Lecture Supplements

Plant Bacteriology

Bacterial Diagnosis-Part 2

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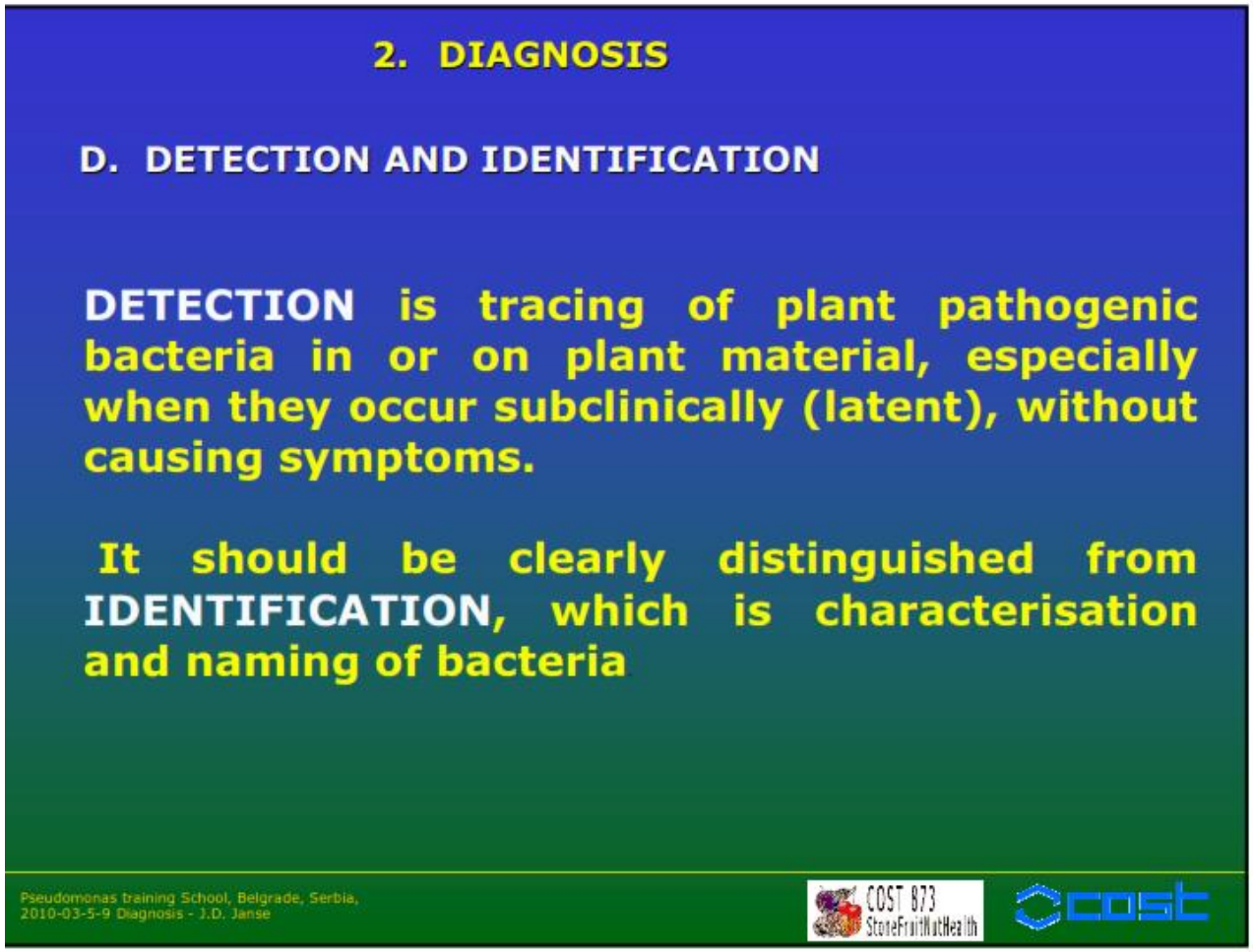


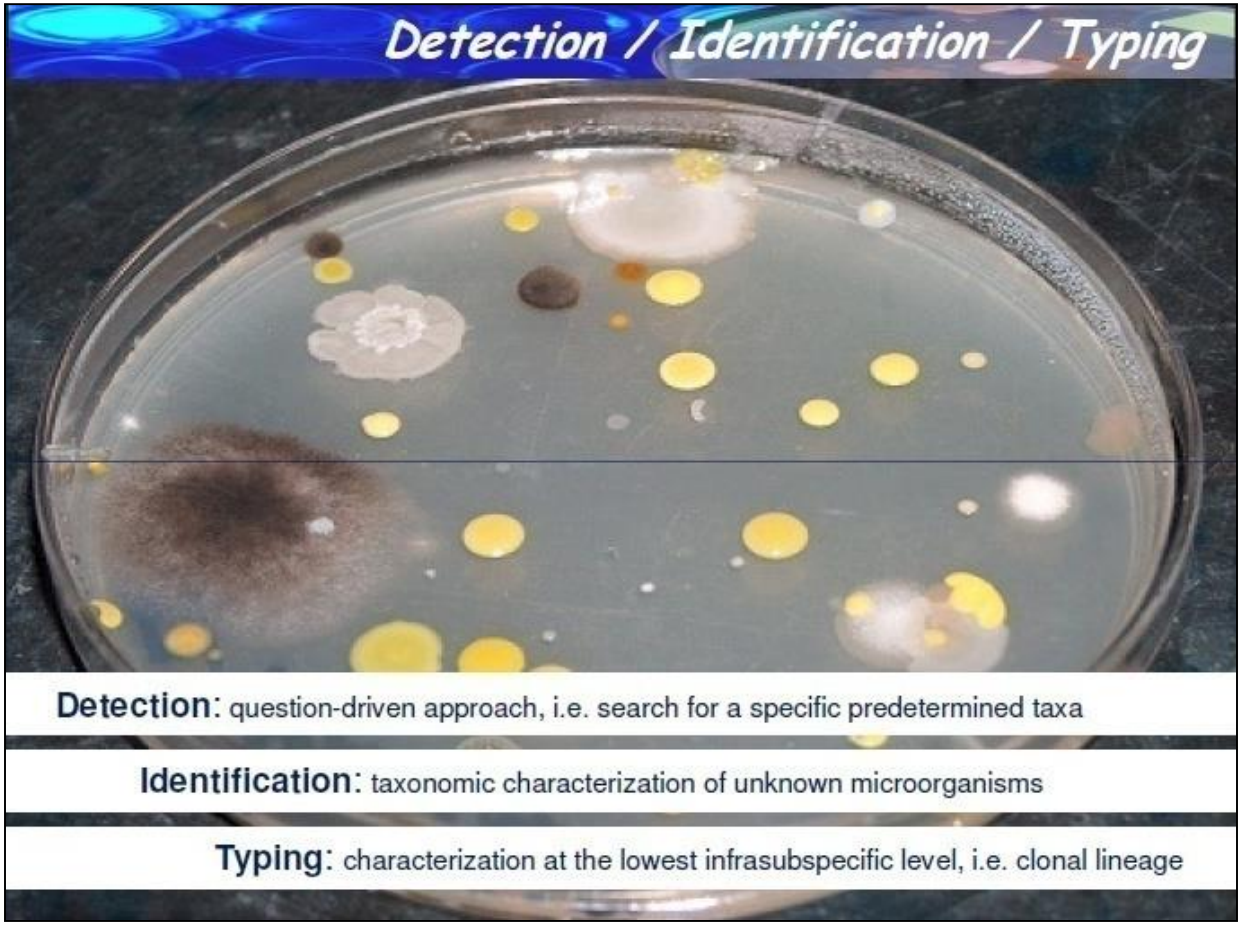
Janse,2010

Diagnosis or Identification:
Detection or Diagnosis

- These terms are often used wrongly!
- If they are fully understood and used correctly, much time and effort can be saved in determining the cause of a plant disease.
- According to López *et al.*,2006 the term "diagnosis" is reserved for identifying the nature and cause of a disease in plants showing symptoms.
- Whereas "detection" is referred to tracing the presence of a target organism in symptomless plant tissues, or in other environmental samples.

Steady,1996;López *et al.*,2010

Diagnosis or Identification:
Detection or Diagnosis



Tavares *et al.*,2010

Diagnosis

- Diagnosis is the process of determining the causal agent of a disease by examination and evaluation of symptoms and detection methods.
- The diagnosis may be:

1. Presumptive i.e. with say a 90% chance of being correct, or
2. Confirmatory i.e. 100% chance of being correct.

- A presumptive diagnosis: Usually comprises isolation plus identification of some sort.
- A confirmatory diagnosis: Requires a host test in addition but increasingly molecular methods are getting close to replacing the need for a host test in some situations.

Stead,1996

Identification

- Identification is an integral part of diagnosis.
- The processes by which unknowns are referred to known taxa.
- Often referred to as determination.
- Starts with a pure culture.
- A diagnosis can usually be made with fewer tests (initial identification tests for common genera) than an identification.
- Among the published manuals, few are invaluable to diagnosticians.
- The manual edited by Schaad *et al.*,2001 covers both diagnosis and identification.

Phylogenetic identification
Advantages

- Simple PCR based system.
- Can use pre-existing database as a reference frame.
- Discrimination of species and sub-species lineages according to length/number of loci.
- Stable reference base with little variation, useful for standardization and inter-laboratory comparison.

Neil Parkinson

Diagnostic information

- The most important pieces of diagnostic information are the symptoms themselves and with an experienced eye, a presumptive diagnosis may be made at this point.

Detection

- Detection methods indicate whether a particular pathogen is present or not.
- Therefore, detection is the process of demonstrating the presence of a specific bacterium in a sample.
- Detection is often based on a single test/method e.g. IF/PCR.
- Detection can give a presumptive diagnosis.

Detection and identification

Modern trends in identification of plant pathogenic bacteria (PPB)

- For good detection and identification, at least 3 detection methods should combine such as:

1. PCR,
2. IF, and
3. Indicator plants.

Microscopic examination of infected tissue

Observing bacteria in diseased tissue

1. Tissue selection

- Lesions are often colonized by secondary organisms that may be either saprophytes living on dead material or secondary pathogens.
- To detect the primary pathogen, select tissue that has recently been colonized.
- Select tissue at or near the active margin of lesions to avoid observing saprophytic bacteria.

2. Cut tissues

- With a single blade razor, slice strips of tissue thin enough to observe microscopically. (Hint: Make oblique sections of stems, fruits, tubers, and flowers.)
- Place the strips on a microscope slide in a drop of water.

Trigiano *et al.*,2006

Microscopic examination of infected tissue

Observing bacteria without staining in diseased tissue

3. Light microscopic evaluation

- Observe the tissue at 400× to 600× magnifications.
- Locate and focus sharply on the interface of the cut edge of the tissue and the water.
- Bacteria are not resolved at this magnification; they are only visible by refracted light and the Brownian movement of cells.
- Reduce the amount of light by either closing the iris beneath the stage or lowering the condenser.
- Bacteria streaming out of the plant tissue will be visible as bright points of light.

Trigiano *et al.*,2006

Histopathology

Microscopic examination of thin sections of infected tissue

Observing bacteria in toluidine blue O stained tissue sections

- **Light microscopic examination**
- If sections are mounted in 0.5% toluidine blue O in 0.1 M phosphate buffer (i.e. 0.696 g Na_2PO_4 and 0.796 g Na_2HPO_4, in 100 ml distilled water), the bacteria are stained a deep blue in 1 min, while plant cells are much paler (Preece, 1978).
- Prompt examination often reveals deep blue bacteria still actively motile.

Bradbury,1998

Histopathology

Microscopic examination of thin sections of infected tissue

Observing bacteria in toluidine blue O stained tissue sections

- **Light microscopic examination**
- Infected tomato tissues embedded in Spurr resin blocks were sectioned with a glass knife on an ultramicrotome for light microscopy.
- Sections (800 nm in thickness) of these tomato samples were placed on a glass slide and heated (40°C) on a slide warmer to adhere these sections to the slide.
- These sections were stained with 1% toluidine blue O for a few seconds at 40°C, washed with distilled water, and examined under a light microscope.

Kim *et al.*,2015

Histopathology

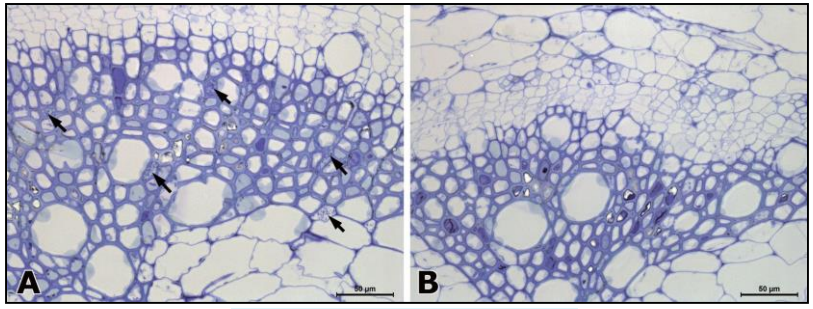
Microscopic examination of thin sections of infected tissue

Observing bacteria in toluidine blue O stained tissue sections

- **Light microscopic examination**
- Light micrographs of transverse sections from the hypocotyls of a susceptible (A) and resistant (B) plant at 3 days after inoculation of *Ralstonia solanacearum*.

A. A large number of bacteria (arrows) were present in the vessel lumen of susceptible tomato (cv. Hoyong) stem at 3 days after inoculation.

B. Transverse section of resistant tomato (IT 201664) stem showed the absence of colonization or vessel reaction.



Kim *et al.*,2015

Histopathology

Microscopic examination of thin sections of infected tissue

Observing bacteria in thionin-orange G stained tissue sections

- **Light microscopic examination**
- Fix material in alcohol-formalin-acetic No. 1 for 48 hours or longer.
- Cut thin hand sections or use sliding microtome for stem sections.
- Wash sections in water I/2 hour to 1 hour.
- Stain in carbol-thionin 1 hour (1% solution of thionin in 5% solution of phenol).
- Wash sections in water 10 to 15 minutes and then in absolute alcohol about 5 minutes.
- Stain in orange G solution only long enough to stain parenchyma walls yellow (1 to 3 minut es). The orange G solution is prepared by mixing equal parts of a saturated solution of oran ge G in clove oil with a saturated solution of the same stain in absolute alcohol.
- Place the section in clove oil about 3 to 5 minutes to remove the excess orange G.
- If the host walls are yellow and the parasite is dark in color after this treatment the section should be mounted in balsam.
- If the host walls are not sufficiently stained by the orange the section may be returned to th e stain for a few seconds.
- Paraffin sections are necessary to show the exact location of bacteria in tissues. They are sc attered over the section during sectioning when free-hand methods or the sliding microtom e are used.

Rawlins,1933

Histopathology

Microscopic examination of thin sections of infected tissue

Observing bacteria in thionin-orange G stained tissue sections

- **Light microscopic examination**
- The tomato plants were inoculated with a *R. solanacearum* suspension of 10^9 cfu/ml.
- Fourteen days after inoculation, 5-mm-long sections were sampled from stems just below the inoculation site (0), at 1cm (+1) or 2cm (+2) above the site, and 1cm (-1) or 2cm (-2) below the site.
- Samples were then fixed in a mixture of formalin, acetic acid and 50% ethanol (1:1:18 in volume) for 48hr or longer, dehydrated through an ethanol and 1-butanol series and embedded in paraffin.
- Transverse sections, 15-20 μm in thickness, were obtained from each sample mounted on a rotary microtome and stained with thionin and orange G7(It was employed both in Stoughton's original formula(1933) and in the modified technique given by Rawlins (1933).
- **Bacteria were stained purple,**
- **lignified cells blue, and**
- **parenchymatous cells yellow to orange.**

Nakaho,1993

Histopathology

Microscopic examination of thin sections of infected tissue

Observing bacteria in thionin-orange G stained tissue sections

- Histopathology of red stripe on rice leaves caused by *Microbacterium* sp.

A. Cross section through very-early-developing, pin-point-sized spot. Bacterial masses (arrow) are observed in the intercellular spaces between parenchymatous cells under stoma (S).

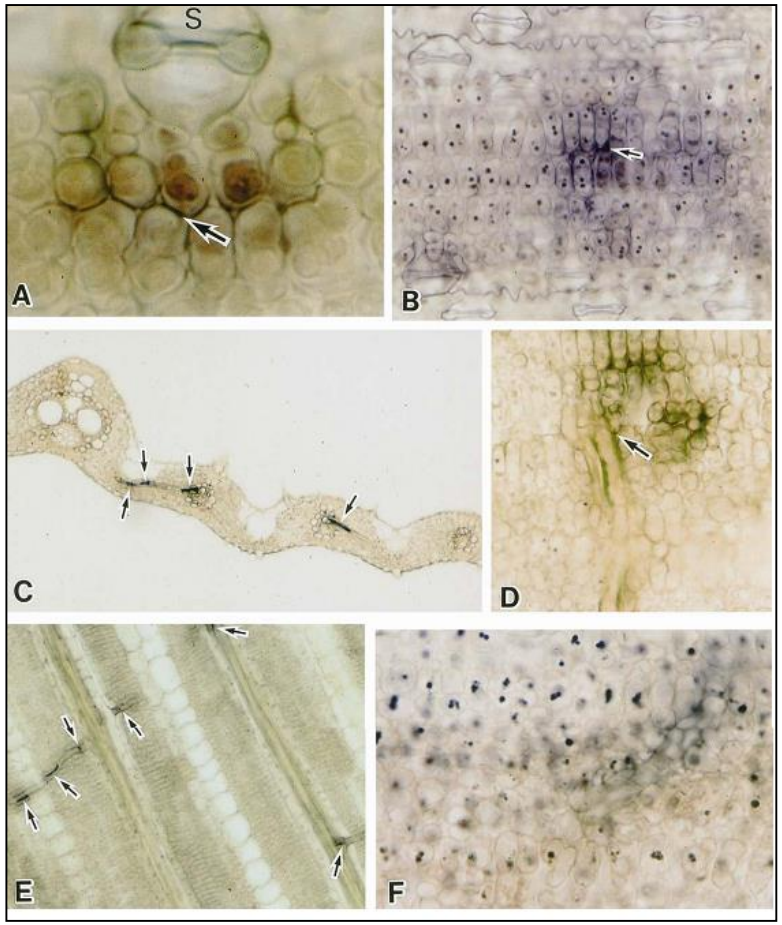
B. Cross section of early-developing orange spot. In the infected parenchymatous tissues, round granules in the protoplasm are stained dark blue. Protoplasm of parenchymatous cells with surrounding bacterial masses (arrow) is discolored and degenerated.

C. Cross section o f typical orange spot. Bacterial masses (arrows) are detected as purple clusters in transverse xylem vessels (connecting strands) connected to longitudinal vascular bundle in area of orange spot.

D. Cross section of leaf with bacterial masses in xylem vessel. Arrow indicates where bacterial mass translocated from parenchymatous tissues through vessel wall into transverse xylem vessels.

E. Longitudinal section parallel to leaf surface with bacterial masses distributed in vascular system of area of orange lesion.

F. Longitudinal section through area of blight symptom on leaf.

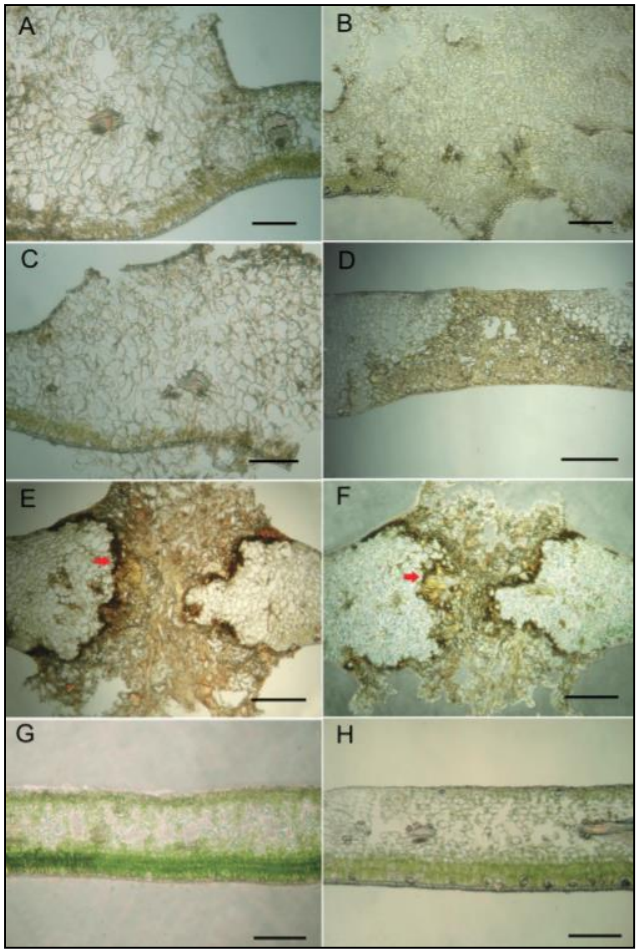


Kaku,2004

Histopathology

Histopathology of lesions induced by *Xanthomonas citri* strains on leaves of Mexican lime (A, C, E, G) and grapefruit (B, D, F, H) 26 days after needle-prick inoculation

- **For light microscopy(A-H):**
- The leaf tissue containing a lesion was excised with a dissection knife 26 days after inoculation by the needle-prick method as described above, and immediately fixed in a 20% gelatin solution, placed onto a frozen metal specimen holder, and sectioned with a Freezing Microtome (model FX-801, Yamato Kohki Industrial Co. Ltd, Japan). Sections were examined under a light microscope.
- **For transmission electron microscopy:**
- Tissues excised from inoculated leaves were immediately fixed in 5% phosphate buffered glutaradehyde for 4 hr at room temperature and post-fixed in 1% osmium tetraoxide solution for 4 hr. Fixed materials were dehydrated with a series of ethanol treatments followed by embedding in LR white resin. Embedded materials were sectioned with Leica Ultracut R (Leica, Ltd, Austria).
- The sections were stained with aqueous uranium acetate and followed by lead citrate, and examined under EM.



Lin *et al.*,2009

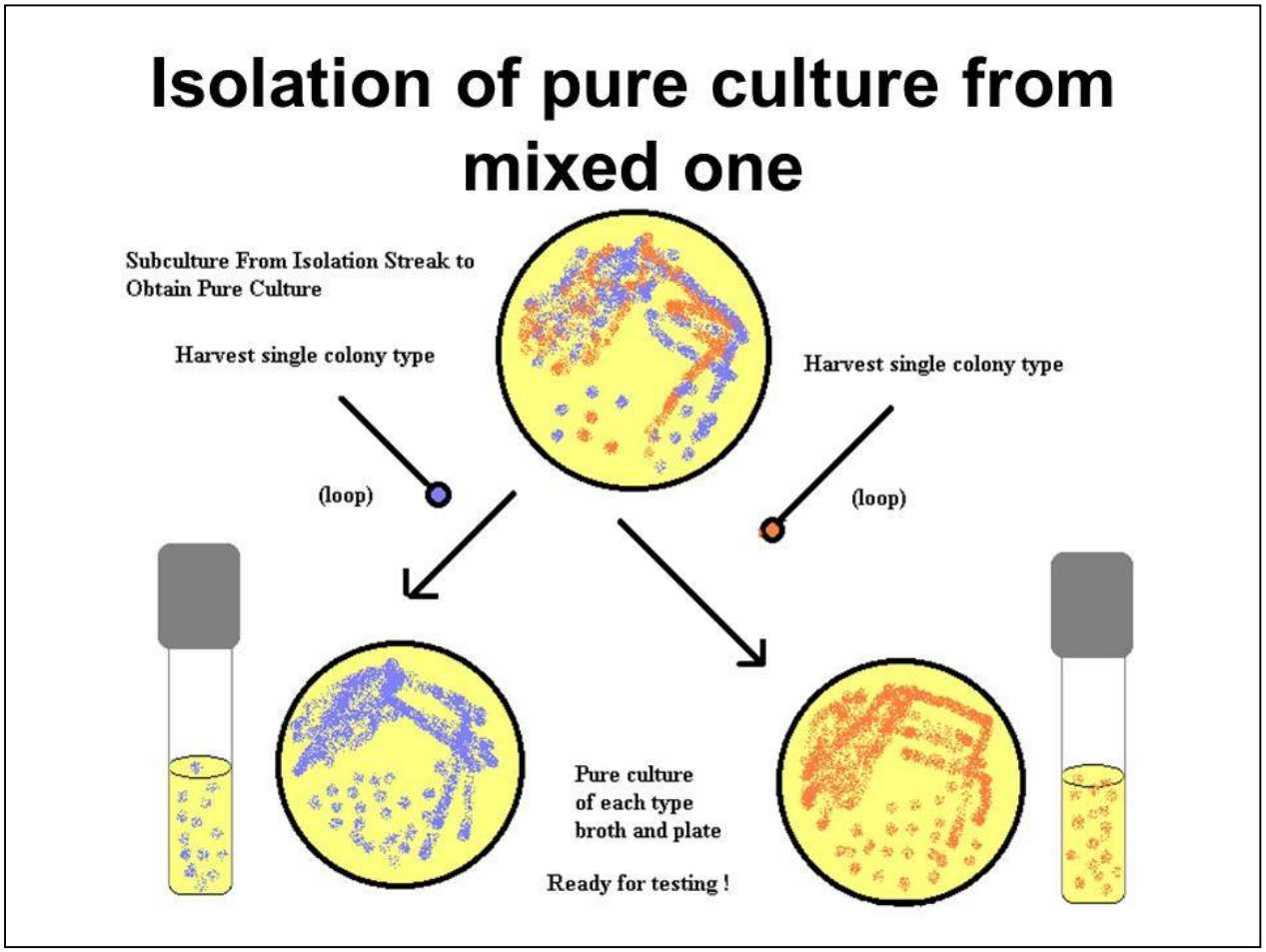
Pure culture

A pure culture is the basis for all further work in identification and diagnosis

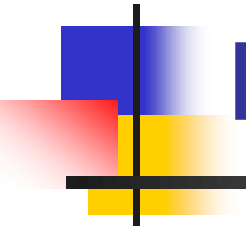
- First, the mixture must be diluted until the various individual microorganisms become separated far enough apart on an agar surface that after incubation they form visible colonies isolated from the colonies of other microorganisms. This plate is called an isolation plate.
- Then, an isolated colony can be aseptically "picked off" the isolation plate and transferred to new sterile medium.
- After incubation, all organisms in the new culture will be descendants of the same organism, that is, a pure culture.

Pure culture

Isolation of a pure culture may be enhanced by providing a mixed inoculum with a medium favouring the growth of one organism to the exclusion of others



Leslie Robinson



Key Tests

Primary tests for characterizing and making initial identifications

Key Tests

1. **Gram stain**
2. **Heat test**
3. **Motility**
4. **O/F test**
5. **Fluorescent pigmentation on KB medium**
6. **Potato soft rot**
7. **Hypersensitivity reaction on tobacco plant**

It is essential that positive and negative control strains be included in each test.

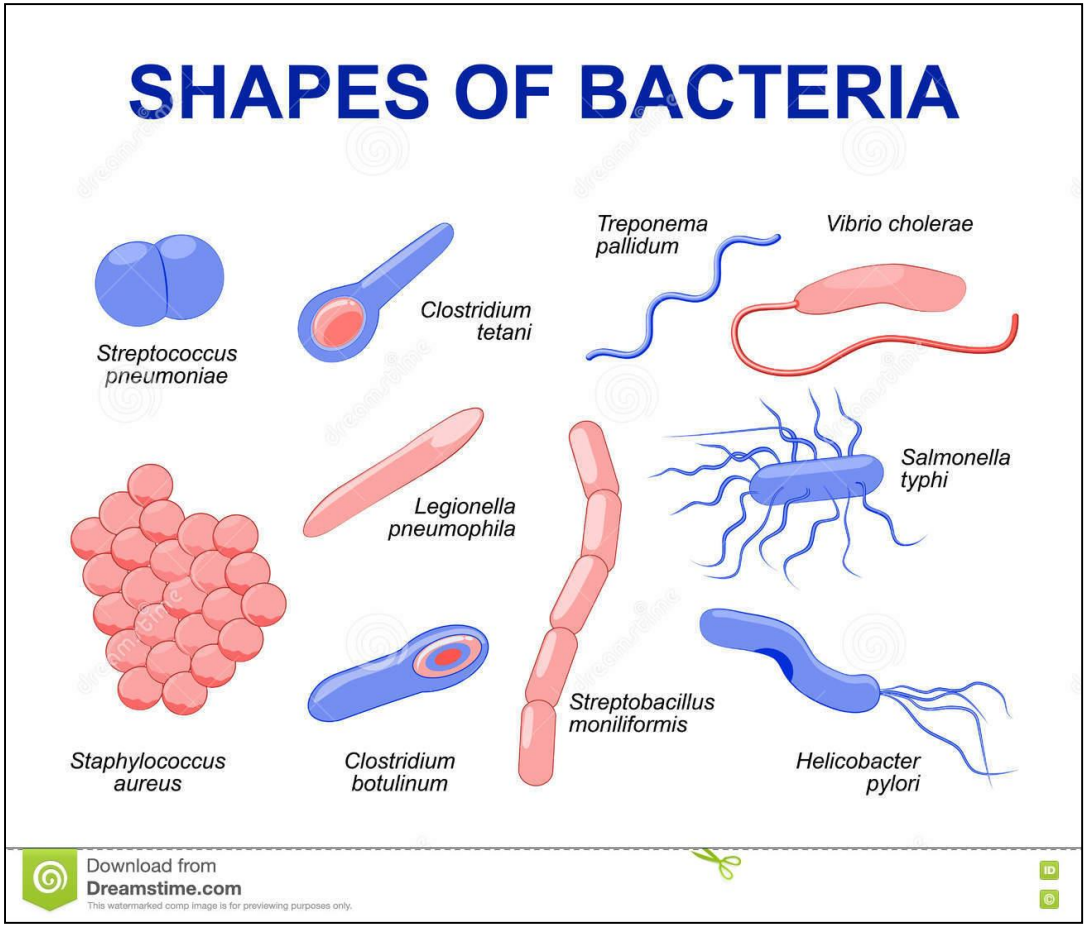
Gram staining
Differential stain

- The Gram staining method, named after the Danish bacteriologist who originally devised it in 1882 (published 1884).
- Differential stains are useful for bacterial identification, contributing to information based on bacterial size, shape, and association.

Hans Christian Joachim Gram
The inventor of the Gram stain

Pacarynuk,2006;..

Shapes of bacteria



Dreamstime

Gram staining
Differential stain

- Based on this, bacteria are differentiated as Gram positive and Gram negative.
- The major pitfall in the Gram stain is the tendency of some gram positive bacteria to decolorize more readily and be perceived incorrectly as gram negative.
- Gram reaction is of a diagnostic value only when applied to prokaryotes which have cell wall, does not yield taxonomically useful information when applied to Mycoplasma group or eukaryotic cells.

Gram staining

Preparation of Gram's Stain Reagents

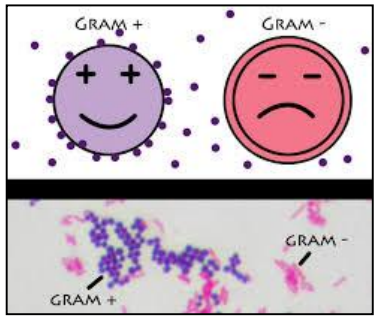
- **Crystal violet solution:** 0.5% aqueous solution (0.5 g in 100 ml Distilled water).
- **Gram's iodine solution:** Iodine 1.0 g; Potassium iodide 2.0 g; Distilled water 300 ml.
- Iodine (Lugol's iodine, IKI) is used as a mordant, which fixes the Gram+ cells so that they will not lose the violet color. It enhances dye to enter through the pore present in the cell wall/membrane.
- **Safranin solution (0.5% w/v):** 0.5% aqueous solution (0.5 g in 100 ml Distilled water).
- Safranin is used primarily as a counterstain. A counterstain is stain that makes cells or structures more visible, when not completely visible with the principal stain.

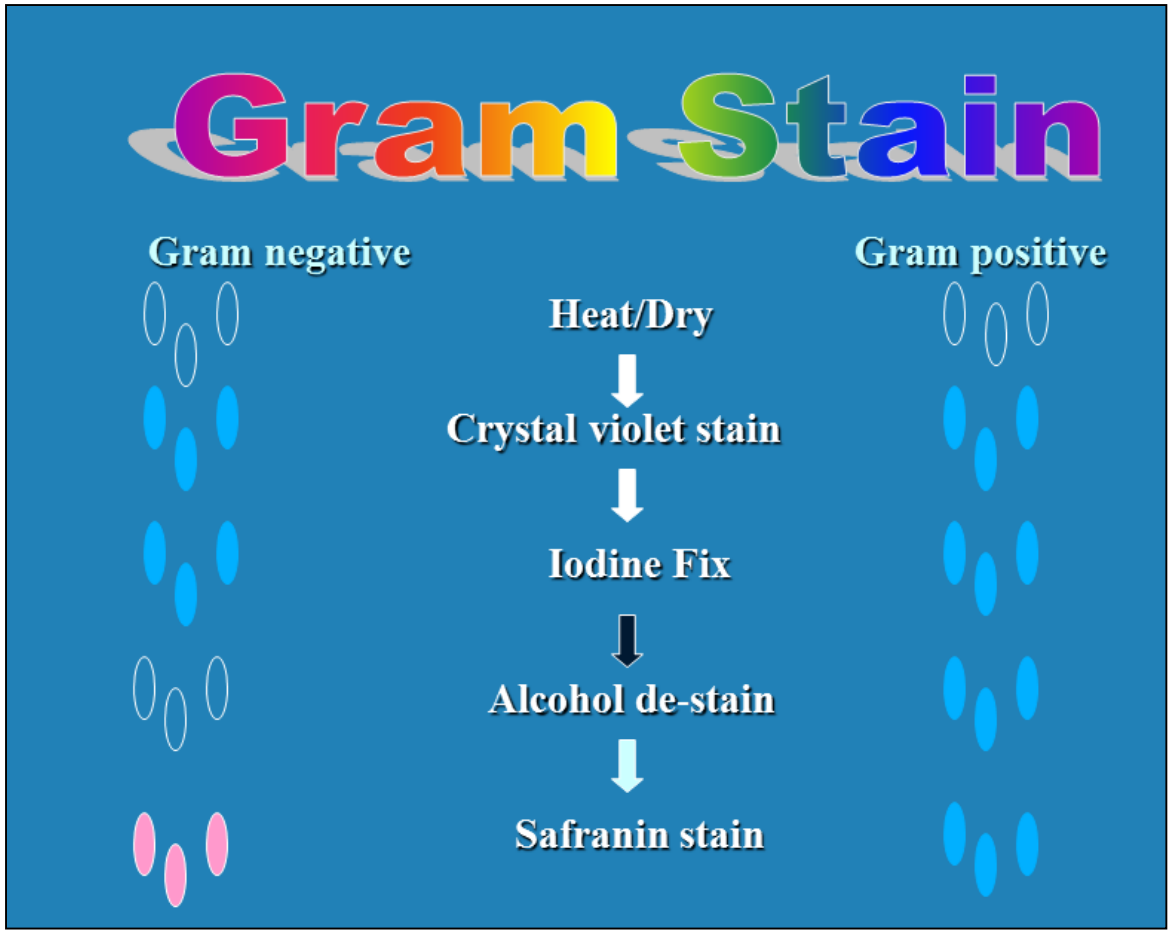
Gram staining
Procedures

- Make a thin smear of the young culture onto glass slide. Some bacteria change their Gram reactions as the cultures age.
- Flood the fixed smear with Gram's crystal violet stain (a basic dye) for 1 ½ minutes.
- Pour off the dye and wash slide with water.
- Flood the slide with Gram's iodine (a mordant) for one minute.
- Pour off the iodine and wash.
- Decolorize the slide with 95% ethanol. Add the alcohol a drop at a time until the material running off the slide is colorless.
- Remove the alcohol at once by washing in cold water.
- Flood the slide with safranin (a counterstain) for 1 ½ minutes.
- Pour off safrarin, wash slide, blot dry.
- Examine slide.

Chan,1997;..

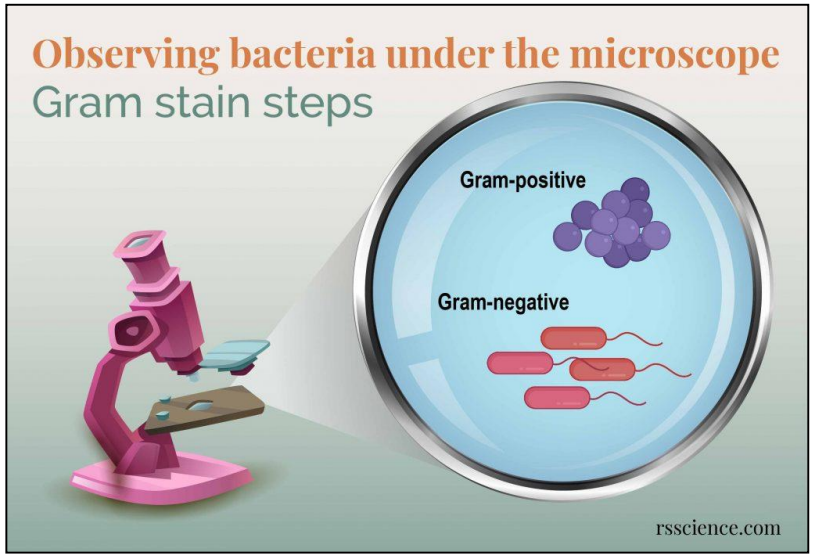
Gram stain reaction

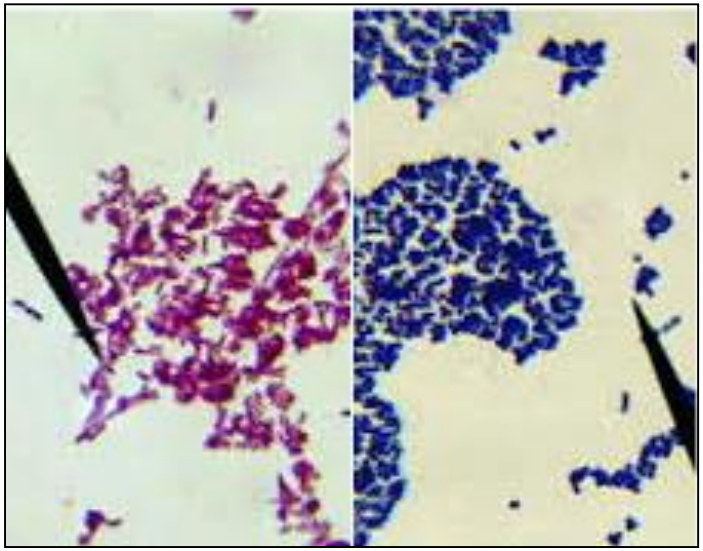




Chan,1997; Briers,2012

Gram stain reaction





Alternatives to Gram staining

Several alternative methods

1. Amino peptidase assay

- G. Cerny,1970 was able to distinguish gram negative from gram positive facultative bacteria by assaying for amino peptidase, a constitutive enzyme found primarily in Gram negative bacteria.

2. KOH test

- The KOH method was originally developed by a Japanese scientist named Ryu in 1938 and performed according to Halebian *et al.*,1981.
- Like the gram stain reaction, the KOH test is based on the differences in the chemistry of the bacterial cell wall. The cell wall of gram negative bacteria is easily disrupted when exposed to dilute alkali solutions.

3. Vancomycin susceptibility test

- Another rapid method is the testing of susceptibility to vancomycin (5 µg). In this test, gram positive bacteria were susceptible to vancomycin (>6 mm zone diameter).

Arthi *et al.*,2003;…

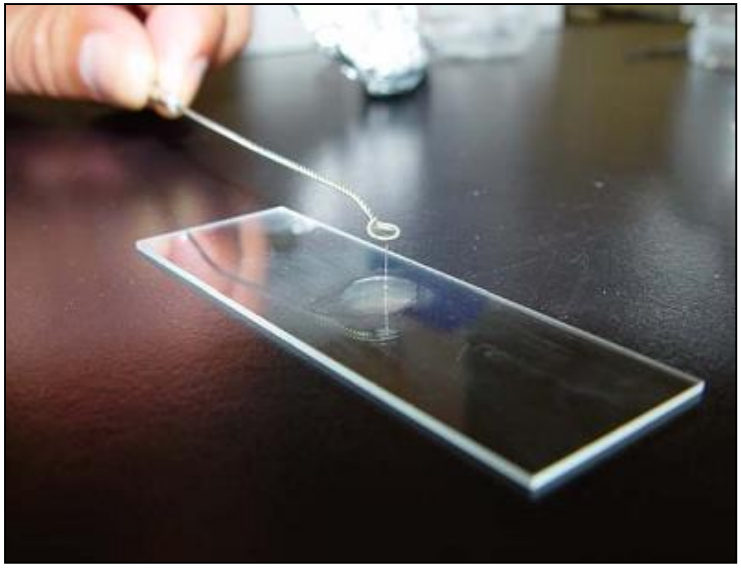
KOH Test

Alternative to Gram staining

Only Gram-negative cells will lyse

KOH String test/String test / DNA stringing test

- A loopful of growth from a colony of the organism was emulsified on the surface of a glass slide in a suspension of 3% KOH.
- The suspension was stirred continuously for 60 seconds after which the loop was gently pulled from the suspension.
- In Ryu's test, Gram negative bacteria are recognized positive by the formation of thread-like slime (DNA) after stirring for 10 sec in a drop of 3% KOH.
- Older cultures may give a Gram-variable reaction.



The Gram-positive wall is more resistant to KOH and remains intact, thus no DNA is released.

Sally Miller,2006;Ownley and Trigiano,2011

KOH Test

This test is useful in cases of doubtful stain results (CAB International,2002)

KOH (potassium hydroxide) Test

- Results correlate with Gram stain
- Strong base damages the cell membranes
  - Gram NEG bacteria: DNA leaks out
  - Gram POS bacteria: DNA stays inside
- Free DNA is viscous, slimy, and *visible* if enough cells are lysed

+ KOH = viscosity = Gram –

– KOH = no viscosity = Gram +

Rogers,2006

Vancomycin Test
Alternative to Gram staining

Vancomycin susceptibility test

- Using an inoculum corresponding to Kirby Bauer technique a lawn culture was made on Mueller Hinton agar.
- Vancomycin discs (5 µg) were placed on the lawn culture and plates were incubated at 37°C overnight.
- Any zone was read as a positive test.
- Gram positive bacteria showed 100% sensitivity to vancomycin.

Arthi *et al.*,2003

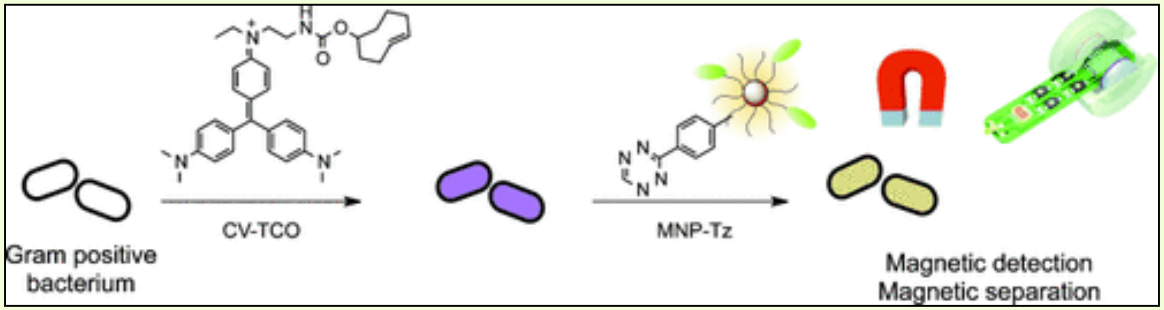
Vancomycin Test
Mode of action

- Vancomycin is a glycopeptide antibiotic, interferes with cell wall synthesis.
- Vancomycin has a large and complex chemical structure, ($C_{66}H_{75}Cl_2N_9O_{24}$.HCl=1485.7) and therefore is unable to penetrate the outer membrane of Gram-negative organisms.
- It inhibits the formation of the peptidoglycan polymers of the bacterial cell wall.
- Unlike penicillin that act primarily to prevent the crosslinking of peptidoglycans that give the cell its strength, vancomycin prevents the transfer and addition of the muramylpentapeptide building blocks that form the peptidoglycan molecule itself.

A magnetic gram stain for bacterial detection

Mode of action

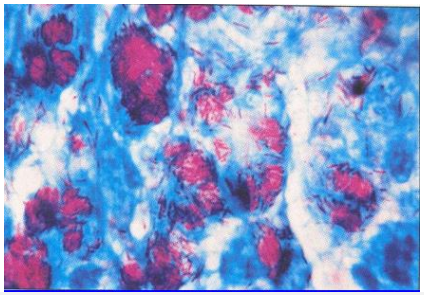
- **Magnetizing**: Bacteria are often classified into Gram-positive and Gram-negative strains by staining with crystal violet (CV).
- The described bioorthogonal modification of CV with *trans*-cyclooctene (TCO) can be used to render Gram-positive bacteria magnetic with tetrazine-functionalized magnetic nanoparticles (MNP-Tz).
- This method allows class-specific automated magnetic detection and magnetic separation.



Budin *et al.*,2012

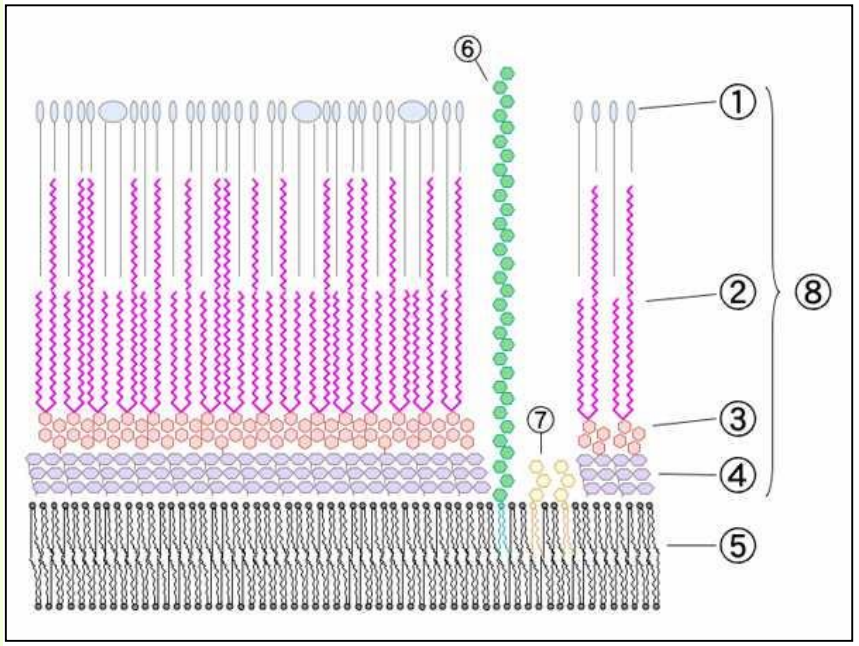
Acid-fast bacteria

Mycobacterial cell wall



Mycobacterium species is stained red, while background is stained blue.

1. Outer lipids
2. Mycolic acid
3. Polysaccharides
4. Peptidoglycan
5. Plasma membrane
6. Molecules involved in evading host immune cells & function.
7. Molecules involved in evading host immune cells & function.
8. Cell wall.

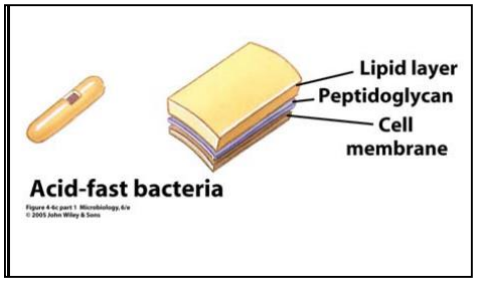


Because of waxy cell wall, they can survive exposure to acids, alkalis, detergents, oxidative bursts, lysis by immune system, and many antibiotics.

Virtual Microbiology Classroom; Kassim,2010

Acid fast staining

Alternative to Gram staining



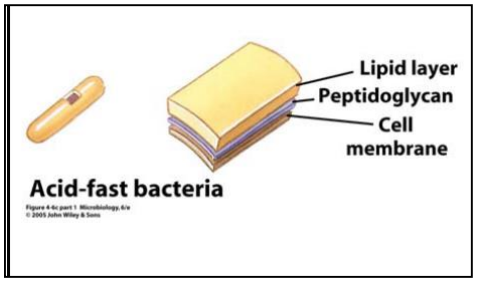
- Some Gram positive bacteria are thick-walled due to waxy cell walls containing mycolic acids (high lipid content).
- Stain based on the mycolic (glycolipid) acid content of the cell wall.
- Their cell wall is thick (not much peptidoglycan) but mainly composed of lipid.
- Most Gram positive bacteria are acid fast negative.
- Acid-fast cell wall (high lipid content) resist decolorization with acid-alcohol and stain red.
- All other bacteria will be decolorized and stain blue.

	acid-fast	nonacid-fast
carbolfuchsin	stained red	stained red -
acid alcohol	remain red	dye removed (colorless)
methylene blue	remain red	stained blue

Biol 2230 Lab Questions 1

Acid fast staining

Alternative to Gram staining



- Cover the smear with a piece of blotting paper and flood with carbol fuchsin.
- Steam for 5 minutes by passing the slide through the flame of a gas burner.
- Allow the slide to cool and wash with water.
- Add the acid-alcohol decolorizing slowly drop wise until the dye no longer runs off from the smear.
- Rinse with water.
- Counterstain with methylene blue for 1 minute.
- Wash with water, blot dry, and observe using oil immersion microscopy.

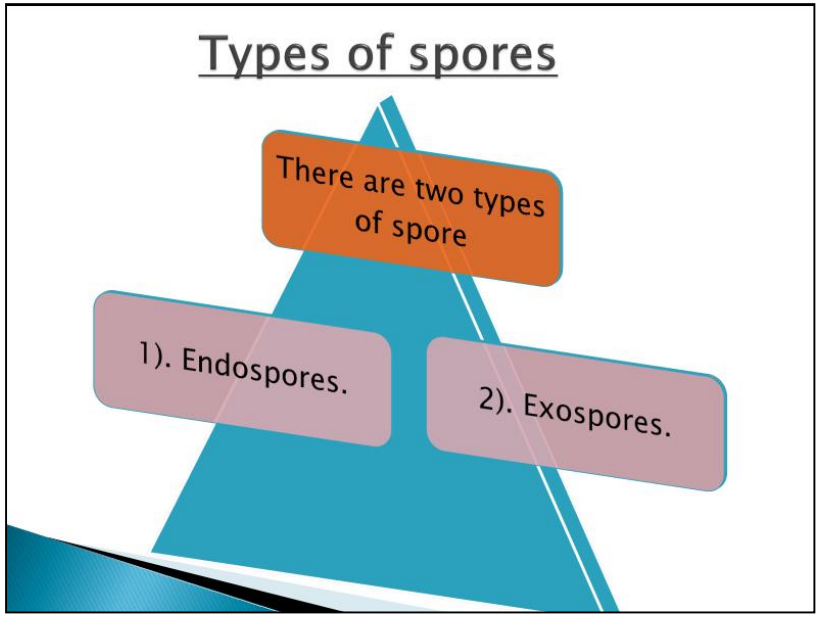
1. **Acid-fast bacteria will appear red.**
2. **Non-acid-fast will appear blue.**

	acid-fast	nonacid-fast
carbolfuchsin	stained red	stained red -
acid alcohol	remain red	dye removed (colorless)
methylene blue	remain red	stained blue

Biol 2230 Lab Questions 1; AP Biology-Benskin

Gram positive bacteria
Spore-forming bacteria
Endospores and exospores

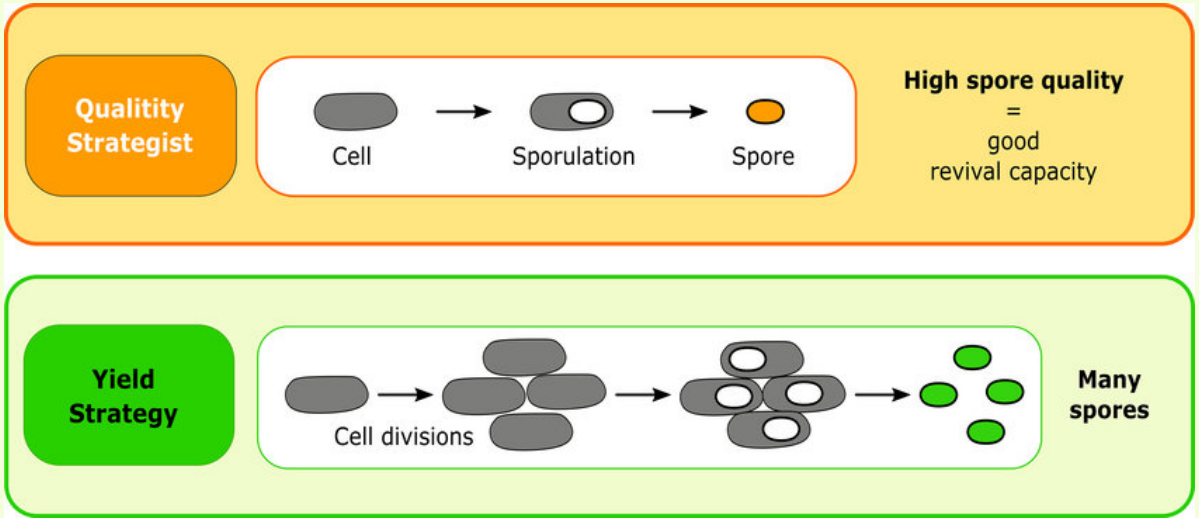
- Two types of reproductive structures or spores are Endospore and Exospore which are produced as stationary or resting systems.



https://www.vedantu.com; Ankur Vashishtha

Gram positive bacteria
Spore-forming bacteria
One cell makes one endospore affair

- Only one spore is formed inside each bacterial cell during sporulation. sporulation that occurs in an organized manner over a period of several hours.
- There are many reports of spores remaining viable over 10,000 years, and revival of spores millions of years old has been claimed.

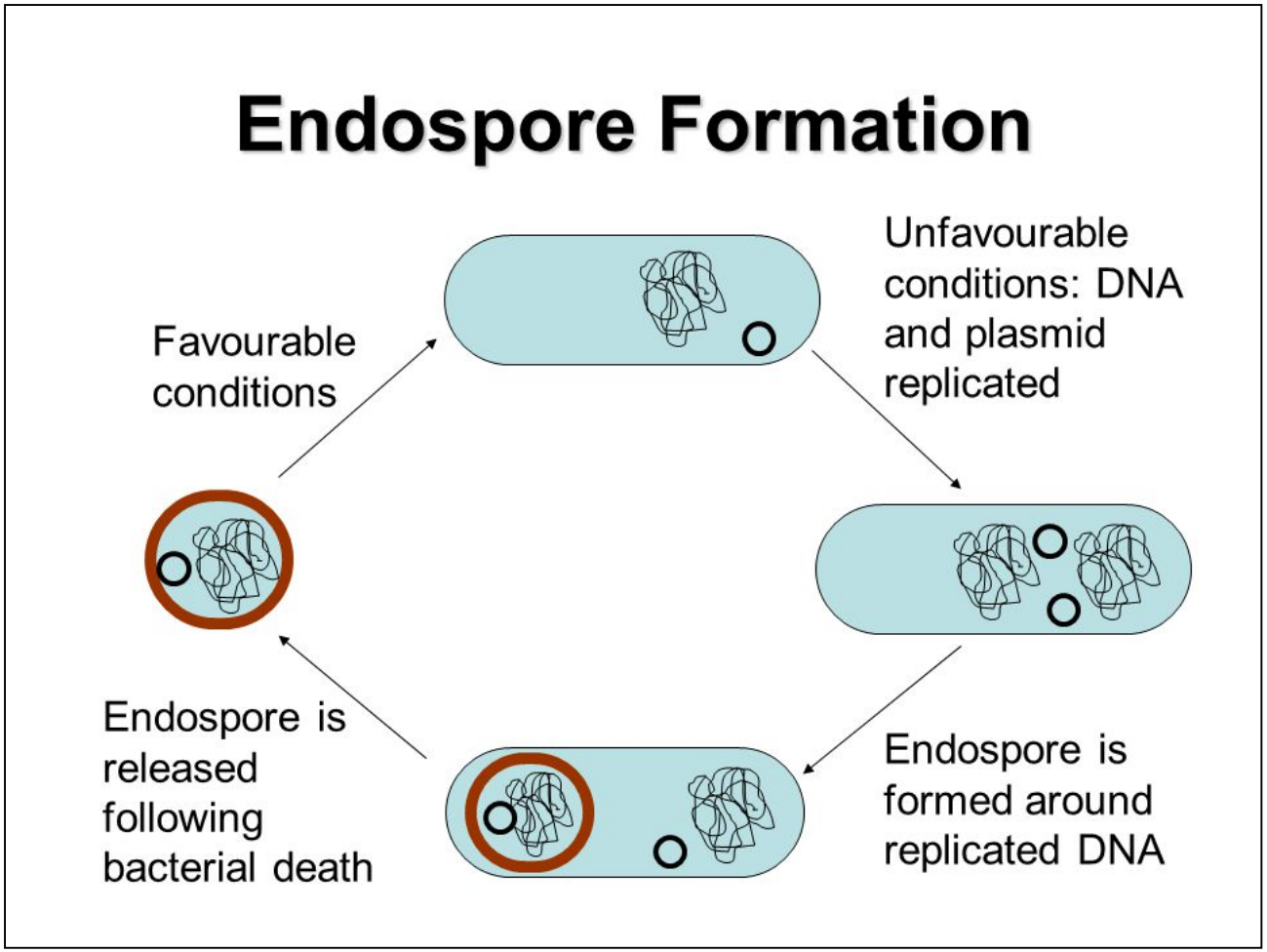


Ilka Bischofs-Pfeifer;..

Gram positive bacteria

Spore-forming bacteria

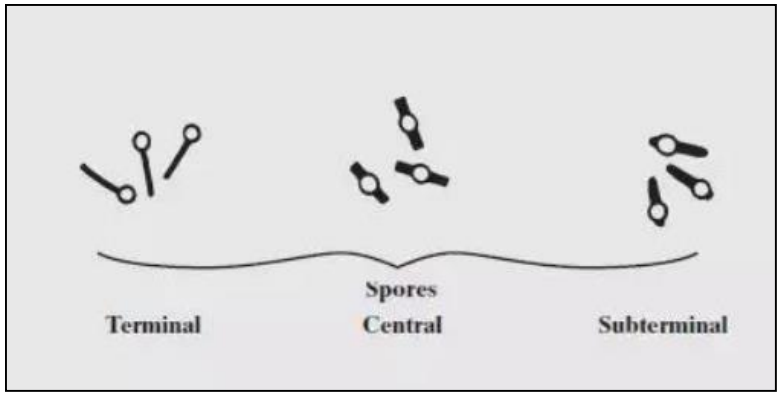
Endospores

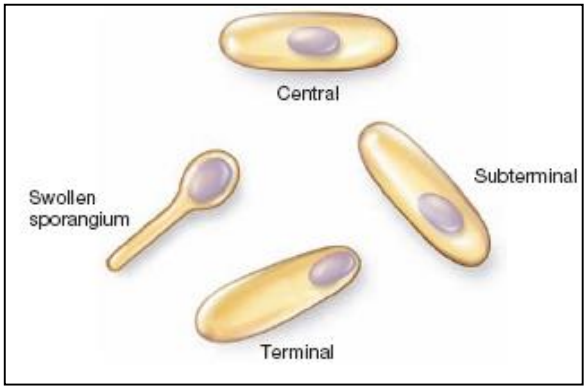


Bacterial endospore

Location and shape of endospores

- Endospores may be formed in a central, terminal, or sub-terminal position in the cell and their shape varies from ellipsoidal to spherical.
- The location of the endospore in the cell is usually characteristic of the species.
- For example, the location and shape of the *Bacillus subtilis* endospore is different from the location and shape of the *Clostridium* endospore.

