria into a culture medium.

isolate: to separate bacterium from an infected plant; the process of acquiring a pure culture of bacterium.

lesion: well-marked area of diseased tissue.

macroscopic: visible to an unaided eye.

medium or culture medium: chemical substrate providing nutrition for bacterial growth in a laboratory.

metabolism: chemical processes in the body of living organism.

microscopic: can be seen only under a microscope.

motile bacteria: capable of independent movement.

ooze: droplet of bateria on the surface of lesions.

opaque zone: dull (not shiny) zone around bacterial growth.

pathogen: bacteria able to cause disease in a host.

pathogenicity: ability of a pathogen to cause disease.

pathovar (pv.): strain of a bacterial species differentiated by pathogenicity in one or more hosts.

PCR (polymerase chain reaction): molecular method for the detection of microorganisms based on the amplification of defined sequences of DNA using specific primers and Taq DNA polymerase.

Petri dish: shallow, circular dish with cover used to grow bacteria in the laboratory.

seedborne bacteria: pathogenic bacteria associated with seed with potential to cause disease of a seedling or older plant.

serology: method for the detection of antigenic substances and the bacteria containing them by using the specificity of the antigen–antibody reaction.

sterilisation: elimination of living cells by heat, chemicals, light from biological media, containers, etc.

subspecies (subsp.): strain of bacterial species defined on the basis of more than one characteristic that distinguishes it from typical representatives of the species.

viable: state of being alive, capable of growth and development.

volunteer plant: plant seeded by chance; self-set plant.

Appendix

Common bacterial diseases of vegetable crops

Crop	Disease	Causal agent
Beans	Common blight	*Xantomonas axonopodis* pv. *phaseoli*
	Halo blight	*Pseudomonas savastanoi* pv. *phaseolicola*
	Bacterial brown spot	*Pseudomonas syringae* pv. *syringae*
Beetroot	Bacterial blight	*Pseudomonas syringae* pv. *aptata*
	Bacterial soft rot	*Erwinia carotovora* subsp. *carotovora*
	Bacterial vascular necrosis and rot	*Erwinia carotovora* subsp. *betavasculorum**
	Crown gall	*Agrobacterium tumefaciens**
	Silvering disease	*Curtobacterium flaccumfaciens* pv. *betae**
Carrot	Bacterial blight	*Xanthomonas axonopodis* pv. *carotae*
	Bacterial soft rot	*Erwinia carotovora* subsp. *carotovora*
Celery	Bacterial blight	*Pseudomonas cichorii**
	Bacterial leaf spot	*Pseudomonas syringae* pv. *apii*
	Bacterial soft rot	*Erwinia carotovora* subsp. *carotovora*
Crucifers	Black rot	*Xanthomonas campestris* pv. *campestris*
	Bacterial leaf spot	*Xanthomonas campestris* pv. *armoraciae*
	Bacterial soft rot	*Erwinia carotovora*
	Varnish spot	*Pseudomonas cichorii*
	Peppery leaf spot	*Pseudomonas syringae* pv. *maculicola*
Cucurbits	Angular leaf spot	*Pseudomonas syringae* pv. *lachrymans*
	Bacterial fruit blotch	*Acidovorax avenae* subsp. *citrulli*
	Bacterial soft rot	*Erwinia carotovora* subsp. *carotovora*
	Bacterial leaf spot	*Xanthomonas cucurbitae**
Lettuce	Bacterial rots	*Pseudomonas cichorii*
		Pseudomonas marginalis
		Xanthomonas axonopodis pv. *vitians*
		Erwinia carotovora subsp. *carotovora*

Onion	Bacterial blight	*Xanthomonas* spp.
	Bacterial internal browning	*Pseudomonas aeruginosa*
	Bacterial internal decay	*Enterobacter cloacae*
	Bacterial leaf spot	*Pseudomonas syringae* pv. *syringae**
	Bacterial soft rot	*Erwinia cartovora*
	Bacterial streak and rot	*Pseudomonas viriflava*
	Slippery skin	*Pseudomonas gladioli* pv. *alliicola*
	Sour skin	*Pseudomonas cepacia*
Pea	Bacterial blight	*Pseudomonas syringae* pv. *pisi*
	Brown spot	*Pseudomonas syringae* pv. *syringae*
Pepper	Bacterial spot	*Xanthomonas axonopodis* pv. *vesicatoria*
	Bacterial soft rot	*Erwinia carotovora* subsp. *carotovora*
Potato	Bacterial wilt	*Ralstonia solanacearum*
	Bacterial soft rot of tubers	*Erwinia carotovora* subsp. *carotovora*
	Erwinia wilt	*Erwinia chrysanthemi*
Spinach	Bacterial soft rot	*Erwinia carotovora* subsp. *carotovora*
Tomato	Bacterial canker	*Clavibacter michiganensis* subsp. *michiganensis*
	Bacterial speck	*Pseudomonas syringae* pv. *tomato*
	Bacterial spot	*Xanthomonas vesicatoria*
	Bacterial stem rot and fruit rot	*Erwinia carotovora* subsp. *carotovora*
	Bacterial wilt	*Ralstonia solanacearum*
	Pith necrosis	*Pseudomonas corrugata*

*Not reported in South Africa.

Contents

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