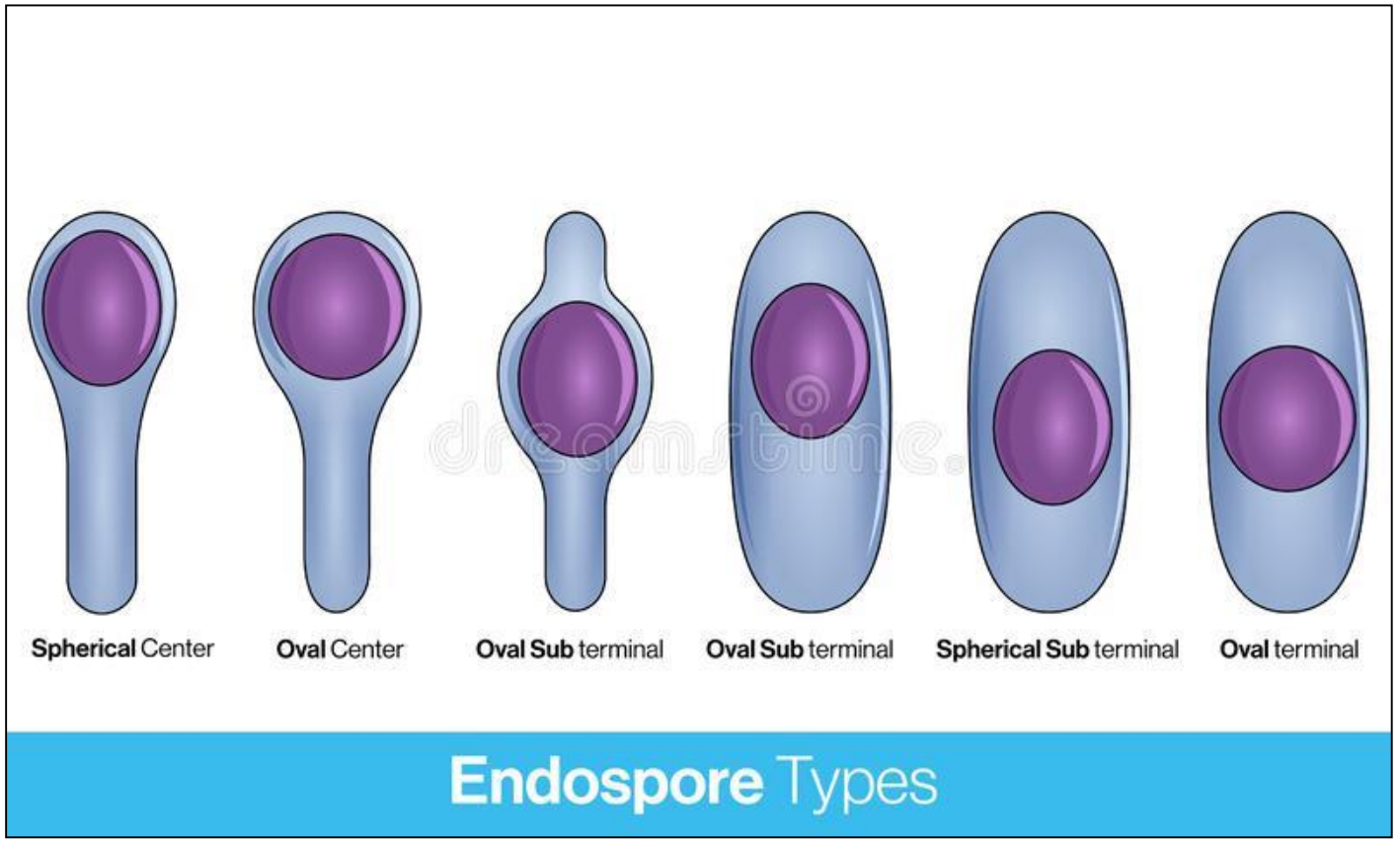




Pacarynuk,2006; Tankeshwar,2015;..

Bacterial endospore

Location and shape of endospores



Dreamstime

Bacterial endospore

Location and shape of endospores

Spore-forming rod shaped bacteria

Almost all Spore-forming bacteria are Gram^{+}

Bacillus – Aerobic

- *Bacillus subtilis,*
- *B. Mycoides*
- *B. Pastturii*
- *B. megaterium*
- *B. Thuringiensis*
- *B. Anthracis*
- *B. Botulinus*
- *B. cereus*

Clostridium – Anaerobic

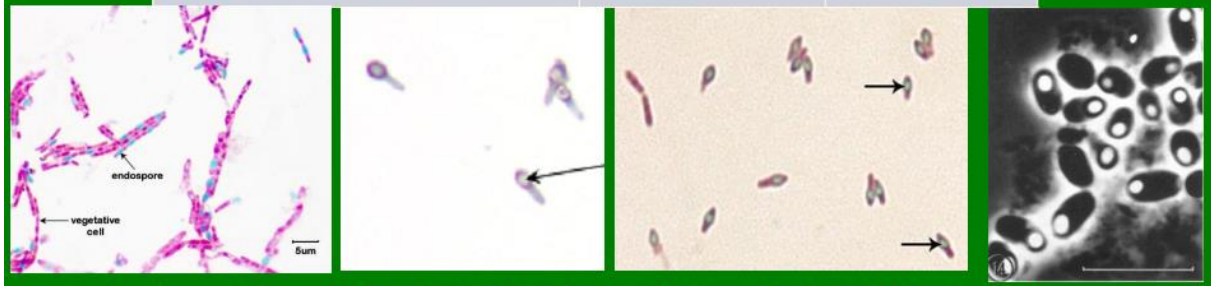
- *Clostridium botulinus*
- *C. butyricum*
- *C. aceticum*
- *C. tetani*
- *C. putrificum*

Bacterial endospore

Location and shape of endospores

Endospore classification- location in cell

Name of bacteria	Location	Shape
Bacillus anthracis	Central	Ovoid
Clostridium tetani	Terminal	Spherical
Clostridium botulinum	Sub-terminal	Spherical
Clostridium perfringens	Sub-terminal	Ovoid
Clostridium difficle	Sub-terminal	Ovoid



Central, ovoid | Terminal, spherical | Sub-terminal, spherical | Sub-terminal, ovoid

J Bacteriol. 1968 Nov; 96(5): 1818–1834

Microbiology, 2017. Openstax, Rice University

Bacterial endospore
Clinical significance- Endospore

- In addition to food poisoning (*Bacillus cereus, Clostridium perfringens* and *C. botulinum*), there are a number of other human illnesses in which spores play a causative role including,

1. wound infections (gas gangene: *C. perfringens*);
2. tetanus: *C. tetani*;
3. wound botulism: *C. botulinum*;
4. intestinal infection: *C. difficile*, and
5. anthrax: *B. anthracis*.

- Spores of *B. anthracis* persisting in soils are the common route whereby animals acquire pulmonary anthrax.

Name of bacteria	Disease
Bacillus anthracis	Anthrax
Clostridium tetani	Tetanus
Clostridium botulinum	Botulism
Clostridium perfringens	Gas gangarene
Clostridium difficle	Pseudomembranous colitis

B. anthracis spores: a) are considered a likely biological warfare agent; and b) were used recently in terrorism incidents in the United States.

Microbiology, 2017; Setlow,2005

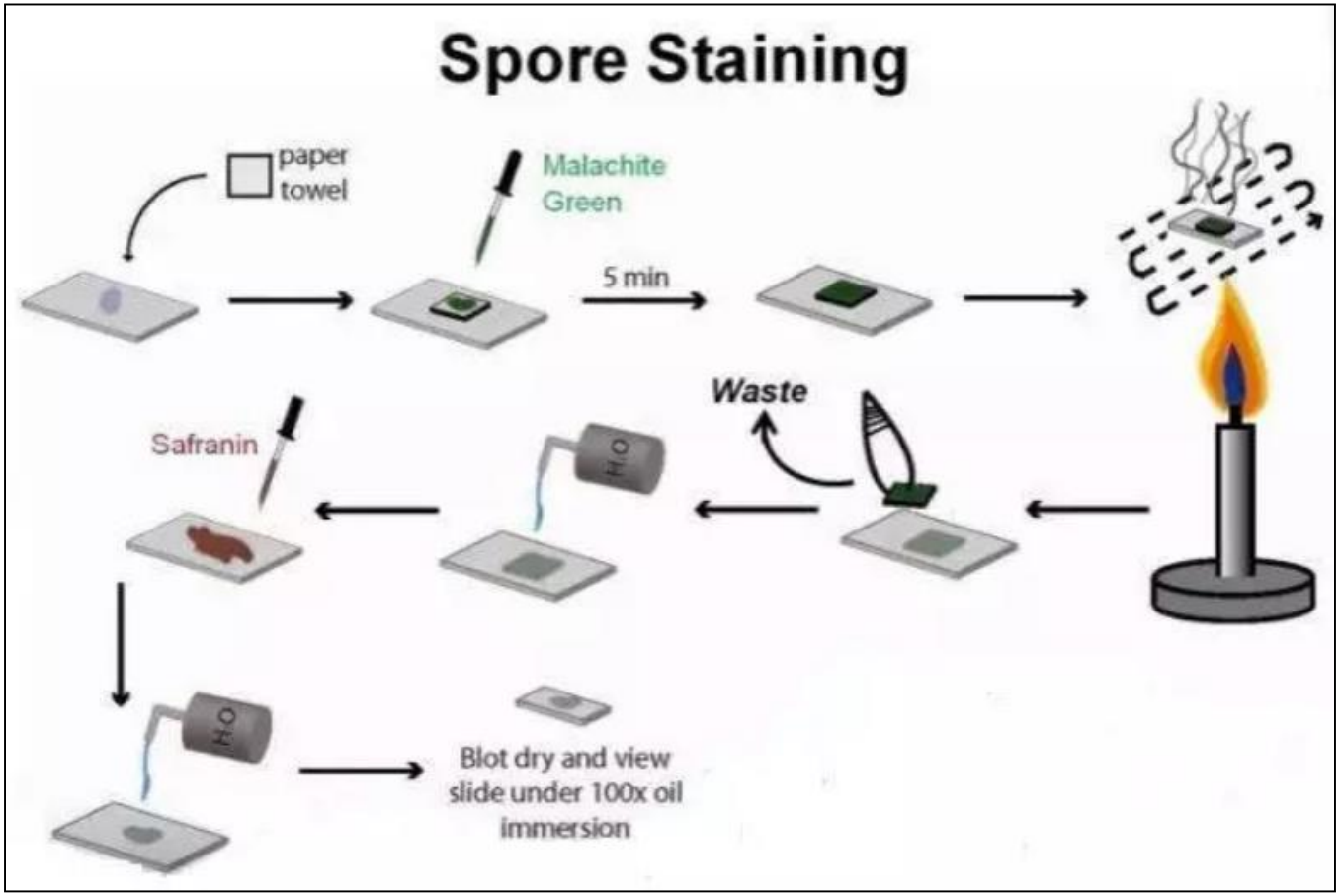
Endospore staining
Procedures

- Prepare smear and heat fix. Cover the dried fixed film with a small piece of paper towel.
- Saturate this with 5% malachite green (Dissolve 5 g of malachite green oxalate in 100 ml of distilled water).
- Pass the Bunsen burner flame under the slide until the stain steams; continue for 5 minutes.
- Add additional stain as needed (Do not allow stain to boil or completely evaporate).
- Allow the slide to cool, then rinse with water.
- Tap over a paper towel to remove excess water
- Counterstain with safranin for 30-60 seconds.
- Rinse slide with water.
- Allow to air dry, and view.

1. Endospores will stain green,
2. The rest of the cell pink.

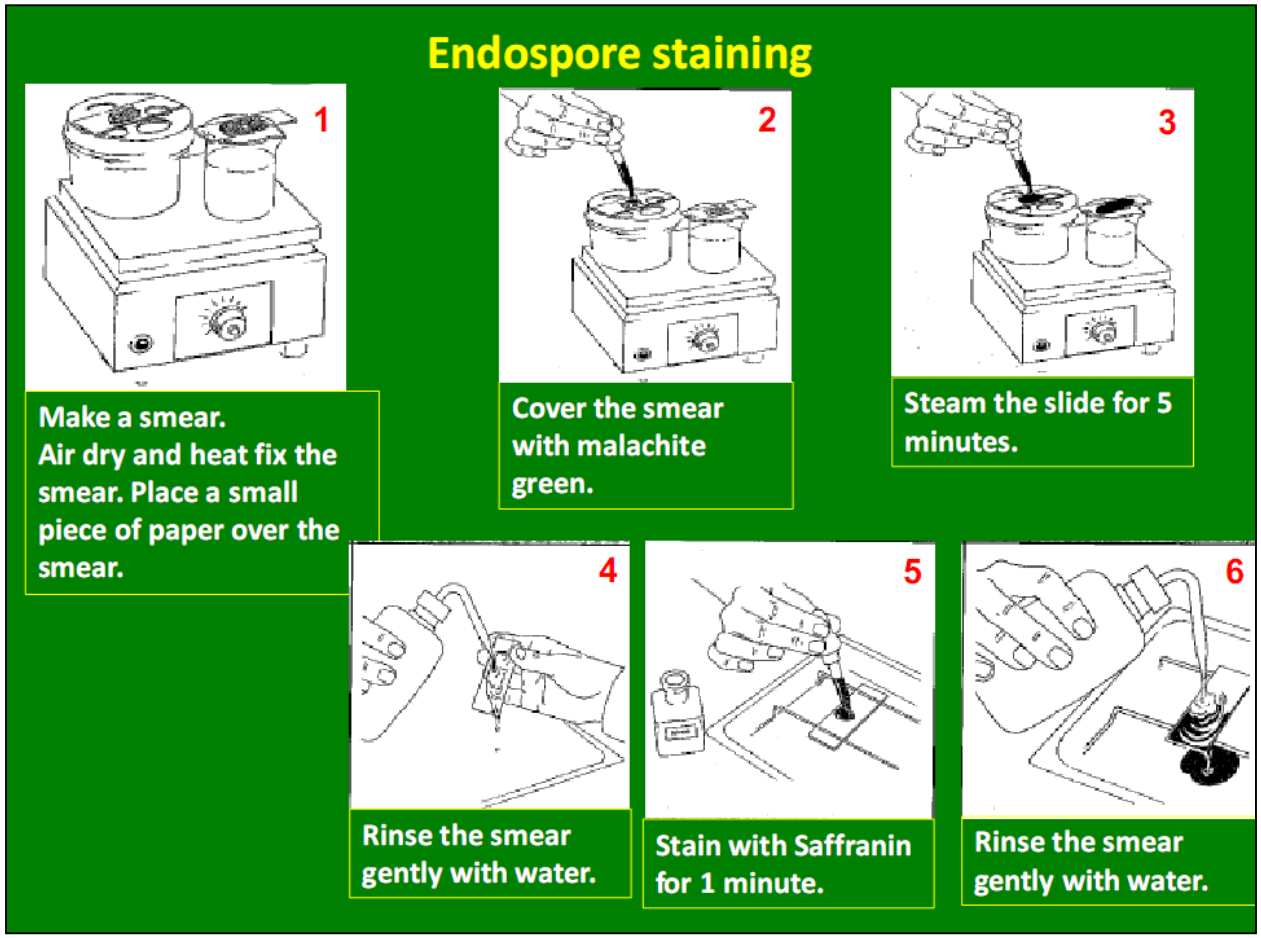
Pacarynuk,2006

Endospore staining
Procedures



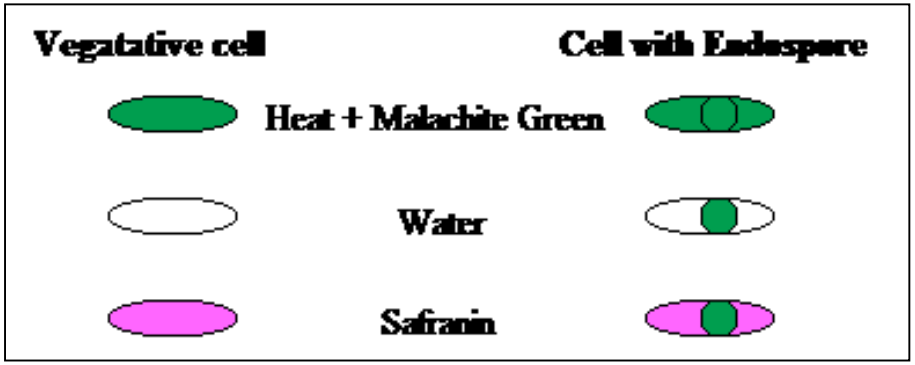
Tankeshwar,2015

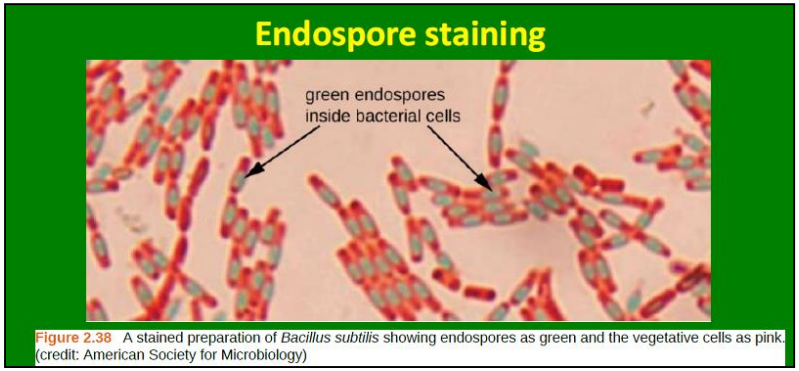
Endospore staining
Modified method



Microbiology, 2017. Openstax, Rice University

Endospore staining
Procedures





Endospores will stain green, the rest of the cell pink.

Microbiology Lab Tutorial;..

Heat test for spores

Alternative method for endospore staining

- In order to test whether a sample of inoculum contains endospores, the sample is exposed to high heat (80°C) for 10 minutes.
- This treatment would kill all cells but endospores would survive and could grow when incubated at an appropriate temperature.

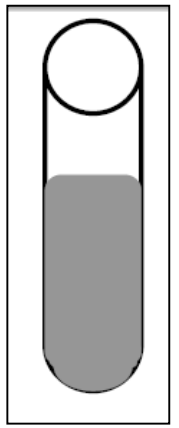
1. If endospores are present, the resultant inoculum in NB medium will give rise to a turbid culture (indicating bacterial growth);
2. If there are no endospores present, no growth is observed.

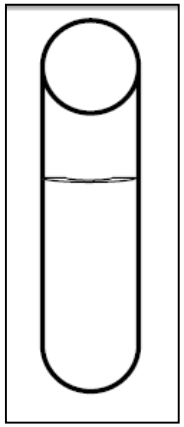
Christopher and Bruno,2003;..

Heat test for spores

Alternative method for endospore staining

- A turbid culture will form following incubation if the original inoculum contained endospores.
- Control tube: uninoculated and Negative result: No growth occurs following extreme heat exposure if the original inoculum did not contain endospores.



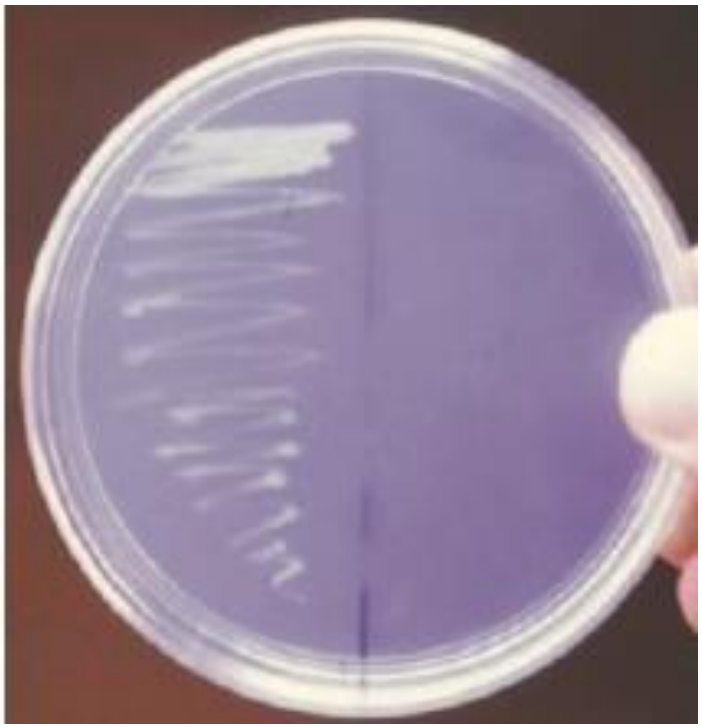


Christopher and Bruno,2003

Heat test for spores

Alternative method for endospore staining

- In this test, bacterial suspensions were exposed to 80°C for 10 minutes.
- One loopful was streaked on NA medium.
- The Growth on the agar plate is limited to the area streaked with bacterial inoculum containing endospore.



Endospore staining

Procedures

Sporulation in normal adverse conditions takes 8hrs-19hrs to complete

- The survival and germination of spores after a heat treatment of 30 min at 100°C is of particular importance because these spores may be considered as very heat-resistant as compared with the spores obtained after a conventional heat shock of 10 min at 80°C.

Most isolates obtained after a heat treatment of 10 min at 80°C were identified as members of the *B. subtilis* group, *B. pumilus*, *B. clausii* and *B. licheniformis*. Isolates with very heat-resistant spores, obtained after a heat treatment of 30 min at 100°C, were identified as members of the *B. subtilis* group, *B. sporothermodurans*, *B. amyloliquefaciens*, *B. oleronius* and *B. pallidus*.

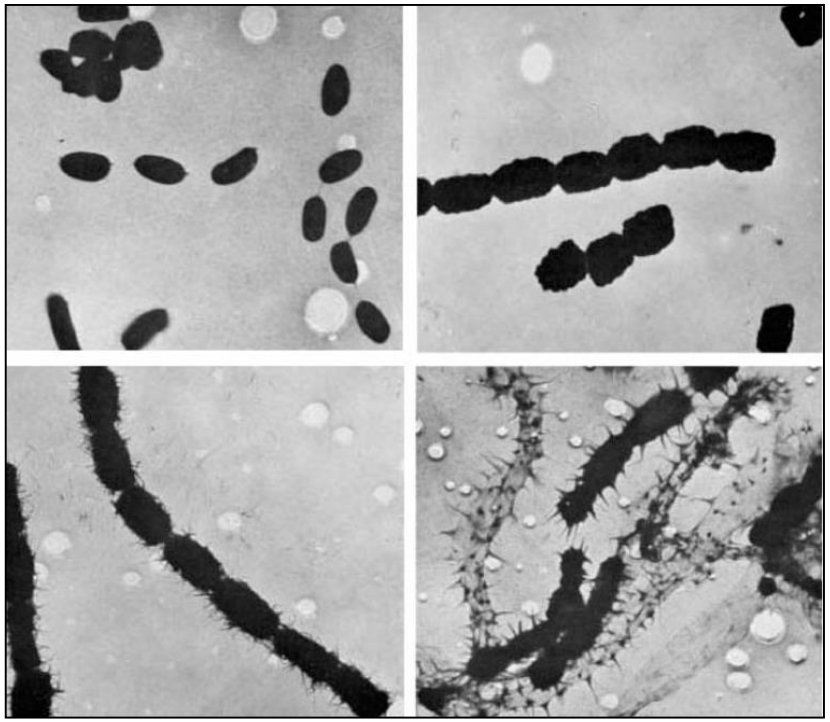
de Paula Pacheco-Sanchez and Rodriguez de Massaguer,2007

Exospore staining

Spore chains of streptomycetes

Long chains frequently having up to 100 spores

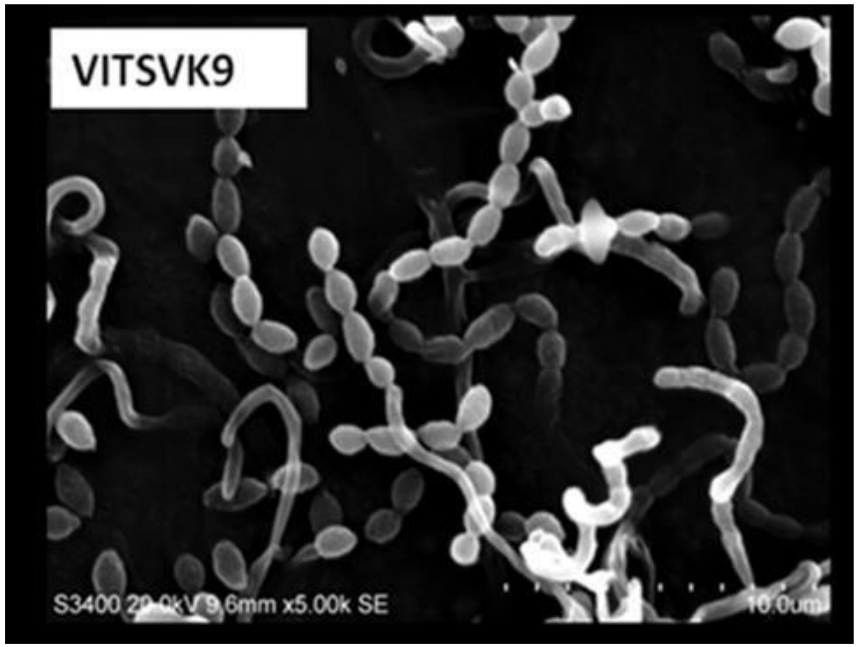
- Electron micrographs of four types of arthrospores (A body that resembles a spore but is not an endospore) of streptomycetes: smooth, warty, hairy and spiny.
- The spores are about 1 m long.



Kumar *et al.*,2011; Li *et al.*,2016

Exospore staining
Streptomyces and related genera

- Spore morphology of *Streptomyces* VITSVK9 spp. observed under SEM.
- Grown in optimized medium at 30°C for 7 days.
- The bar represents 10 µm.



Saurav and Krishnan,2010

Exospore staining
Streptomyces and related genera

- Stain the bacterial preparation on a glass slide for 2 min with 2:2:1 mixture of 1% Bismarck brown, 0.1% toluidine blue, and a saturated solution of Ammonium sulfate $(NH_4)_2SO_4$.
- Wash with water, and mount under a microscope.

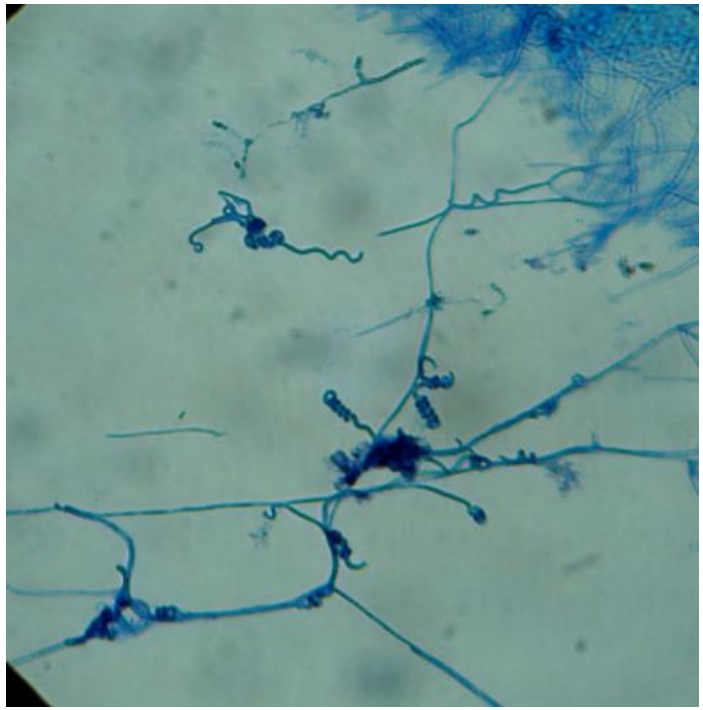
1. The hyphae stain bright yellow,
2. while the spores are blue.
3. Red brown granules can be seen in the hyphae.

- A blue stain may be picked up by some nonsporulating aerial hyphae.

Exospore staining

Streptomyces and related genera

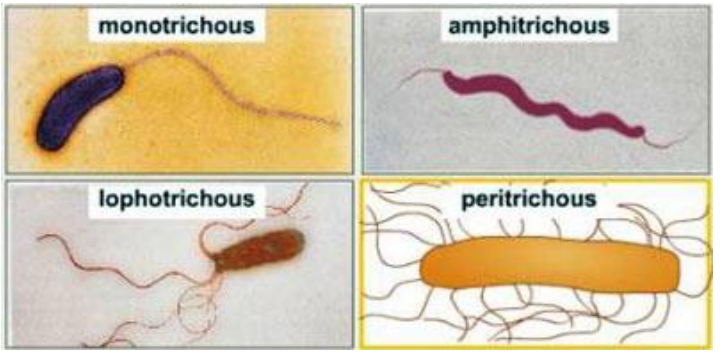
- The cover slips were also withdrawn and mounted on the glass slide having one drop of methylene blue (0.3 g in 10 ml distilled water).
- The cover slips were fixed with feviquick(glue) and observed under light microscope.

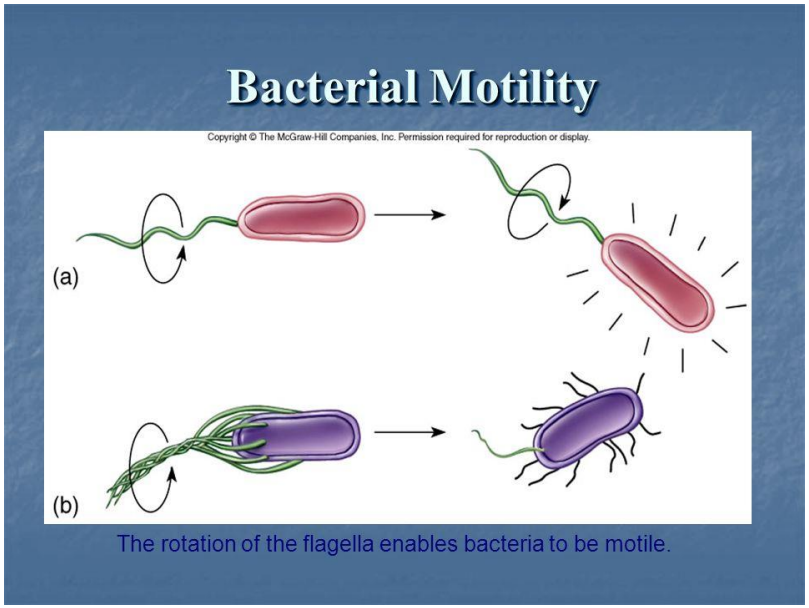


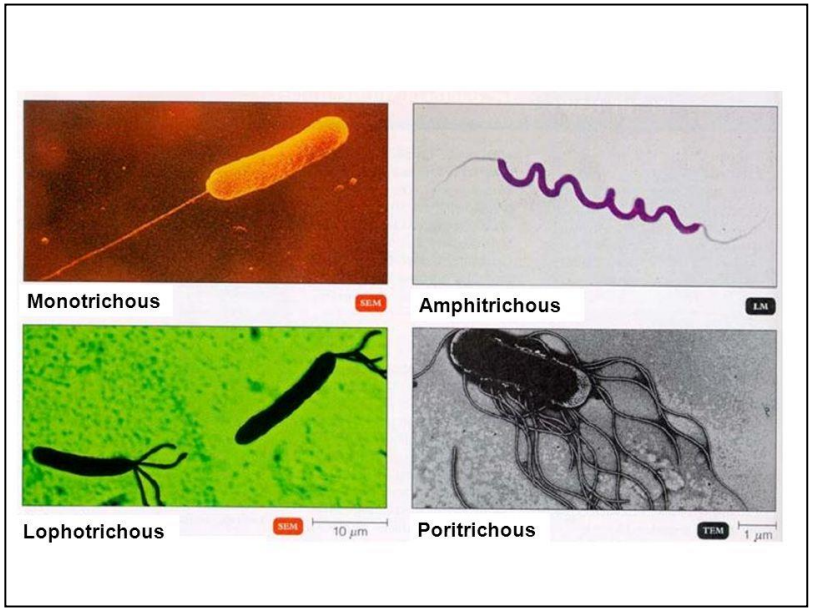
Spore chain morphology of *Streptomyces* sp. (Light microscopy, 1,000).

Motility test

Bacterial flagella

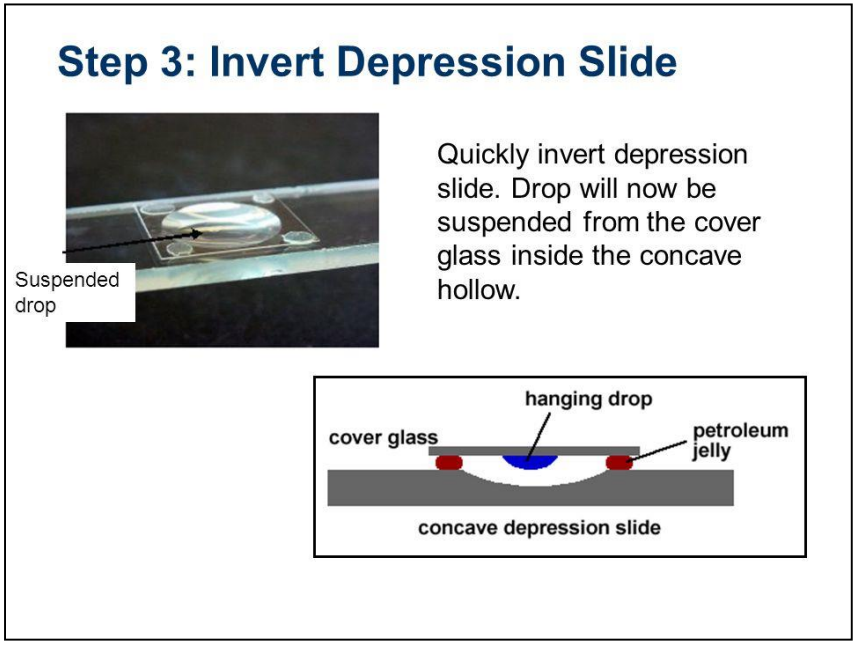






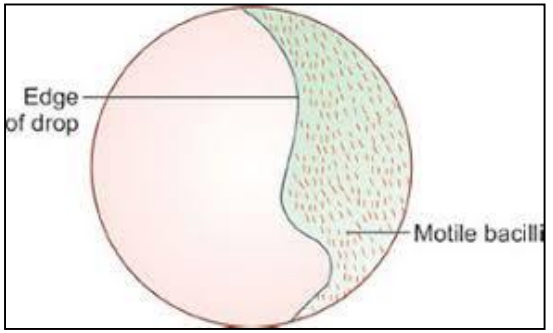
Motility test
Hanging drop slide

1. Place a drop of the bacterial culture (optimally from a young broth culture) in the middle of a cover slip.
2. Place a thin line of petroleum jelly around the edge of the cover slide.
3. Turn the depression slide upside-down (depressed area facing down) and gently touch the cover slide.
4. The jelly holds the cover slip to the slide and also keeps the suspension from drying out.
5. Now flip the entire microscope slide/cover slip combination over.



Reynolds,2005; Christine Shreve

Motility test
Hanging drop slide

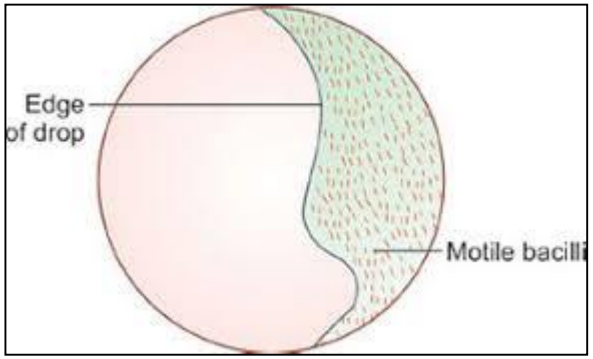


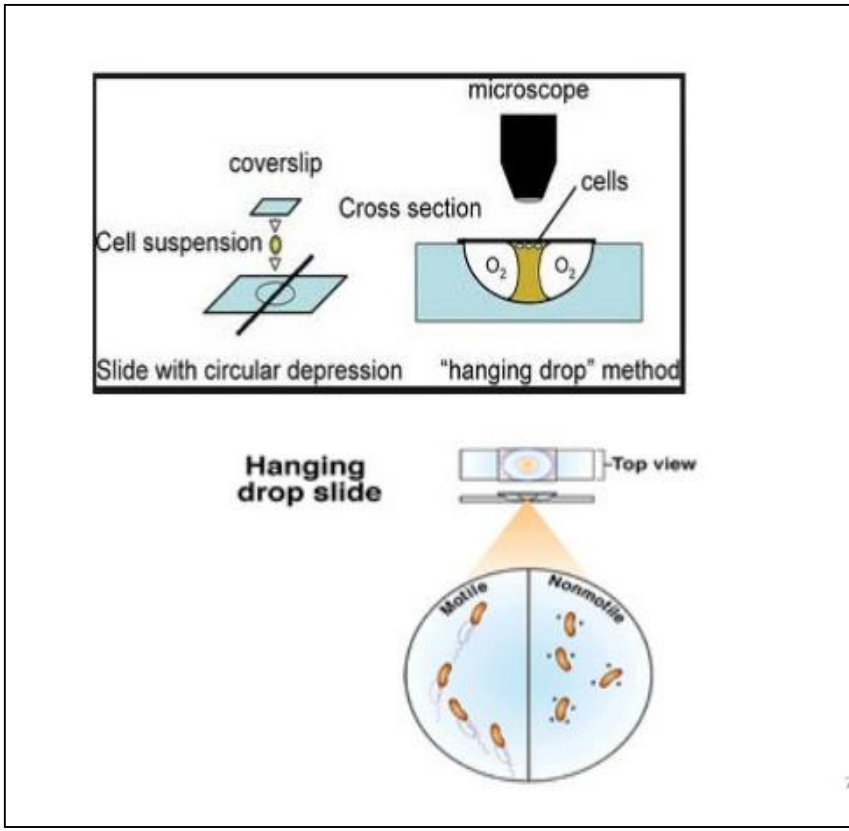
- If you use the regular light microscope, focus initially with the 10X objective, switching to the 40X objective and then (but only if needed) the 100X, oil-immersion objective.
- Adjust the light with the iris diaphragm; optimum results are achieved with a relatively low light intensity.
- Alternately, excellent results can be had by the use the phase microscope with the 40X (middle) objective lens in place.
- Do not be misled by Brownian motion.

Motility test

Hanging drop slide

Under microscope





Hamutumwa,2016;..

Motility test

Motility media

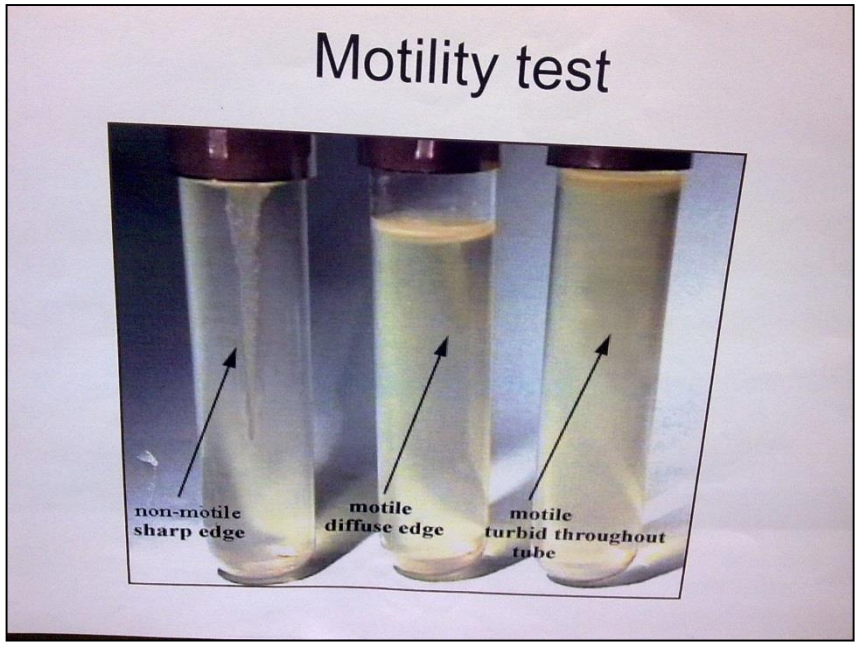
- **For *Bacillus***
- Gelatin 8 g
- Peptone 10 g
- Beef extract 3 g
- NaCl 5 g
- Agar 4 g
- H_2O 1 L
- Distribute into tubes (10 ml) and autoclave.

- **For all bacteria**
- Peptone 3 g
- Yeast extract 5 g
- Agar 2.5 g
- H_2O 1 L
- pH 7.2
- Distribute into tubes (10 ml) and autoclave.

Motility test

Motility Medium

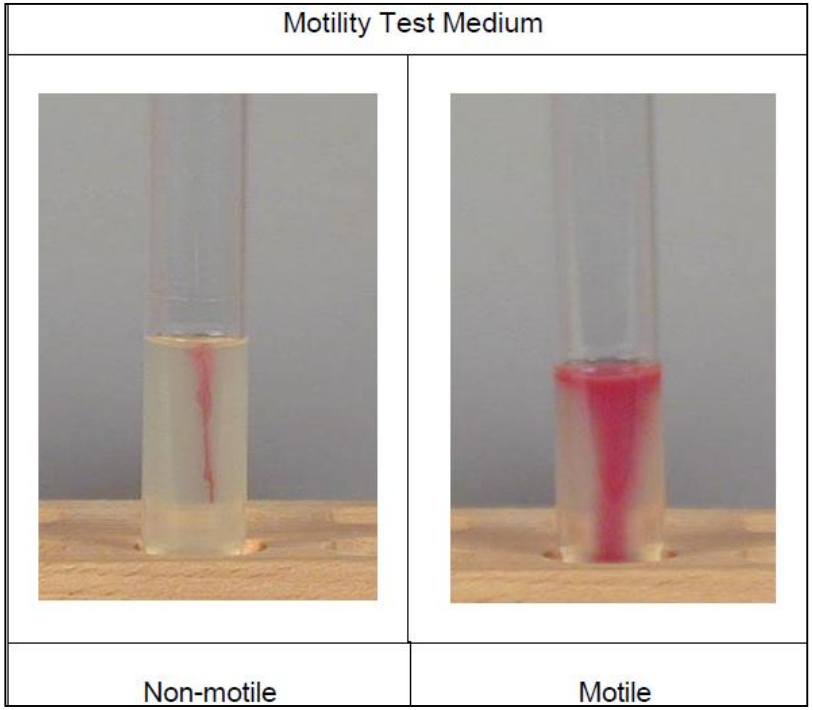
- **A semisolid medium with no dye incorporation:**
- The agar concentration (0.3%) is sufficient to form a soft gel without hindering motility.
- Stab inoculate to a depth of 5 mm.
- After incubation, if turbidity (cloudiness) due to bacterial growth can be observed away from the line of the stab, it is evidence that the bacteria were able to swim through the medium.



Grace Smith;..

Motility test
Motility Medium

- **Motility agar with tetrazolium dye:**
- The same description from above applies to this medium, BUT with the added, helpful colored dye tetrazolium which turns red as a result of the bacteria metabolizing.
- The more bacteria present at any location, the darker red the growth appears.



Kaiser,2009

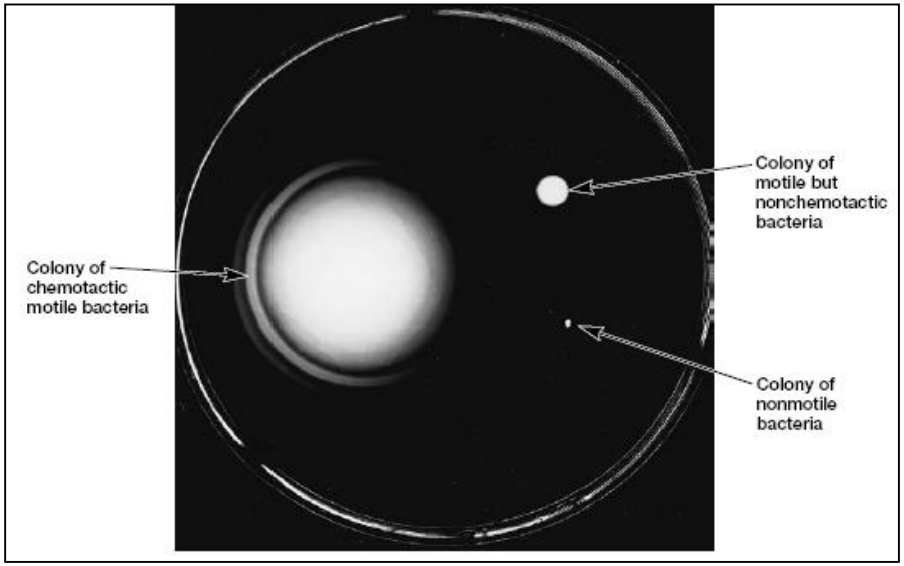
Motile colonies
Swimming(motility) test

- The medium for swimming assays was composed of:
- 1% tryptone (Difco),
- 0.5% NaCl, and
- 1% agar.
- Briefly, the plates were inoculated in the centre with a sterile toothpick and incubated for 16 h at 25°C (Deziel *et al.*,2001).
- Motility or swimming was assessed by observation of the circular turbid zone formed by bacteria migrating away from the point of inoculation.

Fonseca *et al.*,2004;

Motile colonies
Bacterial chemotaxis

- Bacteria do not always move aimlessly but are attracted by such nutrients as sugars and amino acids, and are repelled by many harmful substances and bacterial waste products.
- Chemotaxis can be demonstrated on an agar plate that contains various nutrients.
- Positive chemotaxis by *E. coli* on the left.
- The outer ring is composed of bacteria consuming serine.
- The second ring was formed by *E. coli* consuming aspartate, a less powerful attractant.
- The upper right colony is composed of motile, but nonchemotactic mutants.
- The bottom right colony is formed by nonmotile bacteria.



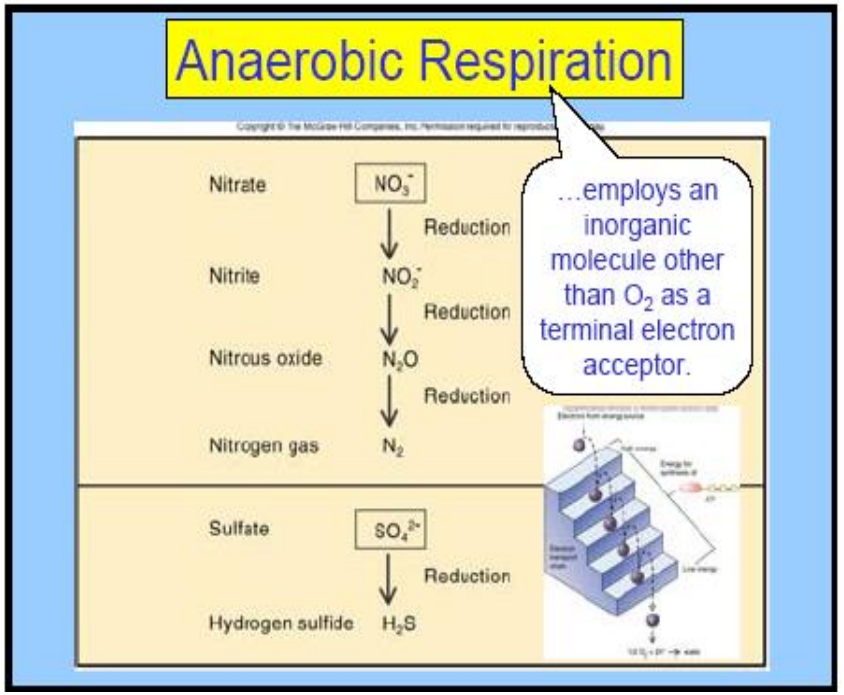
Prescott,2006

Oxidation/Fermentation test
O/F test

- Aerobes (obligate): Extract energy from a compound only in the presence of oxygen.
- Anaerobes (obligate): Extract energy only in the absence of oxygen.
- Facultative organisms: Can break down organic compounds either in the presence or absence of oxygen.

Aerobic/Anaerobic growth

- Bacteria requiring molecular oxygen for growth are called aerobic.
- Those growing only in the absence of oxygen are called anaerobic,
- Those growing in the presence or absence of oxygen facultative anaerobic and
- Those growing at low oxygen tensions, like some lactic acid bacteria, microaerophylic.



Aerobic/Anaerobic growth

Hugh & Leifson medium

O/F test

- Peptone 2 g
- KH_2PO_4 0.3 g
- NaCl 5 g
- Agar 3 g
- Bromothymol blue (1%) 3 ml
- H_2O 1 L
- pH=7.1
- Autoclave and then add 1% filter-sterilized glucose solution aseptically to the basal medium.
- Distribute into sterile tubes.
- Inoculate two tubes with each strain and cover one tube with a thin layer of sterile liquid paraffin.
- A change to yellow color in unsealed medium(acid pH, less than pH 6.0) indicate acid production from glucose (oxidative or respiratory metabolism).

Needles, a straight wire, are mainly used for stabbing bacteria vertically into deep tube of gelatin/nitrate, arginine, etc.

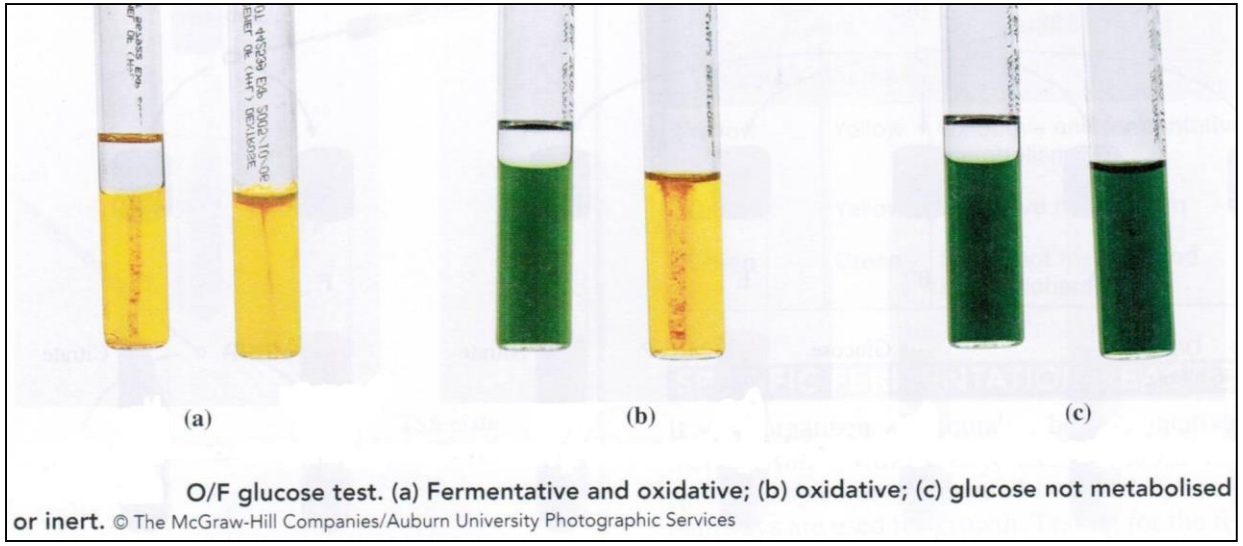
Oxidative/Fermentative Test
O/F Test

- In case of aerobic bacteria, after 48 h a yellow color (right) appears in the test tube that was not sealed with mineral oil.
- A colour change from blue to yellow in both tubes is recorded as positive for anaerobic growth (fermentation).
- In F-tube, a little yellow discoloration just under the paraffin should be considered as doubtful (Janse,2010).
- Organisms that cannot break down the carbohydrate aerobically or anaerobically, e.g., *Alcaligenes faecalis*, produce an alkaline reaction in the open tube and no change in the covered tube.

Oxidative/Fermentative Test

O/F Test

Open (Aerobic)Tube	Covered (Anaerobic) Tube	Metabolism
Acid (Yellow)	Alkaline (Green)	Oxidative
Acid (Yellow)	Acid (Yellow)	Fermentative
Alkaline (Green)	Alkaline (Green)	Non saccharolytic (glucose not metabolized or inert)



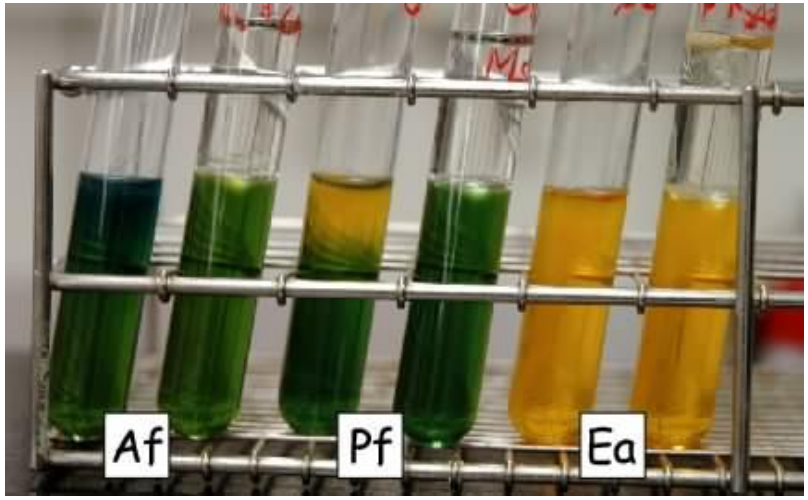
O/F glucose test. (a) Fermentative and oxidative; (b) oxidative; (c) glucose not metabolised or inert.

Oxidation/Fermentation test

O/F test

- There are three types of reactions possible:

1. Microbes that are incapable of utilizing glucose will have a alkaline reaction (blue color) at the top of the aerobic tube (the one not covered with mineral oil) and no reaction in the anaerobic tube (Af; *Alcaligenes faecalis*).
2. Oxidative microbes, a small amount of acid produced, turning the top of the aerobic tube yellow (Pf; *Pseudomonas fluorescens*).
3. Those capable of fermentative metabolism will grow in both tubes and turn the medium yellow due to the production of acid while growing anaerobically (Ea; *Enterococcus aerogenes*).



Virtual Microbiology

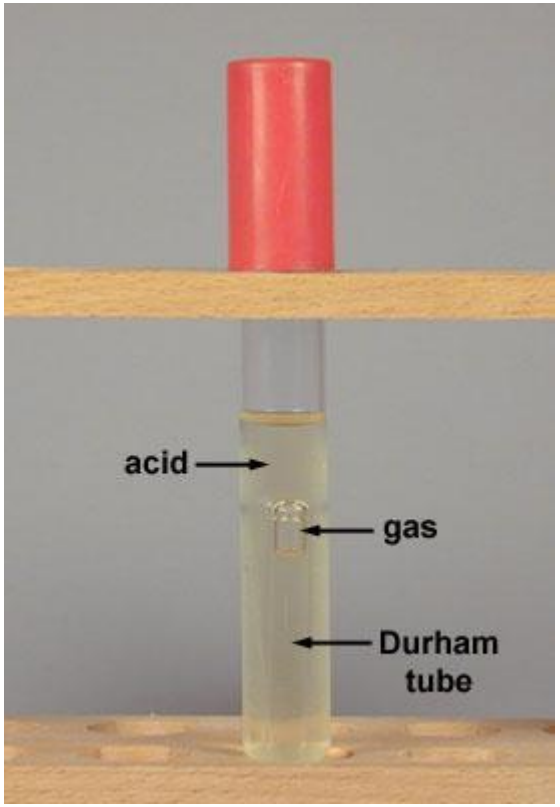
Oxidation/Fermentation test

Gas production

Gas bubble (arrow) seen in a Durham tube

- When carbohydrates are fermented as a result of bacterial enzymes, the following fermentation end products may be produced:

1. Acid end products, or
2. Acid and gas end products.
3. If gas is produced along with the acid, it collects in the Durham tube as a gas bubble.
4. If the carbohydrate is not fermented, no acid or gas will be produced and the phenol red will remain red.



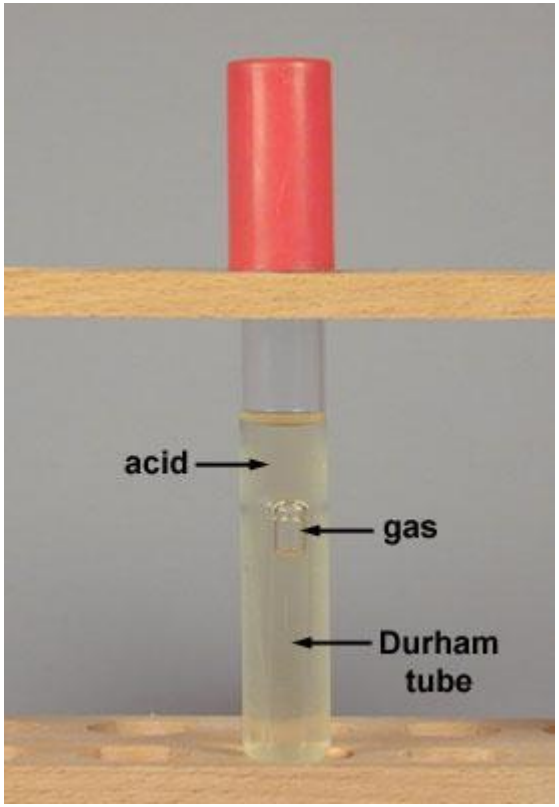
Kaiser,2009

Oxidation/Fermentation test

Gas production

Gas bubble (arrow) seen in a Durham tube

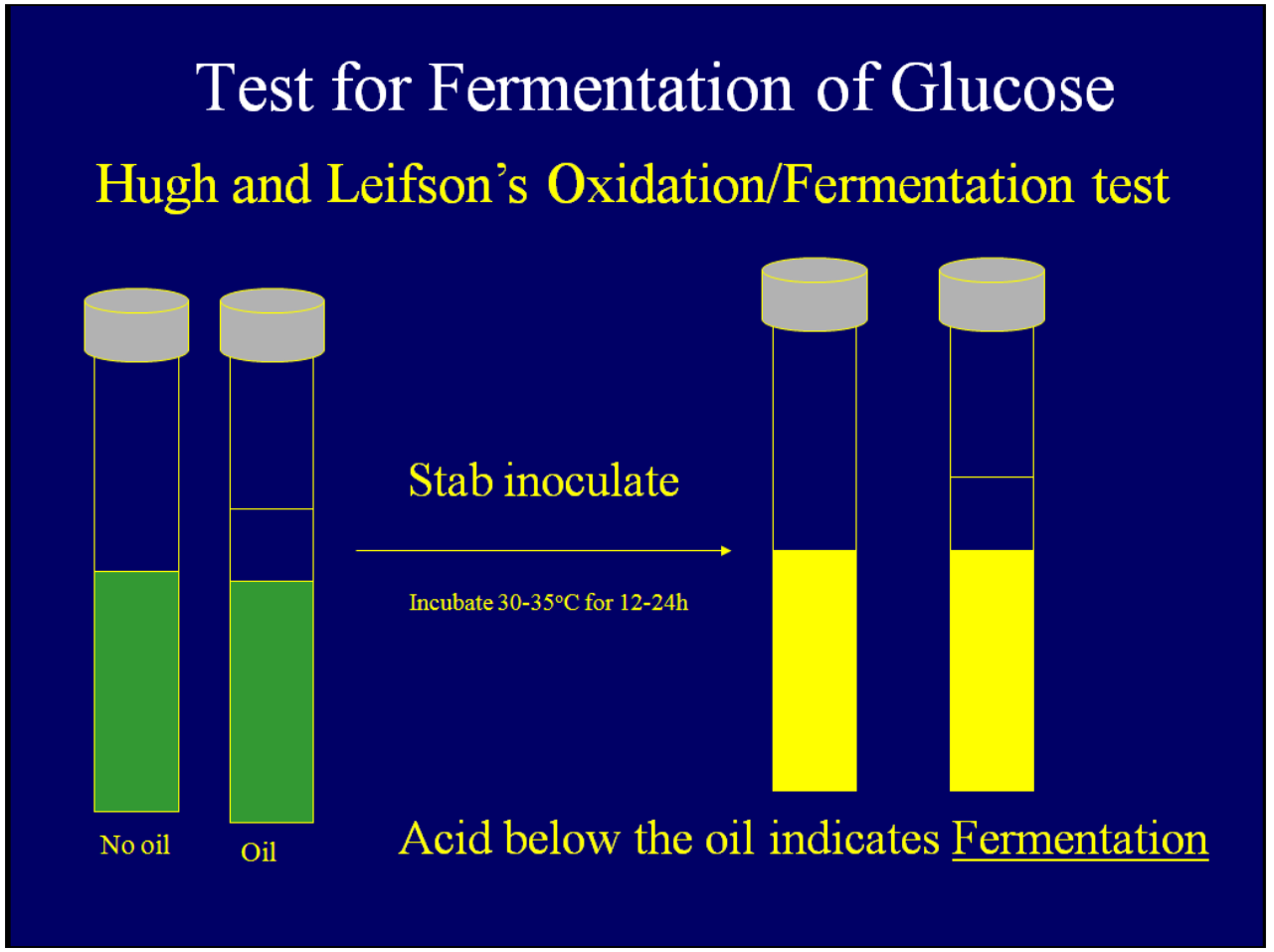
- **Solution A**
- Bacto peptone (Difco) 10.0 g
- Bromocresol purple 0.7 ml 1.5 % solution
- Dist.water 1000 ml
- **Solution B**
- Glucose 10.0 g
- Dist. water 1000 ml
- Place one Durham-tube upside down into each test tube filled with 4.5 ml of solution A and autoclave.
- Filter sterilize solution B and add 0.5 ml to each test tube after it has been cooled down to about 45-50°C.



Kaiser,2009; ...

Oxidation/Fermentation test

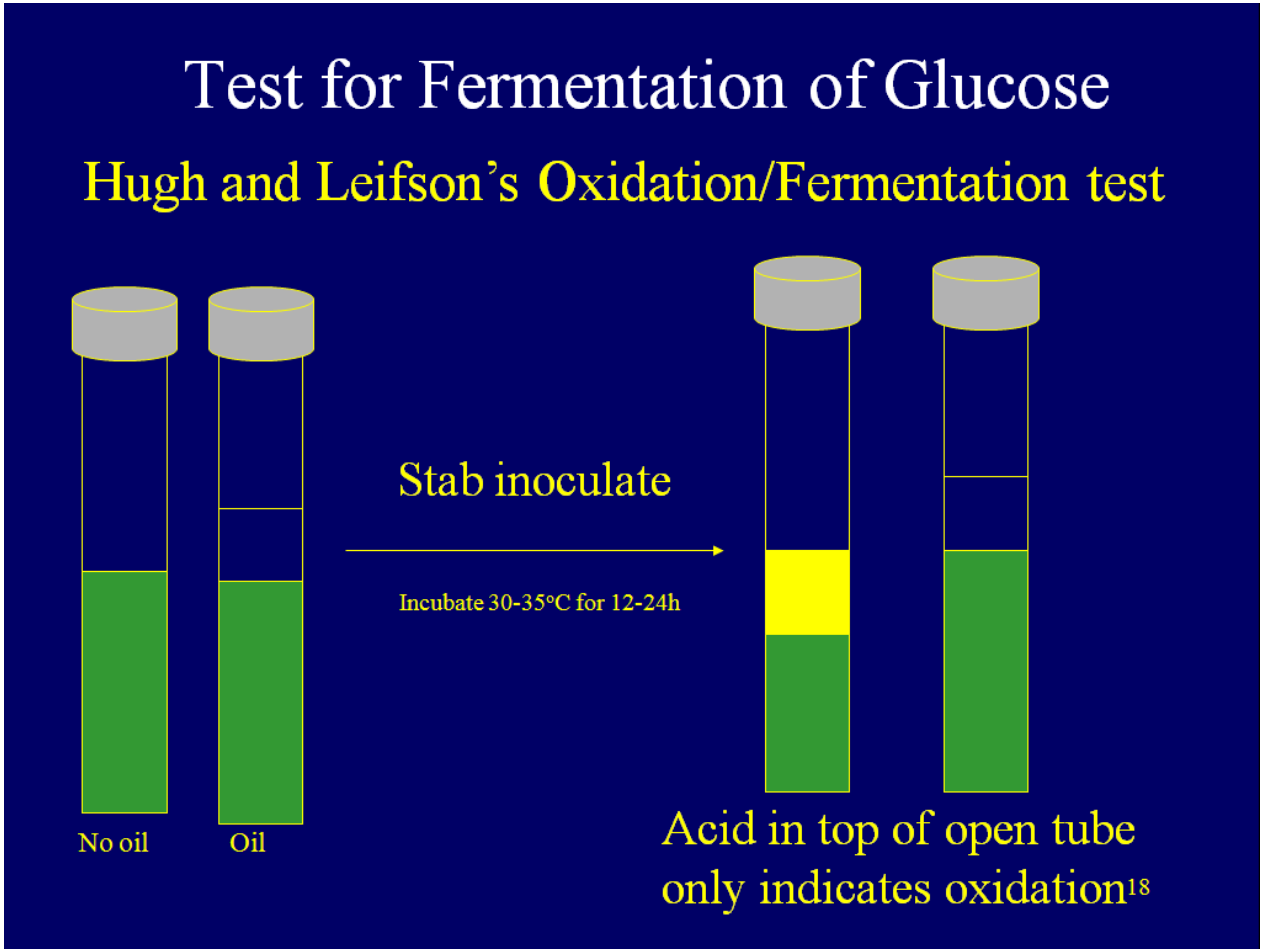
O/F test



Mckay,T.

Oxidation/Fermentation test

O/F test



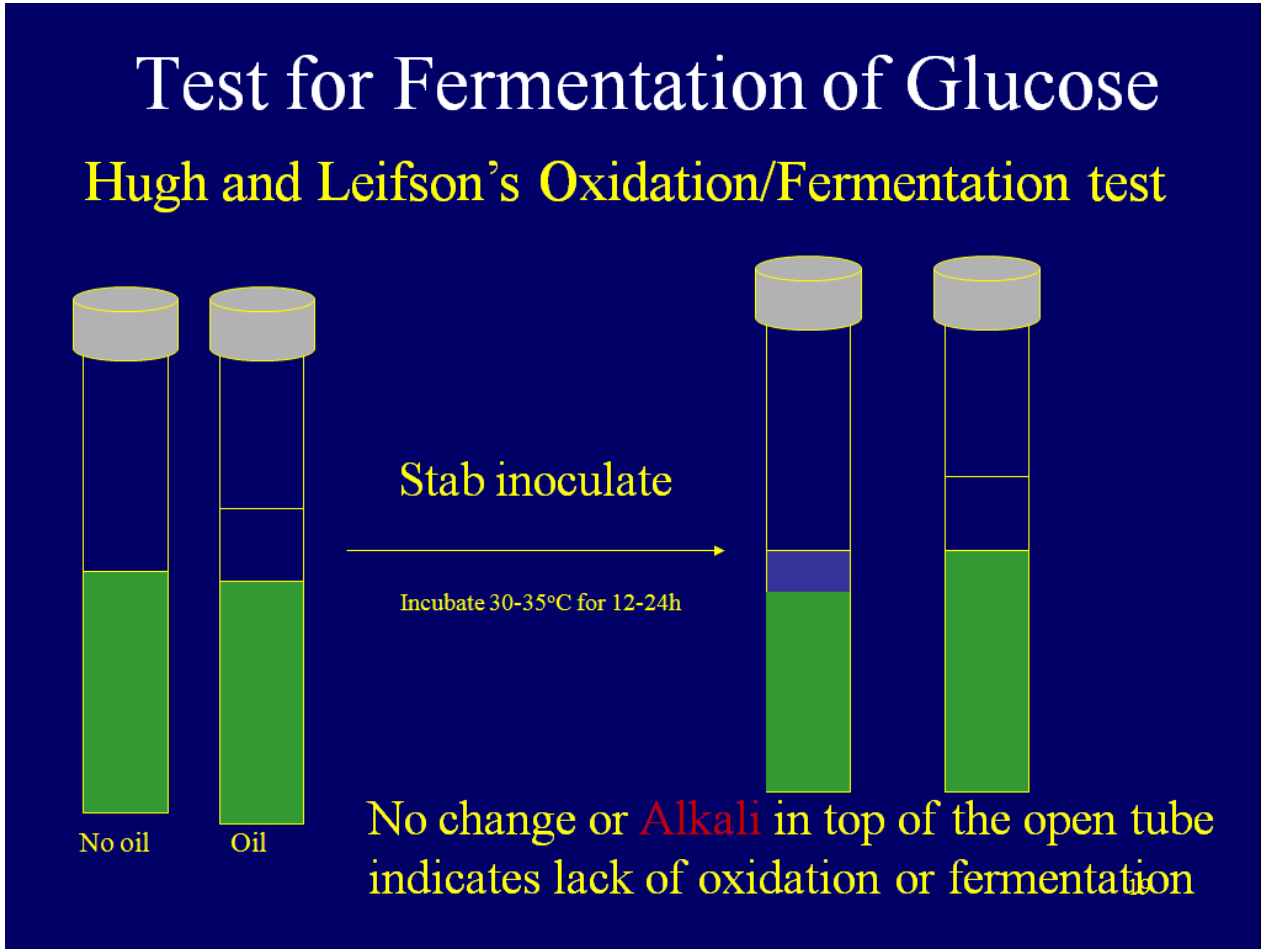
Mckay,T.

Oxidation/Fermentation test
O/F test

- Hugh and Leifson O/F media contains 0.2% peptones and 1% sugar.
- The low peptone, sensitive pH indicator and pH buffer is perfect for obligate aerobes, because a slight acidification makes the media turns yellow.
- Remember that obligate aerobes produce more alkaline products from peptone than do enteric.
- This is because these bacteria that do not produce acid usually cause the medium to turn dark blue in the presence of oxygen (due to alkali from peptones) and no change in the absence of oxygen.
- In the latter, pH increase from amino acid deamination can neutralize any acid produced from carbon fermentation=false(-).

Oxidation/Fermentation test

O/F test



Mckay,T.

Oxidative/Fermentative Test

Alternative medium

One tube test

- Exceptions:
- In some cases there may be a slight change to alkaline in the open tube, indicating that bacterium does not metabolize glucose or alkaline breakdown products of peptone degradation buffered weak acid production from the carbohydrate.
- In such cases which are rare, repeat the test in Ayers *et al*., medium plus 0.05% yeast extract instead of peptone, NaCl and K_2HPO_4 of O/F medium(Lelliott and Stead,1987).
- Very rarely, bacteria are inhibited by bromthymol blue, and if this is suspected the indicator can be added after incubation and growth.

Modified Ayers A medium: $NH_4H_2PO_4$ 1 g; KCl 0.2 g; $MgSO_4$ 0.2 g; Agar 12 g; 0.05% yeast extract; Bromothymol blue 75 ml (solution 0.2%); Distilled water to 1 L. Adjust pH to 7.0-7.2.

Aerobic/Anaerobic growth

Semisolid agar method

Two more alternative media

- Method of Smibert and Krieg, 1994 was used.
- Autoclave a narrow culture tube that has been filled to 60% of its capacity with an appropriate culture medium containing 0.2% agar.

1. After the medium has cooled to 45°C, add the inoculum, mix to distribute the organisms uniformly and then allow the agar to solidify.
2. Alternatively, inoculate the medium by stabbing with an inoculating needle after the agar has gelled; this avoids the mixing that otherwise might add dissolved O_2 to the medium.

https://www.sciencedirect.com › topics › agricultural-and-biological-sciences

Aerobic/Anaerobic growth

Semisolid agar method

Two more alternative media

- Tryptone 10 g
- Yeast extract 5 g
- K_2HPO_4 5 g
- Glucose 1 g
- Agar 3 g
- H_2O 1 L
- pH 7.2

Nutrient Yeast Dextrose Agar

- Nutrient both 23 g
- Yeast extract 5 g
- Dextrose (Glucose) 10 g
- Agar 10 g
- H_2O 1 L

- Distribute into tubes (10 ml) and autoclave.
- Inoculate each tube with a single strain when the medium is in fluid condition (45°C).
- Do not use heavy inoculum to permit discrete colonies.
- Rotate to mix inoculum with the medium and cool.
- Incubate 48 h to observe where bacteria can grow in tubes of a nutrient mixture.
- Upon inoculation strict areobes will be found to grow upon the surface and in the upper layer only, microaerophiles will grow best just a few mm below the surface, facultative anaerobes will grow throughout the medium, and strict anaerobes will grow in the depths, if at all (Shaffer,1975).

Aerobic/Anaerobic growth

Another alternative medium

Fluid Thioglycollate Medium

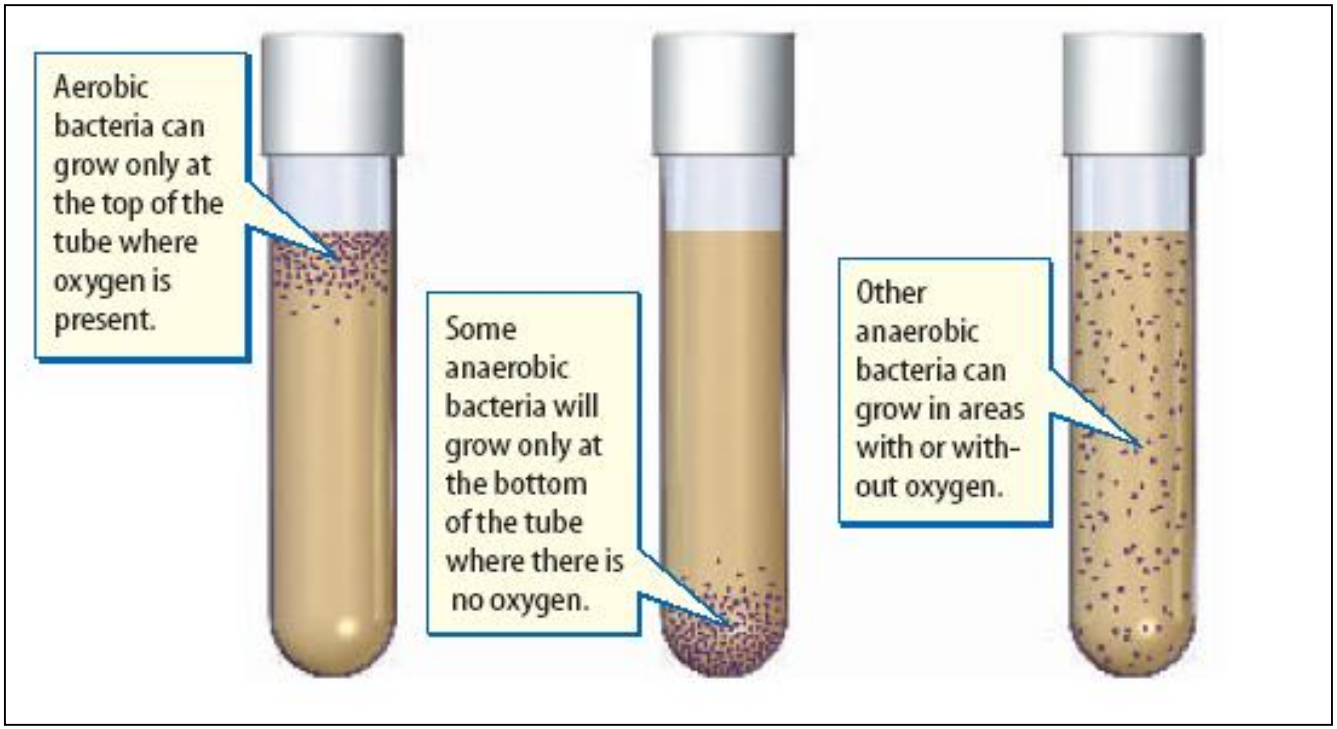
- The medium contains glucose, cystine, and sodium thioglycollate to lower the oxidation-reduction potential.
- The oxygen tension is high at the surface of the media (allowing the media to grow) and decreases toward the bottom of the media (for anaerobic growth). Resazurin (a dye) causes the media to turn pink in the presence of oxygen.
- Procedure:
- Boil and cool media with the screw cap loose
- Inoculate media with the organism using a wire loop. DO NOT SHAKE THE MEDIA.
- Incubate at optimum temperature for 24 hours.
- Interpretation:

1. Aerobe- Growth at the top of the media;
2. Facultative- Growth throughout the media;
3. Anaerobe- Growth at the bottom of the media.

Aerobic/Anaerobic growth

Alternative method for routine O/F test

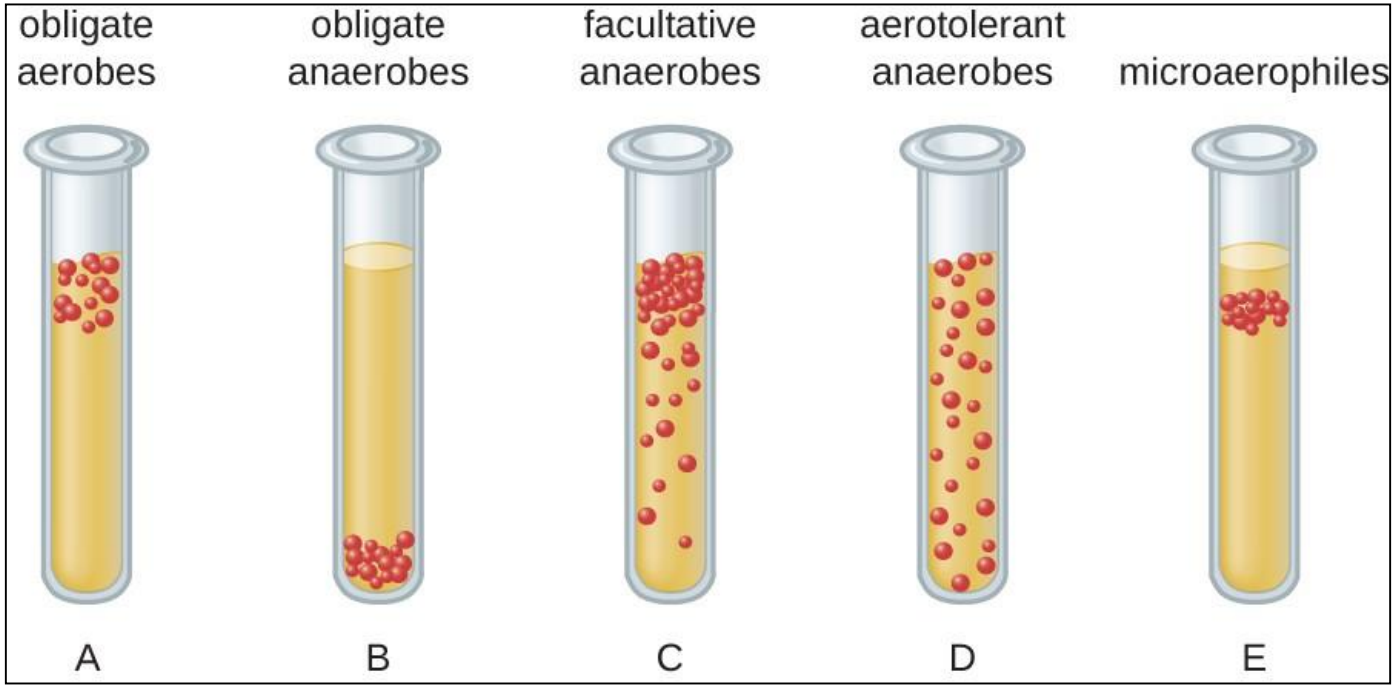
- Observing where bacteria can grow in tubes of a nutrient mixture shows you how oxygen affects different types of bacteria.



Aerobic/Anaerobic growth

Alternative method for routine O/F test

- Diagram of bacterial cell distribution in thioglycolate tubes. The medium contains glucose, cystine, and sodium thioglycollate to lower the oxidation-reduction potential.



Lumen Learning

Oxidative/Fermentative Test

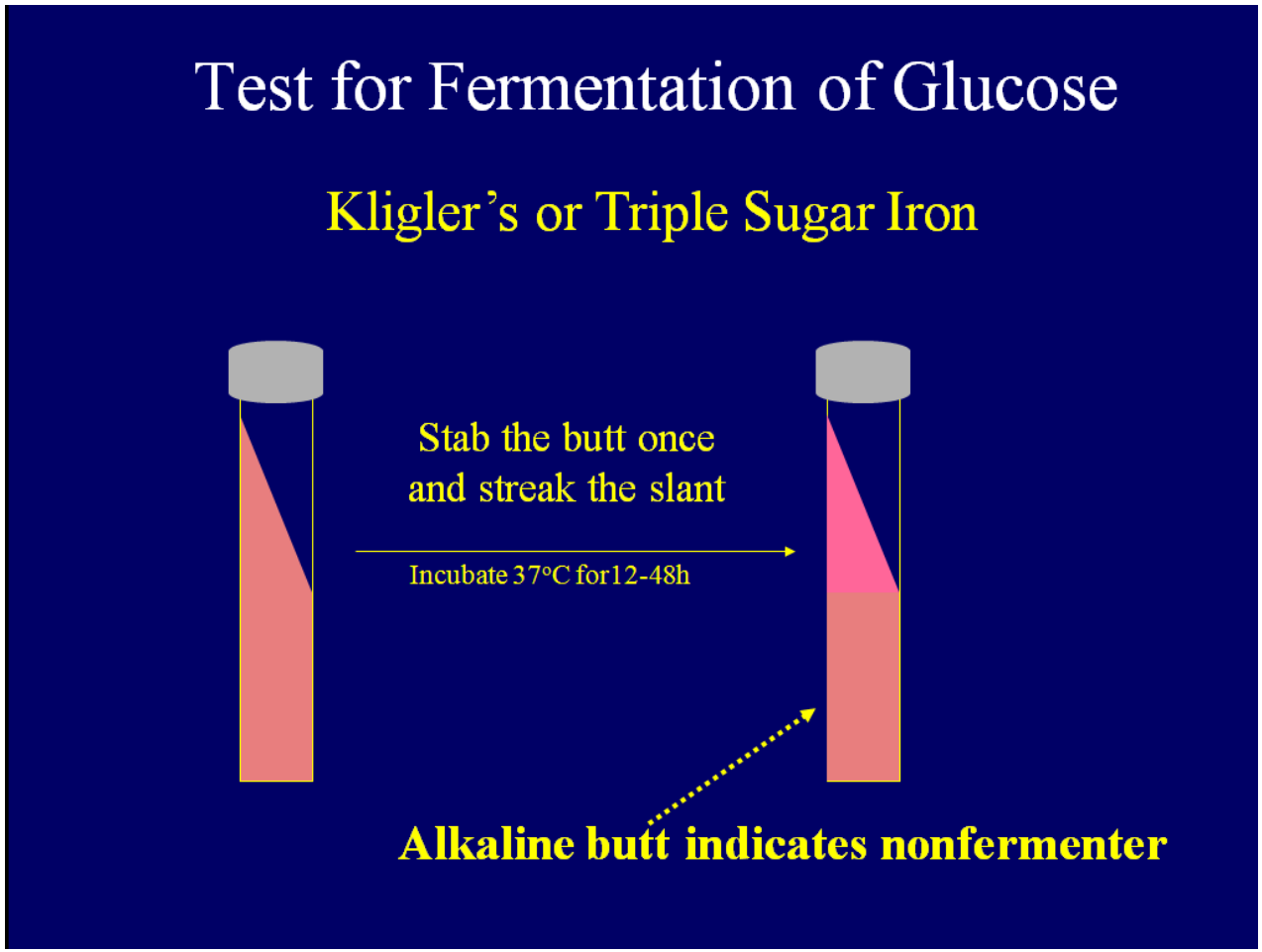
Alternative medium for routine O/F test

Fermentation media such as KIA or TSI

1. Most fermenters (anareobes) produce acid in the bottom of Kligler's iron agar (KIA) or Triple sugar iron agar (TSI).
2. Whereas, non fermenters (obligate aerobes) often fail to produce acid in KIA or TSI but produce an alkaline bottom.

Oxidative/Fermentative Test

Alternative medium



Mckay,T.

KIA or TSI media

Alternative media for O/F test and H_2S production

- Kligler's iron agar (KIA) and Triple sugar iron agar (TSI), is used for the determination of:

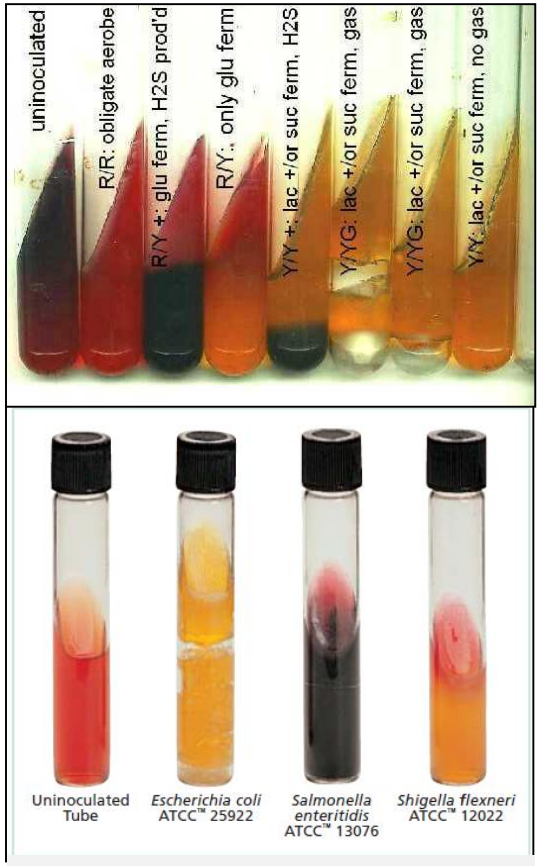
1. Carbohydrate fermentation, and
2. Hydrogen sulfide production.

- Kligler's iron agar (KIA) contains dextrose and lactose whereas TSI Agar contains three sugar formulation i.e. dextrose, lactose and sucrose.
- Carbohydrate fermentation is detected by the presence of gas and a visible color change from red (control) to yellow (treated).
- The acid reaction (yellow) is maintained in the butt of the tube because it is under lower oxygen tension.
- The production of hydrogen sulfide is indicated by the presence of a precipitate that blackens the medium in the butt of the tube.

KIA or TSI media

Alternative media for O/F test and H_2S production

- A yellow (acidic) color in the slant and butt indicates that the organism being tested ferments dextrose, lactose and/or sucrose (anaerobic).
- A red (alkaline) color in the slant and butt indicates that the organism being tested is a nonfermenter (aerobic).
- Hydrogen sulfide production results in a black precipitate in the butt of the tube.



Combination of carbohydrate fermentation, gas production and hydrogen sulfide production were observed.

Composition of KIA or TSI media

Triple sugar Iron Agar (TSI Agar)	(grams/L)
Beef extract	3
Yeast extract	2
Peptone	15
Proteose Peptone	5
Lactose	10
Glucose (dextrose)	1
Sucrose	10
Ferrous Ammonium sulfate	0.2
Sodium chloride	5
Sodium thiosulfate	0.3
Phenol red	0.024
Agar	13
pH	7.4

Kligler Iron Agar(KIA)	(grams/L)
Beef extract	3
Yeast extract	2
Peptone	15
Proteose Peptone	5
Lactose	10
Glucose (dextrose)	1
Ferrous Ammonium sulfate	0.2
Sodium chloride	5
Sodium thiosulfate	0.3
Phenol red	0.024
Agar	13
pH	7.4

Pathogenicity and virulence

Pathogenicity traits

1. Soft rot of potatoes
2. Hypersensitivity test on tobacco
3. Carrot disc assay
4. Koch's Postulates

Robert Hermann Koch (11 December 1843 – 27 May 1910) was a German physician and microbiologist who developed Koch's postulates. As one of the main founders of modern bacteriology, he identified the specific causative agents of tuberculosis, cholera, and anthrax and gave experimental support for the concept of infectious disease which included experiments on humans and other animals.

Virtual Microbiology;Wikipedia.2019

Pathogenicity and virulence

Pathogenicity traits

Colony-forming unit (CFU or cfu) and cells/ml

- Colony-forming unit (CFU or cfu) is a measure of viable bacterial or fungal cells. In direct microscopic counts (cell counting using haemocytometer) where all cells, dead and living, are counted, but CFU measures only viable cells.
- For convenience the results are given as:

1. CFU/mL (colony-forming units per milliliter) for liquids, and
2. CFU/g (colony-forming units per gram) for solids.

Pathogenicity and virulence

Pathogenicity traits

Colony-forming unit (CFU or cfu) and cells/ml

- The CFU/ml can be calculated using the formula:
- cfu/ml = (no. of colonies x dilution factor) /volume of culture plate.
- For example, suppose the plate of the 10^6 dilution yielded a count of 130 colonies.
- Then, the number of bacteria in 1 ml of the original sample can be calculated as follows:
- Bacteria/ml = (130) x (10^6) = 1.3 × 10^8 or 130,000,000.
- The usual estimate is that OD_{600}=1 (1 cm path length) represents on the order of 10^9 CFU/ml.

Tissue maceration

Vegetable slices inoculation method

Potato soft rot

- Wash potato tubers, carrots, onions, and cut into slices about 7 mm (1/4 inch) thick.
- Put about 1/8 inch of sterile water in a Petri dish that contains two disks of sterile filter paper and add the vegetable slices.
- Make a slight cut in the center of each slice, and inoculate with a loopful of cells (ca. 10^6 CFU/ml) from the culture.
- Include a non-inoculated slice for each treatment used.
- Incubate at 20-27°C for 48 h and probe the tissue surrounding the inoculation site with sterile needle to determine whether decay and tissue maceration has occurred.
- Note: Maceration ability dose not prove pathogenicity of the bacterium in a natural environment (Schaad *et al.*,2001).

Tissue maceration

Vegetable slices inoculation method

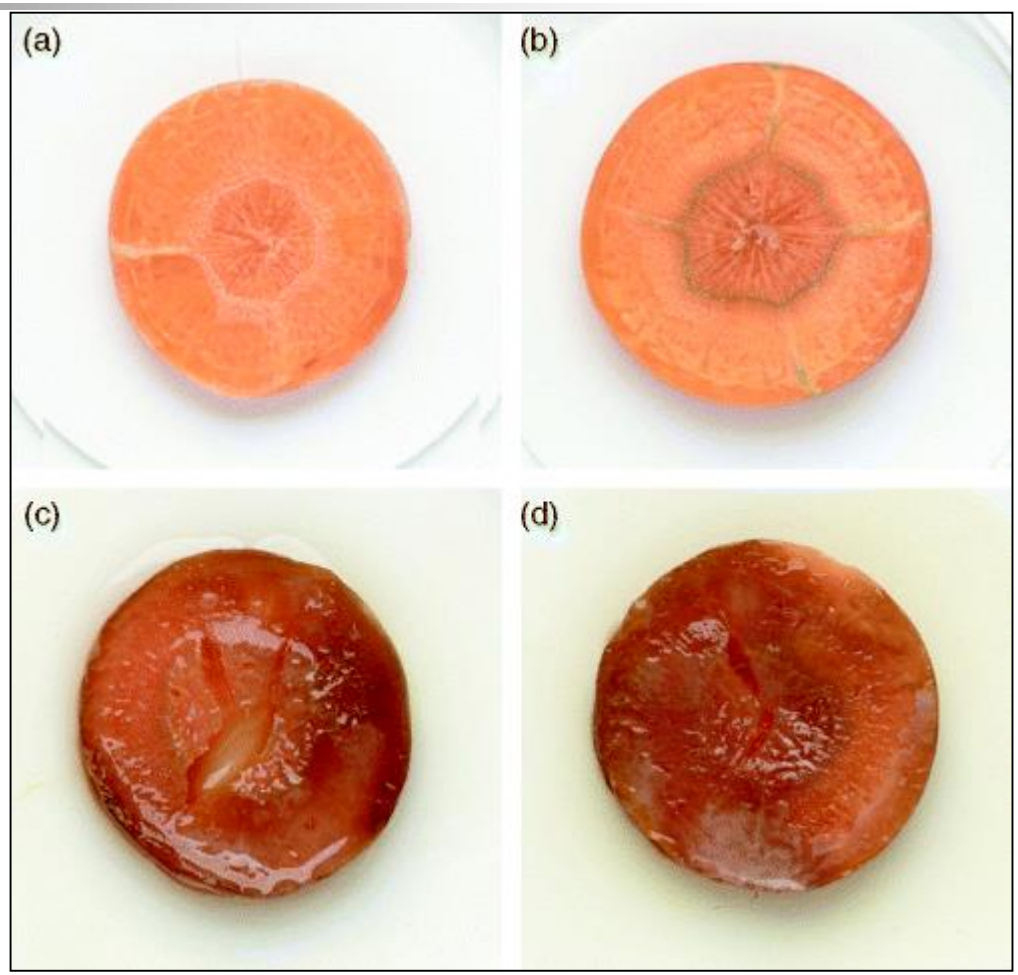
Potato, oninon and carrot soft rot

Tissue maceration

Carrot soft rot

Cold-tolerant *Pseudomonas viridiflava* and *P. marginalis* causing severe carrot postharvest bacterial soft rot during refrigerated export from New Zealand

Carrot tissue pathogenicity bioassay showing tissue degradation after 48 h caused by application of cultured bacteria: (a) uninoculated LB; (b) nonpathogenic *P. fluorescens*; (c) *P. viridiflava* NZCX09; (d) *P. marginalis* NZCX27.



Godfrey and Marshal,2002

Hypersensitivity Reaction (HR) on tobacco

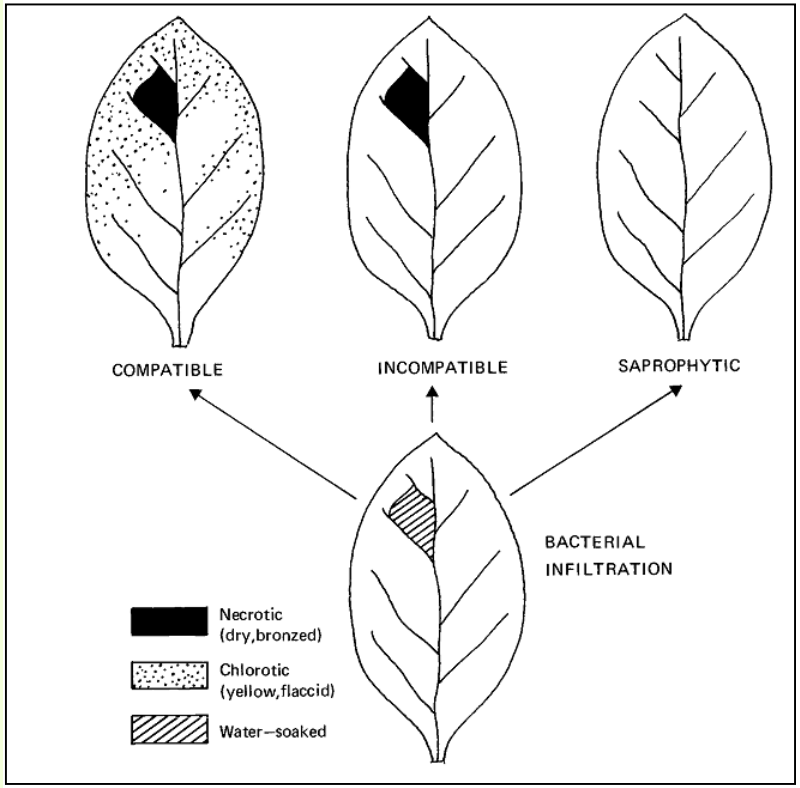
- Most plant pathogens are capable of inducing a hypersensitive reaction (HR), resulting in a rapid death of local plant cells and entrapment of the pathogen.
- This, in effect, limits the spread of infection.
- Collapse of host tissue in the infiltration area within 48 hours indicates the bacterium is likely a pathogen for another host.
- Yellowing or browning without collapse is not a positive reaction.

1. Non-pathogens only causes some yellowing after several days or no reaction at all.
2. Tobacco pathogens give a pathogenic reaction that is slower; the necrosis is usually much darker in colour.

Vidaver and Lambrecht,2004; Lellitt and Stead,1987

Hypersensitivity Reaction (HR) on tobacco

- The inoculum was carefully infiltrated into the parenchyma tissues.
- Leaf response to bacterial infiltration.
- Interpretation of experiment by Klement *et al.*,1964.
- Local infiltration of leaf tissue leads to compatible, incompatible or saprophytic reactions (shown about 5 days after inoculation), depending on bacterial species or pathovar.



Sigee,1993

Pathogenicity and virulence

Dickeya zeae strains isolated from rice, banana and clivia rot plants show great virulence differentials


clivia

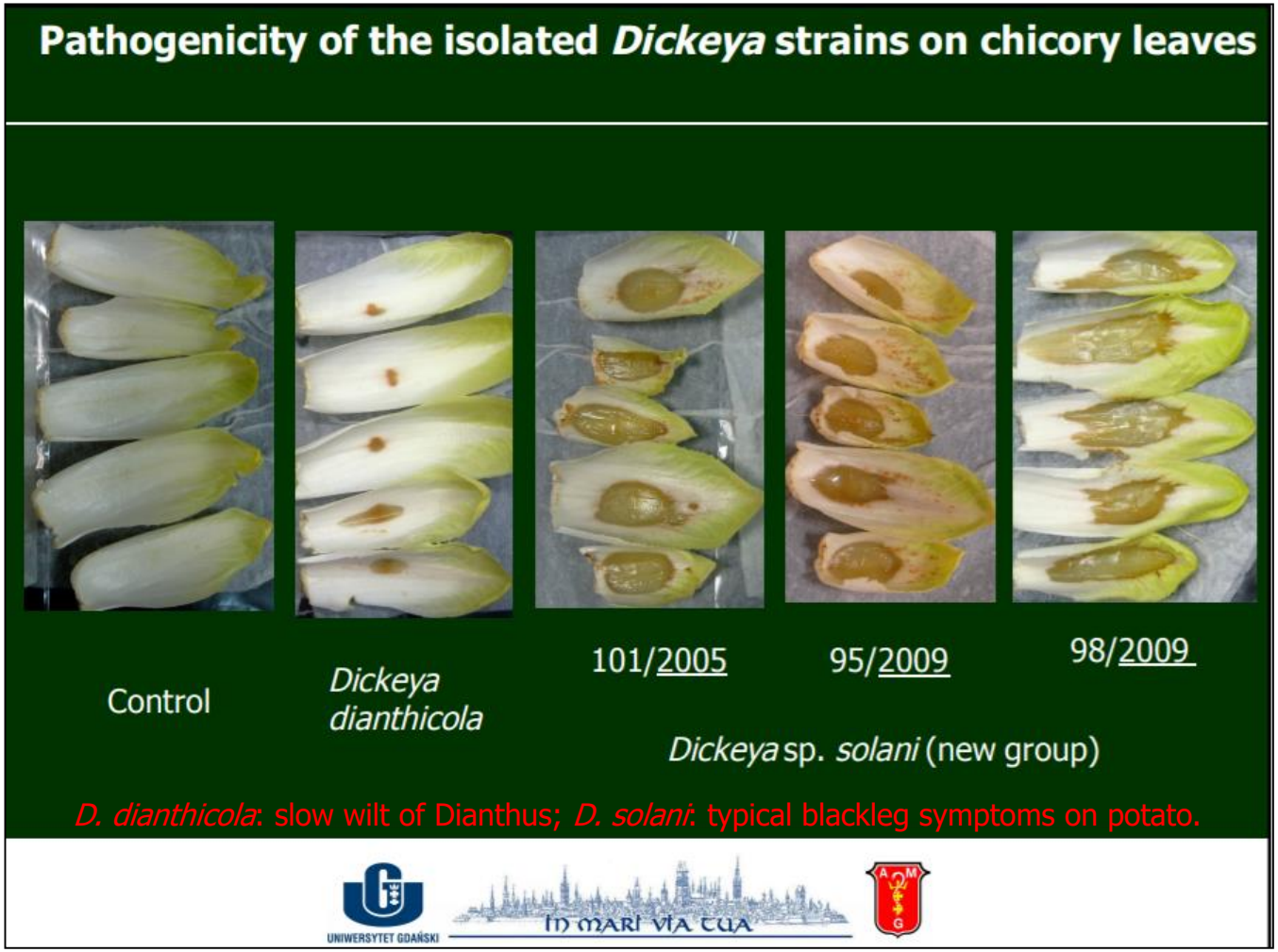
Inoculated plant			Inoculation amount, time	Diseased area (mm^2)					
Class	Species	Organ		EC1	MS2	MS3	JZL1	JZL2	JLZ7
Dicots	*Cucumis sativus*	Fruit	2 μL, 24 h	57.22 ± 7.70	107.97 ± 42.00	65.46 ± 7.88	0	0	0
	Benincasa hispida	Fruit	2 μL, 24 h	1140.57 ± 15.55	1336.50 ± 3.32	1252.61 ± 18.71	0	0	0
	Brassica pekinensis	Petiole	2 μL, 12 h	56.81 ± 3.14	49.83 ± 2.73	42.40 ± 3.17	0	0	0
	Raphanus sativus	Tuber	2 μL, 24 h	156.89 ± 2.60	106.72 ± 2.03	99.84 ± 9.76	0	0	0
	Daucus carota	Tuber	2 μL, 24 h	274.83 ± 18.74	266.70 ± 8.98	231.42 ± 14.11	0	0	0
	Solanum tuberosm	Tuber	2 μL, 24 h	173.33 ± 4.28	211.05 ± 20.54	171.45 ± 6.07	0	0	0
	Lycopersicon esculentum	Fruit	100 μL, 2 d	908.22 ± 5.95	904.75 ± 9.80	945.81 ± 13.77	0	0	0
	Solanum melongena	Fruit	100 μL, 2 d	66.52 ± 2.95	61.83 ± 1.67	86.72 ± 0.85	0	0	0
	Capsicum annuum	Fruit	2 μL, 24 h	101.79 ± 8.81	106.65 ± 10.76	191.42 ± 10.55	0	0	0
Monocots	*Oryza sativa*	Stem	200 μL, 7 d	720.62 ± 21.48	575.71 ± 29.53	539.35 ± 17.77	452.67 ± 19.53	413.01 ± 12.64	499.85 ± 12.09
	Musa sapientum	Stem	200 μL, 7 d	1110.83 ± 19.23	1358.89 ± 17.78	1201.88 ± 19.91	407.90 ± 12.48	377.89 ± 13.71	464.23 ± 11.06
	Clivia miniata	Leaf	200 μL, 24 h	1643.86 ± 6.94	2195.85 ± 7.06	1239.95 ± 7.55	1807.41 ± 9.52	1693.21 ± 20.27	1594.90 ± 14.66
	Allium cepa	Bulb	2 μL, 24 h	185.83 ± 21.68	194.27 ± 18.73	119.90 ± 11.29	0	0	0
	Zingiber officinale	Tuber	2 μL, 24 h	134.37 ± 14.17	86.92 ± 6.75	87.31 ± 12.23	47.44 ± 7.16	65.81 ± 6.11	81.33 ± 13.96
	Gladiolus gandavensis	Stem	200 μL, 7 d	254.16 ± 9.05	168.17 ± 9.23	171.59 ± 5.92	36.75 ± 1.62	36.38 ± 2.39	36.85 ± 3.05
	Colocasia esculenta	Tuber	2 μL, 5 d	461.847 ± 15.55	247.31 ± 15.31	117.34 ± 8.00	81.83 ± 6.00	40.46 ± 5.54	79.48 ± 3.91
	Alocasia macrorrhiza	Stem	200 μL, 7 d	2860.90 ± 65.15	2763.17 ± 30.46	2633.75 ± 35.94	1316.86 ± 17.97	1430.45 ± 32.58	1381.58 ± 15.23

Results showed that the JZL strains could not propagate in potato or cabbage tissues after inoculation for 12 h and 24 h, while EC1, MS2 and MS3 strains grew rapidly in potato in 12 h, and slowly in cabbage.

Hu *et al.*,2018

Pathogenicity test

Pathogenicity of the isolated *Dickeya* strains including *D. solani* on chicory leaves

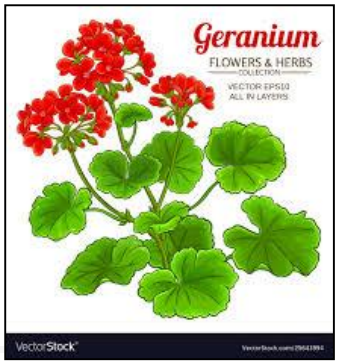


Dianthus

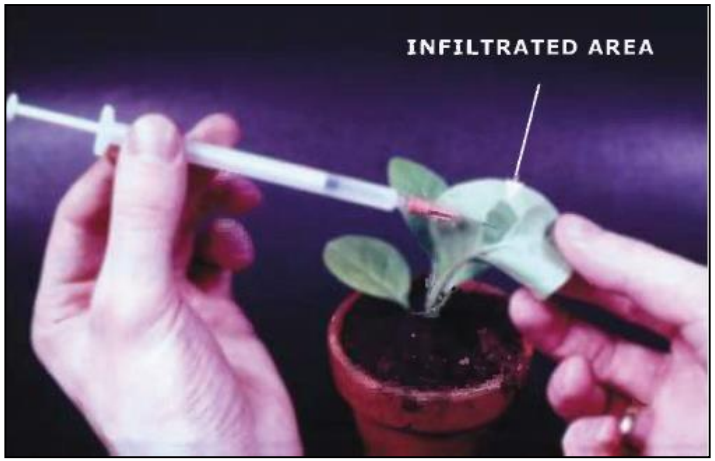
Hypersensitivity test

On tobacco, Four O'clock and geranium

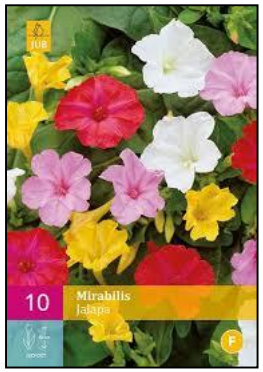
hrp genes and inoculum density



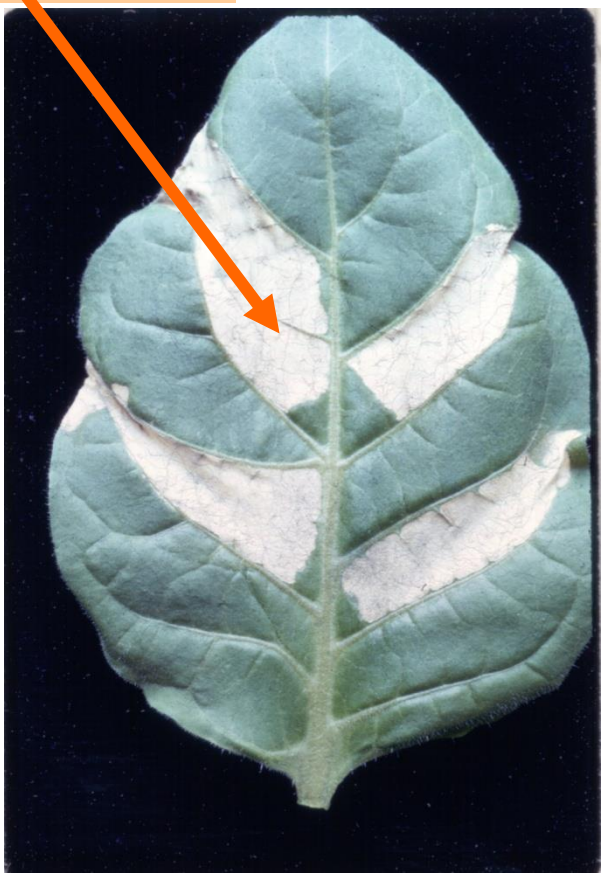
- Hypersensitivity test on:
1. Tobacco
2. Four O-Clock
3. Geranium
- Bacteria with *hrp* genes cause HR;
- Saprophytes do not.
- 10^{6-7} cells/ml
- Gently force bacteria into intercellular space using syringe.



Hypersensitivity test
On tobacco and Four O'clock



- Tobacco (*Nicotiana tabacum*) is frequently used in HR tests because its large leaf panels are easily infiltrated, but Four O'clock (*Mirabilis jalapa*) may be used for some Gram-positive bacteria.



Hypersensitivity test on tobacco
Mode of action

Hrp test in tobacco

Inject bacterial suspension into tobacco leaf. If hypersensitive reaction occurs, then the bacterium possess a 'Type III secretion 'system.

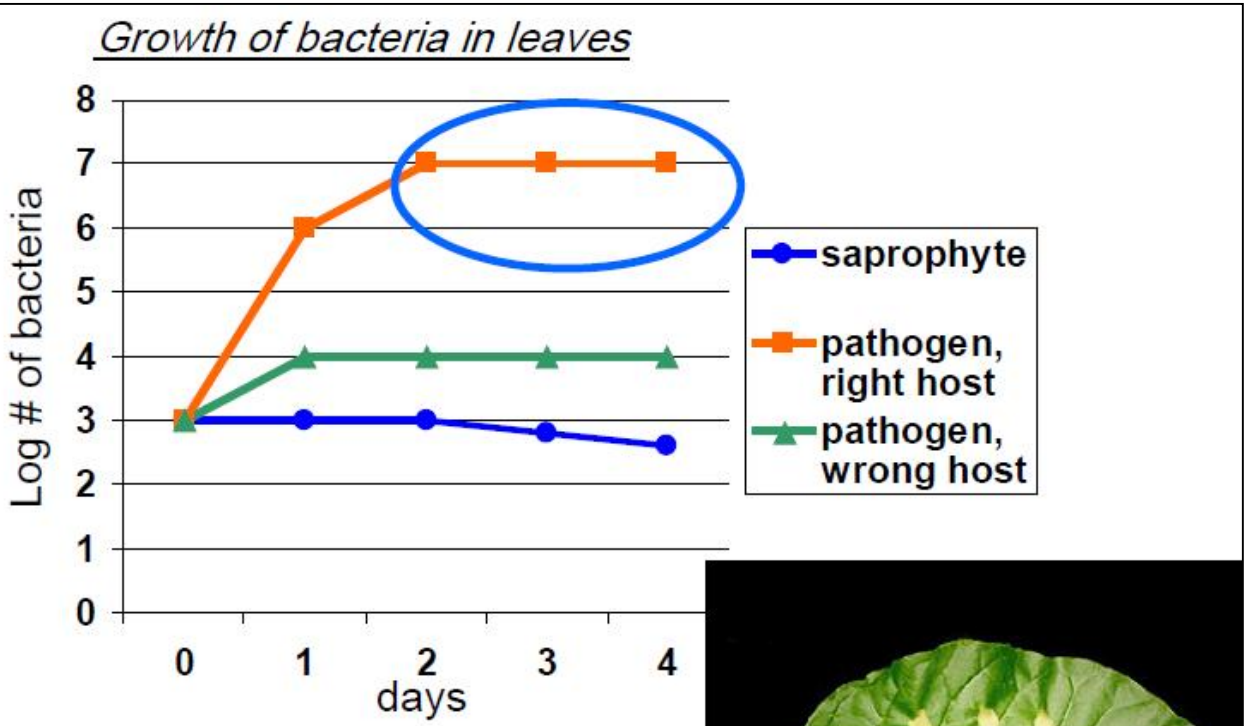
Type III secretion systems are used by bacteria to inject virulence factors into host cells.

Many bacterial pathogens of both plants and animal possess a Type III system.

'Hrp' means hypersensitive response, pathogenesis-related

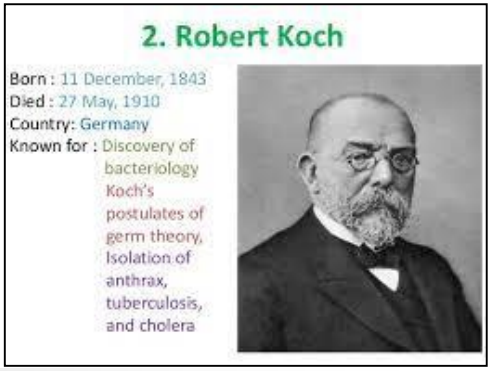
Hypersensitivity test on tobacco

Pathogens vs. nonpathogens



The Hypersensitive Response (HR) - rapid collapse of plant cells in response to an incompatible pathogen

Koch's Postulates



Koch's Postulates

1. Pathogen must be found in the host in every case.
2. Pathogen must be isolated from the host and grown in pure culture.
3. When placed in a healthy host, pathogen produced in pure culture must cause the disease in the host.
4. Pathogen must be isolated from the new host and shown to be the original pathogen.

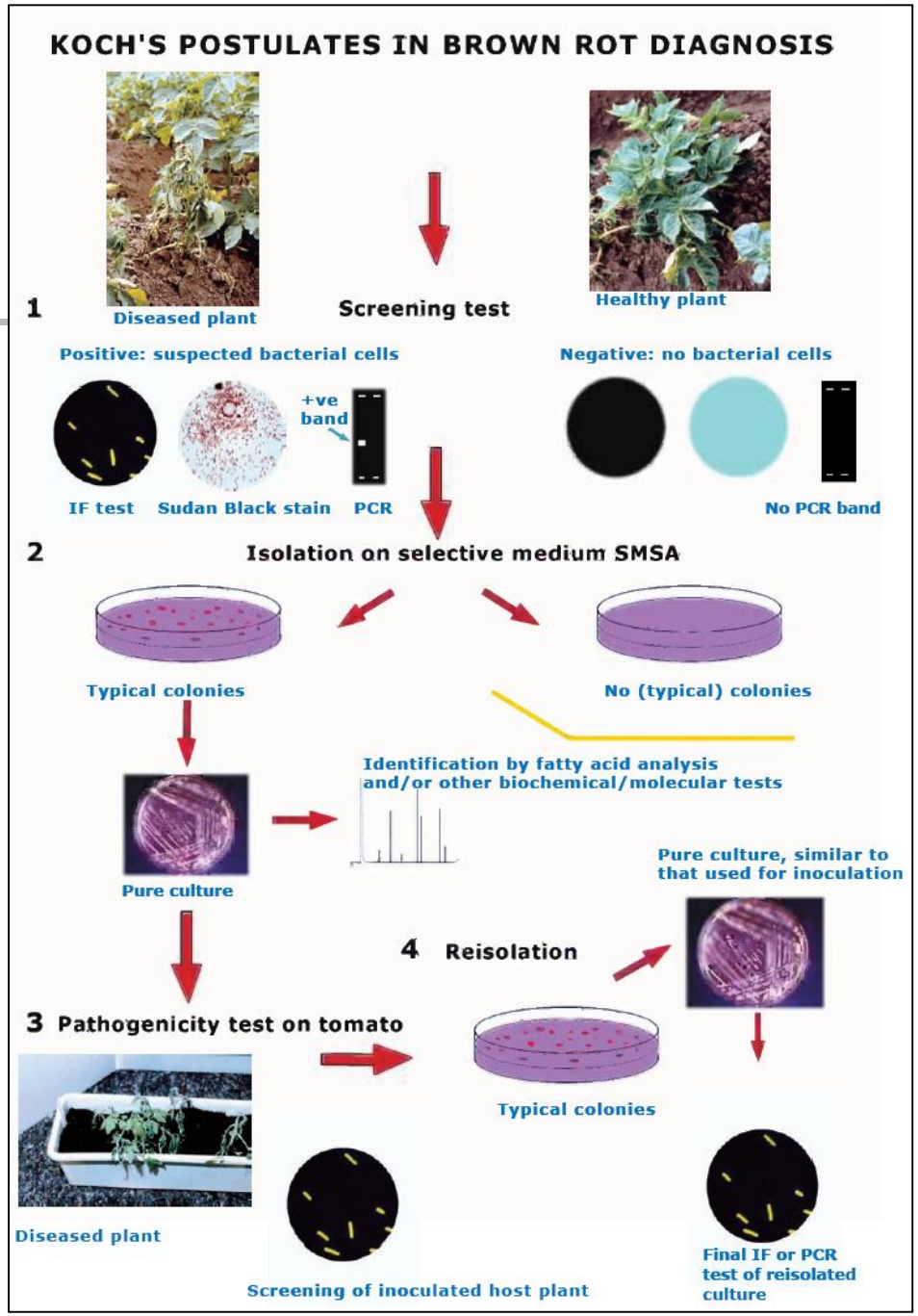
Clark,1996

Example of application of Koch's postulates in modern phytobacteriology

- Diagnosis of potato brown rot caused by *Ralstonia solanacearum* via:

1. Screening using tests such as immuno-fluorescence (IF) staining, simple Sudan Black stain and DNA (PCR) that demonstrate bacteria in tissues;
2. Isolation of the pathogen and identification using biochemical methods;
3. Final confirmation by inoculation into a suitable host plant (tomato);
4. Reisolation from inoculated, diseased plants with a final IF or PCR test on reisolated culture.

Janse,2006



Pathogenicity test using different inoculation methods

- Pathogenicity tests have never been standardizied and subsequently investigators display considerable individuality in their design.
- This is especially so with the genus *Pseudomonas* since it represents a diverse group of bacteria that causes many different kinds of diseases.
- The routine techniques for this and other plant pathogenic bacteria are:

1. Wound inoculations by a blade or needle;
2. Spray and incubation under moist conditions;
3. Wound inoculation by carborundum;
4. Forced intromission of bacteria into plant tissues with a pressure sprayer, and vaccum infiltration.

Schaad,1988

Pathogenicity test using different inoculation methods

Xanthomonas campestris pv. *campestris*

- Six different methods are:

1. Spraying the inoculums with hand atomizer (Klement, 1968);
2. Carborundum abrasion method (Leben *et al*., 1968);
3. Multineedle pricking method (Andrus, 1948; Starr and Dye, 1965);
4. Injection infiltration method (Klement, 1963);
5. Hydathodes inoculation method (Robeson *et al*., 1989), and
6. Scissor clipping were studied to find out the efficiency of different methods in disease development.

Maji and Nath,2015

Pathogenicity test using different inoculation methods

Xanthomonas campestris pv. *campestris*

- The experiment was done using five leaves for each plant with each bacterial inoculation method in a completely randomized design.
- Each leaf was considered as a replicate of each treatment.
- Inoculated plants were covered with polythene bags and kept under high humid condition for 48 h and then kept under natural condition.
- The plants were watered and regularly observed for disease development.

Maji and Nath,2015

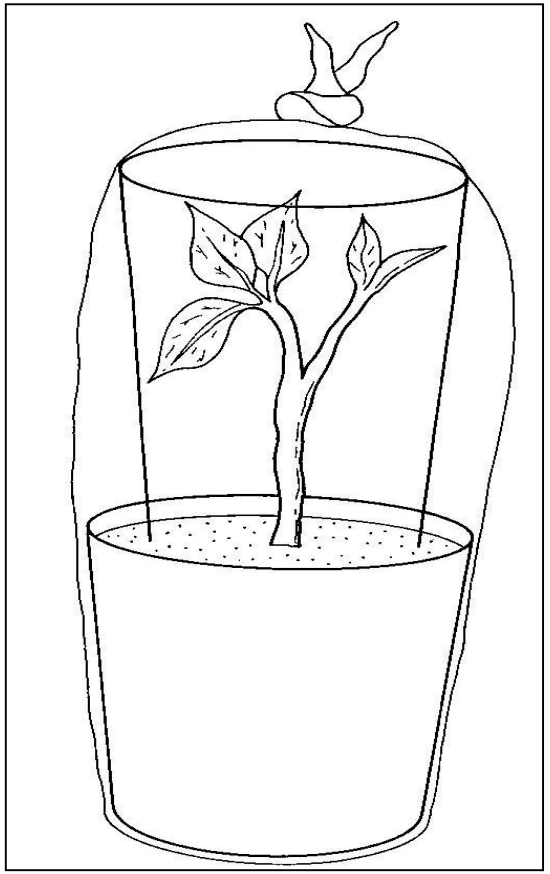
Plant/Pathogenicity Test
Determination of pathogenicity

- For beneficial purposes or as pathogens, populations of 10^6 CFU (colony-forming units/ml) or higher are normally required for bacteria to function as biological control agents or cause infectious disease.

Humid chamber made by covering pots and plants with a plastic bag to induce symptoms caused by a pathogenic bacteria.

Host tests for leaf and stem spot pathogens

- Young host plant incubated in a polythene sleeve with a wire frame to prevent the plant touching the polythene.



Lelliott and Stead,1987

Plant/Pathogenicity Test

Standard precautions in pathogenicity tests

- This strategy can be time-consuming (days, weeks, or months).
- A pure culture of bacteria recovered from diseased tissue is artificially inoculated into the same or related cultivar or another susceptible host species, in an effort to reproduce the same disease symptoms.
- The bacteria should then be reisolated and compared with the inoculant culture.

1. In a pathogenicity test a negative control (plants inoculated with sterile buffer solution only) should always be included.
2. Where necessary also a positive control (using a known pathogenic strain of the pathogen) should be included.

Plant/Pathogenicity Test

Standard precautions in pathogenicity tests

- Many bacterial pathogens can cause different reactions (necrosis, discoloration, eruptions and HR) on non-host plants when high dosages of inoculum(10^7 CFU/ml) are used.
- Some of these reactions on non-host plants may be misinterpreted as a true pathogenic response.
- **To avoid these:**
- Select an a appropriate inoculation technique which most closely simulates the natural method of inoculation or infection.
- Also use relatively low dosages of bacteria in inoculation (10^3 to 10^5 CFU/ml) when spraying or infiltrating plants. This minimize most artificial reactions.

Lelliott and Stead,1987; Schaad,1988; Fahy and Persley,1983

Plant/Pathogenicity Test

Standard precautions in pathogenicity tests

- However doses between 10^7 and 10^8 cells/ml are generally used to increase the chance of success and hasten onset of visible symptoms.
- Some metabolic products of bacteria growth in or on culture media can produces reactions in test plants that can be confusing.
- **To avoid this:**
- To avoid this use suspensions of young bacterial growth, washed rapidly off agar media, as inoculum.

Lelliott and Stead,1987; Schaad,1988; Fahy and Persley,1983

Pathogenicity Assays
Details of the assays

1. Inoculum preparation

- From a freshly restreaked, isolated colony, inoculate either an agar dish or broth.
- Incubate the dishes statically or the broth cultures with shaking (150 to 200 rpm/min) at a temperature similar to the temperature at which the inoculated plants will be incubated.
- Grow the bacteria until colonies form on the agar (48 to 96 h) or the broth culture reaches the stationary phase of growth (turbidity reaches an optical density (OD) of 0.7 to 1.0 at 550 to 600 nm as read on a colorimeter with an optical path of 1.0 cm).
- Suspend bacteria from colonies in water such that OD at 550 to 600 nm=0.7 to 1.0.
- Dilute broth-grown or agar-grown bacterial suspensions 100-fold with water.
- The final concentration of bacterial cells will be approximately 10^6 to 10^7 colony forming units (CFU)/ml.
- Use this suspension for inoculation immediately.

Trigiano *et al.*,2006

Pathogenicity Assays
Details of the assays

2. Inoculation methods

- Inoculation methods vary with the organs affected by the pathogen.
- Inoculations should be made on immature, rapidly developing plant tissues.
- e.g. partially expanded leaves, emerging shoots, immature pods or other fruits(Fahy and Persley,1983).

Trigiano *et al.*,2006

Pathogenicity Assays
Details of the assays

- **Carborundum abrasion method (Leben *et al.*, 1968):**
- The plants were inoculated with help of cotton swab on both the surfaces of leaves.
- The cotton swab was soaked in inoculums containing carborundum powder (300 mesh) for making gentle injury and application of inoculums simultaneously.

Maji and Nath,2015

Pathogenicity Assays
Details of the assays

- **Leaves:**
- Using a sprayer (window cleaner sprayer from grocery store disinfested with 70% isopropanol and rinsed carefully with freshly distilled water), adjust the spray to a fine mist and spray the underside (abaxial) surface of the leaf until watersoaked areas are obvious.
- Water-soaked areas are where the bacterial suspension has been forced through stomata into the leaf, which appear darker and more translucent to light than surrounding leaf tissue.
- **Flowers:**
- With a microliter pipettor, deliver 10:1 of bacterial suspension to the area of the nectartodes.
- Replant in fresh potting medium and water.

Trigiano *et al.*,2006

Pathogenicity Assays
Details of the assays

- **Stems:**
- Mildly stress the plants for water before inoculation.
- Stress plants until leaves are slightly flaccid without being so wilted that they do not revive with watering.
- Place a drop of bacterial suspension (ca 10^6-10^7 cfu/ml) at the junction of a leaf petiole and the stem(leaf axil).
- With a hypodermic syringe, the inoculum is pricked/perforated into the stem.
- Take care the needle not to pierce the opposite side of the stem.
- Remove the needle and water the plant.
- The bacterial suspension will be drawn into the plant through the needle wound by transpiration.

Lelliott and Stead,1987; Trigiano *et al.*,2006

Pathogenicity Assays
Details of the assays

- **Stem:**
- In order to fulfill the pathogenicity test for *B. nigrifluens* , 20 µl of the bacterial suspensions were placed in wounds (about 1 cm long) made in the bark of walnut stems with a scalpel.
- The wounds were protected with parafilm.
- Sterile water, instead of bacterial suspension, was used for control plants.

Moretti *et al.*,2007

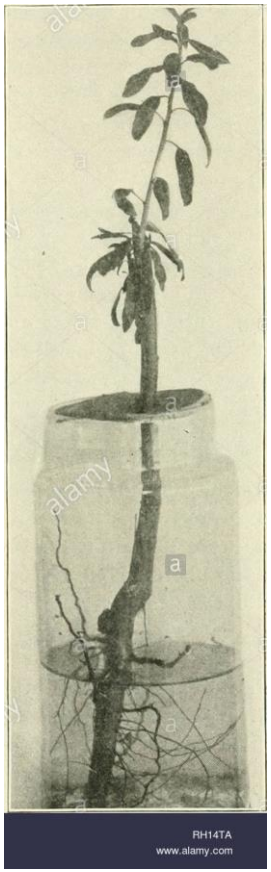
Pathogenicity Assays
Details of the assays

- **Stem cuttings:**
- Although not strictly pathogenicity tests, use of detached organs and fragments is a convenient and valuable means of screening likely pathogens and confirming some diagnosis.
- Many pathogens will produce spreading lesions and dieback on young, detached shoots standing in water.
- Stand cutting in a bacterial suspensions (10^6-10^7 cfu/ml) for a few minutes before striking in a suitable sterilized potting medium and incubating for symptom production.

Fahy and Persely,1983; Lelliott and Stead,1987

Pathogenicity Assays
Details of the assays

- **Stem cuttings:**
- Two healthy seedlings of almond were cut back to small cuttings and placed in jars partially filled with water.
- Incisions were made at the crown a few inches above the water.
- These jars were kept in the laboratory for two months, the water was changed about twice a week, and as the room was kept at a fairly low temperature the growth of the plants was not seriously disturbed.
- Nineteen days after inoculation the first evidences of the gall became visible on one of the plants.



Agriculture Arizona,1898

Pathogenicity Assays
Details of the assays

- **Tubers, rhizomes, and fruits:**
- Fill the eye of a sewing needle (#7 sharp) with either bacteria from a colony on an agar dish or a turbid broth culture (OD at 550 to 600 nm=0.7 to 1.0) and press the eye of the needle 0.5 to 1.0 cm into the organ, rotate the needle and remove.
- **Roots:**
- Uproot young, mildly water-stressed seedlings and gently rinse their roots free of potting medium.
- Immerse their roots in a bacterial suspension (10^6 to 10^7 CFU/ml) for 1 to 5 min.
- Replant in fresh potting medium and water.

Trigiano *et al.*,2006

Pathogenicity Assays
Details of the assays

3. Symptoms

- Incubate inoculated plants in a moist chamber for 48 to 72 h.
- Remove plants to a shaded area in the glasshouse and observe for symptom development.
- **Anticipated Results:**

1. Blighting and rotting occur in 2 to 6 days,
2. Leaf spots appear in 5 to 14 days,
3. Wilting occurs in 10 to 21 days, and
4. Proliferation of plant tissue happens in 14 to 28 days.

- Well-watered plants often do not wilt.
- Mildly water-stress the plants by increasing the rate of transpiration with a small electric fan.

Trigiano *et al.*,2006

Plant associated microorganisms

- **Endophytic bacteria**
- **Contaminating bacteria**
- **Soft rot bacteria**
- **Yellow pigmented bacteria**

Plant associated microorganisms
Primary vs. opportunistic pathogens

- Pathogens can be classified as either:

1. Primary pathogens, or
2. Opportunistic pathogens.

- A primary pathogen can cause disease in a host regardless of the host's resident microbiota or immune system.
- An opportunistic pathogen, by contrast, can only cause disease in situations that compromise the host's defenses, such as the host's protective barriers, immune system, or normal microbiota.
- Sometimes a primary infection, the initial infection caused by one pathogen, can lead to a secondary infection by another pathogen.

Plant associated microorganisms
Primary vs. opportunistic pathogens

- **Opportunistic pathogens:**
- Do not usually cause disease unless the plant is immunocompromised. e.g.
- *Pseudomonas aeruginosa*, and
- *Enterobacter* spp.
- The latter can be found in a wide range of environments including water, sewage, soil, and on vegetables.
- Some species are opportunistic pathogens.
- The major opportunistic pathogens from this group are *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas*, *Burkholderia* and *Ralstonia* spp.

Valvano,2006

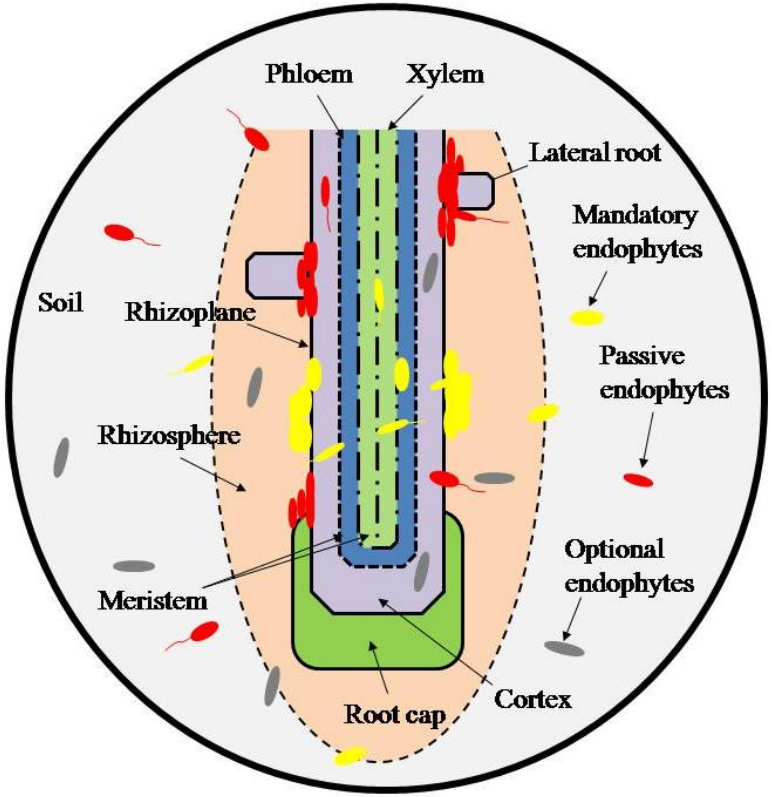
Endophyic bacteria
Endophytes

- Endophytes are organisms, often fungi and bacteria, that live between living plant cells.
- The relationship that they establish with the plant varies from symbiotic to bordering on pathogenic.
- Sometimes extremely unusual and valuable organic substances are produced by these endophytes that are sources of novel chemistry and biology to assist in helping solve not only human health, but plant and animal health problems also.

Endophyic bacteria

Niches and routes of entry of microorganisms in the roots of plants.

- The roots are considered the main gateway to microorganisms and this part of the plant has the highest frequency of endophytic bacteria.
- The enzymatic activity is a factor which facilitates the penetration of these microorganisms in the plant, which may be transmitted by seed.



Langner dos Santos *et al.*,2018

Endophyic bacteria
Endophytes

- Accumulating evidence suggests the ubiquity in plants of bacterial endophytes, most of which are currently unculturable (Zinniel *et al.*,2002).

1. These largely unexplored communities likely affect disease development (Araújo *et al.*,2002).
2. Endophytes are also potential biocontrol agents, or delivery systems for anti-pathogenic compounds such as:

- anti-QS compound ambuic acid that target quorum sensing and inhibits the biosynthesis of the cyclic peptide quormones of *Staphylococcus aureus* and *Listeria innocua*).

Allen *et al.*,2009; Araújo *et al.*,2002; Strobel,2018

Endophyic bacteria
Endophytes of citrus plants

- Example of endophytic bacterial populations on symptomatic or asymptomatic branches of citrus plants:
- *Bacillus pumilus*,
- *Curtobacterium flaccumfaciens*,
- *Enterobacter cloacae*,
- *Methylobacterium* spp.,
- *Nocardia* sp.,
- *Pantoea agglomerans*, and
- *Xanthomonas campestris.*

Allen *et al.*,2009; Araújo *et al.*,2002

Bacterial flora
Fresh vegetables

- The predominant bacteria with fresh vegetables such as carrots, spinach, cucumber, lettuce were:
- **Gram positive bacteria:**
- *Curtobacterium*
- *Staphyloccocus*
- *Enterococcus*
- **Gram negative bacteria(plant pathogenic):**
- *Pseudomonas cichorii* and *P. fluorescens*
- *Agrobacterium rhizogenes*
- *Stenotrophomonas maltophilia*
- *Pantoea agglomerans*
- 60% of isolates were belonged to Enterobacteriaceae such as:
- *Enterobacter*
- *Klebsiella*
- *Serratia*

Izumi *et al.*,2004

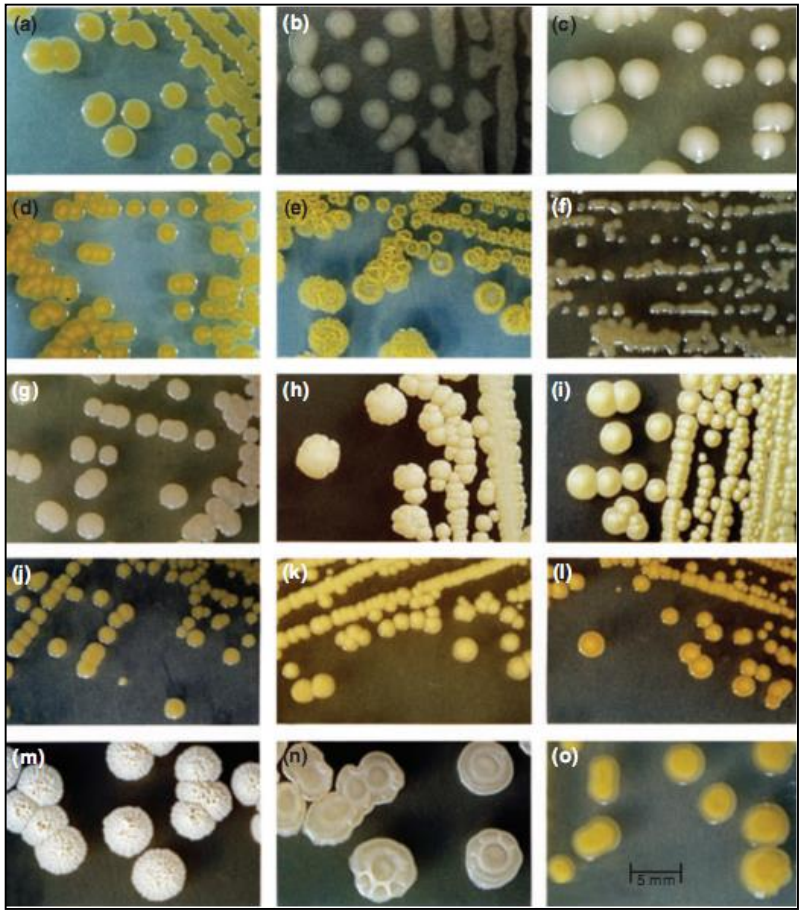
Surface and endophytic bacterial flora
Seeds of rice plants

- The predominant bacteria on and inside of matured rice seeds:
- **Endophytic bacteria:**
- *Acidovorax*
- *Paenibacillus*
- *Pantoea*
- **Surface bacteria:**
- *Stenotrophomonas maltophilia*
- *Agrobacterium*
- **Both inside and outside of the seeds:**
- *Curtobacterium*
- *Methylobacterium*
- *Bacillus*
- *Micrococcus*
- *Xanthomonas*
- *Sphingomonas*

Mano *et al.*,2006

Endophytic rice seed-associated bacteria
Their role in pathogenicity and biological control

- (a) *Pantoea stewartii* LMG 20115, (b) *Pantoea dispersa* LMG 20116, (c) *Enterobacter cloacae* LMG 20117,(d) *Pseudomonas aeruginosa* LMG 20125, (e) *Pseudomonas oryzihabitans* LMG 20126, (f) *Xanthomonas* sp. LMG 20137, (g) *Burkholderia glumae* LMG 20138, (h) *Staphylococcus gallinarum* LMG 20176,(i) *Micrococcus luteus* LMG 20178, (j) *Curtobacterium flaccumfaciens* LMG 20194, (k) *Clavibacter michiganense* LMG 20187, (l) *Cellulomonas flavigena* LMG 20188, (m) *Bacillus pumilus* LMG 20162, (n) *Bacillus subtilis* LMG 20163 and (o) *Paenibacillus polymyxa* LMG 20164.

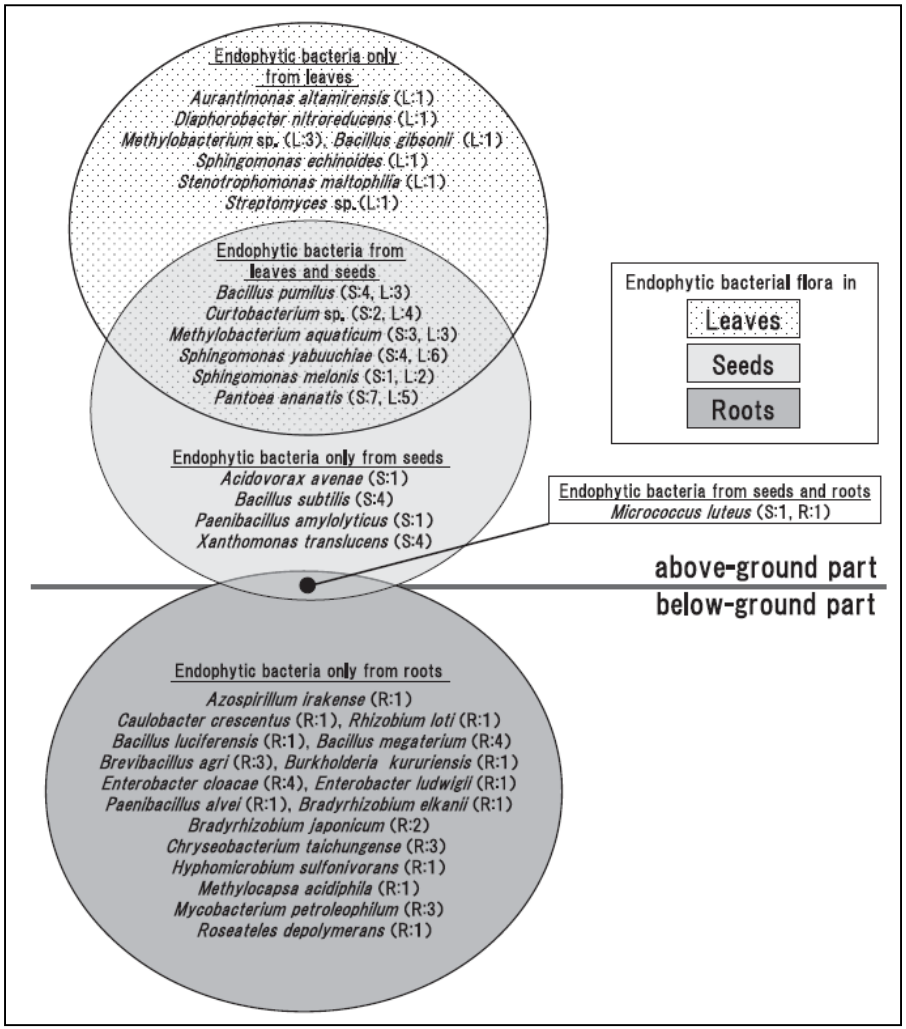


Cottyn *et al.*,2009

Endophytic bacterial flora

Leaves, seeds and roots of rice plants

- Comparison of the culturable endophytic bacterial floras in leaves, seeds and roots of rice plants (*Oryzae sativa*).
- About 30 strains were isolated from each part of the plant and identified by 16S rRNA gene sequences.



Mano and Morisaki,2008

Endophytic bacterial communities
Tomato cultivars

- *Sphingomonas yanoikuyae*
- *Pseudomonas pseudoalcaligenes*
- *Serratia marcescens*
- *Bacillus megaterium*
- *Paenibacillus polymyxa*
- *Bacillus pumilus*
- *Bacillus cereus*
- *Pseudomonas fluorescens*
- *Arthrobacter globiformis*

Feng *et al.*,2013

Endophytic bacteria
Functions

- A large number of plant endophytic bacteria reside in plants which establish harmonious and close relationships with their hosts resulting from co-evolutionary processes.
- Endophytes offer a wide range of benefits to plants such as:

1. Promoting growth,
2. Reducing disease severity inducing plant defense mechanisms inducing plant defense mechanisms,
3. Producing anti-herbivory products,
4. Biologically fixing nitrogen and
5. Increasing plant mineral uptake.

Feng *et al.*,2013

Contaminating micro-organisms
Contaminant bacteria

- Plates are usually contaminated with the following bacteria:
- Contaminant bacteria/yeast: are found essentially everywhere in the environment. e.g.

1. Swarming/gliding bacteria e.g. *P. aeruginosa*.
2. *Achromobacter*
3. *Acinetobacter*
4. *E. coli*
5. *Bacillus* spp.
6. Yeasts.

Swarming motility occurs in many organisms usually on softer semi-solid and solid wetting surfaces.

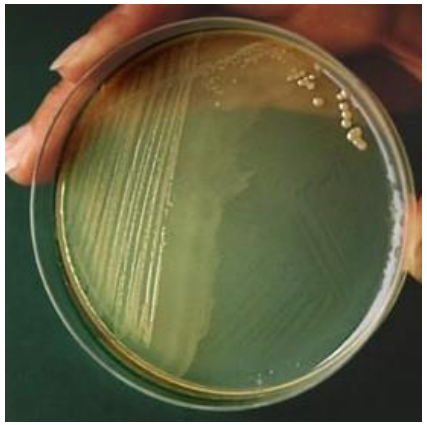
Swarming bacteria

Proteus found in the intestines of man and animals

- Concentric rings of growth on a suitable agar media.
- Swarming occurs in various bacteria such as:

1. *Proteus* (swarm cells causes food poisoning.
2. *Serratia*
3. *E. coli*.

Singleton,2004

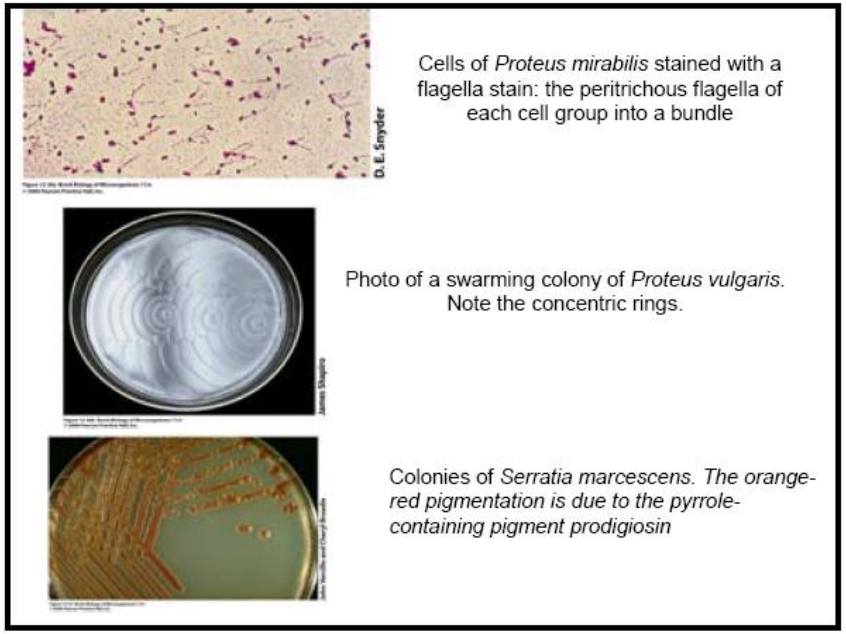


Proteus vulgaris

Swarming bacteria

Proteus mirabilis

- *Proteus*, an enteric bacterium forms unusual swarming patterns on agar plates that have a concentric ring shape.
- *Proteus mirabilis* a pathogen of the urinary tract) swarms in concentric circles.
- This unusual pattern is caused by alternating phases of rapid motility and less motile growth.



Cells of *Proteus mirabilis* stained with a flagella stain: the peritrichous flagella of each cell group into a bundle

Photo of a swarming colony of *Proteus vulgaris*. Note the concentric rings.

Colonies of *Serratia marcescens*. *The orange-red pigmentation is due to the pyrrole-containing pigment prodigiosin*

Diversity_1.pdf,2004

Gliding Bacteria

- Bacterial gliding is a process whereby a bacterium can move under its own power.
- This process does not involve the use of flagella, which is a more common means of motility in bacteria.
- Gliding is prominent in:

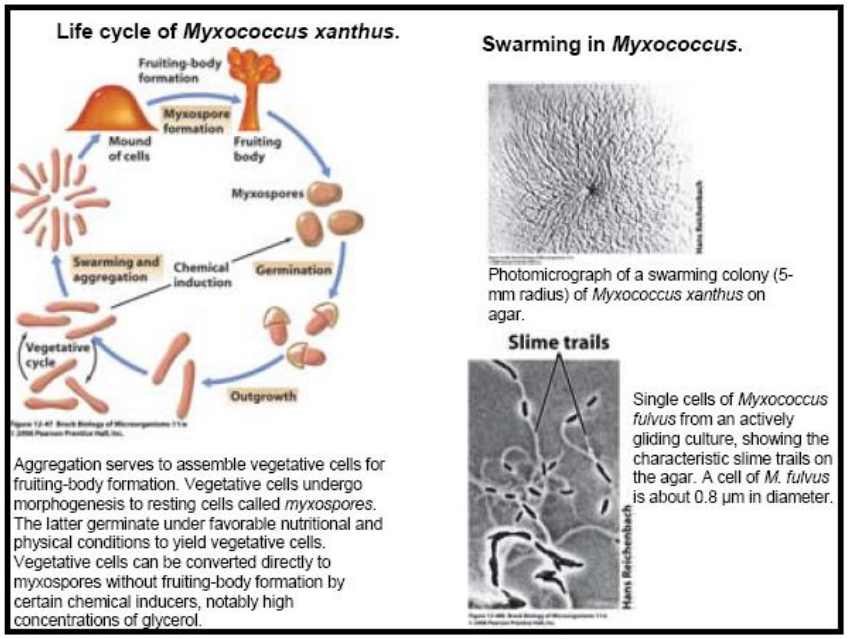
1. Cyanobacteria,
2. Myxobacteria, and the
3. Cytophaga-flavibacteria.

Cyanobacteria are the peculiar bacteria that are half-plant/half-animal. Regardless of the animal aspect of bacteria, cyanobacteria are photosynthetic autotrophs.

Gliding Myxobacteria

Myxococcus xanthus

- The myxobacteria are a group of Gram negative, thin rod shaped organisms.
- *M. xanthus* is also one of the most genetically tractable myxobacteria.
- It is a soil bacterium.
- *M. xanthus* cells are as single cells or in swarms.
- Fruiting bodies are dome shaped structures of approximately 100,000 cells.
- They were taxonomically lumped together with *Cytophaga* and their relatives.

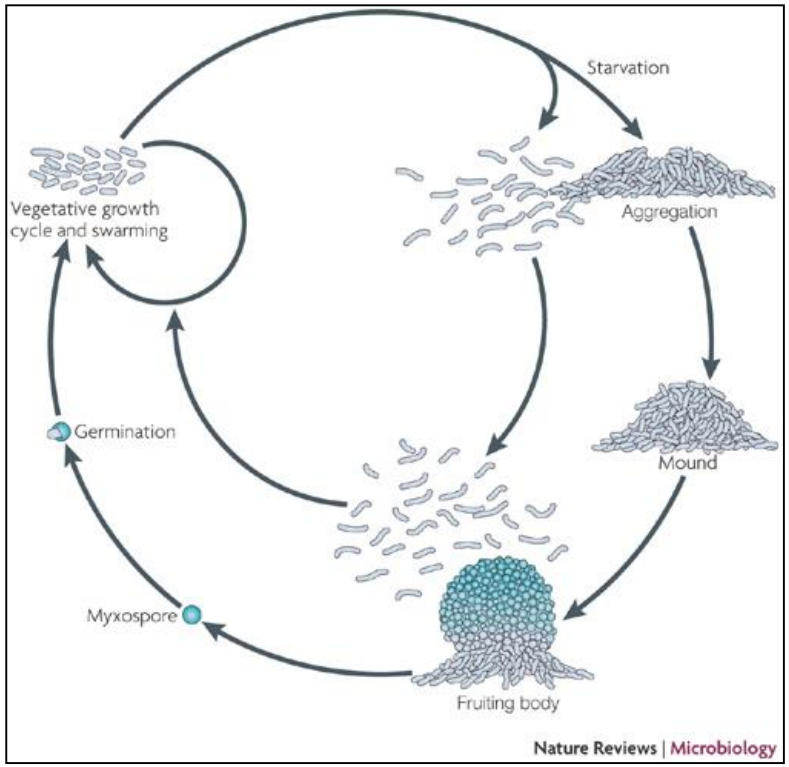


Diversity_1.pdf.2004;..

Gliding Myxobacteria

Myxococcus xanthus

- *Myxococcus xanthus* cells are usually found on solid substrates.
- Upon starvation, cells aggregate at discrete foci to form mounds and then macroscopic fruiting bodies.
- The rod-shaped cells in the fruiting bodies undergo morphogenesis and form spherical spores (myxospores) that are metabolically inactive and partly resistant to desiccation and temperature.
- When nutrients become available, the spores germinate and complete the life cycle.



Zusman *et al.*,2007

Gliding Bacteria

The genus *Lysobacter*

Xanthomonadaceae

- *Lysobacter* spp. are ubiquitous in soil and water. Also found in plant rhizosphere soils.
- *Lysobacter* spp. are related to other myxobacteria because of gliding motility.
- This genus was distinguished from other myxobacteria by it being non-fruit forming and having high G+C content.
- *Lysobacter* is now grouped in γ-proteobacteria, and belonging to the family Xanthomonadaceae.
- *Lysobacter* is very closely related with the genera *Xanthomonas*, *Stenotrophomonas*, *Pseudoxanthomonas*, *Thermomonas* and *Xylella* by phylogenetic analysis.

Yin,2010

Gliding Bacteria

The genus *Lysobacter*

Characteristics

- *Lysobacter* spp. also display a number of traits that distinguish them from other related bacterial genera such as *Xanthomons* and *Stenotrophomonas maltophilia*.
- These are including:

1. Positive oxidase activity,
2. 28°C optimum growing temperature (No or min. growth at 37°C),
3. Varying cell length (2 to 70 μm),
4. High genomic G+C content (typically ranging between 65-72%), and
5. The lack of flagella (negative in flagella motility test).

Yin,2010

Gliding Bacteria

The genus *Lysobacter*

Other characteristics of the genus compared with *Xanthomonas*

Traits	*Lysobacter*	*Xanthomonas*
Gram reaction/cell shape	-ve, rod cell length (2 to 70 µm)	-ve, short rod (2 µm)
Colony color/texture	highly mucoid, cream-colored, pink, or yellow-brown; many strains also produce a brown, water-soluble pigment	Usually yellow non-diffusible pigment. Few white, brown ,non mucoid
Motility	-	+
O/F	Aerobe	Aerobe
Indole	-	-
VP-MR	-	-
NO_3-NO_2	+	-
H_2S produced	V	+
Oxidase	+	- or weak
% NaCl tolerance	2-3	1
Initial pH for growth	5->10	1
Gelatinase	+	+/-
Chitin degradation	Mostly +	-
Pathogenicity	Non-pathogenic	Often plant pathogenic

Yin,2010,Bergyes Manual,2005

Gliding Bacteria

The genus *Lysobacter*

Biocontrol agents

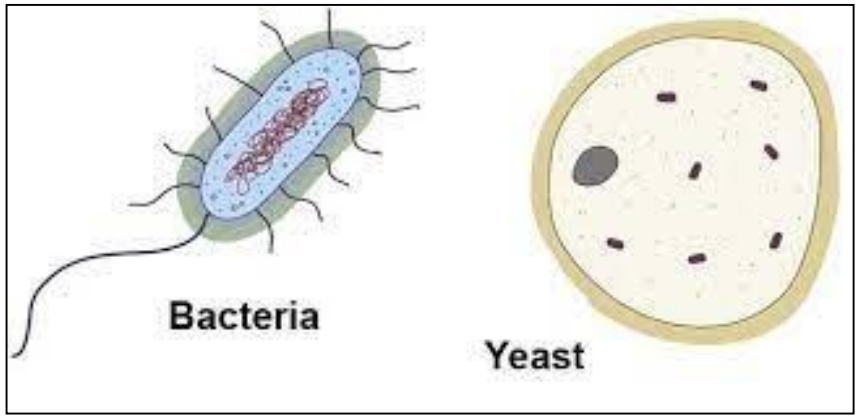
- Strains of *Lysobacter* spp. were reported to have broad spectrum antagonism in vitro against bacteria, fungi, unicellular algae and nematodes.
- The mechanisms associated with this bacterium are:

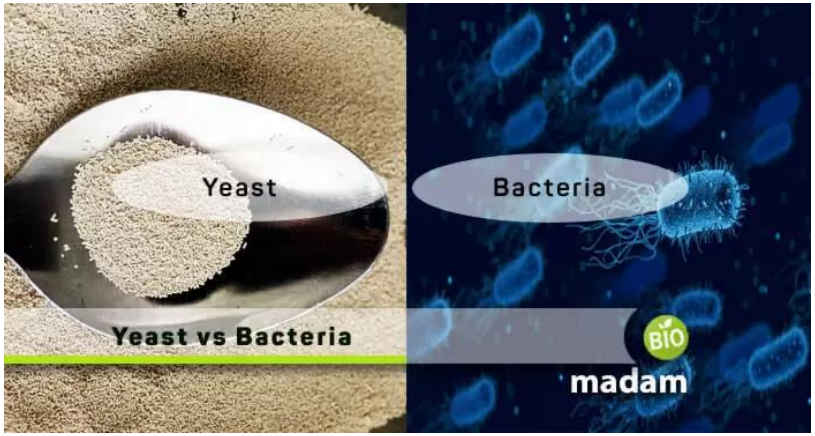
1. Antibiotic production,
2. Extracellular enzyme activities such as protease and chitinase,
3. Induced resistance, and
4. Hyperparasitism.

Yin,2010

Yeasts
Description

- It is quite easy to grow yeasts in the laboratory on a variety of complex and synthetic media.
- *Saccharomyces* stain gram-positive.
- Most yeasts grow very well between pH 4.5 and 6.5.
- Division usually by budding.
- Most yeasts are aerobes.



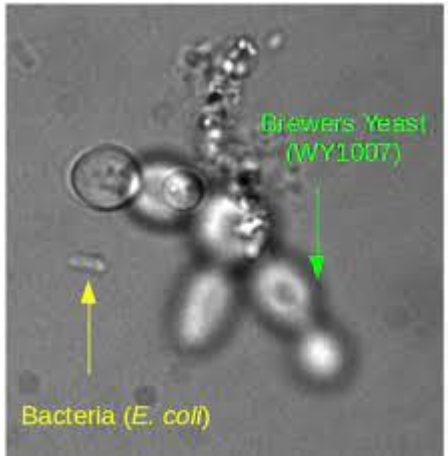


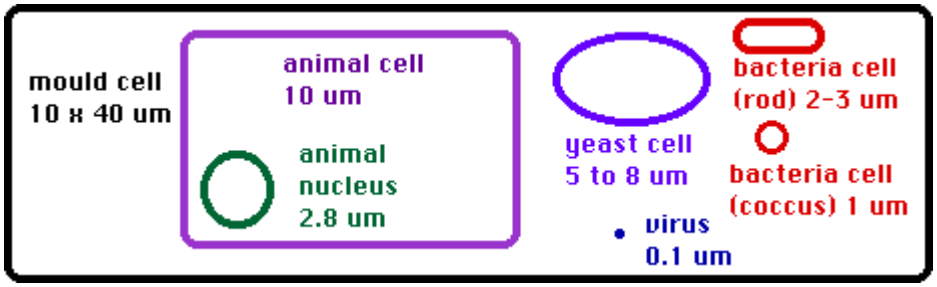
Walker,2004; Chelsea Rogers;..

Yeasts

Cell size and shapes

- Yeast-cell size can vary widely, depending on the species and condition of growth.
- Typical bacterial cell size (2 to 5 µm).
- Some yeasts may be only 2-3 µm in length, whereas others may attain lengths of 20-50 µm.
- Cell width appears less variable, between 1 and 10 µm.





Yeasts
Colonies

- Yeasts generally are more larger colonies than that of bacteria
- The yeast have a flattened edge and appear glossy mucoid in the center whereas the bacterial colonies do not have a flattened edge and is smooth edged mostly.
- The yeast colonies look fused mostly whereas bacterial may appear more separate and distinct.
- The Yeast generally give a distinct cheesy-kind of smell or bakery kind of smell.

Yeast colonies

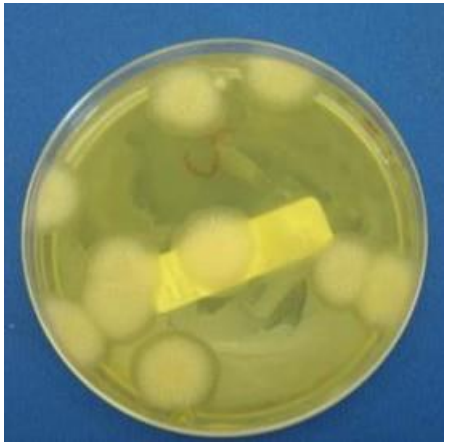
- To identify yeasts, first examine the colony color, shape and texture.
- The colonies may be:

1. Black to brown in color or moist mycelial in texture.
2. Pink to red, on incubation, satellite colonies will be formed.
3. If the colony is white or cream color, perform a germ tube test.

Satellite colonies are very small colonies that have not taken up the plasmid and that form around a large colony that has taken up plasmid."

Yeast colonies

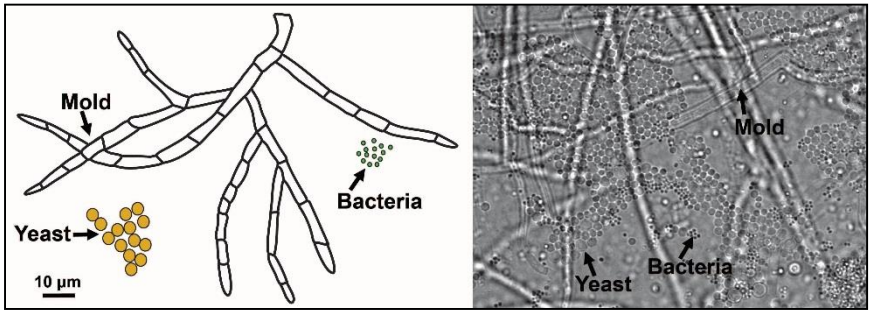
- Yeast colonies generally look similar to bacterial colonies.
- Some species, such as *Candida*, can grow as white patches with a glossy surface.

		
Candida albicans is a type of yeast that can grow on the surface of skin.	Round yeast colonies	Pink yeast satellite colonies

Shijun Liu,2008, Science Buddies

Yeasts

Colony and mycelium

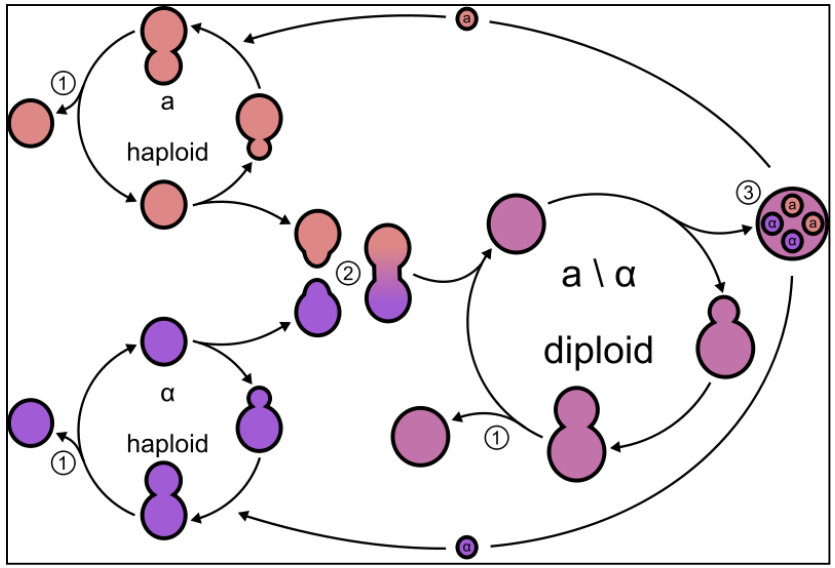


Walker,2004;..

Yeasts
Life cycle

- Yeasts, like all fungi, may have asexual and sexual reproductive cycles.
- The most common mode of vegetative growth in yeast is asexual reproduction by budding.
- The yeast cell's life cycle:

1. Budding
2. Conjugation
3. Spore.



Wikipedia

Universal medium for yeasts

YM

- Yeast extract 3.0 g
- Malt extract 3.0 g
- Peptone 5.0 g
- Glucose 10.0 g
- Agar 15.0 g
- Distilled water 1000 ml
- pH= 6.8 ± 0.2
- Autoclave at 121°C for 15 minutes.
- For *Brettanomyces* species add 0.5-1% $CaCO_3$ to the above medium.
- For osmophilic species add 20-60% sucrose.

Malik,1991

Yeast staining
Procedure

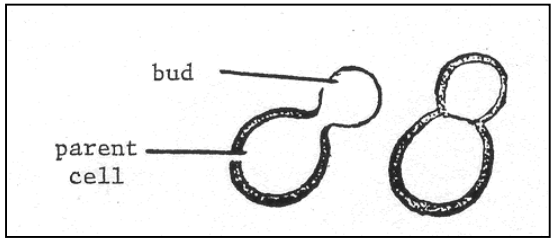
1. Prepare a wet mount of the cells using a drop of methylene blue.
2. Carefully place a cover slip on the cell/stain mixture.
3. View the cells noting size and shape.

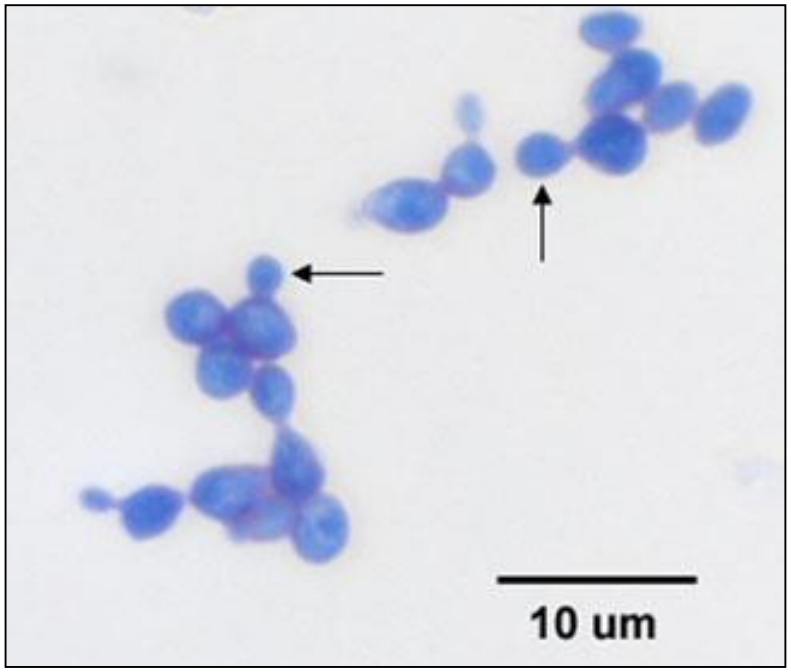
- If you look carefully, you should be able to see budding cells.

Pacarynuk,2006

Staining of *Saccharomyces cerevisiae*
The budding yeast used for bread-making

- Yeasts are fungi that grow as single cells, producing daughter cells either by:
1. budding (the budding yeasts) or by
2. binary fission (the fission yeasts).
- Note budding yeast (arrows).





Kaiser,2009;..

The genus/species
Stenotrophomonas maltophilia
Human and plant associated bacterium

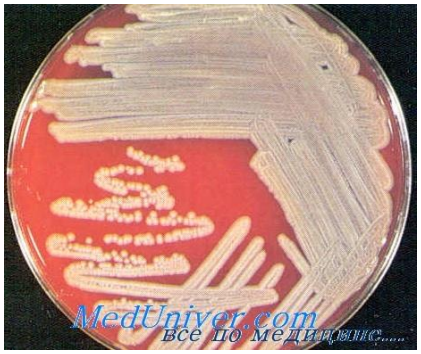
- *Stenotrophomonas maltophilia* (formerly *Pseudomonas* (until 1993) then *Xanthomonas* (until 1995) – reclassified due to nucleic acid homology).

1. *S. maltophilia* is the only species of *Stenotrophomonas* known to infect humans (associated with respiratory infections in humans),
2. Whereas its closest genetic relatives are plant pathogens.

- It is frequently isolated from soil, water, animals, plant matter, and hospital equipment.

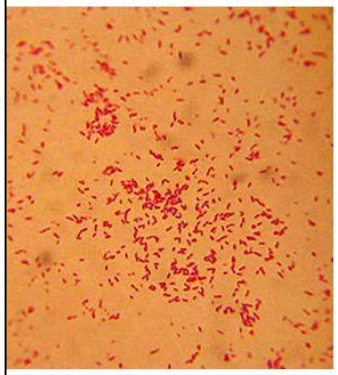
van der Lelie; Mckay,T.; De la Maza,L.M.; Mano *et al.*,2006; Lewis and Zass,2017

Xanthomonadaceae bacterium *Stenotrophomonas maltophilia*



Stenotrophomonas maltophilia

http://www.scielo.cl



http://clinicalmicrobiology.stanford.edu

http://www.microbelibrary.org

Stenotrophomonas maltophilia is a long name, but it is possible to learn it easily: it is narrow-nutrition-unit maltose-loving, so it is a „bacterial panda“, chewing maltose instead of bamboo ☺.

It is maltose loving, so it is a bacterial panda, chewing maltose instead of bamboo.

The genus/species
Stenotrophomonas maltophilia

- Cells of *S. maltophilia* are straight or slightly curved nonsporulating gram-negative bacilli that are 0.5 to 1.5 µm long.
- They occur singly or in pairs and do not accumulate poly-β-hydroxybutyrate as intracellular granules.
- They are motile by means of several polar flagella.
- The colonies are smooth, glistening, with entire margins and are white to pale yellow.
- The solid media used were Columbia agar with sheep blood, chocolate agar, McConkey agar, Candida chrom agar (Brilliance), and malt agar.

Denton and Kerr,1998; Goncalves-Vidigal *et al.*,2011

The genus/species
Stenotrophomonas maltophilia

- Yellow or green non-fluorescent usually non-diffusible pigment.
- It can produce a tan to brown pigment (De la Maza).
- Gram-negative. Catalase positive. H_2S production positive.
- *S. maltophilia* R551-3 from *Populus* represented the second most commonly found endophytic species in poplar.
- *S. maltophilia* R551-3 from *Populus* represented the second most commonly found endophytic species in poplar.

van der Lelie; Mckay,T.; De la Maza,L.M.; Mano *et al.*,2006; Lewis and Zass,2017

The genus/species
Stenotrophomonas maltophilia

- Historically *S. maltophilia* has been of interest for its use in biological control of plant pathogens and bioremediation.
- The mechanisms associated with this bacterium are:
- Antibiotic production,
- Antifreeze activity(AFP),
- Extracellular enzyme activities such as protease and chitinase, and
- Rhizosphere colonization potential.

van der Lelie; Mckay,T.; De la Maza,L.M.; Mano *et al.*,2006; Lewis and Zass,2017; Denet *et al.*, 2018

The genus/species

The growth characteristics of *Stenotrophomonas maltophilia*

An Emerging Global Opportunistic Pathogen.

S. maltophilia is an environmental bacterium found in aqueous habitats, including plant rhizospheres, animals, foods, and water sources.

Growth characteristic	Reaction
Straight or curved rods, 0.5 by 1.5 μm	
Oxidase	+/−
Catalase	+
Methionine is required for growth	+
Optimum growth temp of 35°C	
No growth at 4°C or 41°C	
Survival at refrigeration temp	+
Motility	+
Nitrate reduction, but nitrate is not used as nitrogen source	+
Indole	−
Lysine decarboxylase	+
Ornithine decarboxylase	−
Methyl red	−
Voges-Proskauer reaction	−
Hydrogen sulfide	−
Citrate	v
Phenylamine deaminase	−
β-Galactosidase (ONPG)	v
Carbohydrate utilization	
Acid production from maltose	+
Acid production from glucose	−
Carbon source for growth	
Adonitol	−
Arabinose	−
β-Hydroxybutyrate	−
Cellobiose	v
Dulcitol	−
Glucose	+
Fructose	v
Galactose	v
Lactose	+
Maltose	+
Mannitol	−
Mannose	v
Rhamnose	−
Salicin	−
Sorbitol	−
Trehalose	+/−
Esculin hydrolysis	+
Gelatin liquefaction	+
Tween 80 hydrolysis	+
DNase production	+
Starch hydrolysis	−
Urea hydrolysis	−

[a] +, >85% of strains positive; v, 16 to 84% of strains positive; −, ≤15% of strains positive; ONPG, *o*-nitrophenyl-β-D-galactopyranoside. Data are from references 48, 79, 169, 258, and 367.

Brooke,2012

The genus/species

Stenotrophomonas maltophilia

Gram reaction/cell shape	-ve, rod
Colony color	Variable yellow
Motility	+
O/F	Aerobe
Lysine	+
Urease	V
NO_3-NO_2	V
Arginine dihydrolyase	-
Oxidase*	+
Mannitol	-
Maltose	+
ONPG	+
DNase	+
Pyrrolidonyl-β-naphthylamide(PYR)	-
Resistant to Polymyxin B disk (330-U)	S(susceptible) /V

* -ve according to De la Maza, L.M. The ortho-nitrophenyl-galactopyranoside (ONPG) test is a rapid test for the detection of bacterial β-galactoside.

Isenberg, H.D.; Mckay, T.

Morphological and biochemical characteristics of chromium resistant *Stenotrophomonas maltophilia* OS4 strain

1	Features	Specifications	Outcomes
2	Appearance	Shape	Short Rod
3		Pigments	+
4		Gram reaction	–
5	Biochemical reactions	Citrate utilization	+
6		Indole	–
7		Methyl red	–
8		Nitrate reduction	+
9		Catalase	+
10		Oxidase	–
11		Voges Proskauer	–
12	Carbohydrate utilization	Glucose	+
13		Mannitol	–
14		Sucrose	+
15	Hydrolysis	Starch	+
16		Gelatin	+
17	Tolerance	Cr (VI)	1,200 µg/ml

Oves *et al.*,2013

Bioactive compounds produced by *Stenotrophomonas maltophilia*

Organism	Source	Compound	Activity
Stenotrophomonas maltophilia R3089	Rhizosphere of rape plants (*Brassica napus* L.)	Maltophilin	Antifungal
S. maltophilia	Oil contaminated soil	Bio surfactant Rhamnolipid	Mosquito larvicidal
S. maltophilia	Nematicidal plants	Hydrolytic enzymes and HCN, phenol oxidation	Anti-trichodorid nematode density on potato
S. maltophilia N5.18		Enhance antioxidant activity	Improved sprouts quality in Soybean
Stenotrophomonas sp. strain SB-K88	Rhizosphere of sugar beet	Xanthobaccins A, B, and C	Suppresses damping-off disease
Stenotrophomonas sp.	Deep sea invertebrates	Antimicrobial activity	Hemolysis of fungus
S. maltophilia S1	Soil bacteria from Japan	Alkaline serine protease	Hydrolyses zein: major protein in maize seeds
S. maltophilia SSA	Roots of *Solanum surrattense* Burm	Phytohormones: IAA, gibberellic acid, *trans*-zeatin riboside, abscisic acid	Enhance growth of *Zea mays* seedlings
S. maltophilia		Dipeptidyl aminopeptidase IV	Substrate with hydro-xyproline residue
S. maltophilia PML168	Temperate intertidal zone	Class B Flavoprotein	Catalytic activity
S. maltophilia D457	Laboratory collection	3,5-dihydroxy benzoic acid and the α-phenyl benzenethanethioic acid	Antimicrobial activity against *E. coli*, *S. aureus, P. aeruginosa*, *Bacillus* spp.
S. maltophilia MUJ	Rhizosphere	Chitinase	Antifungal: *Rhizoctonia, Fusarium, Alternaria*.
S. maltophilia AVP27	Chili rhizosphere soil	IAA, ammonia, phosphatise, HCN	Promote growth of chili plant
S. maltophilia W81	Sugarbeet rhizosphere	Chitinase, protease	Inhibit growth of *Pythium ultimum*
S. maltophilia PD3533	Eggplant rhizosphere	Chitinase/protease	Suppress potato brown rot fungus
S. maltophilia	Rhizosphere of oilseed rape	Lytic enzymes	Antifungal: *Rhizoctonia solani*, *Verticillium dahliae*
X. maltophilia	Cucumber root and bark media	Antifungal activity	*Rhizoctonia* and *Trichoderma*
X. maltophilia	Maize rhizosphere in France Pyrrolnitrin		Antifungal: *P. ultimum*, *F. culmorum*

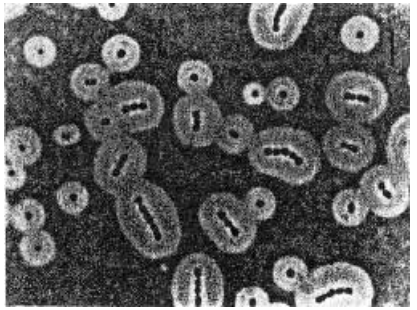
Mukherjee and Roy,2016

The genus *Achromobacter*

- *Achromobacter*, include all nonpigmented, non-spore forming Gram-negative rods, and aerobic saprophytes.
- These unpigmented bacteria are confused with *Pseudomonas* cause spoilage foods.
- Motile with 1-20 sheathed flagella arranged peritrichously.
- Oxidase, denitrification and catalase are positive.
- Urease, DNase, phenylalanine deaminase, lysine and ornithine decarboxylase, arginine dihydrolase, and gelatinase negative.
- Isolated from human clinical samples.
- It can also be isolated from plants where it shows endophytic and plant growth promoting characteristics.
- *Achromobacter xylosoxidans* isolated from wheat plant.

Prabhat and Kumar, 2009; Wittmann *et al.*,2014

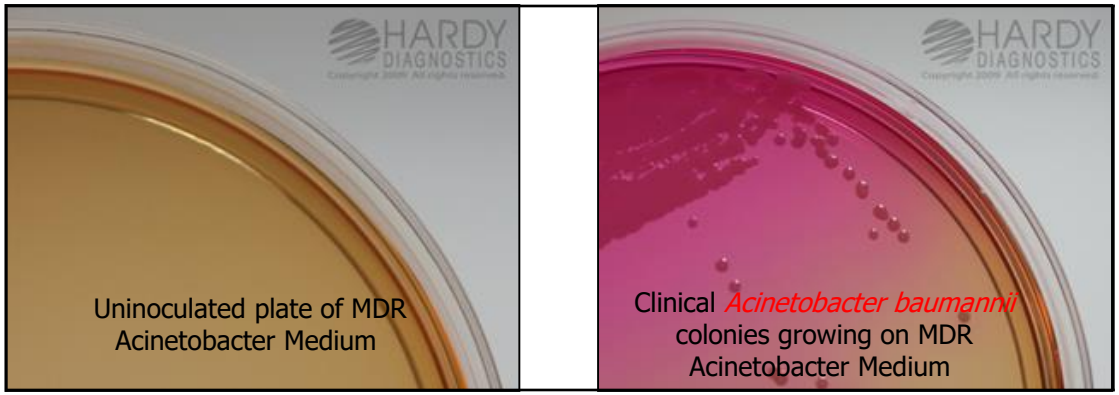
The genus *Acinetobacter*



- Nonmotile achromobacters be considered species of the newly defined genus *Acinetobacter*.
- Nonpigmented Gram-negative, strictly aerobic, encapsulate, non-motile rods.
- Cells occur typically in pairs, and occasionally in chains.
- Oxidase-negative; catalase-positive; usually nitrate negative,
- They are important soil organisms, where they contribute to the mineralization/degradation aromatic compounds such as phenols.
- Plant growth promoting traits of *Acinetobacter* species isolated from rhizosphere of *Pennisetum glaucum* were reported.
- Uncertain pathogenicity.
- Most frequently saprophytic, occurring naturally in soil, water, sewage, foods such as raw vegetables and even on human skin as contaminants.

The genus *Acinetobacter*

- On MDR Acinetobacter Medium or Leeds Acinetobacter Medium, *Acinetobacter* spp. will produce light pink mucoid colonies with a pink to mauve (a moderate purple, violet or lilac color) color diffused into the medium.
- *Stenotrophomonas maltophilia* and *Burkholderia cepacia* will also produce light pink colonies with a pink to mauve diffused into the medium.



Hardy Diagnostics

MDR and Leeds Acinetobacter Medium:

Casein Acid Hydrolysate	15.0 gm
Soy Peptone	5.0 gm
Sodium Chloride	5.0 gm
Fructose	5.0 gm
Sucrose	5.0 gm
Mannitol	5.0 gm
Phelylalanine	1.0 gm
Ferric Ammonium Citrate	0.4 gm
Phenol Red	0.02 gm
Selective Agents**	0.035 gm
Agar	12.0 gm

Ingredients per liter of deionized water.
Final pH 7.0 +/- 0.2 at 25 degrees C.
** MDR Acinetobacter Medium contains additional selective agents.

Acinetobacter spp.

The biochemical characteristics of *A. baumanii* and *A. lwoffii* strains isolated from clinical and environment samples

Test, substrate	*A.baumanii* (n=17)	*A.lwoffii* (n=7)
Morphology	coccobacilli	coccobacilli
Motility	nonmotile	nonmotile
Fermentative or oxidative	O	NO
Catalase	+	+
Oxidase	-	-
Growth on		
MacConkey agar	+	+
SS agar	V	V
Acid from:		
Glucose	+	-
Xylose	+	-
Mannitol	-	-
Sucrose	-	-
Galactose	+	-
Manose	+	-
Rhamnose	+	-
Lactose	+	-
Maltose	V	-
Esculin hydrolisis	-	-
TSI acid:		
Slant	-	-
Butt	-	-
H_2S: on TSI	-	-
Simmons citrate	+	V
Urea,Christensen	V	-
Nitrate reduction	-	-
Methyl red	-	-
Voges-Proskauer	-	-

Key reactions: O= oxidative; NO = nonoxidizer; + = positive reaction; - = negative reactions; V= variable reactions.

Constantiniu *et al.*,2004

Acinetobacter sp.

Isolated from papaya plant infected with dieback disease

- *Acinetobacter* sp. was isolated from papaya dieback disease that caused dark spot at fruit surface.
- This symptom also shown by that:

1. Citrus canker pathogens *Xanthomonas axonopolis* pv. *citri* (now XCC), and
2. *Xanthomonas axonopodis* pv.*aurantifolii* (now Xca).

- This will lead to rupture of the epidermis, which significantly favors pathogen spreading and disease dissemination.

Hisam *et al.*,2009;..

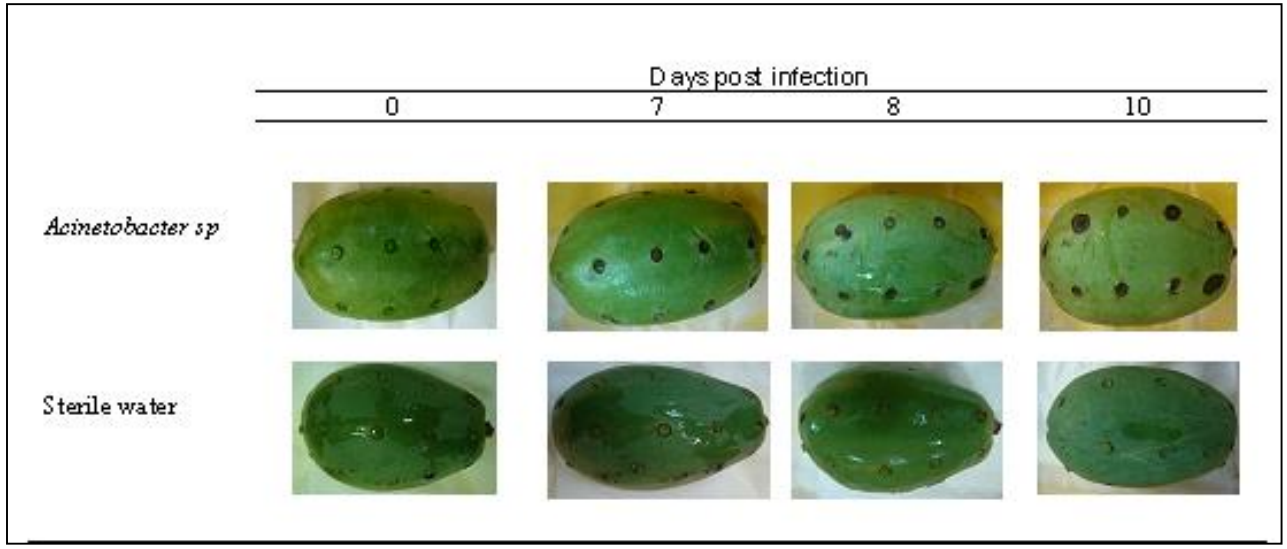
Acinetobacter sp.
Papaya fruit lesion

- Healthy immature papaya fruits at 70 days after anthesis were selected for bacterial inoculation.
- The papaya fruits were washed with 10% Clorox and rinsed three times with sterile water.
- The fruits were air-dried and the skin was core out using core borer (size 6 mm diameter) and inoculated with bacterial suspension (measured the OD_{600} nm=1).
- The inoculated fruit were sprayed with sterile water to maintain humidity until the symptom developed.

Hisam *et al.*,2009

Acinetobacter sp.
Papaya fruit lesion

- A lesion at papaya fruit surface after inoculation of *Acinetobacter* sp.
- The lesion become obvious after 10 day post inoculation (dpi).



Hisam *et al.*,2009

Association of *Acinetobacter baumannii* with a top rot phase of sugarcane red stripe disease in India

- Bacterial red stripe caused by *Acidovorax avenae* subsp. *avenae* is one of the most important and economically a dreadful diseases in sugarcane.
- Leaf stripe and top rot phases, occur singly or together under field conditions.
- The top rot form of the disease is the most economically damaging.
- Bacterial top rot caused by *Acinetobacter baumannii* was observed for the first time from India.

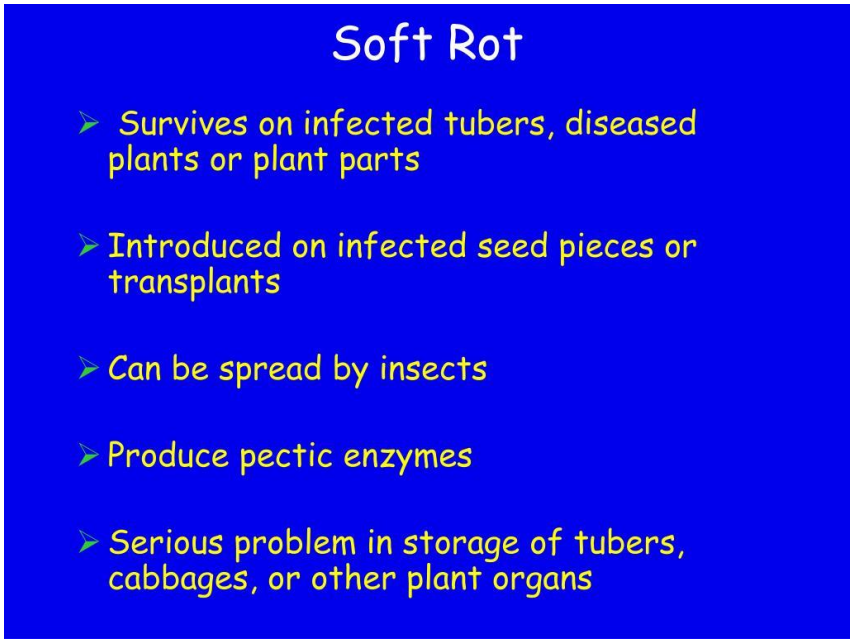
Patro *et al.*,2006

Decay diseases

Soft rot bacteria

- Many plant pathogenic bacteria produce one or more pectic enzymes.
- The rates at which these enzymes are produced and the efficacy with which they can degrade pectin substances varies with the type of bacterium producing them.
- The major soft-rotting bacteria belong to:

1. *Pectobacterium*, and
2. *Dickeya* spp.



Decay diseases
Soft rot bacteria

1. *Pectobacterium* and *Dickeya* spp. along with *Pseudomonas* spp. account for over 90% of soft rot of fresh produce while in storage or at markets.
2. Less than 10% of soft rot of fresh produce found at the markets could be caused by:

- *Xanthomonas*,
- *Cytophaga*,
- *Bacillus*, or
- Other unidentified genera.

Sapers *et al.*,2006

Decay diseases
Soft rot bacteria

- Soft rot of fresh produce can be caused by diverse groups of bacteria including:
- ex. *Erwinias* (*Pectobacterium* and *Dickeya* spp.)
- *Pseudomonas*
- *Xanthomonas*
- *Clostridium*
- *Bacillus*
- *Cytophaga*, and
- Other unidentified genera.
- *Bacillus*, *Clostridium* spp., *Cytophaga* sp. and *Klebsiella planticola* and *K. pneumoniae* are considered as secondary rot organisms.

Decay diseases

Soft rot bacteria

Pectolytic bacilli

- Pectolytic bacilli including *Bacillus polymyxa* and *B. subtilis* can cause soft rot in a wide variety of crops including potatoes, tomatoes, carrot, onion, and cucumber grown at elevated temperatures from ambient to 37°C.

Bacilli generally are more easily isolated from soil than from plant roots (Ambrosini and Passaglia,2017).

Decay diseases

Soft rot bacteria

Pseudomonas spp. and *Chryseobacterium* sp.

- Other bacterial species that cause soft rot include:
- *Pseudomonas cichorii*
- *P. marginalis*
- *P. viridiflava*
- These bacteria attack different plant parts (leaves, stems, bulbs, roots, etc.) in different stages of the disease.
- Two new plant pathogens were also introduced:
- *Pseudomonas veronii*, and
- *Chryseobacterium indologenes*.
- These two pectolytic bacteria associating with soft rot of calla lily (*Zantedeschia* spp.) tubers are described for the first time as plant pathogens (Mikiciński *et al*.,2009).

Phenotypic characteristic of pectolytic bacteria isolated from calla lily tubers

Pseudomonas veronii **(strains 6M& 10M);** ***Chryseobacterium indologenes*** **(strain 1M);** ***Chryseobacterium*** **sp. (strain 5M);** ***Pseudomonas marginalis*** **(strains 7M& 8M)**

	Symbol of strain				Reference strains		Type strains[a]		
Test	2M	4M	9M	853	EccUG	0201 EchTor	*P. carotovorum* subsp. *carotovorum*	*P. chrysanthemi*	*P. carotovorum* subsp. *atrosepticum*
Gram reaction	–	–	–	–	–	–	–	–	–
Staining									
KOH reaction	–	–	–	–	–	–			
Morphology of cell	Rod	Rod	Rod	Rot	Rod	Rod	Rod	Rod	Rod
Colour of colony on NAS medium	White-grey	Yellow	White-grey	White-grey	White-grey	White-grey	ND	ND	ND
Tobacco HR	+	+	+	+	+	+	ND	ND	ND
Spore production	–	–	–	–	–	–	–	–	–
Fluorescent pigment	–	–	–	–	–	–	ND	ND	ND
Oxidase	–	–	–	–	–	–	–	–	–
Oxidative/ Fermentative Test	O/F	O/F	O/F	O/F	O/F	O/F	O/F	O/F	O/F
Catalase	+	+	+	+	+	+	+	+	+
Levan	–	–	–	–	–	–	ND	ND	ND
Arginine dihydrolase	–	–	–	–	–	+/–	–	d	–
Indol production	–	–	–	–	–	+	–	+	–
Nitrite from nitrate	+	+	+	+	+	+	+	+	
Growth at 41°C	–	–	–	–	–	+	40°C – 37°C +	40°C ND 37°C +	40 and 37°C–
5% NaCl tolerance	+	+	+	+	+	+	V	–	ND
Hydrolysis of:									
Gelatin	–	(+)	–	–	–	+	Gelatinase +	Gelatinase +	Gelatinase–
Starch	–	–	–	–	–	–	–	–	ND
Acid production from:									
Glucose	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	–	+	–	+
Lactose	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	ND	+
Sorbitol	–	–	–	–	–	–	–	–	–

+, Positive; –, negative; (+) slowly, after 3 weeks; ND, no data; d: 11–89% of strains are positive, v: strain instability (not equivalent to 'd'),
[a]Type strains according to Brenner et al. (2005).

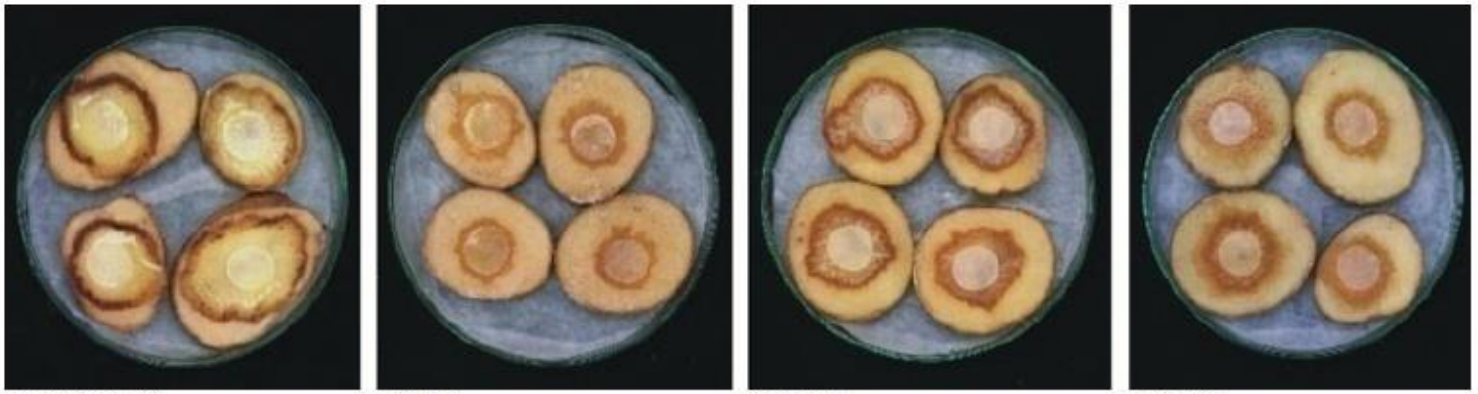
Mikiciński *et al.*,2009

Phenotypic characteristic of pectolytic bacteria associated with soft rot of dieffenbachia

***Reference strains:**
853 – *Pectobacterium carotovorum* subsp. *carotovorum*,
LMG 8337 – *Chryseobacterium indologenes*),
RIPF X02 – *Xanthomonas axonopodis* pv. *dieffenbachiae*.

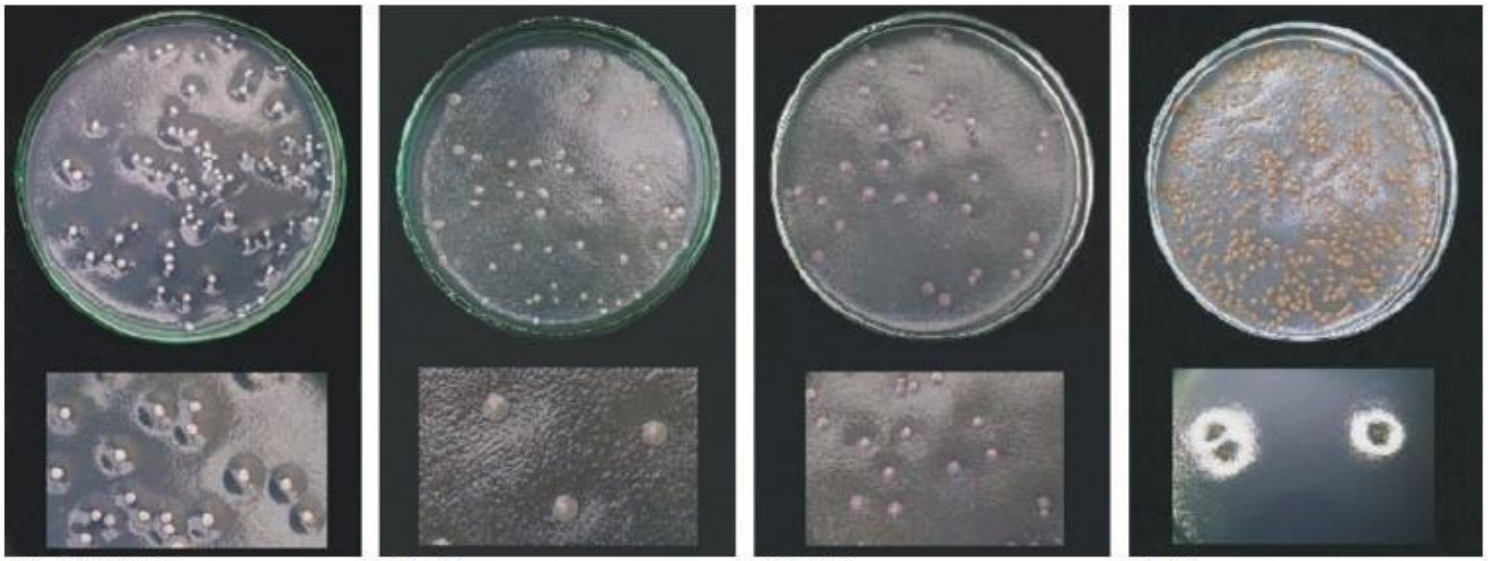
Test	Gray-white colony		Orange colony			
	DLO1.1	853*	2DLO2.3	DLO2.2	LMG 8337*	RIPF X02*
Gram reaction:						
staining	+	–	–	–	–	–
KOH reaction	+	–	–	–	–	–
L-alanine aminopeptydase	+	–	–	–	–	–
Spore production	+	–	–	–	–	–
Cell morphology	rod	rod	rod	rod	rod	rod
Fluorescent pigment	–	–	–	–	–	–
Oxidase	–	–	+	+	+	–
Catalase	+	+	+	+	+	+
Oxidative/fermentative test (O/F)	O/F	O/F	O	O	O	O
Levan	–	–	–	–	–	–
Hydrolysis of:						
gelatin	+	–	+	+	+	+
starch	–	–	+	+	+	–
aesculin	–	+	–/+	+	–/+	+
Nitrate reduction	+	–	–	–	–	–
Arginine dihydrolase	–	–	–/+	–	–	–
Acid production from:						
D-glucose	+	+	+	–/+	–/+	+
lactose	–	+	–	–	–	–/+
D-mannitol	+	+	–	–	–	–
D-sorbitol	–	–	–	–	–	–
D-raffinose	–	+	–	–	–	–/+
D-xylose	+	+	+	–	–	–/+
Pectolytic activity on CVP	+	+	+	+	–	+

Mikiciński *et al.*,2010



Isolates 2M, 4M (*Pectobacterium carotovorum*) | 7M, 8M (*Pseudomonas marginalis*) | 6M, 10M (*Pseudomonas veronii*) | 1M, 5M (*Chryseobacterium indologenes*)

Fig. 1 Potato tuber slice test of pectolytic activity using filter paper discs soaked with inoculum of tested isolate



2M, 4M, 9M, 853 (*Pectobacterium carotovorum*) | 7M, 8M (*Pseudomonas marginalis*) | 6M, 10M (*Pseudomonas veronii*) | 1M, 5M (*Chryseobacterium indologenes*)

Fig. 2 Pectolytic activity of studied isolates on CVP medium

Mikiciński *et al.*,2009

Pathogenicity test

Flavobacterium spp. and *Chryseobacterium* sp.

- **Pathogenicity test on detached dieffenbachia leaf petioles:**

A. Negative control,

B. Inoculation with *Chryseobacterium vrystaatense* (isolates DLO2.2), and

C. Inoculation with two *Flavobacterium* spp. (isolates 2DLO2.3).

Mikiciński *et al.*,2010

Bacterial association with rotting
Primary pathogens

Brenneria quercina	Rotting of acorn
Trinickia caryophylli (ex. *Burkholderia caryophylli*)	Stem rot/wilt of carnation
Burkholderia gladioli pv. *agaricicola*	Soft rot of mushrooms (*Agaricus bisporis*)
Chryseobacterium indologenes	Soft rot of calla lily tubers
Clavibacter m. susbsp. *sepedonicus*	Bacterial ring rot of potato
Dickeya chrysanthemi	Bacterial soft rot on wide hosts
Dickeya paradisiaca	Brown-black root rot of banana
Enterobacter cloacae	Garden onion bulb rot
Enterobacter dissolvens	Corn stalk rot
Erwinia persicina	Red-brown fruit rot of cucumber, banana & tomato
Erwinia psidii	Branch, flower and fruit rot of guava, Eucalyptus
Erwinia rhapontici	Crown rot of rhubarb; pink seed of bean, pea & wheat
Janthinobacterium agaricidamnosum	Soft rot of *Agaricus bisporus*
Pantoea ananatis	Fruit let brown rot of pineapple
Pantoea ananatis subsp. *uredovora*	A common plant pathogen of fruits and vegetables causing soft rot diseases

Bacterial association with rotting
Primary pathogens

Pantoea stewartii* subsp. *indologenes	**Rot of pineapple**
***Pantoea cypripedii* (ex. *Pectobacterium cypripedii*)**	**Brown rot of cypripedium and other orchids**
Pectobacterium cacticida	**Bacterial necrosis of giant cactus**
P. atrosepticm	**Black leg disease on potato, tomato and chicory**
P. betavasculorum	**Soft rot of beet**
P. carotovorum* subsp. *carotovorum	**Rotting of potato and sugar beet; soft rot of calla lily tubers**
P. carotovorum* subsp. *odoriferum	**Odorous soft rot of Endive and chicory**
P. wasabiae	**Rotting of Japanese horse-radish**
Pseudomonas cichorii	**Lettuce rot**
Pseudomonas marginalis* pv. *alfalfae	**Brown rot of alfalfa or Lucerne plants**
Pseudomonas marginalis* pv. *marginalis	**Brown rot of lettuce; Soft rot of calla lily tubers**
Pseudomonas marginalis* pv. *pastinacae	**Parsnip root rot**
Pseudomonas syringae* pv. *lapsa	**Bacterial stalk rot of field corn and sugarcane**

Note: *Pectobacterium wasabiae* was transfer to *Pectobacterium parmentieri* sp. nov. (Khayi *et al.*,2016)

Bacterial association with rotting
Primary pathogens

Pseudomonas viridiflava	**A multi host plant pathogen: Leaf rot of cauliflower, stem rot of poppy, internal stem rot of tomato, Grape panicle rot, etc.**
Pseudomonas veronii	**Soft rot of calla lily tubers**
Ralstonia solanacearum	**Brown rot of potato**
Streptomyces ipomoeae	**Soil rot of sweet potato**
Xanthomonas campestris* pv. *campestris	**Black rot of crucifers**
Xanthomonas citri* pv. *malvacearum	**Bacterial blight/Boll rot of cotton**
Xanthomonas melonis	**Soft rot of fruit of watermelon**
Xanthomonas pisi	**Root rot of pea**

Yellow-orange pigmented bacteria
Plant Pathogenic Bacteria

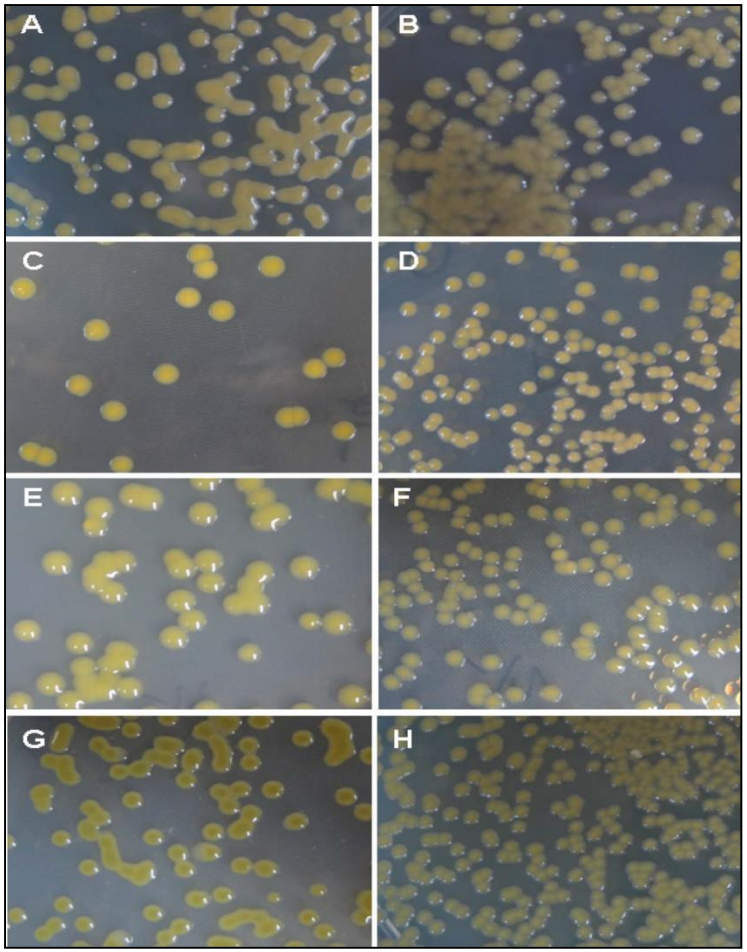
- *Chryseobacterium indologenes*: orange
- *Clavibacter*: cream/yellow
- *Curtobacterium*: yellow
- **Cytophaga*/ *Flexibacter* or *Myxobacteria*: yellow/orange
- * *Flavobacterium*: yellow/orange
- *Gluconobacter*: White with yellow or brown centre
- *Hydrogenophaga*: yellow
- *Leifsonia*: non-pigmented/yellow
- *Nocardia*: Orchre-yellow
- *Pantoea*: cream to orange-yellow
- *Pseudomonas fluorescens* bv. II: Cream to yellowish with greenish centre
- *Rathayibacter*: yellow
- *Rhizobacter*: white/yellowish
- *Rhodococcus*: orange
- *Sphingomonas*: yellow
- *Xanthomonas*: yellow
- *Xylophilus*: pale yellow

Chryseobacterium

*Common in soil, water and from other sources

Colony morphology of the yellow pigmented bacteria from rice seed

- Colony morphology of seed-borne non-pathogenic, yellow-colony forming bacteria isolated from rice seeds on Suwa's medium after incubation at 30°C for 96 hrs:
- (A) *X. oryzae* pv. *oryzae* (leaf blight),
- (B) *X. oryzae* pv. *oryzicola* (leaf streak),
- (C-H) similar to the colony morphology of *Xoo* and *Xoc*.



Lee and Vera Cruz,2014

Flavobacterium
Yellow-orange bacterium

- Yellow to orange pigmented species.
- Cell Shape: Straight rods
- Flagella arrangement: None
- Salt tolerance (% NaCl, w/v): Not determined
- Maximum temperature (°C): 42
- Nitrate reduced to nitrite: Differs among species
- Anaerobic growth with nitrate: Negative for all species
- Indole: Differs among species
- Urease: Differs among species
- Esculin hydrolyzed: Positive for all species
- Cause discolorations on surfaces of meat, butter, poultry, milk, etc.
- Starch hydrolysis in pectolytic (*Flavobacterium*) *Cytohaga* spp. is negative.

Bergey's Manual of Systematic Bacteriology
Second Edition, Volume Two, Part B, 2005.

Pathogenicity test
Dipping Method
Flavobacterium defluvii and *F. johnsoniae* group

- The Dip method is the easiest and most uniformed of all of the inoculation.
- Dieffenbachia leaf petioles were disinfected with 50% ethanol, washed in sterile distilled water and cut in 5 cm long segments.
- The base of each segment was dipped up to 1 cm into a 10^9 cfu per 1 ml suspension of the isolate tested in a sterile test tube.
- The top of each test tube was covered with aluminum foil.
- Left: inoculation of isolate 2DLO2.3.
- Right: negative control.

Mikiciński *et al.*,2010;..

Phenotypic characteristics
Flavobacterium spp.

- *Flavobacterium* species have been isolated from diverse habitats such as fresh- and salt-water, soil, sediment, sea ice, diseased fish and microbial mats.
- Gliding motility typical of the *Cytophaga -Flavobacterium* group, is not observed in *F. frigoris* & *F. micromati*.
- 1, *F. degerlachei*
- 2, *F. micromati*
- 3, *F. frigoris*
- 4, *F. gillisiae*
- 5, *F. pectinovorum*
- 6, *F. saccharophilum*.

Characteristic	1	2	3	4	5	6
Growth on:						
Trypticase soy agar	+	(+)	+	+	+	+
Nutrient agar	+	+	−	+	+	+
Growth at 25 °C on agar	+	(+)	(+)	(+)	+	+
Flexirubin-type pigment	−	−	−	−	+	+
Voges–Proskauer reaction	−	+	−	−	ND	ND
Glucose utilization	+	−	+	+	+	+
Acid from carbohydrates	−	−	−	+	+	+
Degradation of:						
Gelatin	−	−	−	−	+	+
Casein	−	−	+	+	+	+
Starch	+	−	+	+	+	+
CM-cellulose	−	−	−	−	+	+
Agar	−	−	−	−	−	+
Alginate	−	−	−	−	+	ND
Pectin	−	−	−	−	+	+
Chitin	−	−	−	+	+	−
DNA	−	−	−	−	+	−
Tyrosine	−	−	+	−	+	+
β-Galactosidase activity	−	−	−	−	+	+
H_2S production	−	−	−	−	ND	+
Nitrate reduction	−	−	v	−	+	+
Mean G+C content (mol%)	34	33	34	32	35	33

Trappen *et al.*,2004

Yellow pigmented bacteria

Characters used to differentiate *Flavobacterium* from three plant pathogenic bacteria

Character	*Xanthomonas*	*Pseudomonas*	*Flavobacterium*[a]	*Pantoea*[E]
Flagella	1 polar	>1 polar	None	Peritrichous
Xanthomonadin	Yes	No	No	No
Fluorescence	No	Yes	No	No
Litmus milk	Alk	Alk	Unchanged	ND
Growth at 40°C	Yes	No	Yes	Yes
Levan from sucrose	Yes	Yes	No	No
H_2S from cysteine	Yes	No	No	No
Oxidase	Negative	Negative[b]	Positive	Negative[c]
Phosphatase	Negative	ND[d]	Positive	ND
Fermentative	No	No	No	Yes
Growth on: 0.1% TTC	No	Yes	Yes	Yes

[a] Some are orange-colored; [b]*P. cichorii* is positive; [c]Colonies of *P. citrea* and some strains of *P. agglomerans* are generally white; [d]ND=Not determined. [E]Strains gave slimy colonies on levan and KB media.

Type and diseases caused by plant bacterial pathogens

All major and minor genera

Phytopathogenic Bacteria

All major and minor genera

1. *Acetobacter*	16. *Gibbsiella*	31. *Pseudomonas*
2. *Acidovorax*	17. *Gluconobacter*	32. *Ralstonia*
3. *Agrobacterium* (*Rhizobium*)	18. *Herbaspirillum*	33. *Rathayibacter*
4. *Bacillus*	19. *Janthinobacterium*	34. *Rhizobacter*
5. *Bradyrhizobium*	20. *Janibacter*	35. *Sphingomonas*
6. *Brenneria*	21. *Klebsiella*	36. *Rhodococcus*
7. *Burkholderia*	22. *Leifsonia*	37. *Samsonia*
8. *Chryseobacterium*	23. *Lonsdalea*	38. *Serratia*
9. *Clavibacter*	24. '*candidatus* Liberibacter'	39. *Spiroplasma*
10. *Clostridium*	25. *Nocardia*	40. *Streptomyces*
11. *Curtobacterium*	26. *Paenibacillus*	41. *Tatumella*
12. *Dickeya*	27. *Pantoea*	42. *Xanthomonas*
13. *Enterobacter*	28. *Pectobacterium*	43. *Xylella*
14. *Erwinia*	29. '*candidatus* Phlomobacter '	44. *Xylophilus*
15. *Ewingella*	30. '*candidatus* Phytoplasma'	

Type and diseases caused by plant bacterial pathogens

Uncommon/unusual/minor/less well known bacteria

Phytopathogenic Bacteria
All minor genera

1. *Acetobacter*	15. *Ewingella*	
	16. *Gibbsiella*	
	17. *Gluconobacter*	
4. *Bacillus*	18. *Herbaspirillum*	32. *Rhizobacter*
5. *Bradyrhizobium*	19. *Janthinobacterium*	33. *Sphingomonas*
	20. *Janibacer*	
	21. *Klebsiella*	
8. *Chryseobacterium*		36. *Serratia*
	23. *Nocardia*	
10. *Clostridium*	24. *Paenibacillus*	
		39. *Tatumella*

- Some *Pantoea citrea* strains (cause pineapple pink disease) were reclassified as *Tatumella*.
- *Chryseobacterium indologenes* causes soft rot of calla lily tubers.
- *Gibbsiella quercinecans* and *Ewingella* cause oak and mushroom diseases, respectively.
- *Janibacter melonis* a Gram+ve bacterium isolated from the inner part of abnormally spoiled oriental melon in Korea.

Diseases caused by minor plant bacterial pathogens

Gram Negative Bacteria

- *Acetobacter* spp.: Marbling disease of pineapple fruit.
- *Gluconobacter oxydans* : Pink disease of pineapple fruit.
- *Herbaspirillum* : Usually vibroid, occasionally helical, motile by 1 to 3 flagella at one or both poles. Strict aerobes, associated with roots. cream, mucoid colonies, aerobic. Mottled stripe of sugarcane.
- *Janthinobacterium* : Rods, polar flagella, grey-white colonies, aerobic): Soft rot of cultivated mushroom *A. bisporus*.
- *Rhizobacter*: Rods, polar and/or peritrichous flagella, white, yellowish colonies, aerobic): Bacterial gall and corky root of carrot (*Daucus carota*).
- *Serratia marcescens* : Rods, peritrichous flagella, non-pigmented colonies, some red, pink or orange, facultative anareobic: Cucurbit yellow vine disease.
- *Sphingomonas*: Rods, 1 polar, lateral subpolar flagella or non-motile, white/yellowish colonies. *S . melonis*, the causal of Yellow spanish melon.
- *Bradyrhizobium*: Rods motile by one polar or subpolar flagellum. *Bradyrhizobium japonicum* is a phytosymbiotic, nitrogen-fixing bacterium on soybean. Under high soil nitrogen conditions, it produces phytotoxic rhizobitoxin and damages its host.

Differential criteria used to characterize the minor bacteria

Combination of rapid tests from different resources

Pathogen	Gram reaction/ cell shape	Colony color	O/F	Motility	Fluorescences	H_2S production	VP	Methyl red	NO_3-NO_2	Indole
Acetobacter aceti	-	Pale to off-white	Aerobe	Motile	-	-			+	-
Chryseobacterium indologenes	-	Yellow-orange	Aerobe	Non-motile	-				+	+/-
Clostridium	+ Rods	Whitish to brownish color	Anaerobe	Motile	-	-			V	
Janthinobacterium agaricidamnosum	- Round ended rods	Beige	Aerobe	Motile	-	+	-		-	-
Gluconobacter oxydans	- Rods	White with yellow, or even brown center	Aerobe	Non-motile/motile	-	-	-		-	-
Klebsiella pneumoniae	- Short rod	Light gray; stickiness	F. anaerobe	Non-motile	-	-	+	-	+	-
Herbaspirillum rubrisubalbicans	- Rods	Cream	Aerobe	Motile	-	-	-		+	-

Differential criteria used to characterize the minor bacteria

Combination of rapid tests from different resources

Pathogen	Gram reaction/ cell shape	Colony color	O/F	Motility	Fluore scence	H_2S produc tion	VP	Methyl red	NO_3-NO_2	Indole
Nocardia vaccinii	+ Filament	Ochre-yellow colonies	Aerobe	Non-motile	-	+			+	-
Rhizobacter dauci	- Rods	White or yellowish white	Aerobe	Motile/no motile	-	+	-	-	+	-
Serratia marcescens	- Rods, swarming	Red	F. anaerobe	Motile*	-	-	+	+/-	-	-
Sphingomonas melonis	- Rods	Deep yellow	Aerobe	Motile	-	-	+		-	-

* Tubes containing semi-solid nutrient agar (agar concentration reduced to 0.75 %, w/v) were stab inoculated and growth was examined visually for motile swarms for up to 5 days.

Marbling disease of pineapple

Pantoea ananatis and *Acetobacter* spp.

Pineapple marbling disease showing red-brown granular flesh with woody consistency

Sindhu *et al.*,2012; Johan,2015

Differential characteristics between closely related members of the genus *Acetobacter*

Strains/species: 1, *A. nitrogenifigens* RG1^T; 2, *A. cibinongensis* LMG 21418^T; 3, *A. orleanensis*, 4, *A. malorum* LMG 1746^T; 5, *A. aceti*, 6, *A. tropicalis*, 7, *A. oeni* B13^T; 8, *A. indonesiensis*, 9, *A. orientalis* LMG 21417^T; 10, *A. estunensis*, 11, *A. cereviseae*. w, Weak; v, variable; ND, not determined; +, positive; –, negative. Data for species 1 and 5 are from this study. Motility data for species 4 and 11 are taken from Cleenwerck *et al.* (2002). All other data are from Silva *et al.* (2006) unless otherwise indicated.

Characteristic	**1***	**2**	**3**	**4**	**5***	**6**	**7**	**8**	**9**	**10**	**11**
Growth in ammonium with ethanol	+	w	–	–	+	–	–	–	–	+	–
Growth in presence of 10 % ethanol	+	–	–	–	–	–	+	–	–	–	ND
Growth on carbon sources											
Maltose	–	–	v	–	v	+	–	+	w	v	–
Methanol	–	–	–	–	–	–	–	–	w	–	–
Growth on YE +30 % D-glucose	+	+	–	–	–	–	–	–	–	–	–
Growth on N-free LGI plate*	+	–	–	–	–	–	–	–	–	–	–
Motility	Polar-flagellation	ND	ND	Non-motile	ND	ND	Peritrichous flagellation	ND	ND	ND	Non-motile
DNA G+C content (mol %)	64.1	53.8–54.5	55.7–58.1	57.2	56.9–58.3	55.6–56.2	58.1	54.0–54.2	52.0–52.8	59.2–60.2	56.0–57.6

Dutta and Gachhui,2006

Minor plant bacterial pathogens

Gluconobacter oxydans

- Strict aerobic rods, never fermentative.
- They are typically ovoid or rod-shaped.
- Non-motile or lophotrichously flagellated.
- Negative for NO_3, gelatinase, oxidase and H_2S production.
- The colonies are circular with a diameter of 3 mm, raised or convex, and are regularly edged.
- They may be white in color, yellow, or even brown towards the middle of the colony.
- They prefer pHs of 5.5-6.0.
- Cause brown rot on ornamental pear and inner fruit decay symptoms in pomegranate fruits.

Minor plant bacterial pathogens
Herbaspirillum rubrisubalbicans

- The genus *Herbaspirillum* (formerly *Pseudomonas*) contains only a single plant pathogenic member, *H. rubrisubalbicans*, which has been described as a "mild plant pathogen" (Baldani *et al.*,1996).
- *Herbaspirillum rubrisubalbicans*, is a mild plant pathogen causing:

1. Bacterial stripe disease of sugarcane,
2. Cereal root disease (particularly *Herbaspirillum seropedicae*),
3. Leaf spot and blight of greenhouse tomato seedlings.

Fegan,2006

Minor plant bacterial pathogens
Herbaspirillum rubrisubalbicans

- Due to the mild nature of the disease caused by *H. rubrisubalbicans* little research has been conducted into the disease.
- Many of species in this genus, including *Herbaspirillum rubisubalbicans*, exhibit the ability to fix nitrogen (endophytic diazotrophic organisms).
- Gram-negative, aerobic, motile by means of several polar flagella.
- Cream-colored colonies on nutrient agar.
- Colonies on 2% glucose-peptone agar are mucoid
- PHB is accumulated.
- Oxidase, gelatinase, amylase, H_2S production, nitrate reduction and Tween 80 hydrolysis were negative.

Obradovic *et al.*,2007;..

Differential phenotypic characteristics of some *Herbaspirillum* species *H. rubrisubalbicans* (strain No.4)

Characteristic	1	2	3	4	5	6	7
Cell size (μm)	0·4–0·5x1·4–1·8	0·6–0·7x1·5–5·0	0·5–0·7x1·4–1·8	0·6–0·7x1·5–5·0	0·4x1·8	0·6–0·8x2·0–5·0	0·5x1·6
Optimal temperature for growth (°C)	25–37	34	30–37	30	25–30	28	28
Optimal pH for growth	6·0–7·0	5·3–8·0	6·0–7·0	6·7–6·8	Neutrophilic	5·0–8·0	NA
API 20NE (50CH) test:							
Nitrate reduction	–	+	+	+	–	–	–
Nitrite reduction	+	–	–	–	+	+	–
Growth on:							
Glucose	–	–	+	+	–	–	+
Arginine	+	(+)	+	+	+	–	–
Mannose	(+)	–	(+)	–	–	–	+
N-Acetyl-D-glucosamine	+	+	+	–	+	+	+
Erythritol	–	–	–	+	+	–	–
Rhamnose	–	–	+	–	–	–	+
Inositol	–	+	–	–	–	–	–
D-Arabinose	–	+	–	+	+	+	+
Galactose	–	–	+	+	–	–	–
DNA G+C content (mol%)	62·9	64–65	61–65	62–63	63·3	60–62	57·0

Ding and Yokota,2004

Minor plant bacterial pathogens
Janthinobacterium agaricidamnosum

- There is a high degree of genotypic similarity between members of the genus *Janthinobacterium* and *Herbaspirillum*.
- *Janthinobacterium agaricidamnosum* sp. nov., a novel bacterium has been found that causes a soft rot disease of *Agaricus bisporus*, the cultivated mushroom(Lincoln *et al.*,1999).

Janthinobacterium agaricidamnosum
Soft rot disease of *Agaricus bisporus*

- Severe rotting of button mushroom (*Agaricus bisporus*), caused by *Janthinobacterium agaricidamnosum*, stimulated by very a high (relative) humidity (88-91%) that is necessary during cultivation of the mushroom.
- The bacterium is easily spread by water and contact.

Janse,2006

Minor plant bacterial pathogens
Janthinobacterium agaricidamnosum

- Low, convex, round, beige colonies with motile cells.
- No pigment was produced initially but in older cultures, a buff, non-fluorescent pigment was seen.
- No growth occurs below pH 5 or in media containing 2.9% NaCl.
- Acid is produced from glucose.
- Oxidase-positive, catalase-positive.
- Indole, VP, aesculin, arginine dihydrolase, urease are negative.
- Nitrate and nitrite are not reduced.
- Assimilation of arabinose and maltose are negative.
- Assimilation of citrate is variable.
- No violet pigment is produced.
- No white line was produced.
- Resistant to penicillin G (10 µg/disc) and vancomycin (30 µg/disc).

Bergey's Manual,2005; Lincoln *et al.*,1999

Minor plant bacterial pathogens
Klebsiella

- Members of the family *Enterobacteriaceae*.
- Gram negative; Nonmotile (except *K. mobilis*).
- Short rod, often oval-shaped; Facultatively anaerobic.
- The *Klebsiella* is suspected when a large, mucoid, light gray, convex, circular colony is seen on agar plates.
- Producing more or less dome-shaped, glistening colonies of varying degrees of stickiness depending on the strain and the composition of the medium.
- The production of the large capsules gives rise to large mucoid colonies of a viscid consistency.
- Oxidase, arginine dihydrolase & indole are negative.
- The Voges-Proskauer test is usually positive. Urease positive.
- Pectate hydrolyzed by *K. oxytoca*.
- These pectolytic plant-associated bacteria (*K. planticola* and *K. pneumoniae*) are rarely associated with soft rots:

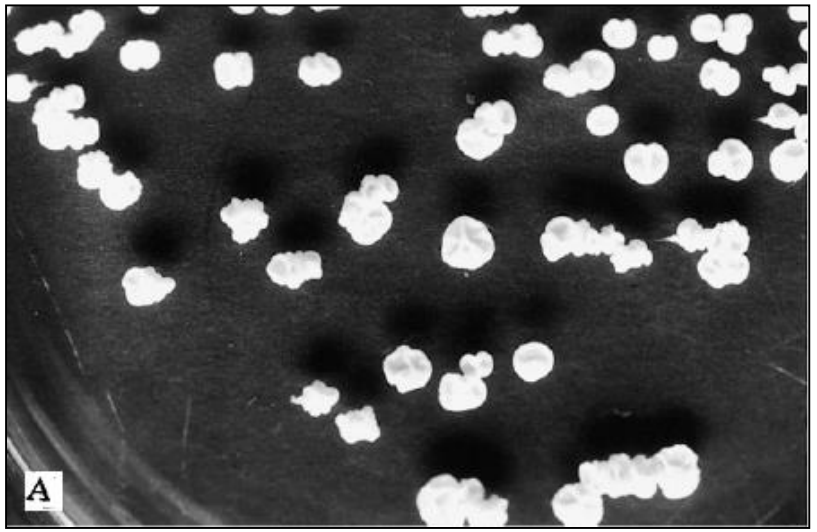
1. *Klebsiella oxytoca* associated with elms bacterial wetwood.
2. *Klebsiella planticola* associated with soft rots.
3. *Klebsiella pneumoniae* associated with soft rots.

Minor plant bacterial pathogens

Galls on carrot roots

Rhizobacter dauci

- Cells stain Gram negative.
- White or yellowish white, plicated, tough, or viscid colonies on agar plates;
- Straight to slightly curved rods, non sporeforming, encapsulated rods;
- PHB are formed;
- Nonmotile or motile by polar or lateral flagella.
- Motile by polar flagella or lateral flagella or both but motile cells are rare in the populations;
- Aerobic;
- No growth at 35°C;
- Growth occurs at pH between 5.0 and 9.0.
- Causing galls on carrot roots.



Plicated colonies of *R. dauci* on DPPG agar

Bergey's Manual of Systematic Bacteriology,2005

Carrot gall

Rhizobacter dauci

Scortichini,2006

Minor plant bacterial pathogens
Rhizobacter dauci

- **Positive for:**
- Oxidase, catalase, urease and lipase (Tween 80).
- H_2S from cysteine and auxin,
- Reduction of nitrate to nitrite.
- Max. NaCl tolerance is 0.7%.
- The type species *Rhizobacter dauci* occurs in soil and is a plant pathogen, causing galls on carrot roots in nature.
- **Negative for:**
- Denitrification, methyl red, and production of arginine dihydrolase, nitrogenase, fluorescent pigment, indole and acetoin formation.
- No HR reaction.

Bergey's Manual of Systematic Bacteriology,2005

Minor plant bacterial pathogens
Bradyrhizobium betae sp. nov

- Rivas *et al.*,2004, isolated several endophytic slow-growing bacterial strains from tumors of two deformed plants.
- Phylogenetic analysis of the DNA regions coding 16S rRNA revealed that these strains belonged to the genus *Bradyrhizobium*.
- Sequence analysis of the 16S-23S rDNA intergenic spacer region indicated that these novel strains formed a homogeneous group different from all *Bradyrhizobium* species previously described.
- They proposed the name *B. betae* sp. nov. for the novel species of *Bradyrhizobium.*

Moliszewska *et al.*,2016

Minor plant bacterial pathogens

Serratia spp.

S. marcescens causes cucurbit yellow vine disease

- *Serratia* spp. is also an opportunistic human pathogen that can be found in plants.
- Some may be red, pink or orange (Holt *et al.*,1994).
- Oxidase, arginine dihydrolase, urease, lipase, indole, H_2S production and phenylalanine deaminase are negative.
- *S. marcescens* is distinguished from other genera of Enterobacteriaceae by its production of three special enzymes DNAse, lipase and gelatinase.
- *S. marcescens* as a soil inhabitant bacterium with high chitinase activity was used as efficient antagonist in biological control of fungal pathogens.

Also See BLOs section in diagnosis-Part 3.

Minor plant bacterial pathogens
Serratia proteamaculans

King Protea (*Protea cynaroides*)

1. *Serratia marcesencs* causing cucurbit yellow vine disease (*Cucurbita maxima*).
2. *S. rubidaea* isolated from plants (vegetables) but mostly coconuts.
3. *S. plymuthica* has been isolated from various plants.
4. *S. proteamaculans* causes leaf-spot disease of the tropical plant King Protea(*Protea cynaroides*).
5. Also causes amber disease in the grass grub pest *Costelytra zealandica*.

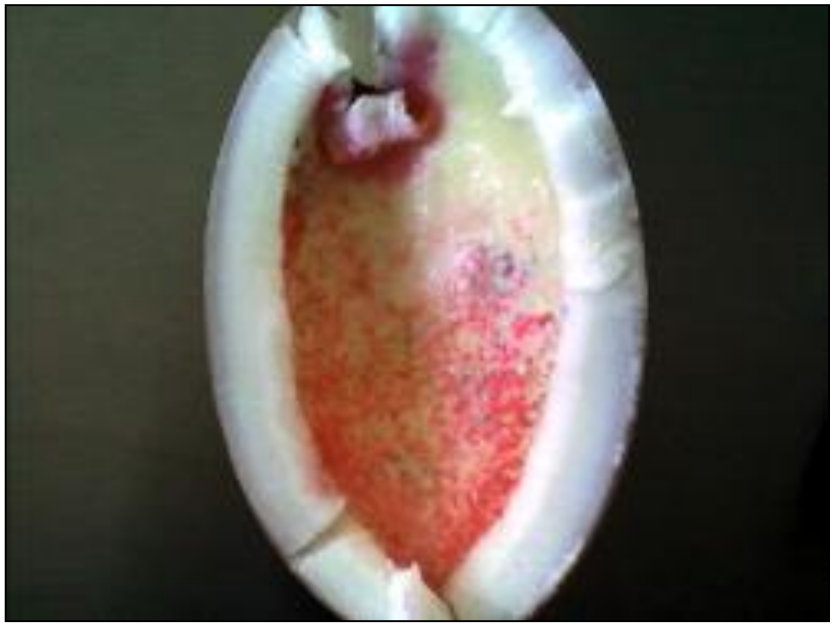
- *Serratia proteamaculans* 568, was isolated as a root endophyte from *Populus trichocarpa* and found to promote plant growth.

ABIS Encyclopedia;..

Serratia spp.

Two species of red-pigmented: *S. marcescens* and *S. rubidaea*

S. marcescens on an agar plate.



S. rubidaea contaminated coconut after opening and slicing.

Wikipedia,2018; ABIS Encyclopedia

Cucurbit yellow vine disease in squash

Serratia marcesencs

Courtesy A. Wayadande

Differentiation of *Serratia* spp. with other genera

	Genus *Serratia*	*Erwinia herbicola*	*Enterobacter cloacae*	'Pectobacteria'	*Klebsiella mobilis*
Carbon source utilization					
L-Rhamnose	−	+	+	v	+
D-Glucosamine	+ or (+)	+	v	v	−
D-Glucuronate	+ or (+)	+	+	−	+
Maltose	+	+	+	−	+
Caprylate	+ or (+)	−	−	−	−
L- and D-Alanine	+ or (+)	v	(+)	−	+
4-Aminobutyrate	+ or (+)	(+)	(v)	−	(v)
L-Proline	+	+	+	−	+
L-Tyrosine	+ or (+)	−	−	−	(+)
Kynurenate	+ or (+)	−	v	−	+
Putrescine	+	−	v	−	+
Yellow pigment	−	+	v	−	−
Gluconate test	+	−	+	−	+
Iodoacetate test	+	+	(+)	−	(+)
Arginine decarboxylase	−	−	+	v	−
Gelatin hydrolysis	+	−	−	v	−
Tributyrin hydrolysis	+ or (+)	−	−	−	−
Tween 60 or 80	+	−	−	v	−
DNAase	+ or (+)	−	−	v	−
Pectin hydrolysis	−	−	−	+	−

+, Over 90 % of strains positive (early reading); (+), over 90 % of strains positive (late reading); −, less than 10 % of strains positive (late reading); v, 10 to 90 % positive (early and late reading); (v), 10 to 90 % positive (late reading only).

Grimont *et al.*,1976

Differentiation of *Serratia* spp. with other genera

	Pigment	ADH	LDC	ODC	Urea hydrolysis	Voges - Proskauer	Lactose fermentation	Cellobiose fermentation	L-arabinose fermentation	D-xylose fermentation
S. entomophila	-	-	-	-	-	+	-	-	-	d
S. ficaria	-	-	-	-	-	[+]	[-]	+	+	+
S. fonticola	-	-	+	+	[-]	-	+	-	+	[+]
S. grimesii	-	+	+	+	-	d	-	-	+	+
S. liquefaciens	-	-	+	+	-	+	-	-	+	+
S. marcescens	d	-	+	+	[-]	+	-	-	-	-
S. nematodiphila	+	+	+	+	-	+	-	-	+	-
S. odorifera 1	-	-	+	+	-	d	d	+	+	+
S. odorifera 2	-	-	+	-	-	+	+	+	+	+
S. plymuthica	d	-	-	-	-	[+]	[+]	[+]	+	+
S. proteamaculans	-	-	+	+	-	[+]	-	-	+	+
S. rubidaea	+	-	d	-	-	+	+	+	+	+
S. ureilytica	-	+	+	+	+	+	-	nd	-	+
S. glossinae *	-	nd	+	+	+	+	nd	-	nd	nd

Legend: + positive 90-100%, - negative 90-100%, [+] positive 75-89%, [-] negative 75-89%, d positive 25-74% of strains, nd - not determined, ADH - arginine dihydrolase, LDC -lysine decarboxylase, ODC - ornithine decarboxylase, * *S glossinae* is not included in ABIS database

Two species of red-pigmented: *S. marcescens* and *S. rubidaea*.

Odour was noted as being either a distinctive odour resembling vegetable matter, unlike the somewhat fishy or faecal odour of most *Serratia* strains (Grimont *et al.*,1976).

Minor plant bacterial pathogens
Sphingomonas spp.

- Type genus of the family *Sphingomonadaceae*.
- *Sphingomonas* is a Gram-negative, nonsporeforming, motile, strictly aerobic bacterium that typically produces non diffusible yellow- or off-white-pigmented colonies.
- Sphingomonads are of biotechnological interest due to:

1. Their ability to degrade various xenobiotic (pollutants) substances,
2. Their potential to produce useful exopolysaccharides and carotenoids.

- *S. melonis*: Brown spots on yellow Spanish melon fruits (fruit spot).

Asker *et al.*,2007;..

The genus *Sphingomonas*
Plant associated species

- Some plant associated species are:
- *Sphingomonas asaccharolytica*
- *Sphingomonas mali*
- *Sphingomonas pruni*
- *Sphingomonas rosa*
- *Sphingomonas roseiflava*

Diseases caused by minor plant bacterial pathogens

Gram Positive Bacteria

- *Bacillus megaterium* : Associated with elms bacterial wetwood.
- *Paenibacillus* : Resemble *Bacillus* and cause rots of terminal buds in date palms. *Paenibacillus polymyxa* causes a brown root rot on the storage roots e.g. ginseng root.
- *Clostridium* spp.: Cause soft rot diseases on root crops such as potato & carrot; Also pectolytic clostridia associating with oak tissue discoloration and bacterial wetwood.
- Some produce pink pigment on potato Infusion Agar (Schaad *et al.*,2001)
- *Nocardia vaccinii* : Causing galls on blueberry plants.

Minor plant bacterial pathogens
Paenibacillus spp.

- *Bacillus* spp. (including *Paenibacillus*, a new described genus which include species once assigned to the genus *Bacillus*) are one of most frequently encountering bacteria from soil that decompose organic materials.
- Some of them cause minor plant diseases: Rot of tubers, seeds, and seedlings and white stripe of wheat.
- They are also known as biocontrol agents for diseases and insect pests.
- *Paenibacillus polymyxa* causes a brown root rot on the storage roots e.g. ginseng root.
- *Paenibacillus polymyxa* was also isolated from calla lily tubers showing soft rot in commercial plantations in Poland.

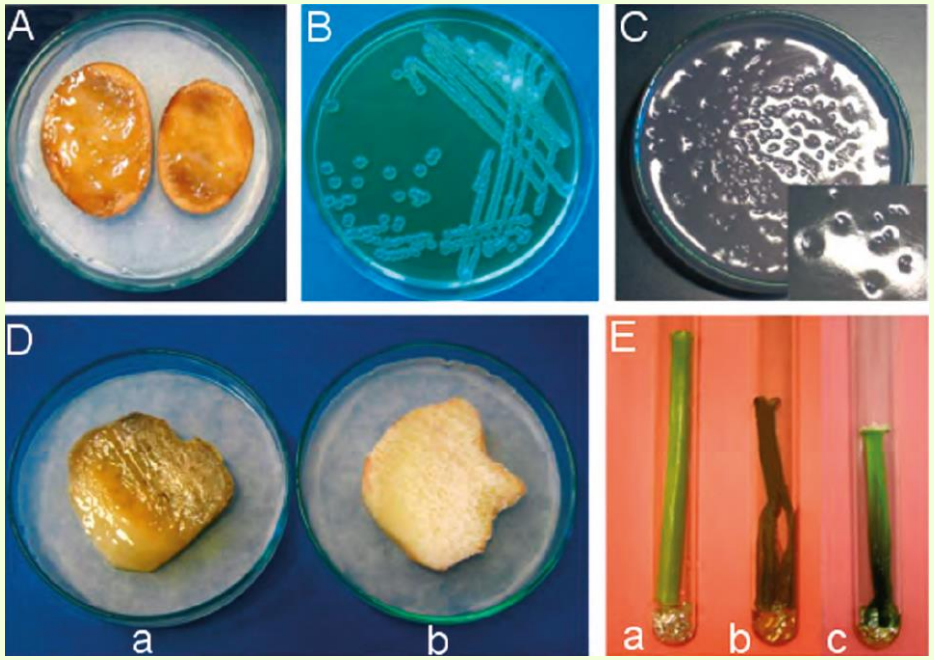
Minor plant bacterial pathogens

Soft rot disease of calla lily

Paenibacillus polymyxa

A. Pectolytic activity of *Paenibacillus polymyxa* isolate 15M on potato slice 24 h after inoculation;
B. Colony morphology of isolate 15M on NAS medium.
C. Characteristic pits around isolate 15M colonies on CVP medium;
D. Rotted calla lily tuber slice 72 h after inoculation with isolate 15M (a), Water control (b);
E. Pathogenicity on calla lily leaf petiole cv. Treasure. Rotted segments 120 h after inoculation with isolate 15M (b, c); water control (a).

Paenibacillus polymyxa pathogen of calla lily.



Mikicinski *et al.*,2010

Minor plant bacterial pathogens
Pathogenicity on calla lily leaf petiole
Paenibacillus polymyxa

- Pathogenicity on calla lily leaf petiole cv. Treasure was carried out on Rotted segments 120 h after inoculation with isolate 15M (b, c); water control (a).
- Leaf petioles of calla cv. Treasure were disinfected with 50% ethanol and cut into 10-cm-long segments.
- The method described by Snijder and van Tuyl (2002), with slight modification, was applied.
- One tip of each petiole segment was dipped for 20 sec in the bacterial suspension (10^8 CFU ml^{-1}) of each of pectolytic isolate and then placed into a sterile glass tube (2 x 20 cm) with water and sterile aluminium foil ball on the bottom to avoid direct contact of the petiole with water.
- The top of each test tube was covered with aluminium foil.
- Each isolate was tested on 30 petiole segments (6 replications x 5 units), which were incubated for 5 days at 24°C.
- The presence of rot was observed and measured each day.

Mikicinski *et al.*,2010

Minor plant bacterial pathogens

Paenibacillus spp.

Plant associated *Paenibacillus* species

- In 1993 Ash *et al*., proposed that members of "group 3" within the genus *Bacillus* should be transferred to the genus *Paenibacillus*, for which they proposed *Paenibacillus polymyxa* as the type species.
- Two of plant associated *Paenibacillus* species are:

1. *Paenibacillus graminis* isolated from plant roots, soil and food.
2. *Paenibacillus odorifer* isolated from plant roots, soil and food.

Comparison of biochemical characteristics of *Paenibacillus* strains *Paenibacillus* spp.

	Paenibacillus sp. CAR114	*Paenibacillus* sp. CAS34	*P. riograndensis* SBR5^T	*P. sonchi* X19-5^T	*P. jilunlii* DSM 23019^T	*P. graminis* DSM 15220^T	*P. polymyxa* ATCC 842^T
Acid production from:							
D-Glucose	+	+	+	+	+	+	+
D-Sorbitol	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Catalase activity	+	+	+	+	+	+	+
Growth at 3% NaCl	V	V	V	V	V	V	+
Growth at 5% NaCl	–	–	–	–	–	–	–
Hydrolysis of casein	+	+	+	+	+	+	+
Hydrolysis of starch	–	+	+	–	–	+	+
Nitrate reduction	+	+	+	+	V	+	+
Voges–Proskauer test	–	–	–	–	–	–	–

Biochemical data obtained in this study. The results of biochemical tests are shown as positive "+," negative "–," or variable "V." Results obtained in our study that contradicted those obtained in other reports are highlighted in gray (more details can be found in Supplementary Table S2).

Sant'Anna *et al.*,2017

Comparison of biochemical characteristics of *Paenibacillus* strains *Paenibacillus* spp.

- **Supplementary Table S4. Comparison of phenotypic characteristics of *Paenibacillus* species among different reports**

Supplementary Table S4. Comparison of phenotypic characteristics of *Paenibacillus* species among different reports.

	Paenibacillus sp. CAR114	*Paenibacillus* sp. CAS34	*P. niograndensis* SBR5^T			*P. sonchi* X19-5^T			*P. jilunlii* DSM 23019^T			*P. graminis* DSM 15220^T						*P. polymyxa* ATCC 842^T					
	1*	1*	1	2*	3	1	3	4*	1	3*	6	1	3	5*	8#	10	11	1	7	8	9	11	12
Acid production from:																							
D-Glucose	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	ND
D-Sorbitol	+	+	+	ND	+	+	-	ND	+	-	V	+	-	-	-	-	-	+	+	+	+	+	-
D-Xylose	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	+	ND	+	+	-	-	ND	ND
Glycerol	+	+	+	+	+	+	-	ND	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	-	-	+	+	+	+	+	+	ND	+	ND	+	+	+	+	+	ND
Maltose	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	ND
Mannitol	+	+	+	+	ND	+	ND	ND	+	+	+	+	ND	+	ND	+	ND	+	+	ND	ND	ND	ND
Sucrose	+	+	+	+	+	+	-	-	+	+	+	+	-	+	-	ND	-	+	+	+	+	+	+
Catalase activity	+	+	+	ND	ND	+	ND	-	+	-	+	+	ND	+	+	ND	ND	+	ND	+	ND	ND	ND
Growth at 3% NaCl	V	V	V	ND	+	V	+	+	V	+	+	V	+	ND	ND	ND	ND	+	ND	+	ND	ND	+
Growth at 5% NaCl	-	-	-	-	ND	-	ND	-	-	-	-	-	ND	ND	ND	ND	ND	-	ND	-	ND	ND	ND
Hydrolysis of casein	+	+	+	-	-	+	+	+	+	-	V	+	-	ND	-	-	-	+	+	-	-	-	+
Hydrolysis of starch	-	+	+	+	+	-	-	+	-	+	+	+	-	+	-	+	ND	+	+	-	-	ND	+
Nitrate reduction	+	+	+	-	-	+	-	-	v	+	+	+	+	+	+	+	ND	+	+	+	+	ND	+
Voges-Proskauer test	-	-	-	-	+	-	+	+	-	-	-	-	-	ND	-	ND	-	-	+	+	+	+	+

Biochemical data obtained from this study and from literature. Asterisks indicate the original report. Underlined numbers refer to data reproduced from other studies, as detailed below. Hash symbol indicates the biochemical profile containing typographic errors. The results of biochemical tests are shown as positive "+", negative "-", variable "V", or not determined "ND". Results obtained in our study that contradicted those obtained in other reports are highlighted in gray.

Sant'Anna *et al.*,2017

Minor plant bacterial pathogens
Clostridium

- Gram positive, spore forming, rod-shaped non-motile bacteria.
- Anaerobic; oxidase negative.
- Some *clostridia* can grow slightly near surface of culture but no *Clostridium* species can produce endospores in presence of oxygen.
- Not able to reduce sulfate to sulfide.
- Capable to actively degrade pectic substances, particularly when the plant tissues were affected prior with aerobic and anaerobic bacteria such as *Pectobacterium*, *Bacillus*, *Dickeya*, etc.

Schaad *et al.*,2001

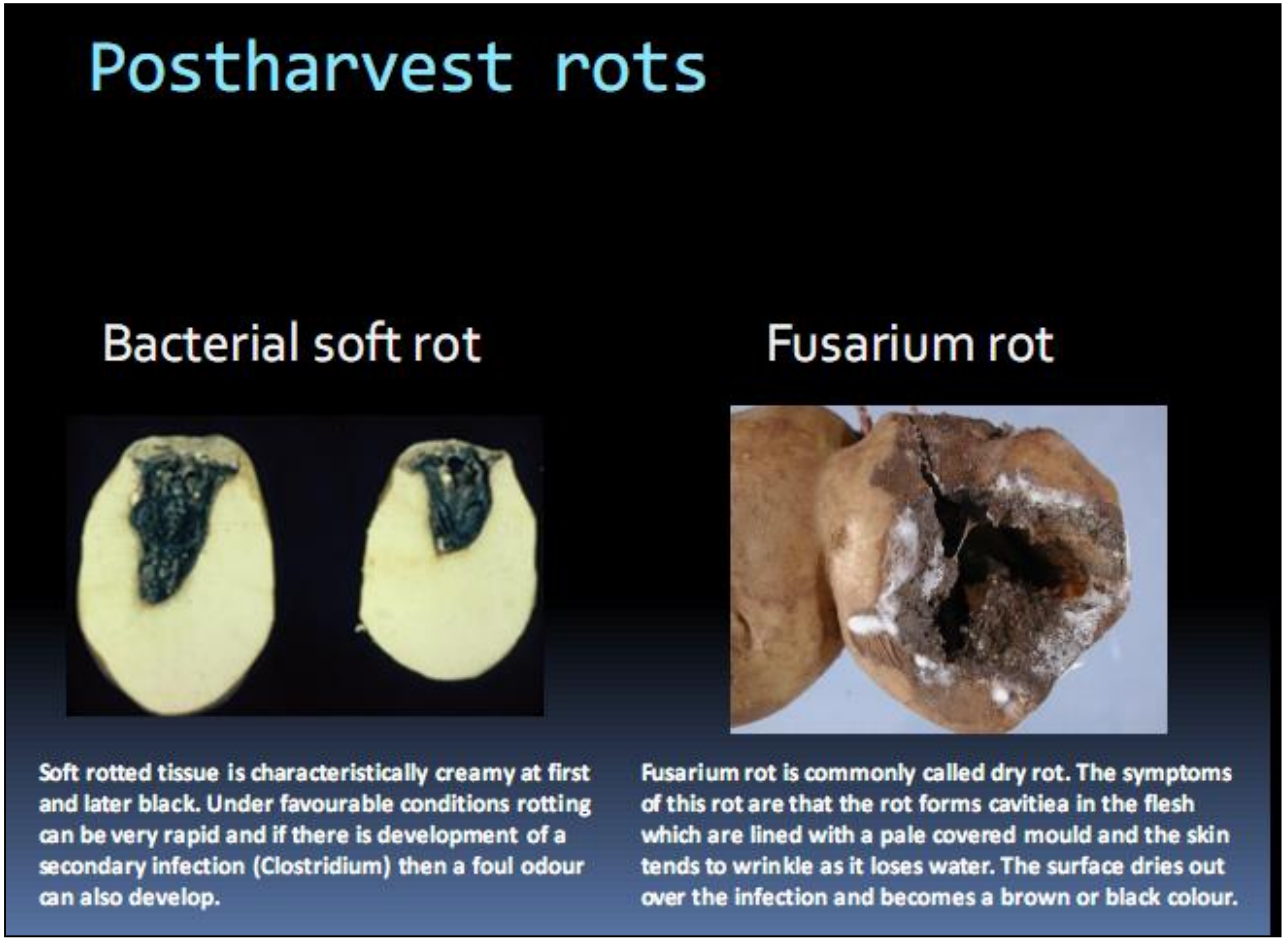
Minor plant bacterial pathogens
Clostridium

- Clostridia have been found in cavity spot of carrots.
- Potato affected by clostridal soft rot show a soft white or creamy rot.
- Causing rot of stored tubers and leaves and wetwood of elm and poplar.
- A pectolytic *clostridium* isolated from wetwood was identified as *C. butyricum*.
- A *clostridium* associated with discolored tissues in living oaks has been described as *C. quercicolum*.

Schaad *et al.*,2001

Minor plant bacterial pathogens

Clostridium



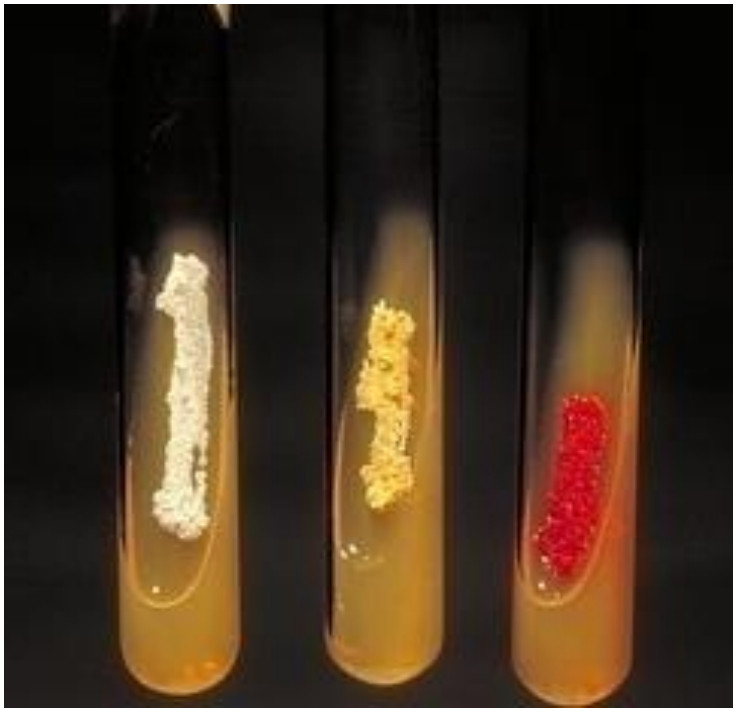
Etienne Gagnon

Minor plant bacterial pathogens

Nocardia spp.

Nocardia vaccinii cause galls on blueberry plants

- *Nocardia vaccinii* cause galls on blueberry plants.
- *Nocardia* are found worldwide in soil that is rich with organic matter.
- Gram-positive branching filamentous aerobic bacteria growing on the surface of and penetrating agar media.
- Ochre-yellow colonies, Some are orange, pink, red, purple brown,.. with smooth, wrinkled surface.
- Soluble brown or yellow pigment may be produced.
- Flagella absent.
- Catalase positive.



Ochre(ow. kuh): golden-yellow or light yellow brown color.

Wikipedia,2007;Holt *et al.*,1994

Minor plant bacterial pathogens

Nocardia vaccinii cause galls on blueberry plants

- Aerobic;
- Partially acid-fast;
- Colonies have sparse aerial mycelia;
- Decomposition of casein, tyrosine and xanthine are negative;
- Catalase, phosphatase, esculin, urease and nitrate reductase are positive.
- Aesculin was hydrolyzed; Acetate ,L-Rhamnose, xylose and mannitol were utilized as sole sources of carbon and energy.
- L-Rhamnose, xylose and mannitol but not adonitol & iso-Butanol were utilized as sole carbon.
- Resistance to lysozyme.

Type and diseases caused by plant bacterial pathogens

Common/major/high-impact/most important bacteria

Phytopathogenic Bacteria
All major genera

		31. *Pseudomonas*
2. *Acidovorax*		32. *Ralstonia*
3. *Agrobacterium* (*Rhizobium*)		33. *Rathayibacter*
6. *Brenneria*		36. *Rhodococcus*
7. *Burkholderia*	22. *Leifsonia*	37. *Samsonia*
	23. *Lonsdalea*	
9. *Clavibacter*	24. '*candidatus* Liberibacter'	39. *Spiroplasma*
		40. *Streptomyces*
11. *Curtobacterium*		
12. *Dickeya*	27. *Pantoea*	42. *Xanthomonas*
13. *Enterobacter*	28. *Pectobacterium*	43. *Xylella*
14. *Erwinia*	29. '*candidatus* Phlomobacter'	44. *Xylophilus*
	30. '*candidatus* Phytoplasma'	

Brenneria quercina was transferred into a new genus/new combination *Lonsdalea quercina*.

Bacteria and Archaea
The two dominant microbes on earth

- Most life on earth is microbial, belonging to the 'Bacteria' and 'Archaea' domains, and to numerous lineages of microbial 'Eukaryota' (e.g. protists).
- Less than 1% of microbes are cultivable, and therefore diversity was vastly underestimated by traditional microbiological methods.
- The known extent of microbial diversity has grown and continues to grow rapidly as sequence-based methods are used to characterize microbes.

Yilmaz *et al.*,2013

Plant Pathogenic Bacteria
Genera/species of pathogenic bacteria

- Of the over 15,000 identified species of bacteria most are saprophytic.
- There are around 200 species of phytopathogenic bacteria:
- Almost all of them are within the plant, on its surface, in plant debris, or in the soil.
- Most phytopathogenic bacteria are aerobic (live in the presence of oxygen), and
- Some are facultative anaerobes which can grow with or without oxygen.

Agrios,2005; Cooper,2006

Type and diseases caused by plant bacterial pathogens

- Plant pathogenic bacteria are found in all parts of the world.
- These are most frequent and severe in tropical and subtropical countries, where warm and humid conditions are ideal for bacterial growth.

Buonaurio,2008

Type and diseases caused by plant bacterial pathogens

- Bacterial diseases are essentially characterized by symptoms of leaf and fruit spots, cankers, blights, vascular wilts, rots and tumors.
- They are most often caused by Gram-negative bacteria belonging to the Proteobacteria phylum, including:

1. *Xanthomonadaceae*,
2. *Pseudomonadaceae*, and
3. *Enterobacteriaceae* families.

Buonaurio,2008

Top 10 plant pathogenic bacteria in molecular plant pathology

The list includes, in rank order

Rank	Bacterial pathogen
1	*Pseudomonas syringae* pathovars
2	*Ralstonia solanacearum*
3	*Agrobacterium tumefaciens*
4	*Xanthomonas oryzae* pv. *oryzae*
5	*Xanthomonas campestris* pathovars
6	*Xanthomonas axonopodis* pathovars
7	*Erwinia amylovora*
8	*Xylella fastidiosa*
9	*Dickeya* (*dadantii* and *solani*)
10	*Pectobacterium carotovorum* (and *Pectobacterium atrosepticum*)

Bacteria garnering honourable mentions for just missing out on the Top 10 include *Clavibacter michiganensis* (*michiganensis* and *sepedonicus*), *Pseudomonas savastanoi* and *Candidatus Liberibacter asiaticus*.

Mansfield *et al.*,2012

Plant Pathogenic Bacteria
Formerly eubacteria

- The genera of the "easily cultured" bacterial group:
- Gram positive:
- *Clavibacter* (prev. *Corynebacterium*);
- *Rhodococcus*, *Clostridium*, *Bacillus*, and
- *Streptomyces* (potential spore former).

"Super" species

- Gram negative:
- *Agrobacterium tumefaciens*
- *Xanthomonas campestris*
- *Pseudomonas syringae*
- *Erwinia amylovora*
- *Pectobacterium carotovorum*
- *Ralstonia solanacearum*

Plant Pathogenic Bacteria

Gram negative/positive bacteria

A: With Cell Walls

i. Easily cultured (~200 species)

- All have a saprophytic stage in nature;
- Generally rod shaped;
- Gram positive and negative.

ii. Not easily cultured

- Probably no saprophytic stage in nature.
- Most are Gram negative. E.g. *Clavibacter* spp., the xylem inhabitating.
- The Gram negative *Xylella fastidiosa*, a fastidious xylem-limited bacterium, grows in xylem and in insect vector causes citrus varigated chlorosis, and related diseases.
- It first cultured in 1978 (Davis *et al.*,1972).

Plant Pathogenic Bacteria
Gram negative/positive bacteria

B. Without Cell Walls

1. Phytoplasma - 300 plant diseases;

- Found in phloem cells;
- Not yet cultured.
- First discovered in 1967(Doi *et al.*,1967)
- Associated with "yellows" diseases.
- Transmitted by insect vectors in which they multiply

2. *Spiroplasma* (*S. citri, S. phoeniceum,S. kunkelii*)

- First discovered in 1972 (Davis *et al.*,1972)
- Several cultured.
- Grows in phloem and in insect vector Cause diseases such as corn stunt previously attributed viruses.
- Transmitted by insect vectors.

S. phoeniceum causes periwinkle yellows. *Candidatus* Liberibacter is a genus of Gram-negative bacteria.

Diseases caused by plant bacterial pathogens

Gram Negative Bacteria

BACTERIA
DIVISION: GRACILICUTES CLASS: PROTEOBACTERIA
Family: Enterobacteriaceae (Gram-negative)
*Erwinia species
Fire blight, soft rot (most common on fleshy vegetables and fruit)
Pantoea species
Stewart's wilt of corn…spread by corn flea beetles
Serratia species (fastidious, phloem-inhabiting)
Yellow Vine Disease of Cucurbits…spread by squash bug
Sphingomonas melonis
Brown spot of yellow Spanish melon fruit

Cuppels,2007

Diseases caused by plant bacterial pathogens

Gram Negative Bacteria

BACTERIA
DIVISION: GRACILICUTES CLASS: PROTEOBACTERIA
Family: Pseudomonaceae (Gram-negative)
Acidovorax species
Leaf spot of watermelon, orchids and corn
*Pseudomonas species
Leqf spots, fruit spots, blights, vascular wilts, soft rots, galls
*Ralstonia species
Wilts of solanaceous crops
Rhizobacter (Bacterial gall of carrot) and Rhizomonas (Corky root of lettuce)
*Xanthomonas species
Leaf spots, fruit spots, blight, vascular wilts, citrus canker
Family: Rhizobiaceae (Gram-negative)
*Agrobacterium (Crown gall)
Family: still unnamed
Xylella (Leaf scorch and dieback on trees and vines)
Xylophilus species (Bacterial necrosis and canker of grape)

Diseases caused by plant bacterial pathogens

Gram Positive Bacteria

BACTERIA
DIVISION: FIRMICUTES CLASS: FIRMIBACTERIA (single-celled)
Bacillus (tuber rot)
Clostridium (tuber rot, wetwood of elm and poplar)
DIVISION: FIRMICUTES CLASS: THALLOBACTERIA (branched)
Arthrobacter (Bacterial blight of holly)
*Clavibacter (bacterial wilts in potato, tomato, alfalfa; ring rot of potato)
Leifsonia (Ratoon stunting of sugarcane)
Rhodococcus (Fasciation of ornamentals and sweet pea)
Streptomyces (Common scab of potato)
MOLLICUTES
DIVISION: TENERICUTES CLASS: MOLLICUTES
Family: Spiroplasmataceae
Spiroplasma (Corn stunt disease, citrus stubborn disease)
Family: still unnamed
Phytoplasma (Yellows diseases, decline diseases in trees, proliferation disease)

Flow Charts
Dichotomous keys

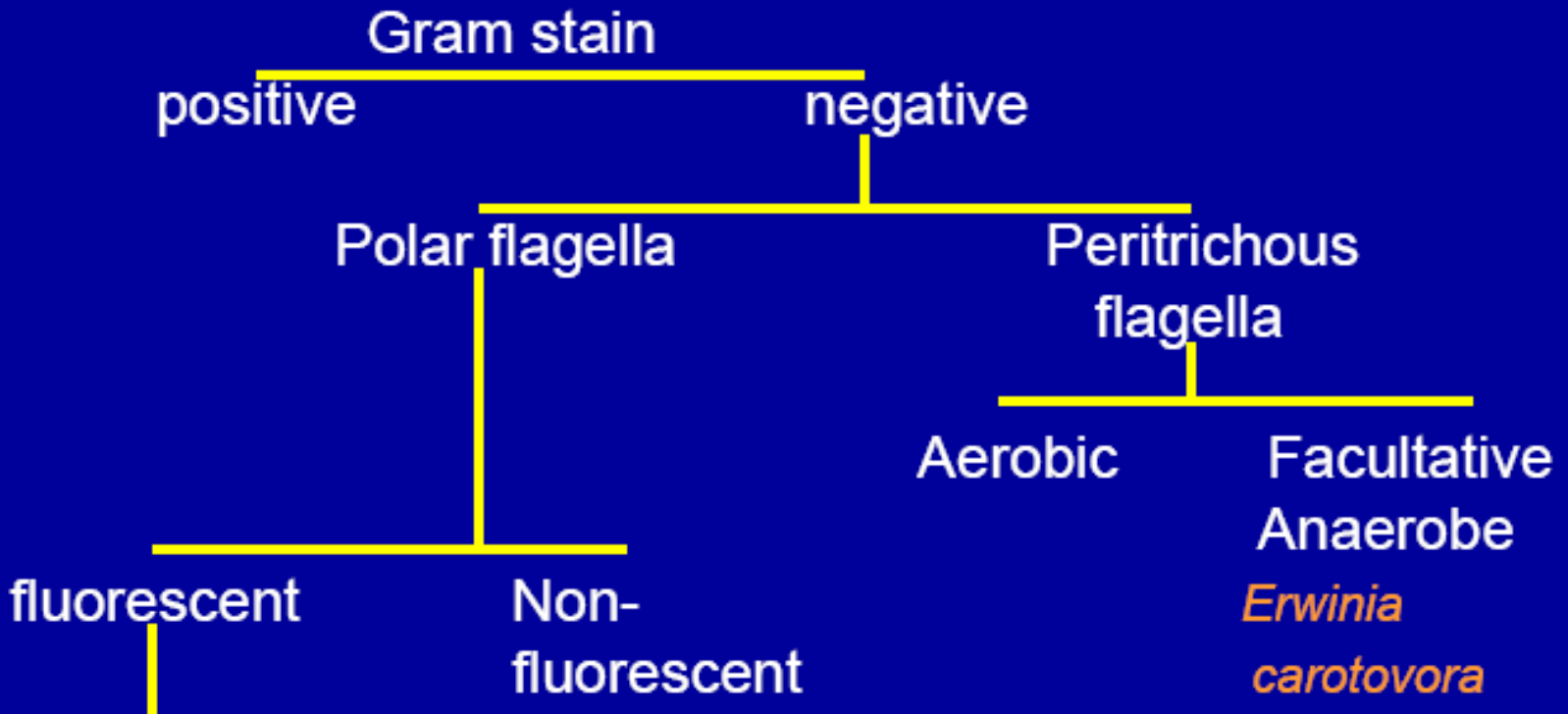
Flowcharts for Identifying Plant Pathogenic Bacteria

The Dichotomous key

- The first thing to prepare for an Unknown Identification exercise is to make a dichotomous key.
- The dichotomous key provides a way to separate bacteria by assessing their morphological and physiological characteristics, which will be valuable in their identification in clinical specimens.
- The dichotomous key presents a logical pathway to identify bacterial isolates without embarking in a very tedious (too long) route of using a great battery of biochemical and physiological tests, resulting in a large expense for the laboratory.

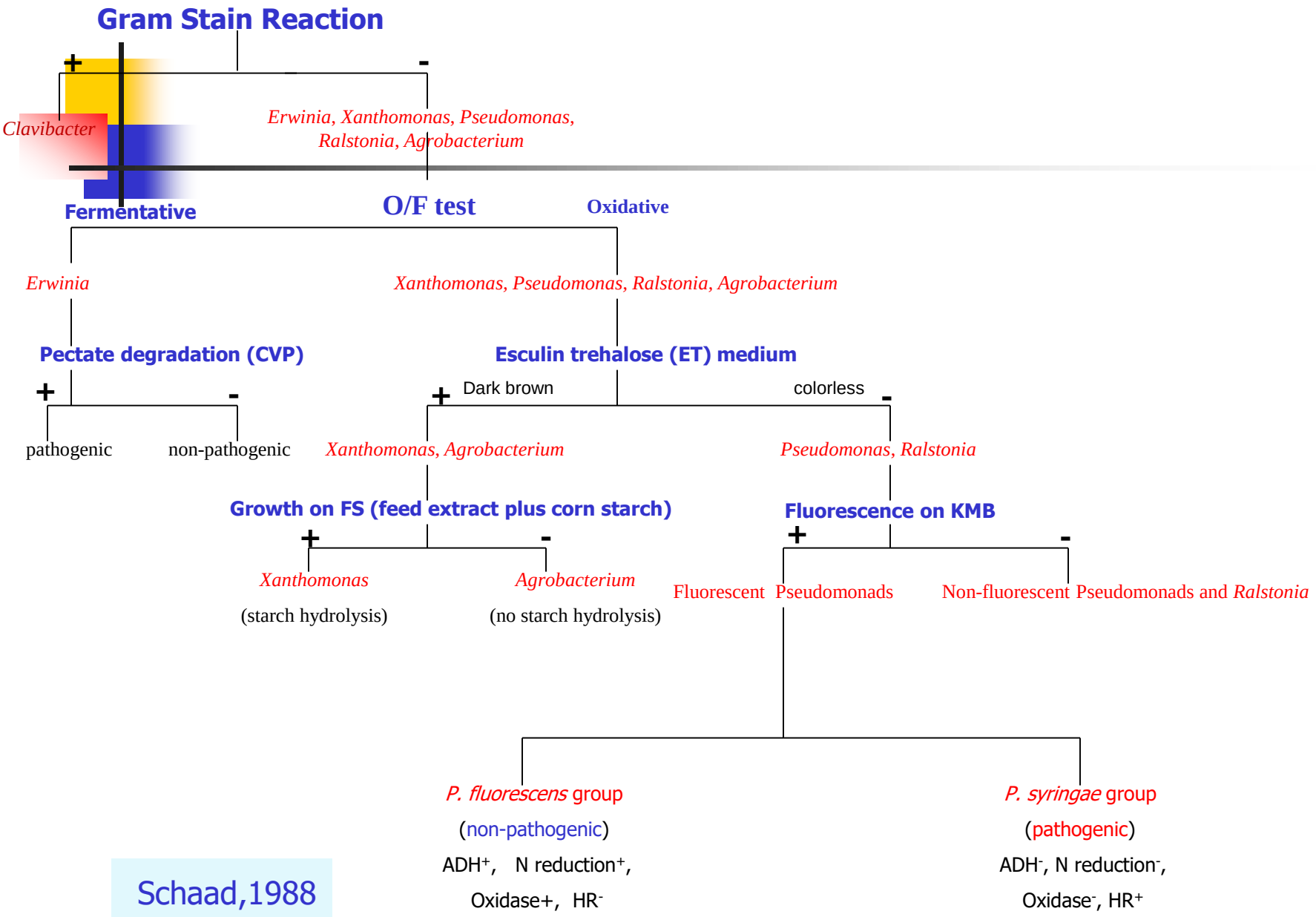
Ginés-Candelaria,2011;..

Identification of bacteria



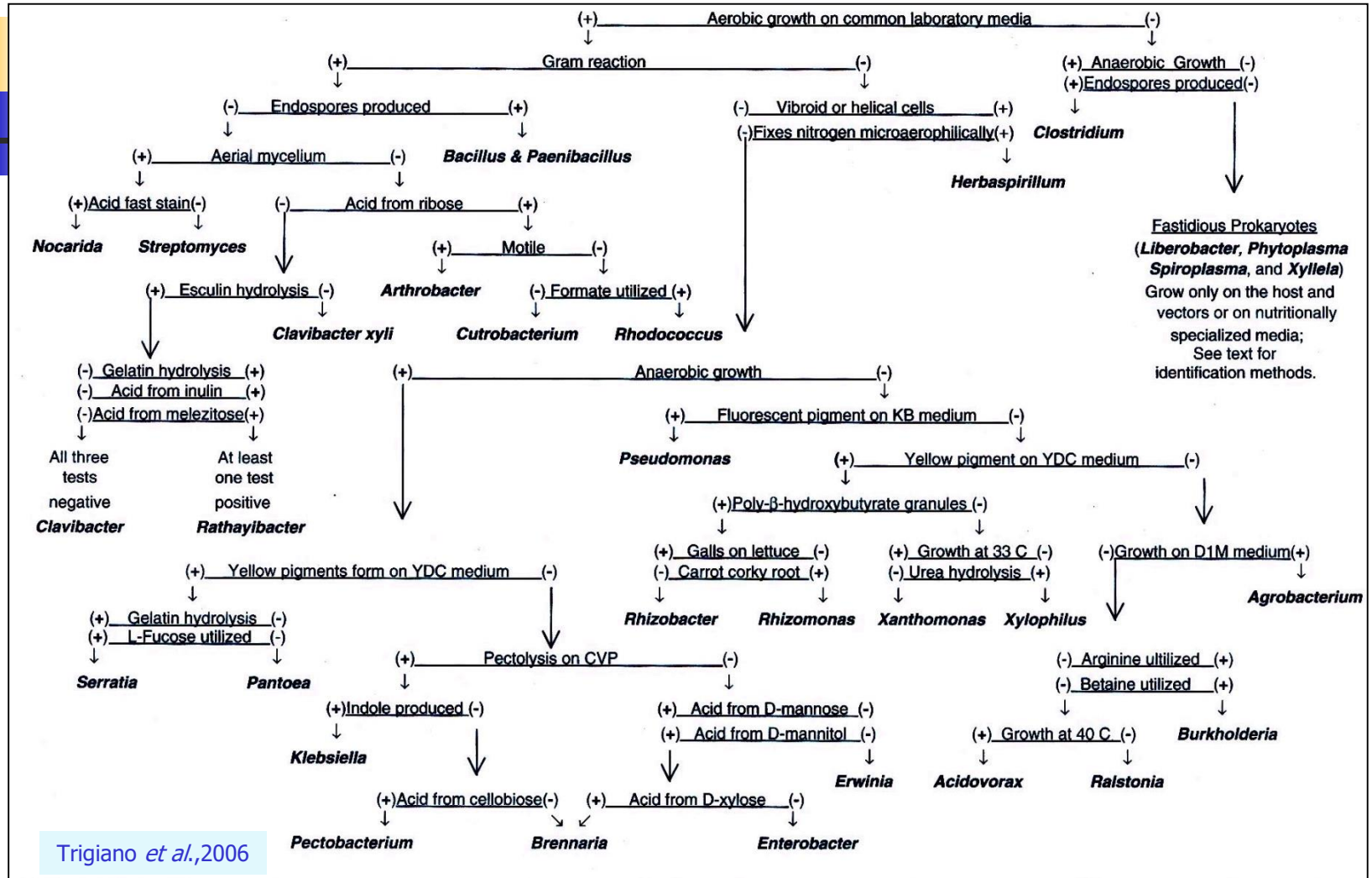
Identification of bacteria is commonly conducted by performing a series of tests and or observations on the bacterial strain

Flow Chart for Identification of Bacterial Genera



Schaad,1988

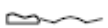
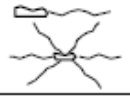
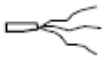
Flow Chart for Identification of Bacterial Genera



Trigiano *et al.*,2006

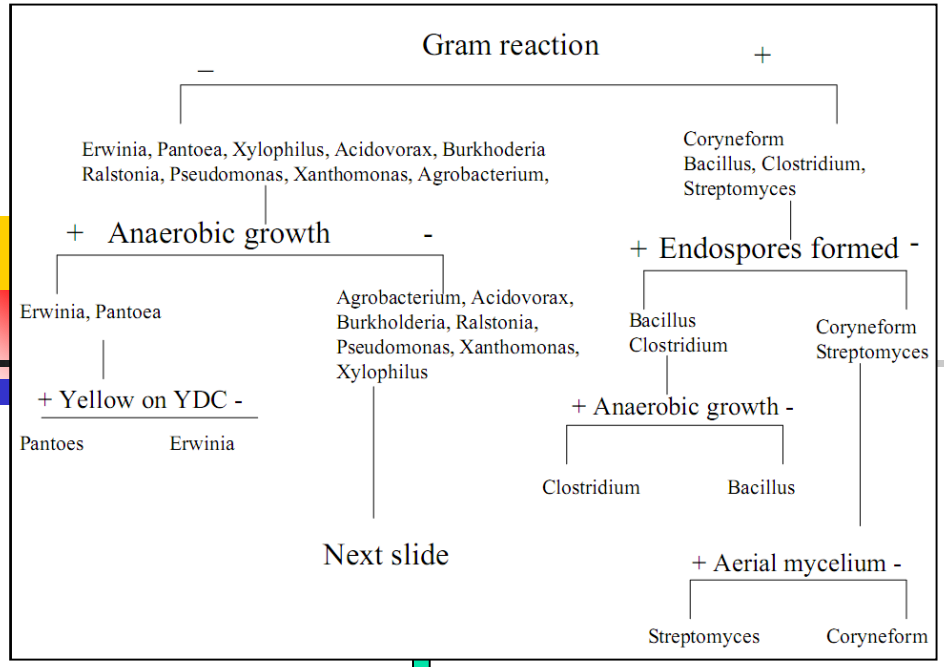
FIGURE 6.2 Flow chart to identify genera of phytopthogenic bacteria that grow readily on common laboratory media [based on Schaad et al. (2001) and Holt et al. (1994)]. Fastidious bacteria are not dealt with here. The genera *Acetobacter* and *Gluconobacter* contain opportunistic bacteria that affect fruit only and are not treated here. This scheme assumes that the bacteria tested are pathogens. In other words, Koch's postulates have been satisfied or approximated.

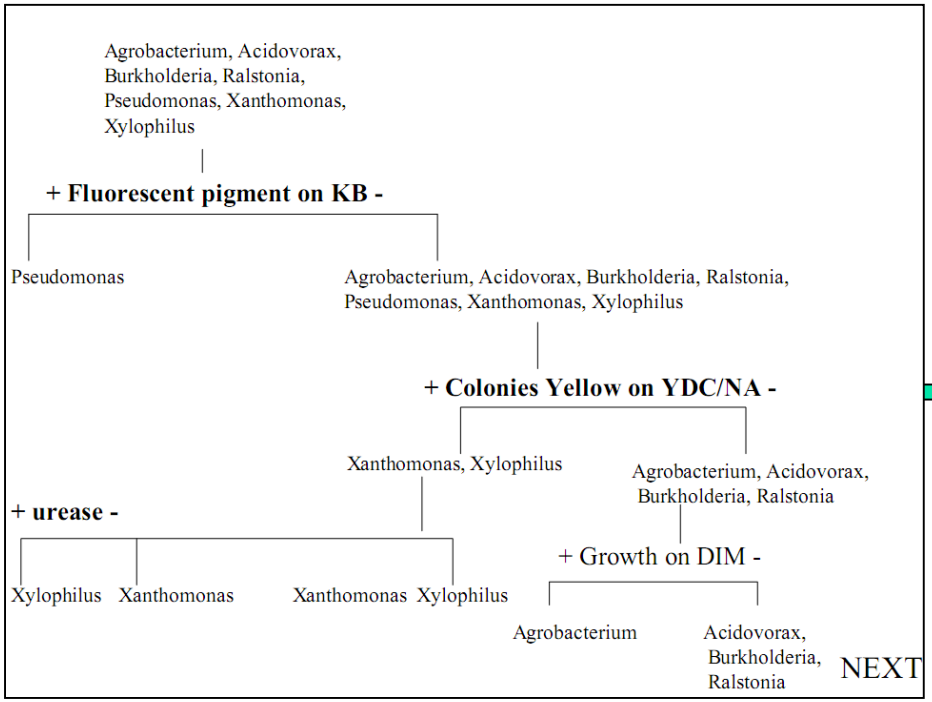
Rapid identification of bacterial genus

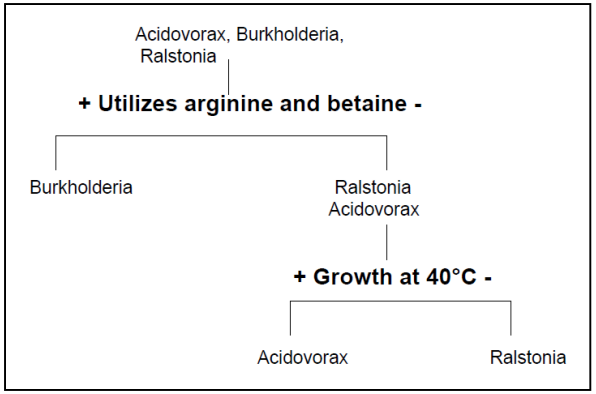
Gram	Utilisation du glucose (Hugh et Leifson)	Mobilit" Flagelles	Fluores-cence	R"duc tion des nitrates	Oxydase	HR sur Tabac	Genres	exemples d'Esp, ces ou de Groupes	Particularit"s
+	Oxydatif ou inadif	-	-	+ ou -	-	+	*Clavibacter*	*C. michiganensis*	Couleur variable suivant subsp. (subsp. nebraskensis : orange)
							Rhodococcus	*R. fascians*	Colonies d'aspect sec, orange vif
							Rhatayibacter	*R. rathayi*	Colonies jaunes
		+ 					*Curtobacterium*	*C. flaccumfaciens*	Couleur variable suivant pv. (pv. *oortii*: jaune)
-	(inacif)	+ 	-	+ ou -	+	+ ou -	*Agrobacterium*	*A. tumefaciens*	Colonies crème muqueuses
								A. vitis	
								Etc…	
	(inacif)	+ 	-	-	-	+ ou -	*Xanthomonas*	19 espèces + 66 pathovars	Colonies jaunes très muqueuses
	Oxydatif ou inadif (oxydatif)		+	-	-	+	*Pseudomonas*	1, 2 de Lelliott *et al.*[1]	Colonies blanc-crème
				+ ou -	+	+		3 *P. cichorii*	
				+ ou -	+	+ ou -		4, 5 (phytopathogènes et saprophytes)	
	(alcalinisant)	+ ou 	-	+ ou -	+	+ ou -	*Acidovorax*	*A. valerianellae*	Colonies blanches généralement ovales
	(inacif)			-	-		*Xylophilus*	*X. ampelinus*	Colonies jaunes, croissance très lente
	(inacif)			+	+		*Ralstonia*	*R. solanacearum*	Colonies crème
	(inactif)			+ ou -	+ ou -		*Burkholderia*	*B. caryophylli*	Colonies crème
	Fermentatif	+ 	-	+ ou -	-	+ ou -	*Erwinia*	*E. amylovora*	Colonies blanches muqueuses
								E. rhapontici	Colonies roses
							Pectobacterium	*P. carotovorum*	Colonies dentelées blanc-crème
							Brenneria	*B. rubrifaciens*	Colonies roses
							Pantoea	*P. agglomerans*	Colonies jaunes (saprophytes)
							Enterobacter	*E. pyrinus*	Colonies blanches

INRA

Manceau,2008







Sally Miller,2006

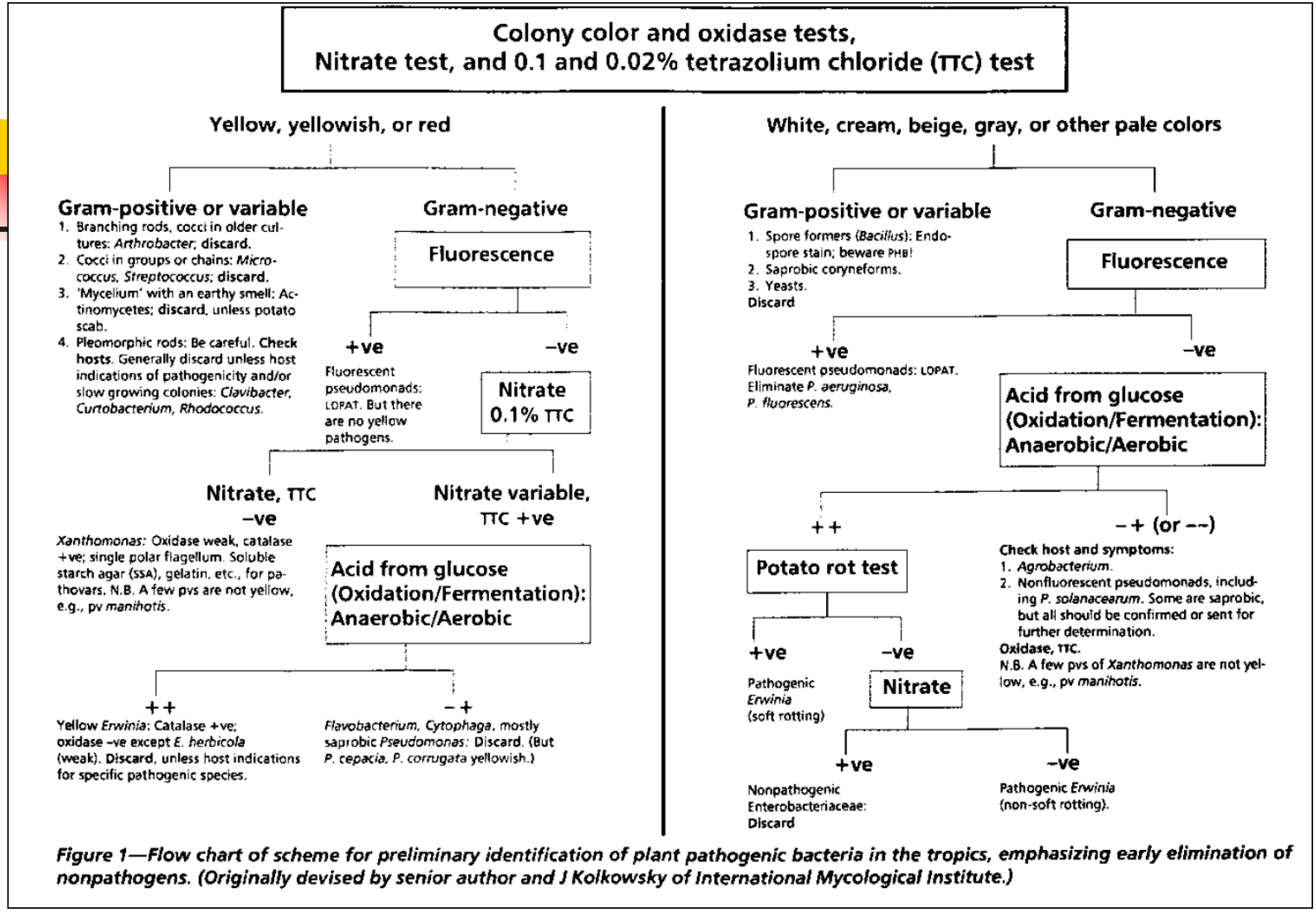


Figure 1—Flow chart of scheme for preliminary identification of plant pathogenic bacteria in the tropics, emphasizing early elimination of nonpathogens. (Originally devised by senior author and J Kolkowsky of International Mycological Institute.)

Mehan and McDonald,1995

Characteristics associated with colonies of pathogens on isolation plates

On three differential media: SNA, Levan and KB

5% sucrose nutrient agar	King's B	Nutrient dextrose agar	Possible pathogen
Whitish, domed, smooth, mucoid (levan).	Whitish-grey, raised with diffusible yellowish green pigment that fluoresces blue-green under ultraviolet light[1]	Whitish-grey, raised, butyrous	Green fluorescent pseudomonads of group Ia, IVa
Whitish-grey, raised	As above	As above	Green fluorescent pseudomonads of group Ib, III, IVb
Convex, smooth, whitish to yellowish with greenish centre	As above	As 5% SNA	Green fluorescent pseudomonads of group II
Convex, smooth or wrinkled, whitish-green to yellowish-brown; diffusible or non-diffusible pigments may be produced	Whitish-grey but often producing pigments which do not fluoresce under ultraviolet light	Convex, smooth or wrinkled, whitish green, yellowish to brown, diffusible or non-diffusible pigments may be produced	Non-fluorescent pseudomonads
High convex to domed, smooth, creamy to yellowish	As 5% SNA. No diffusible pigment.	High convex to domed smooth, mucoid, creamy to yellow; brown diffusible pigment produced rarely.	Xanthomonads
Raised, convex or domed, smooth mucoid (occasional levan production[2]) usually white to whitish-grey	Raised or convex, whitish-grey which do not fluoresce under ultraviolet light	Raised or convex, whitish-grey	Soft rot erwinias
Convex, domed, smooth, mucoid, yellowish-orange	Raised or convex, smooth white-yellowish[3]	Raised, convex to domed, mucoid, yellowish-orange	Coryneform

[1] Occasionally atypical, non-fluorescent forms of some pathogens of Group I are isolated; but they are common in *P. syringae* pv. *morsprunorum*, and are the rule in *P. s.* pv. *persicae*.
[2] *Erwinia amylovora* produces whitish, domed, smooth, mucoid levan colonies.
[3] Gram positive. Pink, blue or violet pigments are sometimes produced

Stead,2008

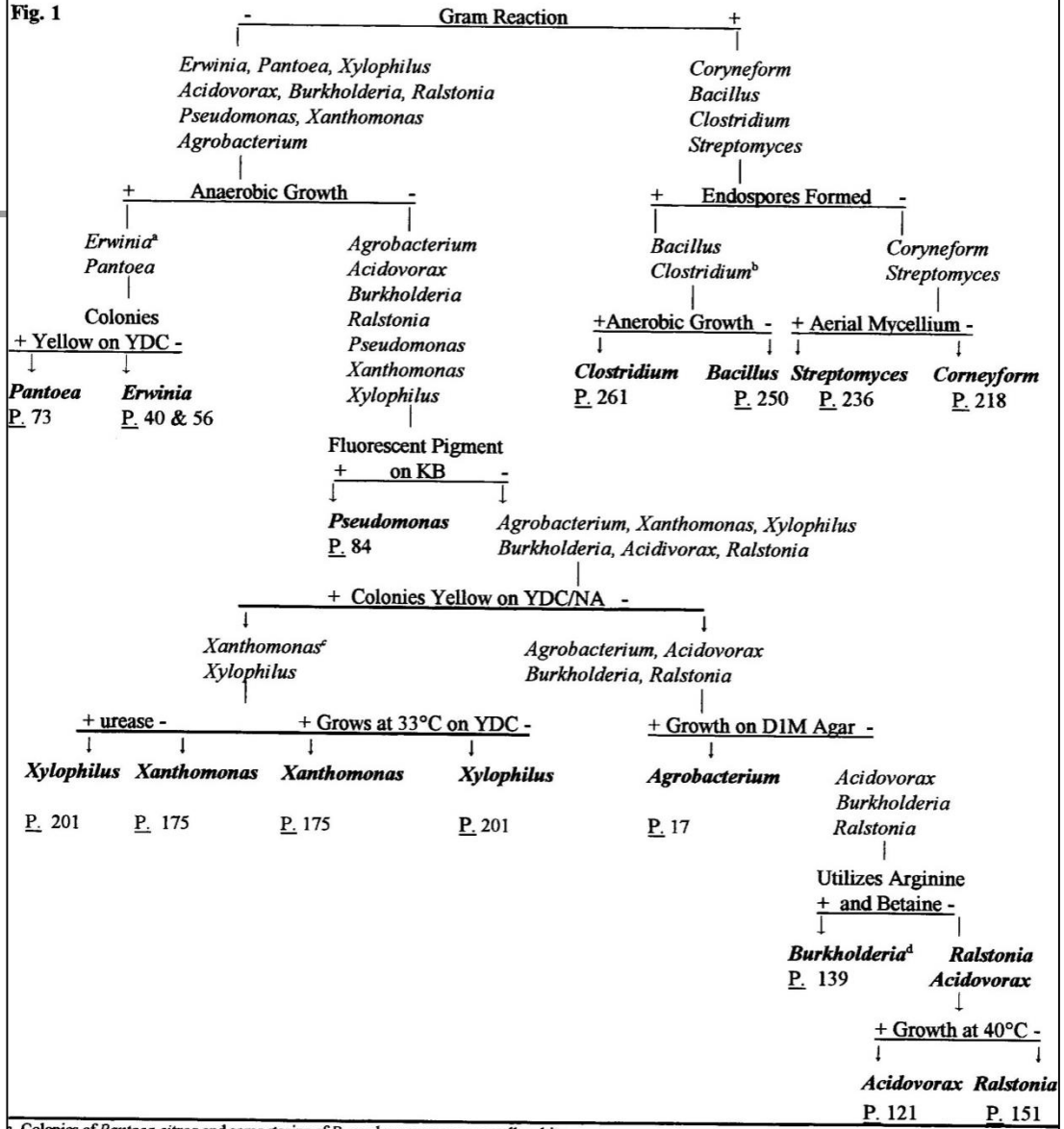
Differentiation of commonly isolated bacteria.

Schaad *et al.*,2001

C. DIFFERENTIATION OF COMMONLY ISOLATED GENERA

The following flow chart (Fig. 1) should be used as a quick guide for identifying a particular species or pathovar:

Fig. 1



[a] Colonies of *Pantoea citrea* and some strains of *P. agglomerans* are generally white.
[b] *Clostridia* cells with spores are swollen, whereas *Bacilli* are not.
[c] Colonies of *Xanthomonas cassavae* and two pathovars of *X. campestris* are white.
[d] *Burkholderia andropogonis* is negative for *arginine* and *betaine*. However, it is oxidase negative, whereas *Ralstonia* and *Acidovorax* are positive.

Characters used to differentiate genera of plant pathogenic prokaryotes that grow on standard media

Character	*Erwinia*	*Pantoea*	*Acidovorax*	*Pseudomonas*	*Ralstonia*	*Burkholderia*	*Xanthomonas*	*Xylophilus*	*Agrobacterium*	*Clavibacter*	*Clostridium*	*Bacillus*	*Streptomyces*
Gram positive	-	-	-	-	-	-	-	-	-	+	+	+	+
Grows anaerobically	+	+	-	-	-	-	-	-	-	-	+	+	-
Grows aerobically	+	+	+	+	+	+	+	+	+	+	-	+	+
Colonies yellow or orange on YDC, or NBY	-	+[a]	-	-	-	-	+[b]	+[c]	-	+[d]	-	-	-
Colonies mucoid on YDC at 30°C	-	-	+	-	+	-	+	-	+	+	ND	ND	-
Fluorescent pigment on KB	-	-	-	+	-	-	-	-	-	-	-	-	-
Diffusible non-fluorescent pigments on KB	-	-	-	-	-	+	-	-	-	-	-	-	-
Urease	-[e]	-	+	-	+	V	-	+	ND	-	ND	ND	ND
Oxidase	-	-	+	-	+	+[f]	-	-	+	-	-	V	+
Grows at 40°C	-	V	+	-	-	+[g]	-	-	-	-	+	+	-
More than four peritrichous flagella	+	+	-	-	-	-	-	-	-	-	V	V	-
Growth on D1M agar	-	-	-	-	-	-	-	-	+	-	-	-	-
Spores formed	-	-	-	-	-	-	-	-	-	-	+	+	-
Aerial mycelium	-	-	-	-	-	-	-	-	-	-	-	-	+

+, 80% or more strains positive after five days; V, between 21-79% of strains positive; -, 80% or more strains negative; ND, not determined.

[a] - Colonies of *Pantoea citrea* and some strains of *P. agglomerans* are generally white.

[b] - Colonies of *X. campestris* pathovars *manihotis* and *mangiferaeindicae* are white.

[c] - *Xylophilus* grows very slowly on these media, but somewhat better on Difco nutrient agar.

[d] - Colonies of *Clavibacter michiganensis* subsp. *sepedonicus* are generally white.

[e] - *Erwinia nigrifluens is positive.*

[f] - *Burkholderia andropogodis* is oxidase negative.

[g] - *Burkholderia andropogodis* and *B. glumae* pv. *agricola* are negative.

Schaad *et al.*,2001

Characters differentiating fastidious and non-culturable plant pathogenic bacteria

Character	*Rhizomonas*[a]	*Xylella*	*Spiroplasma*	Phytoplasma	Pholem-limited(BLOs)[b] or Liberibacter
Growth on S medium	+	-	-	-	-
Growth on PW medium	-	+	-	-	-
Possess cell wall	+	+	-	-	-
Growth on Serum agar	-	-	+	-	-
Helical morphology	-	-	+	-	-

[a]The genus *Rhizorhapis* gen. nov. was proposed to replace the illegitimate genus names *Rhizomonas* and *Sphingomonas*. *Rhizorhapis suberifaciens* is a slow-growing bacterium causes corky root of lettuce. It grows on semi-selective medium (S-medium).

[b]A new medium designated Liber A has been designed and used to successfully cultivate all three '*Candidatus* Liberibacter' spp. as bacterial-like organism (BLO).

Schaad,1988;Francis *et al*.,2014;..

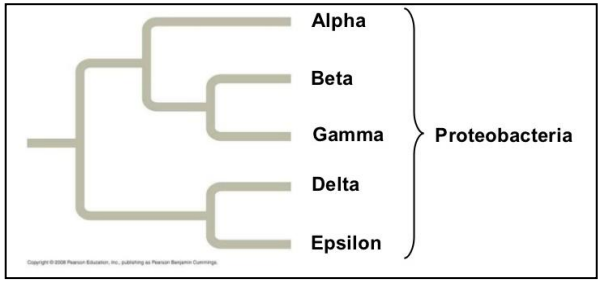
Characteristics of the major groups of phytopathogenic bacteria

Gram-Negative Bacteria

Domain: Bacteria
Phylum: Proteobacteria

- Within the domain Bacteria, the phylum Proteobacteria constitutes at present the largest and phenotypically most diverse phylogenetic lineage.
- Proteobacteria consist of 5 major classes:

1. Alphaproteobacteria
2. Betaproteobacteria
3. Gammaproteobacteria
4. Deltaproteobacteria
5. Epsilonproteobacteria



- All cultivable Gram-negative plant pathogenic prokaryotes occur within the alpha, beta and gamma subdivisions of the Phylum Proteobacteria based on DNA sequencing.

A new class within the phylum Proteobacteria, Acidithiobacillia classis nov., was proposed by Williams and Kelly,2013.

Domain: Bacteria
Phylum: Proteobacteria

Some selected plant diseases caused by alpha, beta and gamma subdivisions of the Phylum Proteobacteria.

The gammaproteobacteria are the largest class in terms of species.

Proteobacterial class and species	Family [a]	Disease (symptoms)
"Alphaproteobacteria"		
Agrobacterium rhizogenes	Rhizobiaceae	Hairy root
Agrobacterium tumefaciens	Rhizobiaceae	Crown gall
"*Candidatus* Liberibacter asiaticus"	in cluster of Rhizobiaceae, Bartonellaceae, etc.	Greening disease on citrus (a phloem-restricted disease)
"Betaproteobacteria"		
Acidovorax anthurii	Comamonadaceae	Leaf-spot on *Anthurium*
Burkholderia cepacia	"Burkholderiaceae"	Soft rot (sour skin on onion)
Burkholderia glumae	"Burkholderiaceae"	Sheath necrosis on rice
Ralstonia solanacearum	"Ralstoniaceae"	Moko disease on banana (vascular wilt)
Xylophilus ampelinus	Comamonadaceae	Necrosis and canker on grapevine
"Gammaproteobacteria"		
Brenneria (Erwinia) salicis	Enterobacteriaceae	Watermark disease on willow
Brenneria nigrifluens	Enterobacteriaceae	Bark canker on Persian walnut (*Juglans regia*)
Erwinia amylovora	Enterobacteriaceae	Fire blight on pome fruit (vascular wilt)
Erwinia stewartii	Enterobacteriaceae	Stewart's wilt on corn (vascular wilt)
Pectobacterium (Erwinia) carotovorum	Enterobacteriaceae	Soft rot
Pseudomonas agarici	Pseudomonadaceae	Spots on mushrooms
Pseudomonas marginalis	Pseudomonadaceae	Soft rot (pink eye) on potato
Pseudomonas savastanoi	Pseudomonadaceae	Galls on olive trees
Pseudomonas syringae	Pseudomonadaceae	Wildfire on tobacco, haloblight on beans, spots on tomato and pepper (blights and spots)
Pseudomonas syringae	Pseudomonadaceae	Canker on stone fruit
Xanthomonas campestris	"Xanthomonadaceae"	Black rot on crucifers (vascular wilt)
Xanthomonas citri	"Xanthomonadaceae"	Canker on citrus
Xanthomonas oryzae	"Xanthomonadaceae"	Blight on rice
Xanthomonas populi	"Xanthomonadaceae"	Canker on poplar trees
Xanthomonas translucens	"Xanthomonadaceae"	Blight on cereals
Xanthomonas vesicatoria	"Xanthomonadaceae"	Spots on tomato and pepper
Xylella fastidiosa	"Xanthomonadaceae"	Pierce's disease (e.g., on grapevine)

[a] According to *Bergey's Manual of Systematic Bacteriology* (Garrity and Holt, 2001). See also Fig. 1. Quotation marks are used for names which have not yet been validated (as of mid 2002).

Alphaproteobacteria

- 5/6 genera contain plant pathogens.

1. *Acetobacter* in *Acetobacteriaceae*
2. *Gluconobacter* in *Acetobacteriaceae*
3. *Sphingomonas* in *Sphingomonadales*;
4. *Agrobacterium* (*Rhizobium*);
5. *Candidatus Liberibacter*.

Betaproteobacteria

- Six genera contain pathogens and these represent 4 of the 5 families in the *Burkholderiales*.

1. *Acidovorax* in *Comamonadaceae*
2. *Burkholderia* in *Burkholderiaceae*
3. *Ralstonia* in *Ralstoniaceae*
4. *Herbaspirillum* in *Oxalobacteriaceae*
5. *Janthinobacterium* in *Oxalobacteriaceae*
6. *Xylophilus* (family not certain).

Gammaproteobacteria

- Three main families:

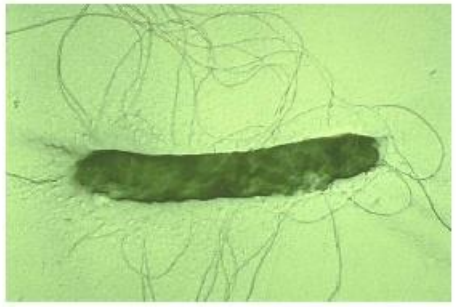
1. *Enterobacteriaceae* – 10 genera containing pathogens.
2. *Pseudomonodaceae* – 1 genus (*Pseudomonas*).
3. *Xanthomonodaceae* – 2 genera (*Xanthomonas, Xylella*).

Identification of the bacterial pathogens

Enterobacteriaceae

Enterobacterial plant pathogens

Disease diagnosis and pathogen diagnostics



The old taxonomic position and nomenclature

- The broadest classification is that of Dye (1968, 1969a, 1969b, 1969c), which separates *Erwinia* into the:

1. Carotovora group (pectolytic soft rots),
2. Herbicola group (the yellow pigmented),
3. Amylovora group (the white nonpectolytic wilt-causing),
4. Atypical group.

Characteristics

The family *Enterobacteriaceae*

1. Gram-negative straight rods;
2. Motile by peritrichous flagella or non-motile;
3. Do not form endospores;
4. Grow in presence or absence for oxygen (facultative anaerobes).

Common diseases caused by ex. *Erwinia* spp.

- Four basic types of plant diseases caused by ex. *Erwinia* spp. comprised of include:

1. Rapid necrosis called necrotic diseases;
2. Progressive tissue maceration called soft-rot diseases;
3. Occlusion of vessel elements called vascular wilt diseases;
4. Hypertrophy leading to gall or tumor formation.

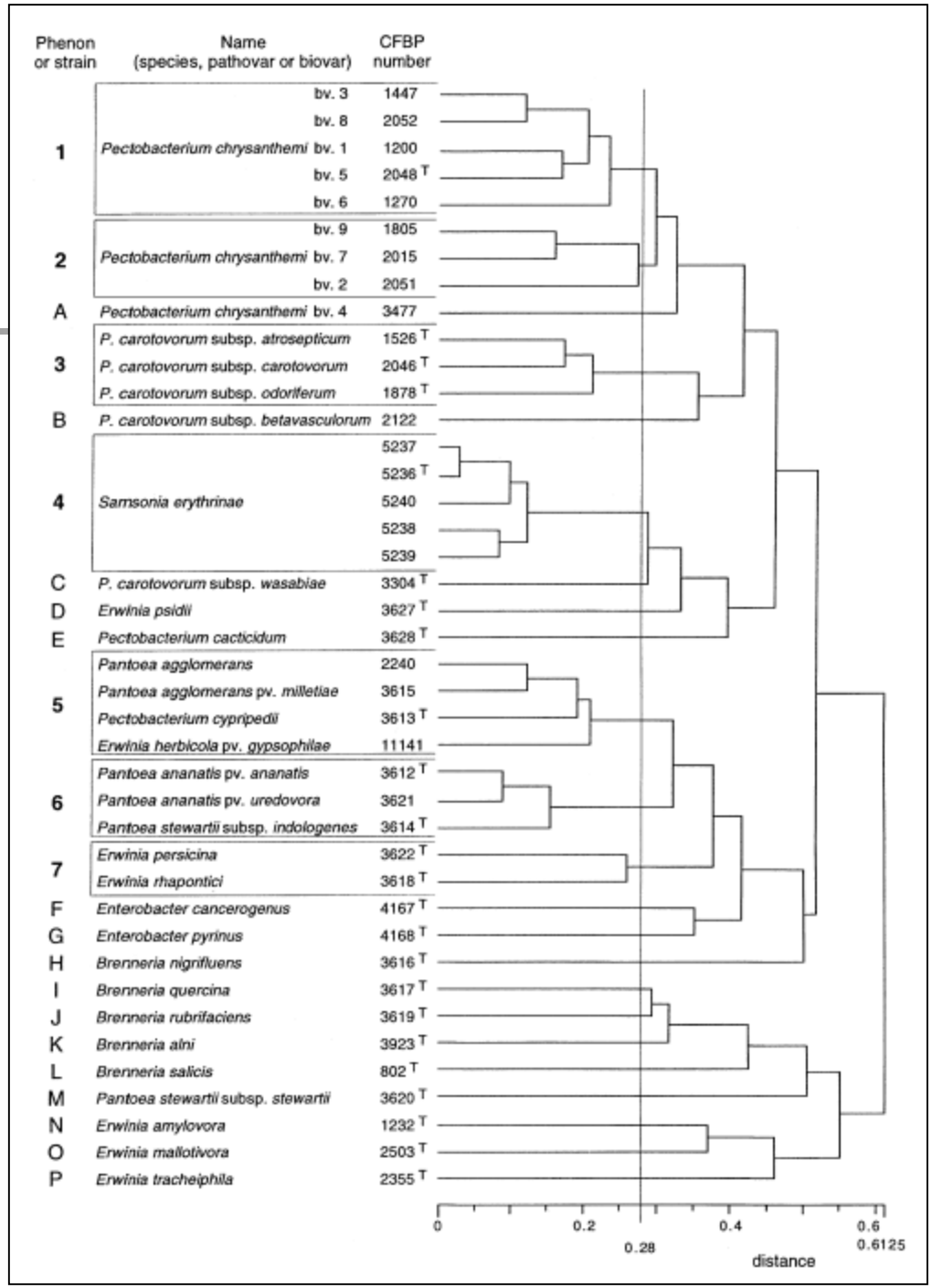
Diseases caused by ex. *Erwinia* spp.

The Prokaryotes (chapter 3.3.15),2006

Species	Disease name	Type of infection	Natural host plant
Necrogenic group emended from *Erwinia* to *Brenneria* (Hauben et al., 1998)			
Brenneria alni Wilson et al., 1957	Canker	Necrogenic	Alder
B. nigrifluens	Shallow canker	Necrogenic	Members of the Juglandaceae
B. paradisiaca	Brown-black root rot	Necrogenic	Banana
B. quercina Hildebrand and Schroth, 1967	Drippy nut and blight	Maceration	Coast live oak (*Quercus agrifolia*)
B. rubrifaciens Wilson et al., 1967	Deep bark canker	Necrogenic	Members of the Juglandaceae
B. salicis (Day 1924) Chester 1939	Bacterial wilt, water mark	Vascular wilt	*Salix* species
Status quo *Erwinia* group			
E. amylovora (Burrill, 1882) Winslow et al., 1920	Fire blight	Necrogenic	Members of the Rosaceae
E. billingia Mergaert et al., 1999	Associated with cankers	Secondary invader	Members of the Rosaceae
E. cypripedii (Hori) Bergey et al., 1923	Brown rot	Maceration	*Cypripedium* and other orchids
E. mallotivora Goto, 1976	Black leaf spot	Necrogenic	*Mallotus japonicus* tree
E. persicinus	Red-brown fruit rot	Necrogenic	Cucumber, tomato, banana
E. psidii	Fruit rot	Necrogenic	Guava
E. pyrifoliae Kim et al., 1999	Leaf and stem blight	Necrogenic	Pear, *Pyrus pyrifolia* cv Shingo
E. rhapontici (Millard) Burkholder 1948	Crown rot	Maceration	Rhubarb
E. tracheiphila (Smith, 1895) Bergey et al., 1923	Cucumber wilt	Vascular wilt	*Cucumis* species
P. carotovora subsp. Carotovora (Jones) Bergey et al., 1923	Storage soft rot	Maceration	Harvested corms, tubers, roots, bulbs
P. carotovora subsp. Chrysanthemi Burkholder et al., 1953	Stem blight, wilt	Vascular wilt	Corn, carnation, chrysanthemum, tropic plants, e.g., Philodendron, Dieffenbachia
P. carotovora subsp. atroseptica (van Hall 1902) Dye 1969	Blackleg	Vascular wilt	Potato roots, lower stems, tubers
P. carotovora subsp. betavasculorum	Soft rot	Maceration	Beet, *Beta vulgaris*
P. carotovora subsp. odoriferum	Soft rot	Maceration	Endive and chicory
P. carotovora subsp. wasabiae	Soft rot of excised petioles	Maceration	Japanese green horse radish
Erwinia species emended to Enterobacter species			
Enterobacter cloacae	Internal yellowing, brown-black discoloration	Diffuse necrogenic	Papaya (*Carica papaya* L.) Onion (*Allium cepa* L.)
En. nimipressuralis	Wet wood	Necrogenic	Elm
En. cancerogenus	Canker	Necrogenic	Poplar
En. dissolvens	Rot	Slow maceration	Corn

Soft-rotting group proposed emendation from *Erwinia* to *Pectobacterium* (Hauben et al., 1998).

Dendrogram of phenotypic distances between the 41 strains of plant-pathogenic *Enterobacteriacae*.



Sutra *et al.*,2001

The current taxonomic position and nomenclature

Phylogenetic and genomic analyses of the order Enterobacterales

- Genome-based phylogeny and taxonomy of the 'Enterobacteriales': proposal for Enterobacterales ord. nov. divided into the seven families:

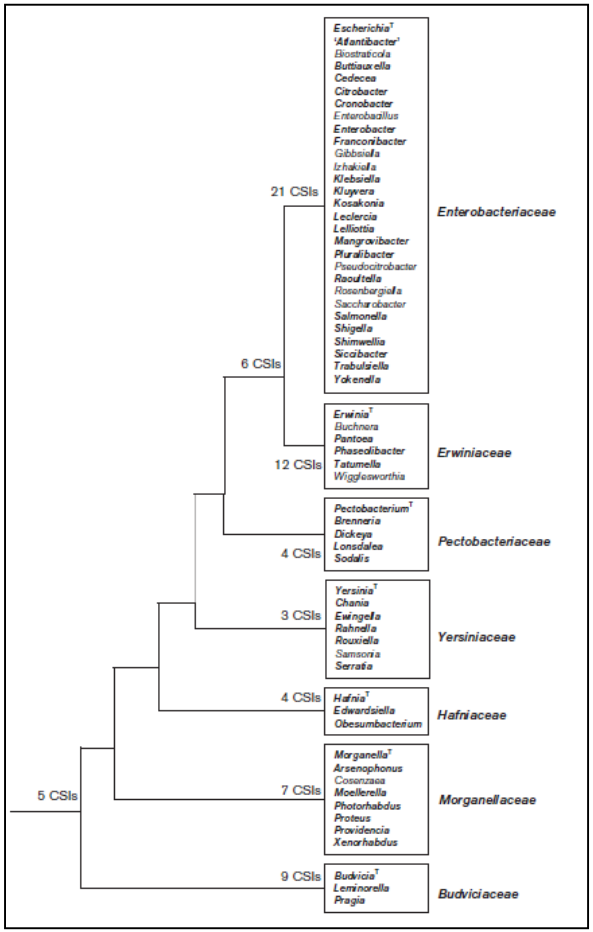
1. Enterobacteriaceae,
2. Erwiniaceae fam. nov.(the *Erwinia-Pantoea* clade),
3. Pectobacteriaceae fam. nov. (the *Pectobacterium-Dickeya* clade),
4. Yersiniaceae fam. nov.,
5. Hafniaceae fam. nov.,
6. Morganellaceae fam. nov., and
7. Budviciaceae fam. nov.

Adeolu *et al.*,2016

The current taxonomic position and nomenclature

Phylogenetic and genomic analyses of the order Enterobacterales

- Phylogenetic reconstructions based on 1548 core proteins, 53 ribosomal proteins and four multilocus sequence analysis proteins, as well as examining the overall genome similarity amongst the members of this order.
- A summary diagram depicting the distribution of identified CSIs(conserved signature insertion/deletion) within the order 'Enterobacteriales' (synonym: Enterobacterales ord. nov.) and the proposed 7 families described in this study.
- Genera which have had their genomes analysed in this study are indicated in bold type.
- The superscript letter T beside a genus indicates that it is the type genus of the family.



Adeolu *et al.*,2016

The current taxonomic position and nomenclature

Phylogenetic and genomic analyses of the order Enterobacterales

- Description of Enterobacteriaceae (Emended description of the family Enterobacteriaceae (Approved Lists 1980):
- The family Enterobacteriaceae contains:

1. the type genus *Escherichia* and the genera:
2. *Atlantibacter*
3. *Biostraticola*
4. *Buttiauxella*
5. *Cedecea*
6. *Citrobacter*

- *Cronobacter*
- *Enterobacillus*
- *Enterobacter*
- *Franconibacter*
- *Gibbsiella*
- *Izhakiella*
- *Klebsiella*
- *Kluyvera*

Adeolu *et al.*,2016

The current taxonomic position and nomenclature

Phylogenetic and genomic analyses of the order Enterobacterales

- Description of Enterobacteriaceae (Emended description of the family Enterobacteriaceae (Approved Lists 1980):
- The family Enterobacteriaceae contains:
- *Kosakonia*
- *Leclercia*
- *Lelliottia*
- *Mangrovibacter*
- *Pluralibacter*
- *Pseudocitrobacter*
- *Raoultella*
- *Rosenbergiella*
- *Saccharobacter*
- *Salmonella*
- *Shigella*
- *Siccibacter*
- *Trabulsiella*, and
- *Yokenella*.

Adeolu *et al.*,2016

The current taxonomic position and nomenclature

Phylogenetic and genomic analyses of the order Enterobacterales

- Description of Erwiniaceae fam. nov. (the *Erwinia-Pantoea* clade):
- The family Erwiniaceae contains:

1. the type genus *Erwinia* (Hauben *et al.*,1998) and
2. the genera *Buchnera* (Munson *et al.*,1991),
3. *Pantoea* (Brady *et al.*, 2010b),
4. *Phaseolibacter*(Halpern *et al.*,2013b),
5. *Tatumella* (Hollis *et al.*,1981) and
6. *Wigglesworthia* (Aksoy,1995).

Note: *Pseudomonas flectens* was transferred to the family *Enterobacteriaceae* as *Phaseolibacter flectens* gen. nov., comb. nov. (Aizenberg-Gershtein *et al.*,2016). *Tatumella* (formerly some *Pantoea* spp.) *Buchnera* is an endosymbiont of aphids and *Wigglesworthia*, endosymbiont of the tsetse fly.

Adeolu *et al.*,2016

The current taxonomic position and nomenclature

Phylogenetic and genomic analyses of the order Enterobacterales

- Description of Pectobacteriaceae fam. nov. (the *Pectobacterium-Dickeya* clade):
- The family Pectobacteriaceae contains:

1. the type genus in the family *Pectobacterium* (Hauben *et al.*,1998) and
2. the genera *Brenneria* (Brady *et al.*,2014a),
3. *Dickeya* (Samson *et al.*,2005),
4. *Lonsdalea* (Brady *et al.*,2012), and
5. *Sodalis* (Dale & Maudlin,1999).

Lonsdalea (formely *Brenneria quercina*)and the genus *Sodalis* found in the hemolymph of the tsetse fly.

Adeolu *et al.*,2016

The current taxonomic position and nomenclature

Number of species and new genera of the family

- The *Enterobacteriaceae*, once almost all incorporated within *Erwinia*, now has 12 genera proposed containing plant pathogenic species:

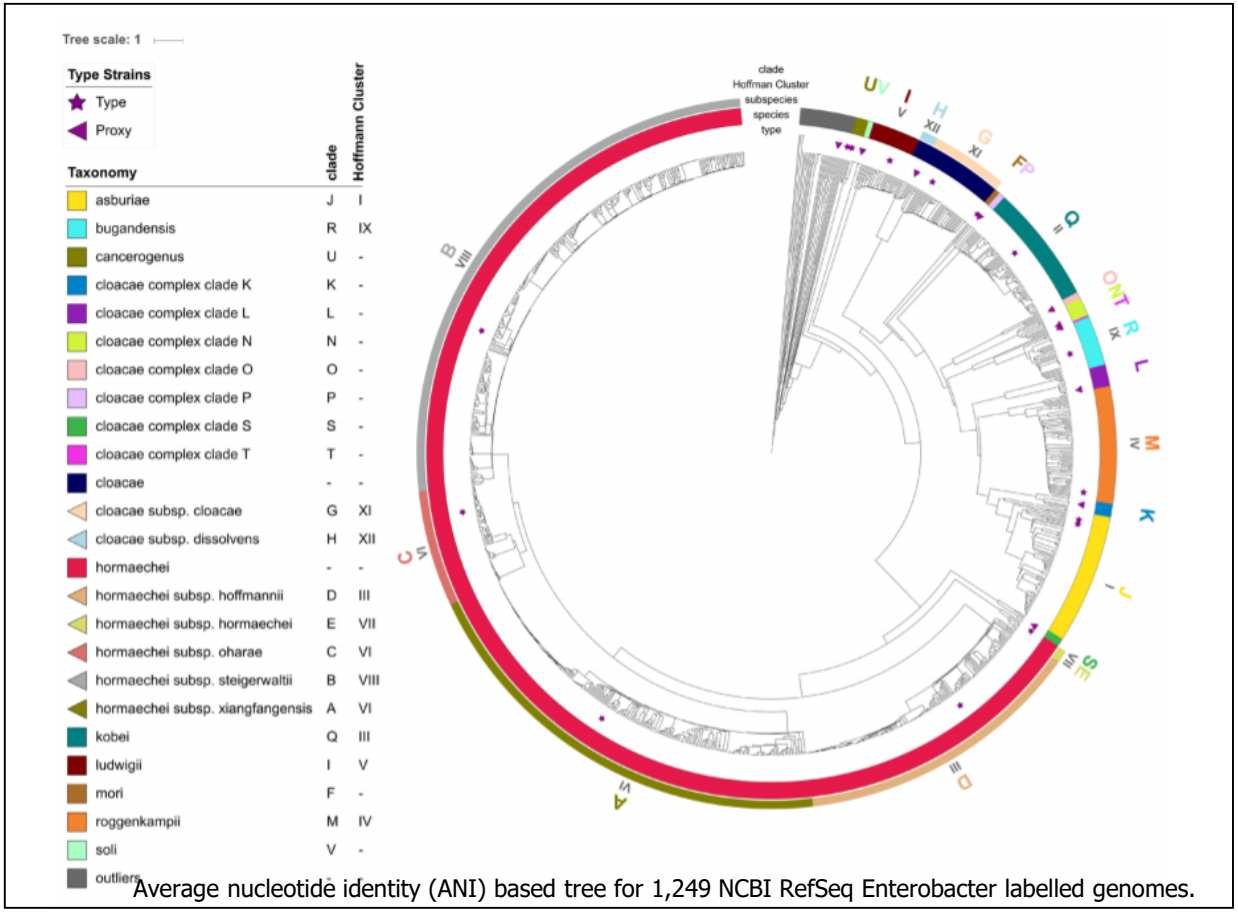
1. *Enterobacter*,
2. *Erwinia*,
3. *Pectobacterium*,
4. *Pantoea*,
5. *Dickeya*,
6. *Brenneria*,
7. *Serratia*,
8. *Samsonia*,
9. *Lonsdalea*,
10. *Ewingella*,
11. *Gibbsiella*,
12. *Candidatus* Phlomobacter

See also BLO section for *Serratia* and *ca.* Phlomobacter description.

Characteristics of
The genus *Enterobacter*

- Facultative anaerobes,
- Motile by peritrichous flagella,
- Rod shaped,
- Some *Enterobacter* spp. such as *E. cloacae* have been emerged in recent years as a phytopathogens of important crops.

Characteristics of
The genus *Enterobacter*



Average nucleotide identity (ANI) based tree for 1,249 NCBI RefSeq Enterobacter labelled genomes.

Sutton *et al.*,2018

Diseases caused by: *Enterobacter* spp.

- *Erwinia* species emended to *Enterobacter* species as follows:

E. cloacae	**Brown-black discoloration of papaya**
***E. ludwigii* (previous termed *E. cloacae*)**	**Enterobacter onion bulb decay**
Enterobacter cancerogenus	**Poplar canker**
Enterobacter dissolvens	**Bacterial corn rot**
Enterobacter mori	**Bacterial wilt of mulberry and kiwifruit**
Enterobacter nimipressuralis	**Wet wood disease of elm & poplar**
***Enterobacter pyrinus* (formerly *Erwinia pirina*)**	**Associated with brown leaf spot disease of pear trees**

Enterobacter complex (*E. cloacae*, *E. asburiae* and *Enterobacter* sp.) causing mulberry wilt disease (MWD) and leaf blight and dieback of Eucalyptus is caused by *Enterobacter cowanii*.

Diseases caused by:
Enterobacter spp.

Garden onion bulb decay caused by *Enterobacter cloacae*

Corn bacterial stalk rot caused by *Enterobacter dissolvens*

USDA Forest Service,2008

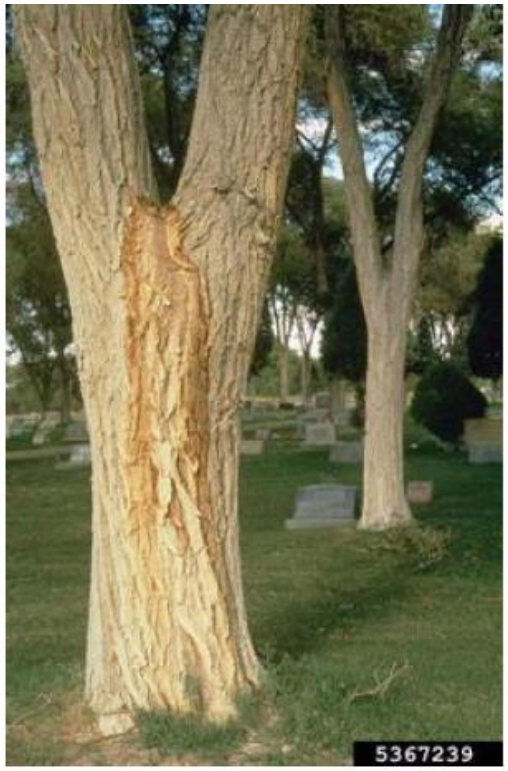
Bacterial wetwood on post oak

Enterobacter nimipressuralis

USDA Forest Service,2008

Bacterial wetwood on elm and poplar

Enterobacter nimipressuralis



American elm

Aspen/poplar

USDA Forest Service,2008

An emerging pathogen of kiwifruit *Enterobacter mori*

- Disease symptom of *E. mori* on plants and its epidemics in kiwifruit orchards of Cangxi-China.

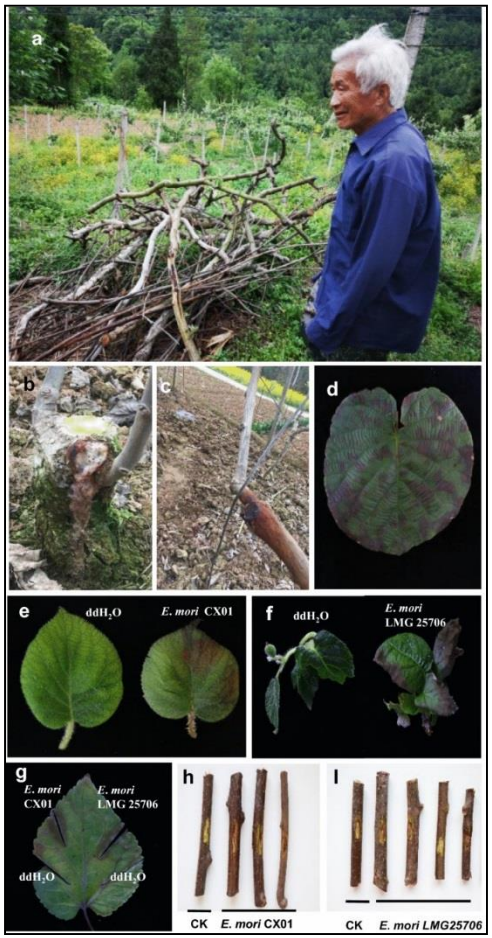
a. High disease incidence caused by *E. mori* led to eradication of whole kiwifruit orchards.

b. The canker-like disease symptoms on the trunk with white exudation.

c. Reddish-brown discoloration under the bark of the trunk.

d. Disease symptom of brown spots on kiwifruit leaf.

- Artificial inoculations(e-I).



Zhang *et al.*,2021

Pure cultures

Enterobacter cloacae, D. chysanthemi and *K. pneumoniae*

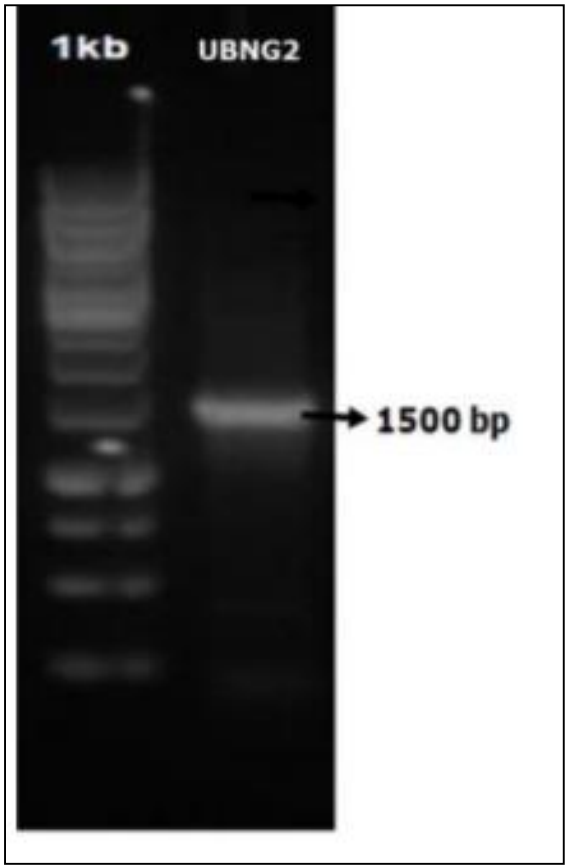
Ganem,2008

Biochemical differentiation of the four species of the genus *Enterobacter*

Test	*Enterobacter cancerogenus*	*Enterobacter cloacae*	*Enterobacter dissolvens*	*Enterobacter nimipressuralis*
Anaerobic growth	+	+	+	+
Oxidase	-	-	-	-
Arginine dihydrolase	+	+	+	+
Indole production	-	-	-	-
MR	-	-	-	-
VP	+	+	+	+
H_2S production	-	-	-	-
Nitrate reduction	+	+	+	+
Urease	-	d	+	-
Gelatinase	-	-	-	+
Esculin hydrolysis	+	d	+	+
Lipase	-	-	d	-
Acid production:				
Melibiose	-	+	+	+
α-methyl-D-glucoside	-	+	+	+
Glycerol	-	d	-	+

Amplification of the 16S rRNA gene *Enterobacter cloacae* in shallot (*Allium cepa*)

- DNA amplification to isolate UBNG21 using universal primers for 16S rRNA with the sequence of bases:
- 8-27F (59 AGAGTTTGAT CCTGGCTCAG-39), and
- 1492-1509R (59 GGTTACCTTG TTACGACTT-39).
- single band with a size of 1500 bp.



Arwiyanto *et al.*,2021

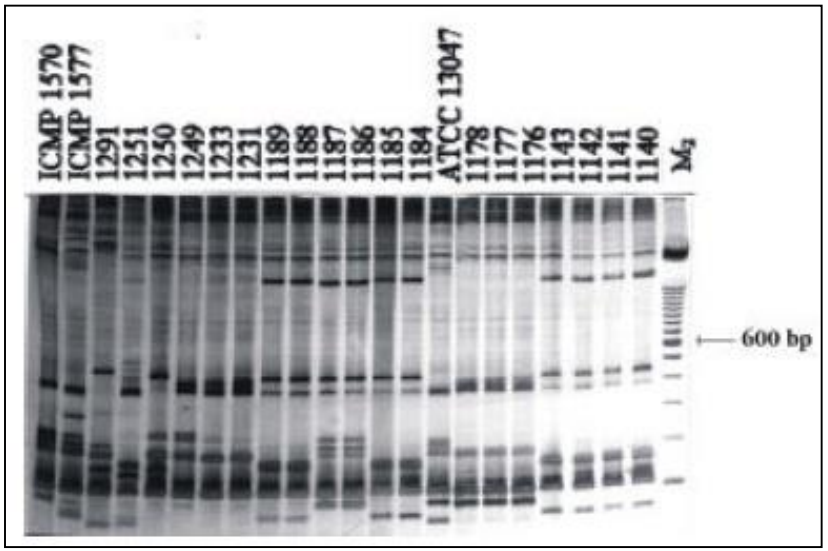
RFLP of the 16S-23S rDNA intergenic transcribed spacer
Enterobacter cloacae

- PCR-RFLP results showed that *E. cloacae* strains isolated from plants were homogeneous presenting close similarity among them.
- Whereas the strains from clinical samples were heterogeneous.

Rodrigues Neto *et al.*,2003

RFLP of the 16S-23S rDNA intergenic transcribed spacer *Enterobacter cloacae*

- The amplification of 16S-23S ITS yielded a unique fragment of 1,000 base pairs approximately.
- Digestion of the PCR products with the restriction enzymes *Dde*I, *Hinf*I and *Sau*3 AI revealed similarity among the majority of *E. cloacae* strains isolated from plants except for 1186 and 1291 strains.



RFLP pattern of the 16S-23S ITS rDNA of *Enterobacter cloacae* and type strains of *E. dissolvens* and *E. nimipressuralis* after digestion with *Dde* I (A) and *Sau* 3 AI (B). M2 100 bp molecular weight marker. The 100 bp DNA Ladder contains 12 discrete DNA fragments ranging in size from 100 bp to 3,000 bp.

Rodrigues Neto *et al.*,2003

Pathogenicity test

Enterobacter cloacae

- Pathogenicity tests were performed on mature onion bulbs to verify if strains isolated from clinical and from vegetables could induce disease symptoms.
- Five onion bulbs were inoculated through a syringe and hypodermic needle with about 0.5 mL of a bacterial suspension in distilled water of each strain with approximately 10^8 colony forming unity per milliliter (CFU/mL) injected in tissues.
- Controls were inoculated with water.
- The inoculated onion bulbs were maintained at 36°C in a humid chamber.

Rodrigues Neto *et al.*,2003

Pathogenicity test

Infection severity scale

Enterobacter cloacae

- Pathogenicity symptoms were recorded after a week according to the following scale:

0= tissues remain undamaged and without discoloration;

1= slight discolored tissues 1 cm around the inoculation point and tissues undamaged;

2= inner scales discolored or flaccid;

3= inner scales flaccid or discolored from top to the basis;

4= flaccid and discolored tissues of half or entire bulb.

Rodrigues Neto *et al.*,2003

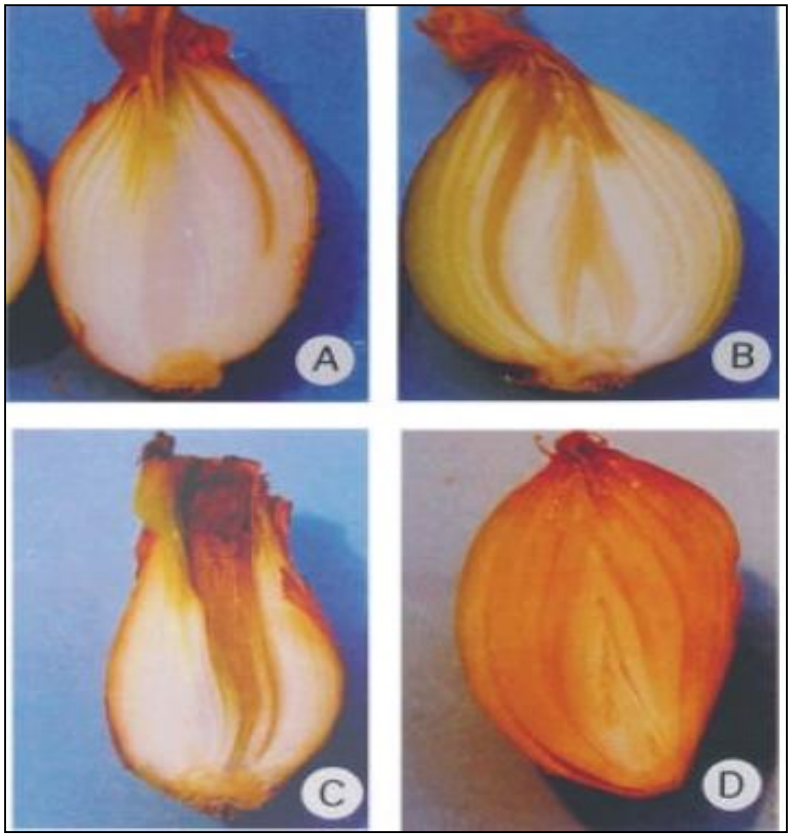
Pathogenicity test

Infection severity scale

Enterobacter cloacae

- Pathogenicity levels of *Enterobacter cloacae* on onion bulbs after artificial inoculation with different strains.

A. strain 1251 (level 1);
B. strain 1184 (level 2);
C. strain 1250 (level 3);
D. strain 1140 (level 4).



Rodrigues Neto *et al.*,2003

Pathogenicity test

Mulberry wilt disease (MWD)

Enterobacter complex (*E. cloacae*, *E. asburiae* and *Enterobacter* sp.)

- To test the pathogenicity of the four MWD strains, laboratory bioassays and whole plant inoculations in the greenhouse were performed.
- For the bioassay, healthy shoots of mulberry 'Nongsang', 15.0 cm long with five leaves, were cut and placed in flasks containing 50.0 ml SDW, and then 0.2 ml aliquots of bacterial cell suspension (1.0×10^8CFU ml^{-1}) were injected into the axils of the plant stem.
- These were compared with injections with SDW or nutrient broth (NB) as controls.
- The inoculated plants were incubated in a growth chamber with 30/25°C day/night temperatures, 95% relative humidity and a 12-h photoperiod.
- For the whole plant inoculations, 3-month old grafted mulberry seedlings (Nongsang × Heyebai) planted in individual pots were inoculated using the same method described above.
- After inoculation, the plants were covered with plastic bags and maintained in high humidity for 24 h, and then put in a greenhouse (25 to 30°C day and 23 to 25°C night with a 10 h photoperiod).

Wang *et al.*,2009

Pathogenicity test

Mulberry wilt disease (MWD)

Enterobacter complex (*E. cloacae*, *E. asburiae* and *Enterobacter* sp.)

- Symptoms of mulberry wilt disease in the field (A-C), bioassay (D, E) and the whole plant inoculation (F–J).
- A. field shoot wilt symptoms; B. mulberry healthy stem; C. dark brown lesions on the stem xylem; D, E. three symptoms of the bioassay; the arrows indicate top necrosis (R2-2, R11-2), discoloration (R18-2, R11-2) and wilt symptoms (R6-2, R18-2) on the mulberry shoots 19 days post inoculation with four MWD isolates.
- F, G. Leaves with wilt and defoliation symptoms on mulberry seedling 15 days post artificial inoculation with *Enterobacter* sp. isolates R2-2, R6-2, R11-2 and R18-2 (typical symptoms marked by arrows);
- H, I, J. Seedling symptoms in type strain LMG2683 (H), R11-2 (I) and R18-2 (J) inoculation; CK indicates control.

Wang *et al.*,2009

The new taxonomic position and nomenclature

The genus *Erwinia*

- The genus *Erwinia* comprises phytopathogenic and non-pathogenic species.
- The species in the "amylovora" group will be considered together with the atypical erwinias and will include:

1. *Erwinia amylovora*
2. *E. mallotivora*
3. *E. papayae*
4. *E. persicina*
5. *E. psidii*
6. *E. pyrifoliae*
7. *E. rhapontici*
8. *E. tracheiphila*

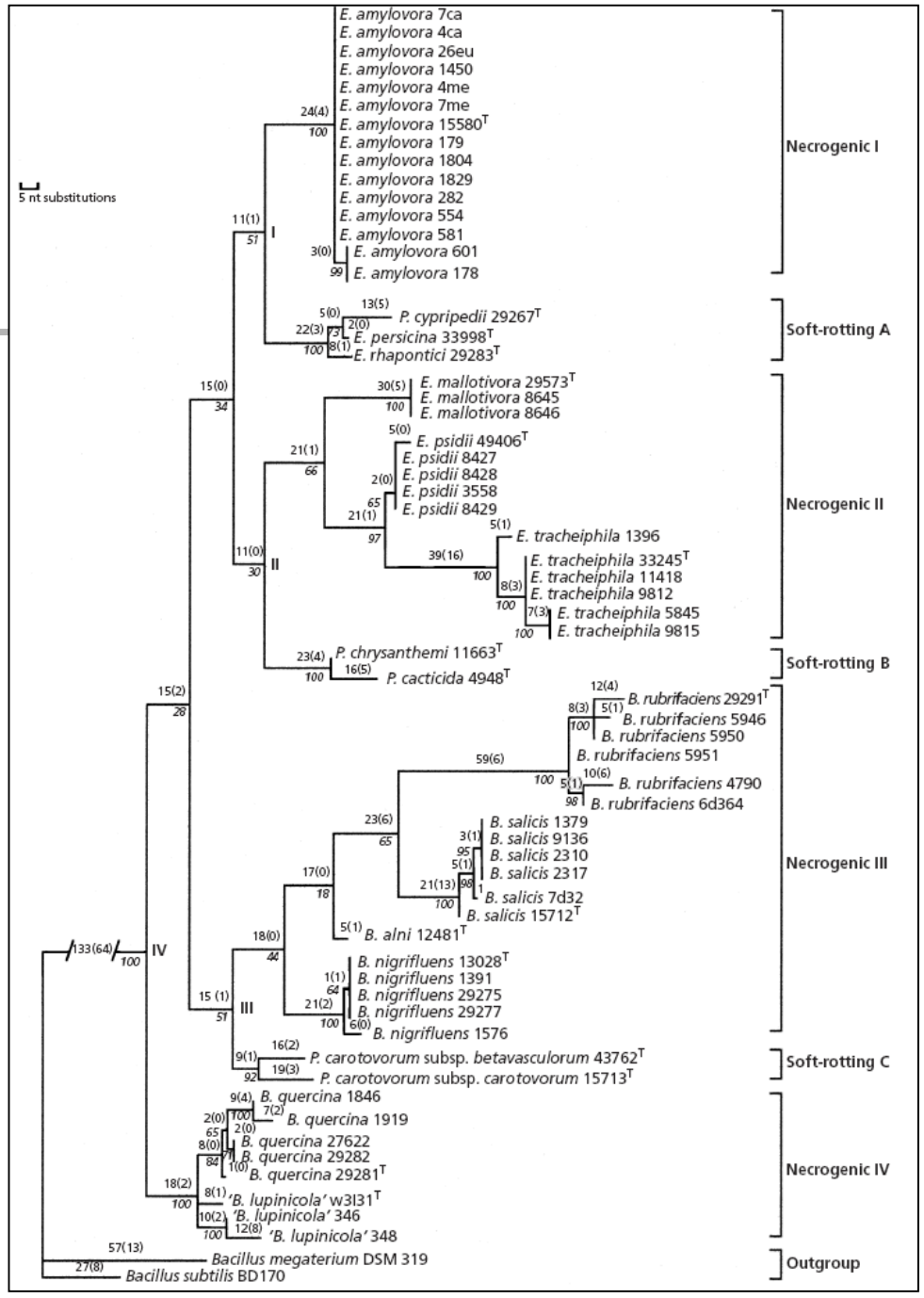
- **Associated bacteria:**

1. *Erwinia tasmaniensis*: A non-phytopathogenic bacterium from apple and pear trees (J.P. Euzéby).
2. *Erwinia toletana*: Associated with *Pseudomonas savastanoi* - Induced tree knots (J.P. Euzéby).
3. *Erwinia billingiae*: A non-phytopathogenic bacterium from apple and pear trees but causes bacterial canker of mango.

Phylogeny

Erwinia and *Brenneria*

- Phylogenetic relationships of necrogenic *Erwinia* and *Brenneria* species based on *gapDH* genes.
- The *gapDH* gene encodes glyceraldehyde-3-phosphate dehydrogenase (GapDH), an essential component of glycolysis.
- The *gapDH* locus has been used to describe the taxonomic positions of several taxa at the species level.



Brown *et al.*,2000

Diseases caused by:
The genus *Erwinia*

- They occur primarily on trees causing various blights, cankers and wilts.
- *Erwinia amylovora*, the fire blight pathogen, is not only the type species of the genus but it also the most important economically.

Diseases caused by:
The genus *Erwinia*

Erwinia amylovora	**Fire blight disease of rosaceous plants**
Erwinia uzenensis	**Black lesions on shoots of European pear trees**
E. papayae	**Bacterial canker of papaya**
E. persicina	**Red-brown fruit rot of cucumber, banana & tomato**
E. psidii	**Bacterial blight and wilt of guava; Fruit rot of guava**
E. pyrifoliae	**Black stem blight(necrotic disease) of Asian pear trees**
E. piriflorinigrans	**Necrotic pear blossoms**
E. rhapontici	**Crown rot of rhubarb and pink seed of bean, pea &wheat**
E. tracheiphila	**Bacterial wilt of cucurbits**
Erwinia billingiae	**Bacterial canker of mango**

Characteristics of
The genus *Erwinia*

- Facultative anaerobes,
- Motile by peritrichous flagella,
- Rod shaped,
- Acid produced from fructose, glucose, galactose, and sucrose.
- Only one species, *E. persicinus* reduces nitrate while the other species are nitrate reduction negative.

Key diagnostic tests
The genera of *Enterobacteriaceae.*
(ex. *Erwinia* spp.)

Test	*E. alni*	*E. amylovora*	*E. mallotivora*	*E. nigrifluens*	*E.paradisiaca*	*E. persicinus*	*E.psidii*	*E. pyrifoliae*	*E. quercina*	*E. rubrifaciens*	*E. salicis*	*E. tracheiphila*
Tobacco hypersensitivity	-[b]	+	+	-	ND	ND	-	+	-	-	-	ND
Pectate degradation[c]	-	-	-	-	+	+	-	-	+	+	+	-
Growth factors required[d]	-	+	+	-	ND	ND	-	ND	+	-	-	+
Pink pigment on YDC	-	-	-	-	ND	+	-	ND	-	+	-	-
Growth at 36°C	+[D]	-	-	+	ND	+	-	-	-	-	-	-
Growth at 39°C	-	-	-	-	+	-	-	-	-	-	-	-
H_2S from cysteine	+	-	-	+	+	+	+	-	+	+	+	+
Urease	+[D]	-	-	+	-	-	-	-	-	-	-	-
Indole test	-	-	-	-	+	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	ND	+	-	-	-	-	-	-
Gelatin liquification	-	+	-	-	-	-	-	-	-	-	-	-
Acid production from[e]:												
Salicin	+	-	-	+	+	+	+	ND	+	-	+	-
K-methyl glucoside	ND	-	-	ND	-	-	+	-	+	+	-	-
Melibiose	-	-	-	+	+	ND	ND	ND	ND	ND	ND	ND
Inositol	-	-	-	+	ND	ND	+	ND	ND	-	+	ND
L-arabinoise	+	v	-	+	+	+	+	+	-	+	-	ND

[a] Data taken from Hao et al. (10), Hauben et al. (11), Neto et al. (21), Schroth and Hildebrand (28), Surico et al. (30).
[b] +, 80% or more strains positive; +[D], 80% or more strains delayed positive; -, 80% or more strains negative; ND, not determined; v, variable.
[c] Tests were made on Paton's media (22). Pitting after 3 days represents a positive test.
[d] All positive species required yeast extract to grow in basal media with glucose. *E. amylovora* requires nicotinic acid for good growth in some minimal media.
[e] After 7 days growth at 27°C in unshaken aqueous solution of 1% organic compound and 1% peptone with bromcresol purple as indicator.

Schaad *et al.*,2001

General characteristics of four plant associated Enterobacteriaceae

Test	*Pectobacterium*	*E. amylovora*/ *Brenneria*	*Pantoea* spp.
Pectate degradation	+	-	-
Yellow pigment	-	-	v
Nitrate reduced to nitrite	+	- (except *E. persicinius*)	v
Gas production from glucose	v	-	-
Anaerobic growth	+	w	+

Distinguishable characteristics of *E. mallotivora* and *E. papaya*

E. mallotivora sp., a new pathogen of papaya in Malaysia

Characteristic	*E. mallotivora* (BT-Mardi)	*E. mallotivora*	*E. papaya*
Blue pigment on King's B agar	-	-	+
Citrate utilization	+	+	-[a]
Reducing substances from sucrose	+	+	-
D-Mannitol	+	+	-
L-Arabinose	-	-	+

[a] More than 70% of the strains negative.

Amin *et al.*,2010

Erwinia spp. associated with pear diseases

Erwinia spp.	Disease incited
Erwinia amylovora	The causal agent of the fire blight disease of rosaceous plants in most countries with broader host range.
Erwinia pyrifoliae	Species that are pathogenic to pear trees Nashi pear (*Pyrus pyrifolia*) and European pear (*Pyrus communis*), include in Korea and Japan. symptoms are indistinguishable from those of fire blight in Asian pear trees.
Erwinia piriflorinigrans	Causal agent of pear blossom necrosis.
Erwinia spp.	Found in Japan. Japanese *Erwinia* spp. cause Japanese bacterial black shoot disease of pear (BBSDP) and bacterial shoot blight of pear (BSBP), respectively. Found on several cultivars of pear trees.

Palacio-Bielsa *et al.*,2012

Reaction of *Erwinia amylovora*-like isolates in various diagnostic tests

- *Erwinia amylovora*-like (Ea-like) isolates originating from symptomatic samples of fire blight host plants.
- These isolates identified as *Pantoea dispersa* and *P. agglomerans*.
- These are white variants of mentioned species that occur less frequently than yellow variants.

Ea-like isolate	PTA-ELISA	IF test	HR test on tobacco	PCR	Optimised PCR	BIOLOG
1	+	+	–	+	–	*P. dispersa*
2	+	+	–	+	–	*P. agglomerans*
3	+	+	–	+	–	*P. agglomerans*
4	+	+	–	–	–	nt
5	+	+	–	–	–	nt
6	+	–	–	–	–	nt
RICP Ea 8/95	+	+	+	+	+	*E. amylovora*

Kokošková *et al.*,2005

Non-pathogenic species

The secondary invaders on pome fruits

Erwinia billingiae and *E. tasmaniensis*

- The species *E. tasmaniensis* and *E. billingiae* are epiphytic bacteria and part of the apple and pear microbiota may represent antagonists for biocontrol of fire blight.
- The non-pigmented *Erwinia herbicola* strains from trees have been reclassified as the novel nonpathogenic species, *E. billingiae*.
- *E. billingiae* was also isolated from bacterial canker disease of mango in Costa Rica(Daniela *et al.*,2021).
- Strains were isolated from stem cankers, diseased blossoms, and immature fruits mainly of rosaceous trees, often in association with plant pathogens, and are considered as secondary invaders rather than primary pathogens.

Bergey's Manual of Systematic Bacteriology,2005;Palacio-Bielsa *et al.*,2012

Erwinia spp. associated with pear diseases

Selected phenotypic characteristics

Characteristics	*E. amylovora*[a]	*E. pyrifoliae*[b]	BSBP *Erwinia* sp.[c]	BBSDP *Erwinia* sp.[d]	*E. piriflorinigrans*[e]	*E. billingiae*[f]	*E. tasmaniensis*[g]
Levan production	+[h]	−	+	ND	+	−	+
Tween 20 hydrolysis	−	−	ND	ND	+	+	−
Nitrate reduction	−	−	−	−	−	+	−
β-Galactosidase	−	−	−	+	+	+	+
Acid production from							
Sorbitol	+	+	+	+	−	+	−
Saccharose	+	+	+	+	+	−	+
Glycerol	−	−	+	+	+	−	−
D-Xylose	+	−	ND	ND	+	+	−
Adonitol	−	−	−	−	+	−	−
D-Mannose	−	−	ND	ND	−	+	−
L-Rhamnose	−	−	ND	ND	−	+	−
Methyl-α D-glucopyranoside	−	−	−	V[i]	+	−	−
Esculin hydrolysis	(+)	−	−	(+)	+	+	−
D-Maltose	−	−	ND	ND	−	+	−
D-Raffinose	−	−	ND	ND	+	−	−
D-Fucose	−	−	ND	ND	+	−	−
D-Arabitol	−	−	ND	ND	−	+	−

Palacio-Bielsa *et al.*,2012

Characteristics of *Erwinia billingiae*

- **Not determined:**
- Levan
- **Positive for:**
- Production of acetoin,
- $N0_3$-NO_2
- **Negative for:**
- Gelatinase
- Production of indole
- Oxidase
- Arginine dihydrolase
- Tween 80 hydrolase
- Urease

Characteristics of Type species
Erwinia amylovora (Burrill) Winslow *et al.*,

- Fire blight restricted to plants belonging to Rosaceae.
- Burrill 1882 - United States.
- North America, part of Central America, Europe, New Zealand.
- **Characteristics:**
- All species exhibit motility;
- Weak growth under anaerobic conditions;
- Inability to reduce nitrate to nitrite;
- Does not produce cell wall degrading enzymes;
- The species is serologically homogeneous.
- Produce two types of EPS:

1. Levan (polyfructan);
2. Amylovoran (acidic exopolysaccharide).

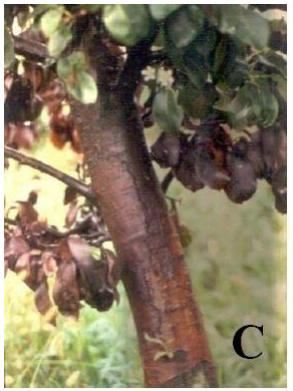
Characteristics of Type species
Erwinia amylovora (Burrill) Winslow *et al.*,

- Tolerance to NaCl: No growth in 5% and higher concentrations of NaCl.
- Optimum pH: 6.8; minimum 4.0-4.4; maximum 8.8.
- Optimum temperature: 30°C; minimum between 3-8°C; maximum 37°C.
- Haemolysis is not produced.
- Catalase is produced.
- Habitat: pathogenic for many species of the family Rosaceae.

Martinec and Kocur *et al.*,1999b

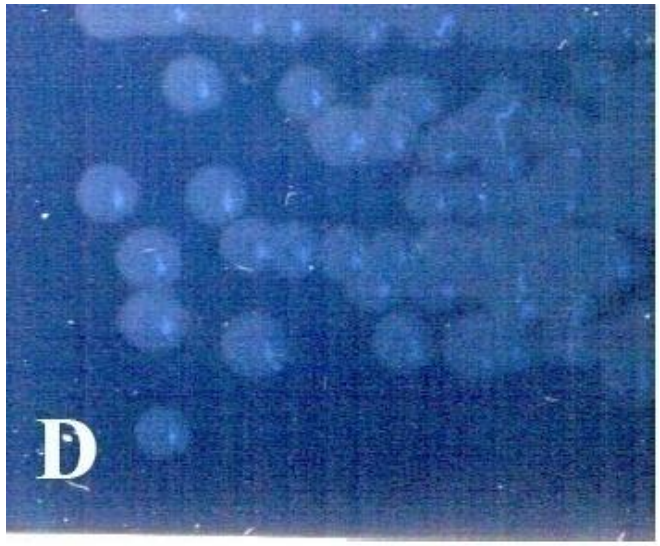
Fire blight symptoms

Erwinia amylovora

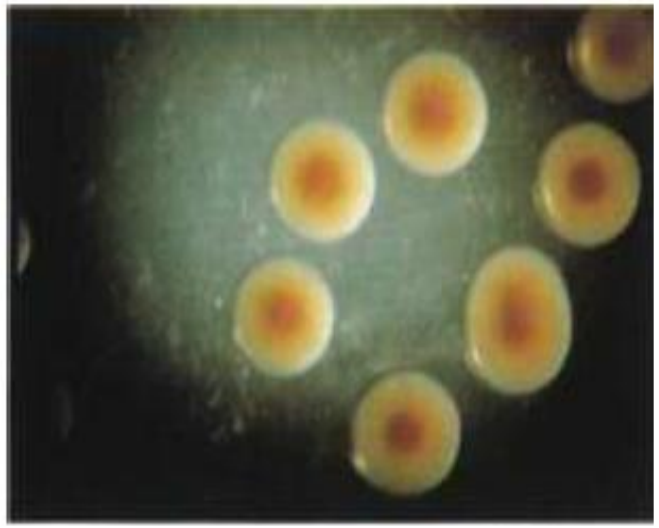
	
Pear blossoms	**Pear shoots**
	
Apple shoot	**Pear trunk Canker**

Bulletin OEPP/EPPO Bulletin 34, 15-157

Colony characteristics of *Erwinia amylovora* on semi selective media



Colonies of *E. amylovora* on CCT.
Crystal violet-Cycloheximide-Tergitol (CCT) medium



Colonies of *E. amylovora* on Miller-Schroth medium(MS)

Bulletin OEPP/EPPO Bulletin **34**, 155-157; Schaad *et al.*,2001

Typical appearance of *E. amylovora* bacterial cultures in the three media

- Typical colony morphology of *E. amylovora* on:

A. King's medium;

B. levan (NSA) medium, and

C. semi selective CCT medium(Crystal violet-Cycloheximide-Tergitol (CCT) medium

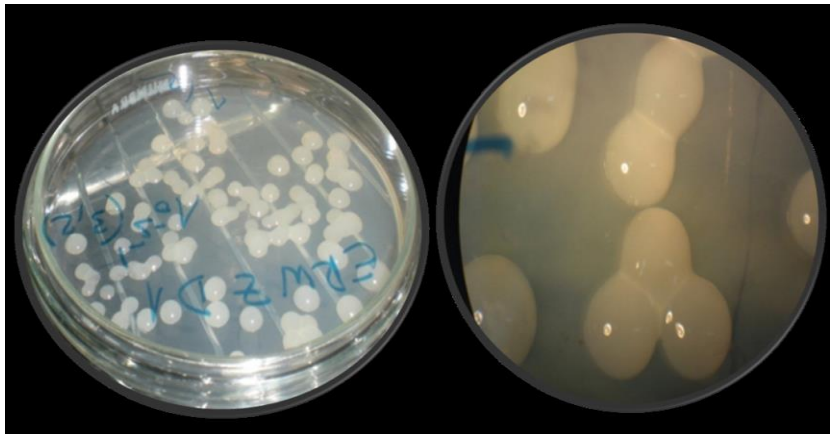
D.

NAS: sucrose nutrient agar medium

Bulletin OEPP/EPPO Bulletin,2013

Typical appearance of *E. amylovora* bacterial cultures on levan medium

- Typical colony morphology of *E. amylovora* on levan (NSA) medium.
- Appearance, color and shape of colonies of *E. amylovora* isolated on Levan medium after an incubation period of 48 h at 25°C.



Bahadou *et al.*,2020

Semi-selective medium

Erwinia amylovora

- **Crystal violet-Cycloheximide-Tergitol (CCT) medium** (Ishimaru & Klos,1984):
- Sucrose 100 g
- Sorbitol 10 g
- Niaproof 1.2 ml
- Crystal violet 2 ml (sol. 0.1% ethanol)
- Nutrient agar 23 g
- D.H_2O to 1 L
- Adjust pH to 7.0-7.2.
- Sterilize by autoclaving at 115°C for 10 min.
- Then prepare: Thallium nitrate 2 ml (1% w/v aqueous solution); cycloheximide 0.05 g (highly toxic reagents, handle with care).
- Sterilize by filtration (0.45 µm).
- Add to 1 L of the sterile medium (at about 45°C).

Bulletin OEPP/EPPO Bulletin **34**, 155-157

API 20E

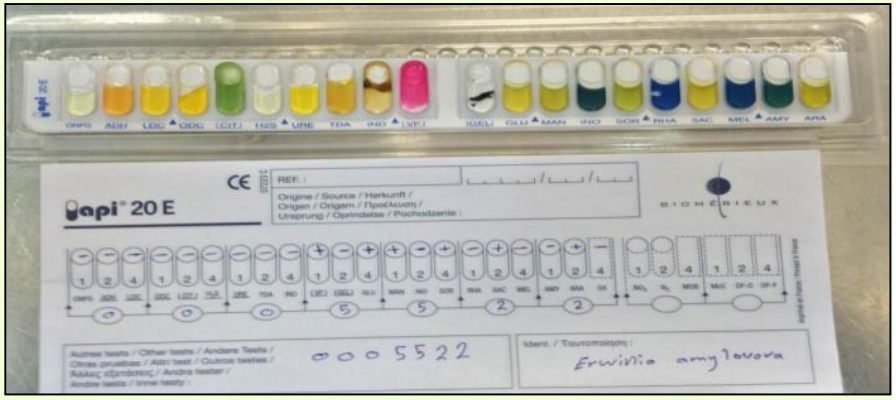
Kit for *Erwinia amylovora*

- Biochemical identification of *E. amylovora* can be obtained by specific profile in API 20 E and API 50 CH strips.
- The manufacturer's instructions should be followed for inoculation of the strip.
- After incubation at 25-26°C, the strips should be read after 24 and 48 h.
- Utilization of the different carbohydrates is indicated by a yellow colour in the well.
- For API 50 CH, a suspension of OD=1.0 should be prepared in PBS, and 1 ml added to 20 ml of Ayers medium.

The API 20E tests

Erwinia amylovora

- Strip containing 20 tests and the profile sheet (0005522) code numbers indicated that pathogen belong to *Erwinia amylovora*.
- Typical readings of *E. amylovora* in API 20E tests after 48 h.



Biochemical characterization of Bulgarian isolates by API 20E in comparison with the type species of *E. amylovora* ATCC 15580.

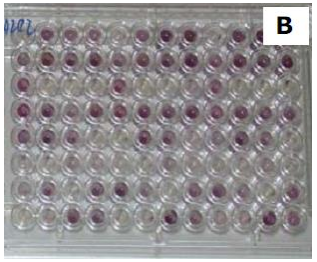
Reaction*	Chokeberry		Strawberry	*E. amylovora*
	2 strains	2 strains	5 strains	ATCC 15580
ONPG	–	–	–	–
ADH	–	–	–	–
LDH	–	–	–	–
ODC	–	–	–	–
CIT	–	–	–	–
H_2S	–	–	–	–
URE	–	–	–	–
TDA	–	–	–	–
IND	–	–	–	–
VP	+	+	+	+
GEL	+	+	+	+
Acid from:				
GLU	+	+	+	+
MAN	+	+	+	+
INO	–	+	+	–
SOR	+	+	+	+
RHA	–	–	+	–
SUC	+	+	+	+
MEL	+	+	+	+
AMY	–	–	–	–
ARA	+	+	+	+
OX	–	–	–	–
API 20E profile	0007562	007762	0007772	0007562

* ONPG, β-galactosidase; ADH, arginine dihydrolase; LDH, lysine decarboxylase; ODC, ornithine decarboxylase; CIT, citrate utilization; H_2S, formation of H_2S;

Bulletin OEPP/EPPO Bulletin 34,155-157; Atanasova *et al.*,2007

Automated Biolog identification system *Erwinia amylovora*

- An identification system based on utilization of 95 carbon sources in a microtiter plate is commercially available (Biolog, US).
- The results from the BIOLOG system were analysed using the compatible software, Microlog™ version 4.20.05, and showed 100% presence of *E. amylovora* (with similarities between 0.916 and 0.959) compared with the positive control (similarity of 0.993).



Bulletin OEPP/EPPO Bulletin **34**, 155 –157; Arsova *et al.*,2017

Immunological test

Antibodies recommended for detection and identification of *E. amylovora*

Antibody	Type	Source
E.amylovora[1]	Polyclonal	Loewe Biochemica, Germany
IVIA EPS 1430[2]	Polyclonal	Plant Print Diagnostics, Spain
IVIA Mab 7A[3]	Monoclonal	Plant Print Diagnostics, Spain
IVIA Mab 8B+5H[4]	Monoclonals	Plant Print Diagnostics, Spain

1 Recommended for detection using IF test (validated in ring tests).
LOEWE Biochemica GmbH.
Mühiweg 2a
D-82054 Sauerlach.Germany

2 Recommended for detection using IF test (validated in ring tests).

3 Recommended for detection using IF test (validated in ring tests).

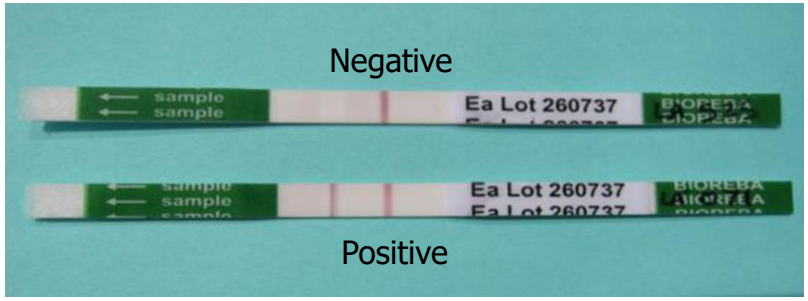
4 Recommended for detection using Enrichment DASI-ELISA test (validated in ring tests).

Immunological test

Ea AgriStrip

Erwinia amylovora

- Ea AgriStrip utilizes antigen-antibody interaction to quickly identify isolates as *E. amylovora* (Bioreba, Reinach, Switzerland).
- Bands appearing on the AgriStrip indicated a positive antigen-antibody reaction targeted specifically to *E. amylovora*.



- The upper Ea AgriStrip is from an assay in which the colony tested was not *E. amylovora*.
- The bottom test is the reaction with a confirmed isolate of the pathogenic bacterium. A positive result should have a red line in both the test and control line regions on the stripe.

Carey,2011

pEA29-PCR
Erwinia amylovora

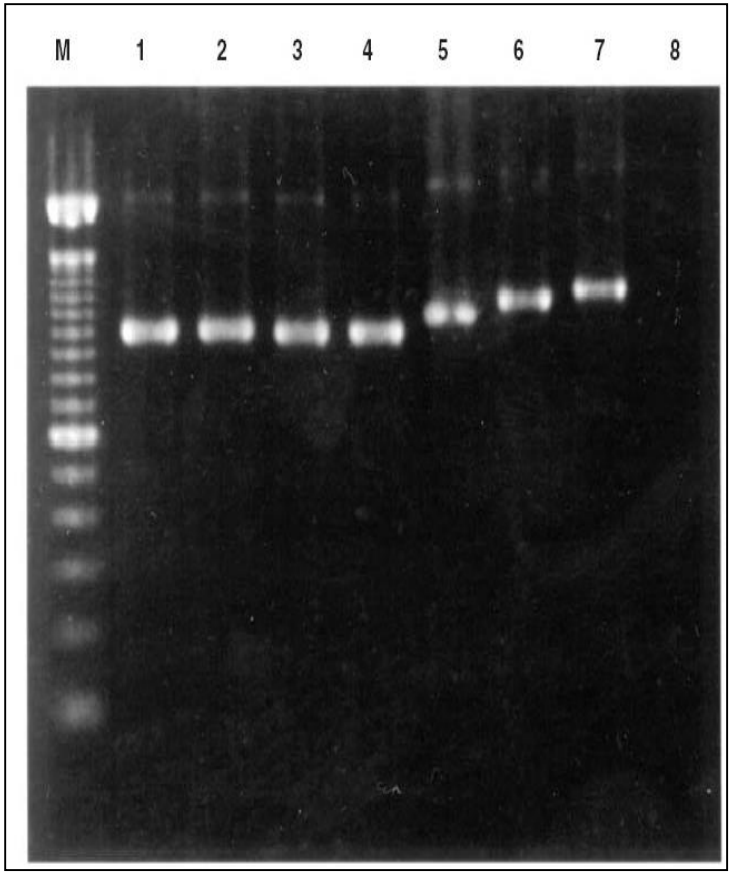
- Detection and identification of the fire blight pathogen, *Erwinia amylovora*, can be accurately done by polymerase chain reaction (PCR) analysis in less than 6 h.
- Two oligomers derived from a 29-kb plasmid which is common to all strains of *E. amylovora* were used to amplify a 0.9-kb fragment of the plasmid.
- This technique could also be used for detection of the fire blight pathogen in extracts of tissue obtained from infected plant material.

Two 17-mer oligonucleotides from the borders of the pEA29 fragment with sequences 5'-CGGTTTTTAACGCTGGG (primer A) and 5'-GGGCAAATACTCGGATT (primer B) were chosen for PCR amplification.

Bereswill *et al.*,1992

pEA29-PCR
Erwinia amylovora

- pEA29-PCR (Bereswill *et al.*,1992) amplification products (1–1.2 kb) generated for the strains listed below demonstrate differentiation among some strains.
- Lane designations:
- M, 100 bp DNA marker;
- 1, E4001A (apple);
- 2, Ea273 (apple);
- 3, Ea416 (*Rubus*);
- 4, Ea528 (*Rubus*);
- 5, Ea510 (*Rubus*);
- 6, Ea546 (Asian pear, Hokkaido, Japan);
- 7, Ea 556 (Asian pear, Hokkaido, Japan);
- 8, water.

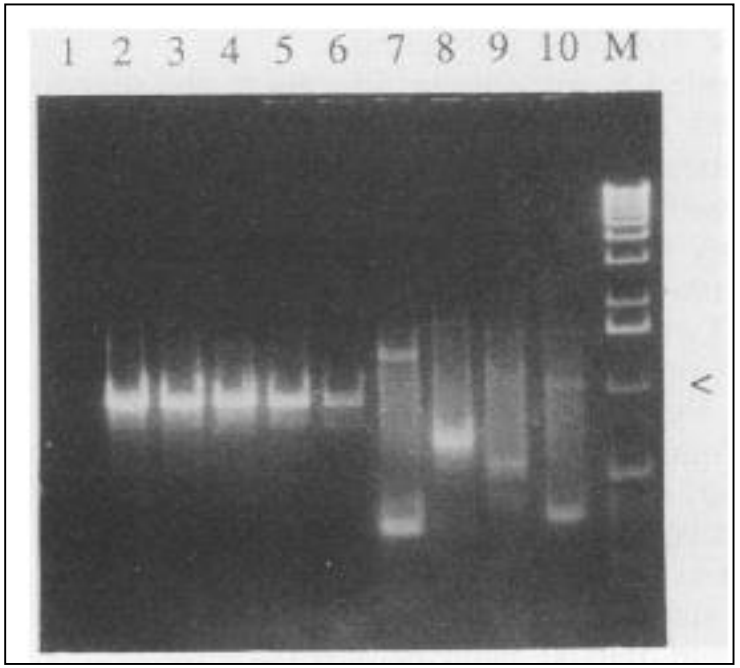


Vanneste,2000

Crude DNA-PCR

Erwinia amylovora

- Lane 1, control without DNA;
- Lanes 2 to 6, crude DNA from *E. amylovora* Eal/79 (1 mg/ml) diluted 10^{-2} to 10^{-6} (a sample of 1 µl applied);
- 7 to 10: 10 ng of crude DNA from:
- *E. herbicola* (strains NZ and 2035);
- *E. carotovora* subsp. *atroseptica* (strain 185);
- *P. syringae* pv. *syringae* (strain 2), respectively.
- M, 1-kb ladder marker DNA (< indicates the size of 1 kb).



PCR of DNA preparations from phytobacteria.

Bereswill *et al.*,1992

Nested PCR analysis

pEA29-nested PCR

- The nested-PCR of Llop et al. (2000) uses two sets of primers, which are combined in a single reaction tube.
- Because of the different annealing temperatures the two PCRs are run consecutively.
- The external primers are those designed by McManus and Jones (1995) and are based on sequences of the pEA29 plasmid.
- The internal primers are those described by Llop et al. (2000).
- The sequences are the following:
- External primers:
  - Forward primer: AJ75: 5′-CGT ATT CAC GGC TTC GCA GAT-3′
  - Reverse primer: AJ76: 5′-ACC CGC CAG GAT AGT CGC ATA-3′
- Internal primers:
  - Forward primer:PEANT1: 5′-TAT CCC TAA AAA CCT CAG TGC-3′
  - Reverse primer: PEANT2: 5′-GCA ACC TTG TGC CCT TTA-3′.

Nested PCR analysis

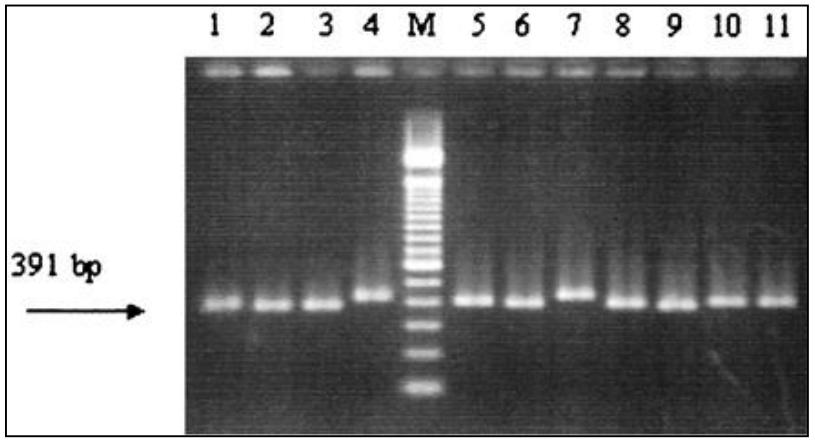
pEA29-nested PCR

- The PCR mixture is composed of: ultrapure water, 36.25 µl; buffer 10×, 5 µl; $MgCl_2$ 50 mM, 3 µl; dNTPs 10 mM, 0.5 µl; AJ75 0.1 pmol/µl, 0.32 µl; AJ76 0.1 pmol/µl, 0.32 µl; PEANT1 10 pmol/µl, 1 µl; primer PEANT2 10 pmol/µl, 1 µl; Taq DNA polymerase 5 U/µl, 0.6 µl.
- A DNA sample volume of 2 µl should be added to 48 µl PCR mix.
- The cycling parameters are:
- a denaturation step of 94 °C for 4 min followed by 25 cycles of 94 °C for 60 s and 72 °C for 90 s.
- This first round PCR is followed in the same thermocycler by a second denaturation step of 94 °C for 4 min and 40 cycles of 94 °C for 60 s, 56 °C for 60 s, and 72 °C for 60 s, with a final elongation step at 72°C for 10 min.
- The expected amplicon size is 391 bp, although variations in size can occur.

Nested PCR analysis

Detection of *Erwinia amylovora* by nested PCR

- Diversity of fragments obtained after amplification following the nested-PCR method in a single closed tube.
- The sizes vary, including:
- The expected 447 bp (lanes 4 and 7);
- 391 bp (lanes 1, 2, 3, and 9);
- Intermediate values (lanes 5, 6, 8, 10, and 11).
- Lane M, marker(100-bp DNA ladder).



Llop *et al.*,2000

Specificity of the nested PCR in a single closed tube compared to that of other nested PCR methods

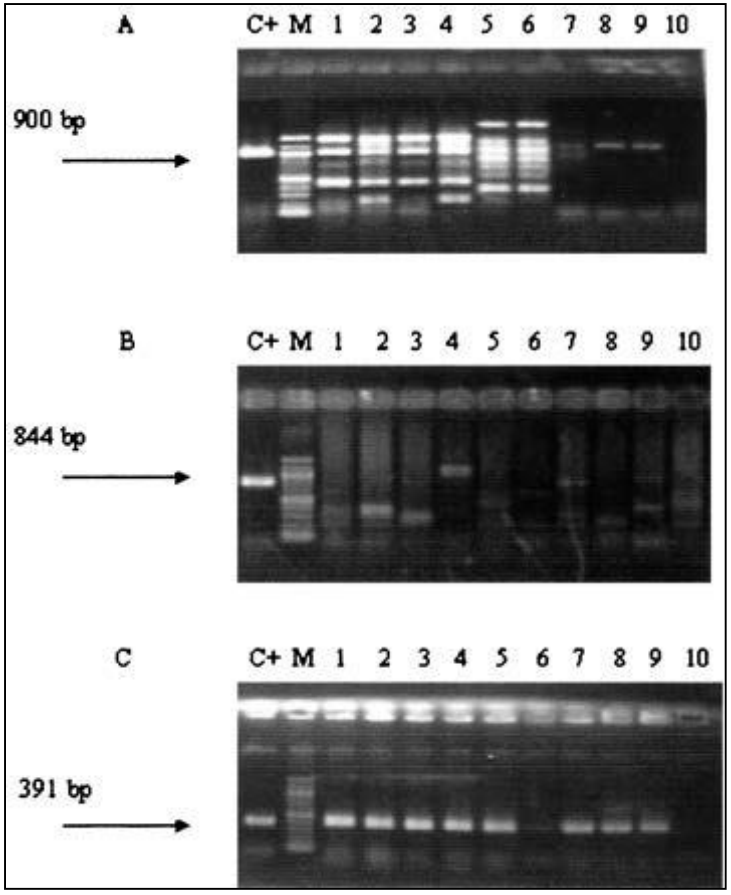
Erwinia amylovora

A. Standard nested PCR procedures: Samples were analyzed by one-round PCR using the primers described by Bereswill *et al.*,1992.

B. Standard nested PCR procedures: The primers described by McManus and Jones,1995.

C. The nested PCR developed in this work.

- Note that the first two pairs of primers produce unspecific amplifications.
- Sample number 6 gave a faint band.
- C+, positive control;
- M, marker (100 bp);



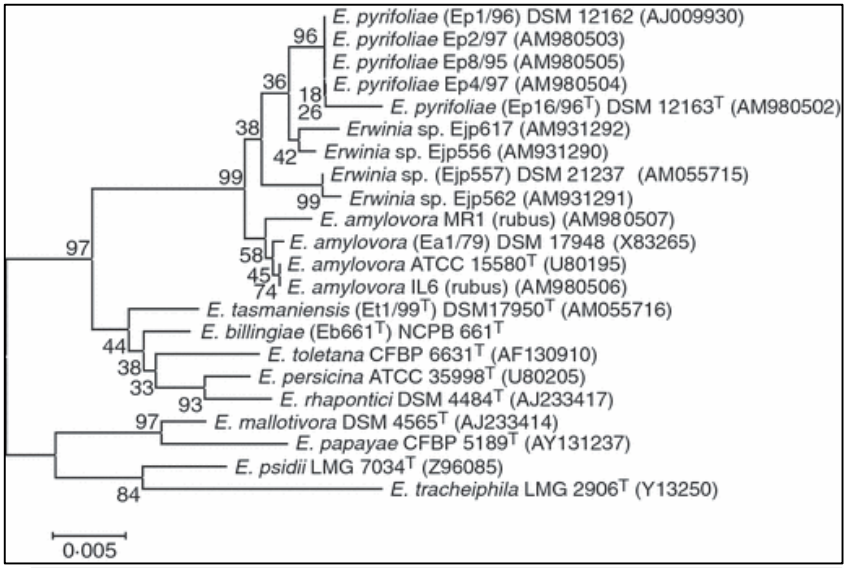
Llop *et al.*,2000

PCR analysis

16S rRNA sequences

Phylogenetic placement of the pear pathogenic *Erwinia* strains from Japan *E. pyrifoliae*

- Dendrogram from 16S rRNA sequences for allocation of *Erwinia* strains from Japan isolated from pear flora as *Erwinia pyrifoliae*.
- Bootstrap values, expressed as a percentage of 1000 replications, are given at branching points (unrooted tree).
- The accession numbers of the nucleotide sequences used in the dendrogram are provided in parenthesis at the end of the lines.



A dendrogram with 16S rRNA sequences of predominantly the type strains of all *Erwinia* species placed the *Erwinia* strains from Japan close together with *E. pyrifoliae* and in a short distance to *E. amylovora* (*Erwinia amylovora* strains form a clade, where the type strain is adjacent to the fruit tree strain Ea1/79 (DSM 17948) and rubus strains MR1 and IL6.
Other *Erwinia*-species such as *Erwinia billingiae*, *Erwinia tasmaniensis*, *Erwinia persicina* and *Erwinia rhapontici* were well separated from *E. pyrifoliae*, the *Erwinia* strains from Japan and *E. amylovora*.

Geider *et al.*,2009

RAPD analysis

Erwinia amylovora

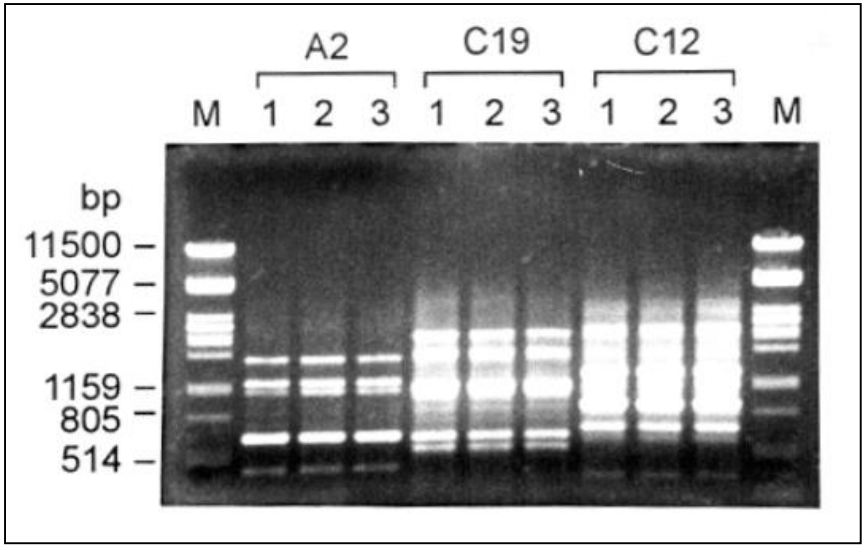
- Total genomic DNA was prepared from 5 ml liquid culture as described by Ausubel *et al.*,1989 or with Genomix kit (Talent, Trieste, Italy).
- PCR assay for amplification of a specific 0.9 kb DNA fragment of plasmid pEA29 was carried out as described by Bereswill *et al.*, 1992.
- For RAPD analysis, PCR amplifications were carried out in 25 µl volume and contained 50 ng of genomic DNA, 2 mM $MgCl_2$, 0.4 M primer, 0.1 U of Taq DNA polymerase, 100 µM (each) dNTP in 10 mM Tris-HCl (pH 9.0), 50 mM KCl and 0.1% Triton X-100.
- Amplification was performed in a thermal cycler programmed for one cycle of 5 min at 94° C, 1 min at 35° C and 2 min at 72° C; 40 cycles of 1 min at 94° C, 1 min at 35C and 2 min at 72C, and a final extension for 5 min at 72° C.
- Genomic diversity of various *E. amylovora* strains was assessed by the RAPD analysis using 37 different 10-base arbitrary primers from kits A,B,C,D and E.
- After PCR, 12.5 µl of the product was electrophoresed in 1.2% agarose gel and visualized by ethidium bromide staining.

Manulis *et al.*,1998

RAPD analysis

Erwinia amylovora

- RAPD patterns generated with *Erwinia amylovora* strains using primers A2, C19 and C12.
- Lane M contains molecular weight markers of lambda DNA digested by P*st*1.
- Numbers 1-3 refer to strains:
- 105/4 (pear),
- Ea1004 (apple) and
- GD1 (quince), respectively.
- All the strains produced the same RAPD patterns with each of primers.

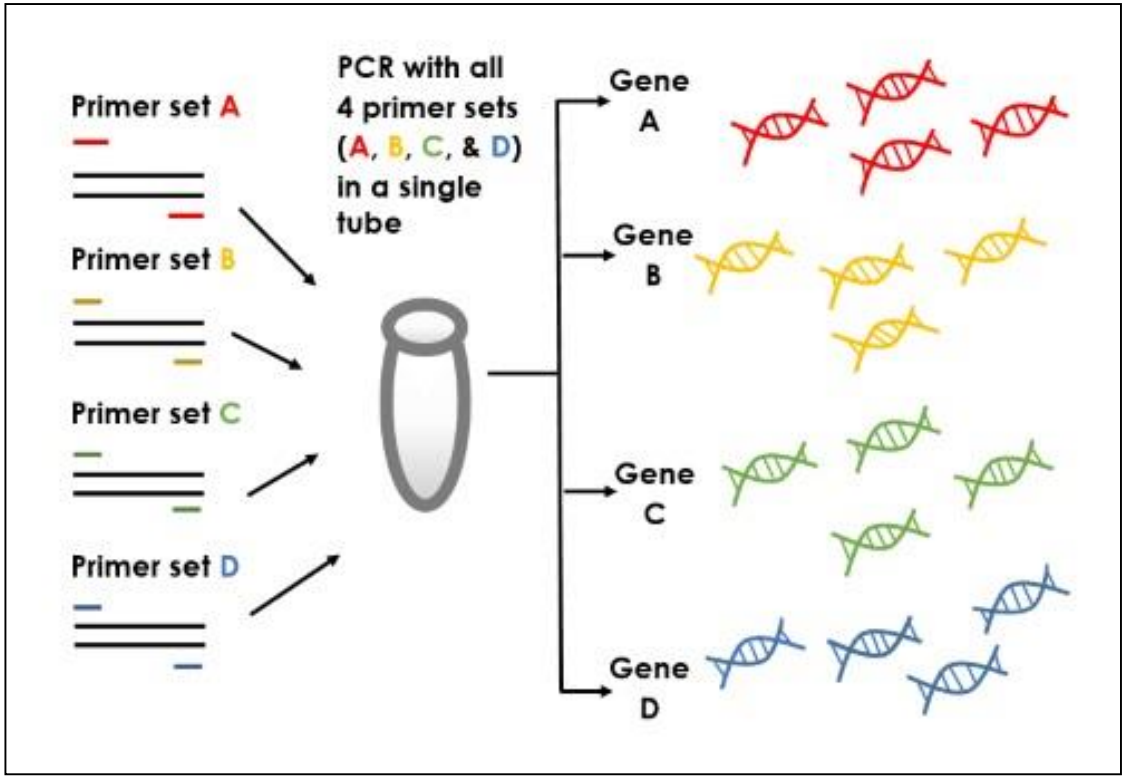


Manulis *et al.*,1998

Multiplex PCR

The simultaneous detection of two or more DNA by multiplex PCR or by multilocus sequence typing (MLSA-MLST)

- The strains were characterized by multiplex PCR and by multilocus sequence analysis/typing (MLSA-MLST) based on seven housekeeping genes.



Duplex PCR
ams-genes and pEA29
Erwinia amylovora

- One-step multiplex PCR for simultaneously identification of the both *ams*-genes and pEA29 in order to identify rapidly *E. amylovora*.
- Two pairs of primers were used:

1. One pair was based on plasmid pEA29 [A (5`-CGGTTTTTAACGCTGGG-3`) and B (5`- GGGCAAATACTCGGATT-3`), and
2. The other was based on chromosomal DNA ams-region:

- AJ245 (5`- AGCTGGCGGCACTTCACT-3`) and AJ246 (5`-CCCCGCACCGTTCAGTTTT-3`), or
- AMSb1 A (5`-GCTACCAGCAGGGTGAG-3`) and AMSb1 B (5` TCATCACGATGGTGTAG-3`).
- The multiplex PCR amplification was performed at the conditions of the single PCR amplification of *ams*-region and a fragment of the pEA29 plasmid.

Bereswill *et al.*,1995; Kabadjova-Hristova *et al.*,2006; Ashmawy *et al.*,2015

Duplex PCR
ams-genes and pEA29
Erwinia amylovora

- All strains were subjected to a duplex PCR analysis using primers based on sequences of the 29-kb plasmid pEA29 of *E. amylovora* AJ75 (5'-CGTATTCA CGGCTTCGCAGAT) and AJ76 (5' ACCCG CCAGGATAGTCGCATA) that amplify an 844-bp fragment (McManus and Jones 1995) and also from the chromosomic ams-region using primers AMSbL (5'- GCTACCAGCAGGGTGAG-3') and AMSbR (5'-TCATCACGATGGTGTAG - 3'), that amplify a 1.6- kbp fragment (Bereswill et al. 1995).
- The PCR amplification was performed using 1 µl of bacterial suspension (10^9 cfu/ml) of *E. amylovora*, treated at 100°C for 10 min.
- The final PCR reaction mix contained 10 pmoles of each primer, 1X Buffer Taq flexi (Promega), 1.5 mM $MgCl_2$, 0.1 mM of each dNTP and 1 U (0.2 µl) of Taq DNA polymerase, in a final volume of 20 µl.
- Sterile water was used as DNA-negative control in every PCR run.

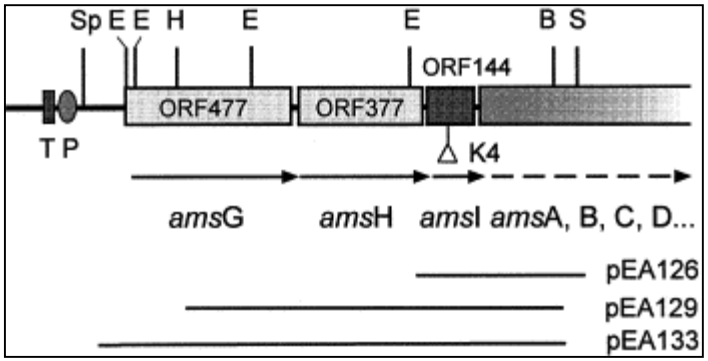
Hannou *et al.*,2013

Duplex PCR

ams-genes and pEA29

Erwinia amylovora

- PCR products were positive and represented the expected length 1.1 and 1.6 kb, for plasmid pEA29 and chromosomal DNA ams–region, respectively.
- Note: A cluster of 12 genes in ams-region coding for *E. amylovora* amylovoran, a complex exopolysaccharide with high molecular weight and act as a key factor of pathogenicity.



Comparison	ΔrcsC/WT	ΔrcsB/WT	ΔrcsC/WT	ΔrcsB/WT
Gene	Pear	Pear	MBMA	MBMA
rcsA	0.25	0.05	2.3	0.02
rcsB	1.25	0.0002	1.15	0.0009
rcsC	0.006	0.67	0.036	0.61
rcsD	0.75	0.56	0.89	0.51
amsA	0.23	0.19	3.0	0.26
amsB	0.27	0.21	2.9	0.33
amsC	0.27	0.17	5.8	0.2
amsD	0.28	0.15	2.8	0.11
amsE	0.33	0.24	4.2	0.18
amsF	0.26	0.17	2.8	0.14
amsG	0.30	0.15	2.9	0.1
amsH	0.29	0.27	3.58	0.35
amsI	0.37	0.30	1.9	0.27
amsJ	0.28	0.31	2.34	0.46
amsK	0.76	1.25	2.01	0.43
amsL	0.52	0.49	2.55	0.68

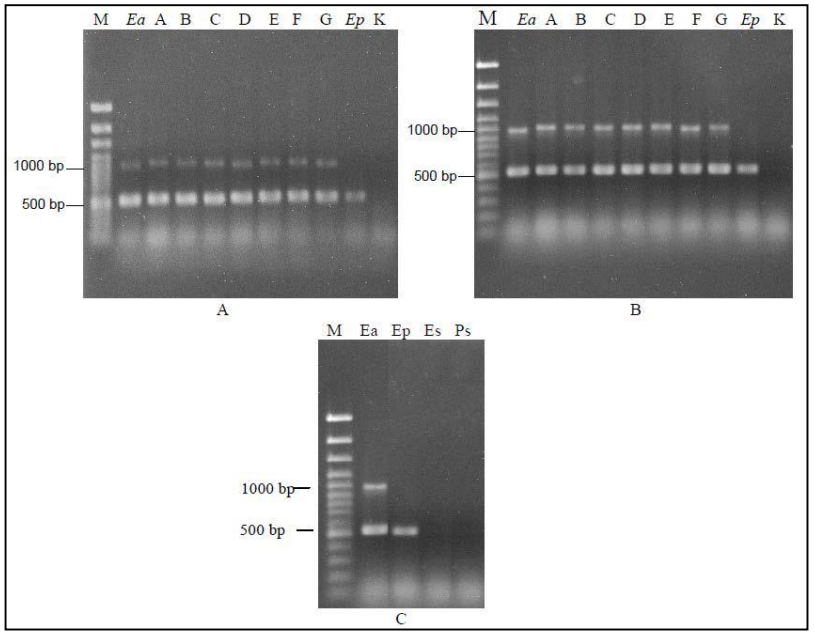
Bugert and Geider,1997; Wang *et al.*,2011; Ashmawy *et al.*,2015

Duplex PCR

ams-genes and pEA29

Erwinia amylovora

- Duplex PCR of the ams-region and the pEA29 plasmid of the *E. amylovora* strains.
- Lanes A-C, strains from chokeberry; lanes D-G, strains from strawberry; *Ea*, type strain of *E. amylovora* ATCC 15580;
- *Ep*, type strain of *E. pyrifoliae* DSM 12163; *Ps*, type strain of *E. stewartii* ATCC 8199; *Ps*, type strain of *P. syringae* pv. *syringae* 2420 NBIMCC; K, control (PCR-mixture without DNA без); M, 100 bp DNA marker.



Kabadjova-Hristova *et al.*,2006

Duplex PCR

ams-genes and pEA29

Erwinia amylovora

- Forty-eight hour-old bacterial colonies were picked-up from Levan agar medium, transferred into Levan broth and incubated at 28°C overnight on a rotary shaker.
- The bacterial cultures were centrifuged for 10 min at 14000 rpm and the pelleted bacterial cells were resuspended in SDW.
- Cells suspensions in SDW were lysed by heating for 15 min at 95°C, quickly cooled on ice and centrifuged for 5 min at 7000×g.
- The obtained supernatants were used to perform amplification by duplex PCR.

Tafifet *et al.*,2020

Duplex PCR
ams-genes and pEA29
Erwinia amylovora

- The duplex PCR protocol was carried out using two pairs of primers:

a) AJ75 (5-CGTATTCACGGCTTCGCAGAT) and AJ76 (5-ACCCGCCAGGATAGTCGCATA), a pair that amplifies an 844-bp fragment of the 29-kb plasmid pEA29 of *E. amylovora* (McManus and Jones, 1995), and

b) AMSbL (5-GCTACCAGCAGGGTGAG-3) and AMSbR (5-TCATCACGATGGTGTAG-3), a pair that amplifies a 1.6-Kb fragment of the chromosomic ams region of *E. amylovora* (Bereswill *et al.*,1995).

- Sterile water and genomic DNA extracted from the French E. amylovora strain CFBP 1430 were used as negative and positive amplification controls, respectively.

Tafifet *et al.*,2020

Duplex PCR

ams-genes and pEA29

Erwinia amylovora

- PCR amplification was performed as described in Hannou *et al.*, 2013.
- The PCR program included an initial denaturation at 94°C for 5 min, followed by 35 cycles of 45 s at 94°C for denaturation, 45 s at 52°C for annealing, and 1 min and 45 s at 72°C for extension; with a final extension at 72°C for 10 min (Hannou *et al.*,2013).
- Amplified fragments were loaded on 1.5% agarose gel to be separated by electrophoresis.

Tafifet *et al.*,2020

Real-time PCR assays
Erwinia amylovora

- Specific and sensitive TaqMan real-time PCR assays were developed targeting chromosomal DNA of *Erwinia amylovora* (*ams* C gene and ITS region).
- These assays increased the reliability of detection of:

1. *E. amylovora* strains, regardless of their plasmid profile, and
2. To differentiate between:

- *Erwinia* spp. strains from Hokkaido island, Japan,
- *Erwinia pyrifoliae* and
- *Erwinia* spp. isolated from necrotic pear blossoms in Spain.

Pirc *et al.*,2009

Primers for *Erwinia* spp.

Erwinia amylovora

- The real-time PCR assays reliably detected at least 10^3 cells mL^{-l} (*c.* four cells per reaction) of the pathogen from blighted woody plant material.
- The real-time PCR assays were targeting chromosomal .... ITS (16S-23S rRNA ITS).
- The primer used for this assays amplify a 79-bp fragment.
- The primers were used:
- ITS15F, TGA GTA ATG AGC GAG CTA AGT GAA G-
- ITS93R, CGC AAT GCT CAT GGA CTC AA-

Pirc *et al.*,2009

Primers for *Erwinia* spp.

PCR primers for *Erwinia* spp.

Specificity	Primer designation	Sequence	Size(bp)
E. amylovora pEA29	A B	(5'-CGGTTTTTAACGCTGGG-3') (5'-GGGCAAATACTCGGATT-3')	1,000[a]
	AJ75 AJ76	(5'-CGTATTCACGGCTTCGCAGAT-3) (5'-ACCCGCCAGGATAGTCGCATA-3')	107
chromosomal *ams* region	AMSbL AMSbR	(5'-GCTACCAGCAGGGRGAG B3') (5'-TCATCACGATGGTGTAG B3')	1,635
	AJ245[b] AJ246	(5'-AGCTGGCGGGCACTTCACT-3') (5'-CCCCGCACCGTTCAGTTTT-3')	519
chromosomal DNA		(5'-CCTGCATAAATCACCGCTGACAGCTCAATGB3') (5'-GCTACCACTGATCGCTCGAATCAAATCGCCB3')	187
chromosomal 23S rDNA gene	EaF EaR	(5'-GCGCAGTAAAGGGTGACAGCCCCGTACACAAAAAGGCAT-3') (5'-CCCTAGCCGAAACAGTGCTCTACCCCCGG- 3')	565
E. pyrifoliae chromosomal *cps*	CPS1L CPS2Rc	(5'-CGCGGAAGTGGTGAGAA-3') (5'-GAACAGATGTGCCGAGTA-3')	1,200
16S rDNA/ITS	Ep16A EpIG2c	(5'-AGATGCGGAAGTGCTTCG-3') (5'-ACCGTTAAGGTGGAATC-3')	700
E. tracheiphila 16S rDNA	ET1 ET2	(5'-TGAGTTCCCGACCAAAT-3') (5'-GGGAGGAAGGGACGCTG-3')	706

[a] The size of the amplified fragment may differ slightly from strain to strain due to the number of 8-bp repeat sequences within the fragment.

[b] Can be combined in the same reaction with primers AJ75/AJ76 to yield the two expected amplification products. These primers cross react with *E. pyrifoliae*

Schaad *et al.*,2001

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

Genus *Erwinia*

Species	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
E. amylovora	**A/B** Plasmid DNA (pEA29)	Conventional	Bacteria, plant (untreated)	Bereswill *et al.*, 1992; Brown *et al.*, 1996	
E. amylovora	**AMSbL/AMSbR** Chromosomal (*ams* genes) region **fD2/rP1** 16S rRNA gene	Conventional	Bacteria (untreated)	Bereswill *et al.*, 1995	Amplification also obtained for pathogenic strains that lack plasmid pEA29.
E. amylovora	**A/B (external)** Plasmid DNA (pEA29) + **AJ75/AJ76 (internal)** Plasmid DNA (pEA29)	Nested	Plant (GeneReleaser)	McManus and Jones, 1995	
E. amylovora	**Ea71** Chromosomal DNA (unknown)	Conventional	Bacteria (untreated), plant (enrichment followed by immuno-capture)	Guilford *et al.*, 1996	Amplifies also pathogenic strains that lack plasmid pEA29.
E. amylovora	**EaF/EaR** 23S rRNA gene	Conventional	Bacteria (proteinase K), plant (PVP and PVPP addition) lysates	Maes *et al.*, 1996a	Amplifies also *Erwinia piriflorinigrans* isolated from necrotic pear blossoms.
E. amylovora	**AJ75/AJ76 (external)** Plasmid DNA (pEA29) + **PEANT1/PEANT2 (internal)** Plasmid DNA (pEA29)	Nested	Bacteria, plant (DNA extraction)	Llop *et al.*, 2000	
E. amylovora	**PEA71** Chromosomal DNA	Conventional BIO	Bacteria (untreated), plant (DNA extraction, GeneReleaser™)	Taylor *et al.*, 2001	Amplifies also pathogenic strains that lack plasmid pEA29.
E. amylovora	See: Bereswill *et al.*, 1992; Llop *et al.*, 2000	Conventional Nested	Bacteria, plant (DNA extraction)	Anon., 2004a	Recommended in the EPPO protocol.
E. amylovora	**P29TF/P29TR (primers)** **P29TM (probe)** Plasmid DNA (pEA29)	Real-Time (TaqMan) (SBYR® Green Master Mix)	Bacteria (lysed), plant (untreated)	Salm and Geider, 2004	

Palacio-Bielsa *et al.*,2009

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

Genus *Erwinia*

E. amylovora	**pEA29A/pEA29B** Plasmid DNA (pEA29) **AJ245/AJ246** Chromosomal *ams* region	Multiplex	Bacteria (DNA extraction)	Kabadjova-Hristova *et al.*, 2006	Amplification also obtained for pathogenic strains that lack plasmid pEA29.
E. amylovora	**A/B** Plasmid DNA (pEA29) **PEANT1/PEANT2** Plasmid DNA (pEA29) **AJ75/AJ76** Plasmid DNA (pEA29)	Conventional	Plant (DNA extraction)	Stöger *et al.*, 2006	
E. amylovora	**E3/E4** Plasmid DNA (pEA29) + **PEANT1/PEANT2** Plasmid DNA (pEA29)	Real-time (duplex format of Scorpion) Nested-Scorpion	Bacteria (DNA extraction)	De Bellis *et al.*, 2007	
E. amylovora	**FER 1-F/FER 1-R** Chromosomal DNA (unknown)	Conventional	Bacteria (boiled)	Obradovic *et al.*, 2007	Amplifies also pathogenic strains that lack plasmid pEA29.
E. pyrifoliae	**EP16A/EPIG2c** 16S rRNA/ITS region **CPS1/CPS2c** *cps* region	Conventional	Bacteria (DNA extraction), plant (untreated)	Kim *et al.*, 2001	
E. pyrifoliae	**EpSPF/EpSPR** Chromosomal DNA (unknown)	Conventional	Bacteria (DNA extraction)	Shrestha *et al.*, 2007	

Palacio-Bielsa *et al.*,2009

Pathogenicity test
Erwinia amylovora

- Inoculation on leaves of detached pear shoots:
- Left, negative control;
- Right, *E. amylovora*.

Bulletin OEPP/EPPO Bulletin **34**, 155-157

Pathogenicity test

Erwinia amylovora

- Symptoms observed in immature pear fruit infection by *E. amylovora*.

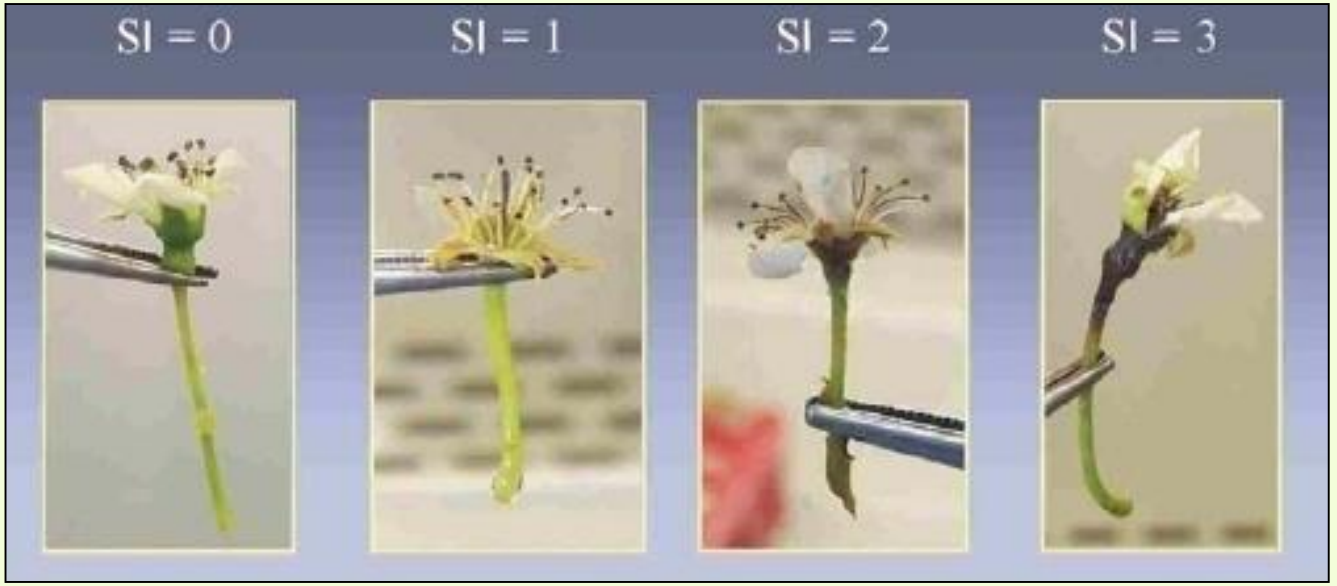
A. oozes
B. Necrosis, or
C. necrosis with oozes.

The cell conc. was adjusted turbidimetrically to 10^7cfu/ml. Absorbance 0.1 at 540 nm=10^8 cfu/ml.

Olamendi,2005

Severity Index Of Blossom Blight
Erwinia amylovora

- Severity Index(SI) of fire blight in detached flowers.
- Levels: 0, no symptoms observed; 1, partial pistil necrosis; 2, total pistil necrosis; 3, necrosis progression through peduncle.



Olamendi,2005

Evaluating fire blight resistance
Erwinia amylovora

- Inoculations were conducted by bisecting the two youngest leaves, on actively growing shoots, with scissors that had been dipped into the inoculum at a concentration of ~ 4.7 x 10^8 cfu/ml prior to each cut.
- Disease assessments were made 40 days after the initial inoculations.
- Lesion length and total length of the current season's growth were measured.
- The severity of infection was calculated as the length of the fire blight lesion as a percentage of overall shoot length.

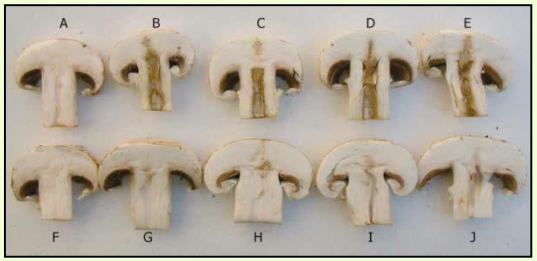
Bell *et al.*,2009

Pathogenicity test

Performed with *A. bisporus* to verify the pathogenicity of *Ewingella americana* and other species of the family of Enterobacteriaceae

- The test of pathogenicity to the host was performed by injecting a buffer-washed bacterium directly into the stipe using healthy cultivated mushrooms.
- After inoculation, the mushrooms were incubated for 3 days at ambient temperature (21-23°C) and examined for symptoms (stipe brown lesions).
- The control treatments consisted of a batch of mushrooms injected with the phosphate buffer solution alone.
- The pathogenic potential of *E. americana* only involved *A. bisporus*, suggesting a certain host specificity.
- These lesions have been attributed to the chitinolytic activity of *E. americana* situated in the stipe where the chitin of the hyphae is diffuse and not well crystallized, making it more susceptible to the action of this enzyme.

A: Inoculated with a phosphate bu!er (control); B: *E. americana* AB/005; C: E. americana AB/007; D: *E. americana* AB/012; E: *E. americana* AB/040; F: *Ent. amnigenus* bgp. 1 AB/013; G: *Ent. amnigenus* bgp. 1 AB/021; H: *K. terrigena* AB/002; I: *Pantoea* spp. bgp.2 AB/003; J: S. rubidaea AB/001.



Reyes *et al.*,2003

Preservation

For routine use

- For routine use, it may be convenient to store cultures in the frozen state.
- *E. amylovora* can be stored in 25% glycerol in a -80° C freezer(Bell *et al.*,2009).
- Short-term (1 year maximum) storage of the isolate can rely on specialized media such as Preservation agar (P agar), which contains per liter 5 g peptone, 5 g sodium chloride, 0.03 g cysteine, and 10 g agar.
- The medium is autoclaved in completely filled screw capped tubes.
- Bacteria are preserved as stab cultures that were allowed to grow for about 4-8 days and then stored at 4°C.

The Prokaryotes (chapter 3.3.15),2006

Characteristics of
The genus *Brenneria*

- Type species – *Brenneria salicis*
- Cause diseases on trees.
- Members within this group are Gram-negative, facultative anaerobes, peritrichous flagella, rod shaped, and acid produced from fructose, glucose, galactose, and sucrose.
- All species exhibit motility.
- Nitrate reduction negative.
- They occur primarily on trees causing various blights, cankers, wilts.
- None of the bacterial species cause a bacterial soft rot.

The current taxonomic position and nomenclature

The genus *Brenneria*

Bacterial species/subspecies (former names)	Bacterial species/subspecies (new names)
Brenneria alni	*Brenneria alni*
B. nigrifluens	*B. nigrifluens*
B. rubrifaciens	*B. rubrifaciens*
B. salicis	*B. salicis*
B. goodwinii	*B. goodwinii*
B. lupinicola	*B. lupinicola*
B. populi	*Lonsdalea quercina* subsp. *populi*
B. quercina	*L. quercina* subsp. *quercina*

Note: Some species in *Brenneria* were transferred into a new genus, *Lonsdalea* gen. nov. such as *Lonsdalea quercina* comb. renamed as *Lonsdalea quercina* subsp. *quercina* comb. nov. Two more subspecies in genus *Lonsdalea* are *L. quercina* subsp. *iberica* and *L. quercina* subsp. *britannica*.

Diseases caused by: *Brenneria* spp.

Brenneria alni	**Bark cankers of alder (*Alnus*) species**
B. goodwinii	**Associated with Acute oak decline**
B. lupinicola	**Drippy pod of mediterranean white lupine (*Lupinus albus*)**
B. nigrifluens	**Shallow bark canker of walnut**
B. rubrifaciens	**Deep bark canker of walnut**
B. salicis	**Wilting and watermark disease in willow (*Salix* ssp.) trees**

Walnut bacterial canker

Brenneria nigrifluens and *B. rubrifaciens*

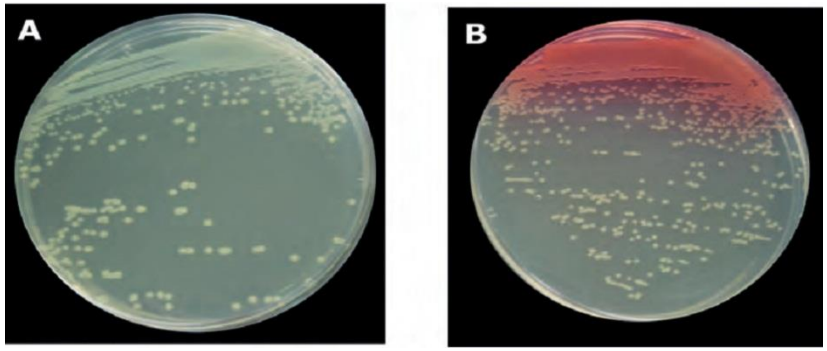
A. Colonies of *B. nigrifluens*

B. Colonies of *B. rubrifaciens*

- on kB (King's B medium) plates after 48 h of incubation at 26-28°C, which are light cream, circular, smooth and raised with entire margins.

A. *B. rubrifaciens* produces the pink soluble pigment when grown on KB medium.

(Photos by E.G. Biosca)



Biosca and López,2012

Distinguishing features of *Brenneria* species

Characteristic	*B. alni*	*B. nigrifluens*	*B. paradisiaca*	*B. quercina*	*B. rubrifaciens*	*B. salicis*
Indole	−	−	+	−	−	−
β-Galactosidase	−	+	+	+	+	+
Pectate degradation	−	−	+	−	+	+
Acid from						
L-Arabinose	+	+	+	−	+	−
Raffinose	−	+	+	−	−	+
Xylose	+	+	+	−	−	−

Based on data from Lelliott and Dickey (1984), Surico et al. (1996), and Hauben et al. (1998a).

The Prokaryotes (chapter 3.3.1),2006

General physiological and biochemical characteristics of type strains of *B. nigrifluens* and *B. rubrifaciens*

Test[a]	*B. nigrifluens* NCPPB 564^T	*B. rubrifaciens* NCPPB 2020^T
Gram stain or KOH 3% test	-	-
Oxidase	-	-
Catalase	+	+
O/F glucose metabolism	+/+	+/+
Nitrate reduction	-	-
Arginine dihydrolase	-	-
Urease	-	-
Indole	-	-
Esculin hydrolysis	+	+
Pectate degradation	-	-
β-galactosidase	+	-
Acid production from:		
L-arabinose	+	+
Raffinose	+	-
Xylose	+	-
Growth at 36ºC	+	-
Growth at 39ºC	-	-

[a] Results after 48 h of incubation at 26-28ºC.

Biosca and López,2012

Distinguishing features of *Brenneria* species

- Phenotypic characteristics that distinguish the five poplar strains from recognized species of the genera *Brenneria* and *Lonsdalea* species:
- 1, *Brenneria populi* sp. nov. (n55); 2, *B. goodwinii* (n59); 3, *B. salicis* (n53); 4, *B. alni* (n55); 5, *B. nigrifluens* (n55); 6, *B. rubrifaciens* (n55); 7, '*B. roseae*' (n57); 8, *L. quercina* (n517).
- n, Number of strains. Data for species 2, 3, 4, 5, 6 and 8 taken from Denman *et al.*, 2012; data for species 7 taken from Brady *et al.*, 2014). +, Positive; 2, negative; V, variable reaction; d, 11–89 % strains positive; NA, not available.

Characteristic	1	2	3	4	5	6	7	8
β-Galactosidase	−	+	+	−	+	+	V	−
Arginine dihydrolase	+	−	−	−	−	−	+	−
Ornithine decarboxylase	−	−	−	−	−	−	+	−
Acid production from (API 50CHB/E):								
L-Arabinose	+	+	−	+	+	+	+	−
Amygdalin	+	+	−	+	−	−	−	−
D-Galactose	+	+	−	+	−	−	+	d
Gentiobiose	d	+	−	−	+	−	−	−
Inositol	d	+	−	−	+	−	+	−
Melibiose	+	+	−	−	+	−	−	−
Potassium gluconate	d	d	+	−	−	−	+	d
Raffinose	+	+	+	−	+	−	−	−
D-Sorbitol	+	+	−	−	+	−	+	−
Trehalose	d*	+	−	+	+	−	−	d
Turanose	−	+	−	+	−	−	−	+
D-Xylose	+	d	−	+	+	−	+	−
Dulcitol	+	−	−	−	−	−	−	−
Utilization of:								
Dextrin	+	−	NA	NA	NA	NA	+	−
Gentiobiose	−	+	NA	NA	NA	NA	−	−
Lactose	−	+	NA	NA	NA	NA	−	−
Maltose	+	−	NA	NA	NA	NA	−	−
L-Rhamnose	+	−	NA	NA	NA	NA	V	−
Xylitol	+	−	NA	NA	NA	NA	−	−
Acetic acid	+	−	NA	NA	NA	NA	−	−
α-Hydroxybutyric acid	+	−	NA	NA	NA	NA	−	−
α-Ketobutyric acid	d	−	NA	NA	NA	NA	−	−
D, L-Lactic acid	+	−	NA	NA	NA	NA	V	V
L-Serine	−	+	NA	NA	NA	NA	+	−
Uridine	+	−	NA	NA	NA	NA	V	−
Thymidine	+	−	NA	NA	NA	NA	+	−
2,3-Butanediol	+	−	NA	NA	NA	NA	−	−

*The type strain is positive.

Li *et al.*,2015

API 20E
Kit for *B. nigrifluens* & *B. rubrifaciens*

- Isolates that were Gram negative, oxidase-negative and with oxidative and fermentative metabolism were submitted to the API 20E procedure.
- *Brenneria nigrifluens* isolates including the reference strains generated a 7-digit code=0005773 in the API 20E system, identical for the type strain of *B. nigrifluens*.
- *B. nigrifluens* cannot be confused with *B. rubrifaciens*, the causal agent of deep bark canker of walnut, which generates different 7-digit codes (0004022 or 0004122).

Moretti *et al.*,2007

API 20E

Kit for *Brenneria* and some other related bacteria

Test	Isolate number and strains							
	6, 10, 12, 13, LMG 2694^T, LMG 5107, LMG 5953	7	36, 43	37, 38	39	40, 41	42	44
β-galactosidase	-	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-	+
Lysine decarboxylase	-	-	-	-	-	-	+	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-
Citrate utilization	-	+	+	+	-	+	-	-
H_2S production	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-
Tryptophane deaminase	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-	-
Acetoin production	+	+	+	-	+	+	+	+
Gelatinase	-	-	-	-	-	-	-	-
Assimilation of :								
Glucose	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+
Inositol	+	-	+	+	+	+	+	-
Sorbitol	+	+	+	+	-	-	+	-
Rhamnose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+
Amygdalin	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-
NO_2 production	-	-	-	+	-	-	+	+
Reduction to N_2	-	-	+	-	+	+	-	+
7-digit code	0005773	1205573	1205773	1204773	1005373	1205373	5005773	3005173
Bacterial identification according to Mergaert *et al.* (1984) codes or API computer service	*Brenneria nigrifluens*	*Pectobacterium cypripedii*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Pantoea agglomerans*	*Pectobacterium rhapontici*	Not identified	*Pantoea* spp.	*Pectobacterium rhapontici*	*Raoultella terrigena*	Not identified

Moretti *et al.*,2007

Fatty acid composition (percentage of peak areas) of type strains of *Brenneria nigrifluens* and *Brenneria rubrifaciens*

Fatty acid class	*B. nigrifluens* NCPPB 564^T	*B. rubrifaciens* NCPPB 2020^T
Saturated fatty acids		
12:0	3.24 (0.29)[a]	3.11 (0.61)
14:0	6.15 (0.10)	7.15 (1.68)
16:0	30.58 (1.82)	29.47 (1.01)
18:0	1.04 (0.73)	0.19 (0.28)
Unsaturated fatty acids		
16:1 w7c	31.04 (0.35)	29.18 (1.22)
18:1 w7c	13.97 (1.25)	12.14 (0.55)
Cyclopropane fatty acids		
17:0	6.63 (0.54)	1.62 (0.02)
19:0	1.29 (0.60)	0
Hydroxy fatty acids		
14:0 3OH	8.99 (0.21)	9.89 (0.24)
Unknown 14.503[b]	0.78 (0.23)	0.42 (0.59)

[a]Mean (%) and standard deviation of two extractions per each strain.
[b]Equivalent chain length relative to the lengths of known fatty acids.

Biosca and López,2012

PCR test

Brenneria nigrifluens

- The 16S rDNA partial sequences of analyzed strains were amplified using the primers:
- P0 (5'-GAGAGTTTGATCCTGGCTCAG-3') and
- P6 (5'-CTACGGCTACCTTGTTACGA-3').
- The 1,359-bp 16S rDNA sequences obtained.
- Additionally, the gyr B gene sequences were generated with primer:
- GyrB-F (5'-MGGCGGYAAGTTCGATGACAAYTC-3') and
- GyrB-R (5'-TRATBKCAGTCARACCTTCRCGSGC-3').
- The gyr B gene sequences of our strains were 100% homologous to the sequences of *B. nigrifluens* deposited in GenBank.

Popovic *et al.*,2013

REP-PCR analysis

Brenneria nigrifluens

- **Procedure:**
- Bacterial isolates were subjected to rep-PCR analysis using the primers REP 1R and REP 2I, according to the procedure of Rademaker and De, 1997.
- This analysis was repeated twice.
- DNA was extracted from bacterial cells grown on Luria-Bertani broth for 16 h at 27±1°C in an orbital shaker at 200 rpm.
- Amplicons were separated by electrophoresis on 1.5% agarose gels in 0.5x TAE buffer (20 mM Tris-acetate, 0.5 mM EDTA, pH 8.0) at 50 V and 4°C for 14 h.
- DNA fingerprints were visualised with a UV transilluminator and their images captured with the EuroClone Photoprint camera system.

Moretti *et al.*,2007

REP-PCR analysis

Brenneria nigrifluens

- Lanes were compared by reading horizontally across the gel image, from bottom to top;
- If a band was present, it was assigned a value of 1 at that location,
- If absent, it was assigned a value of 0.
- The presence/absence of bands was collated into a binary data matrix.
- Cluster analysis was performed on similarity matrices, which were produced using:

1. Dice coefficient (Dice, 1945), or
2. Jaccard coefficient (Jaccard, 1908) and subjected to the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm, using NTSYSpc software, version 2.1.

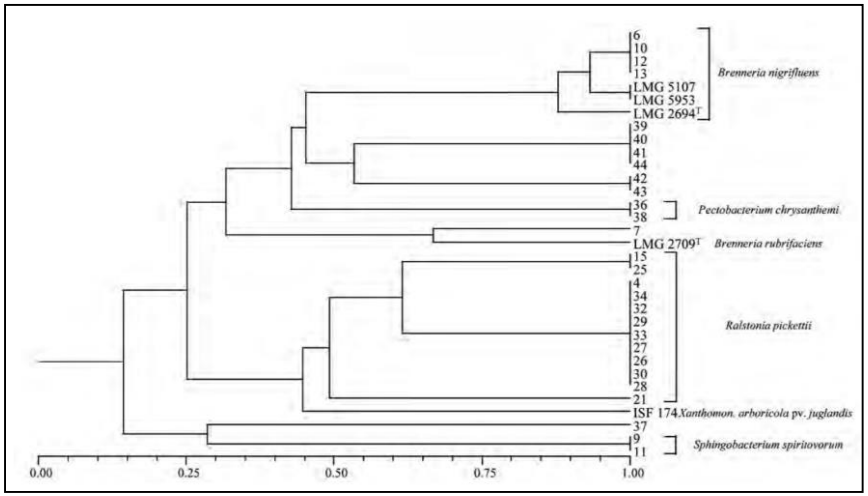
- To establish robustness of clusters, the cophenetic value was obtained using the NTSYSpc software.

Moretti *et al.*,2007

REP-PCR analysis

Brenneria nigrifluens

- The genomic REP-PCR profiles consisted of bands ranging in size from 300 to 2000 bp.
- Seventeen reproducible, clearly resolved bands were scored for UPGMA analysis.
- Dice's coefficient and Jaccard's similarity index yielded very similar dendrograms (data not shown).



Dendrogram derived from REP-PCR fingerprint data, obtained by cluster analysis (UPGMA) and Dice's coefficient.

Moretti *et al.*,2007

PCR test

Brenneria rubrifaciens

- Primers BrAF/BrAR (designed from sequence of asparagine synthase gene) and, 2BrIF/2rBIR (designed from sequence of the autoinducer synthase gene involved in rubrifacine production), previously reported by McClean and Kuepfel (2009) that are shown in Table below.
- They yield amplicons of 537 and 671 bp, respectively.

Name	Sequence (5´- 3´)	References
Forward primer F1	CCTGCGCCATGTTGCCAGATCGCTAT	Loreti *et al.*, 2008
Reverse primer C3	ACCTGAGTAGCAGTTTCGACTATTT	(*B. nigrifluens*)
Forward primer BR1	CAGCGGGAAGTAGCTTGCTACTTTGCCGG	
Reverse primer BR3	TGAAAAAGTCTCTCTTAAACCTTTCC	
Forward primer GSP1F	TAGTGTTGCATTAGCCGATTTAG	
Reverse primer GSP1R	GCATTTAAAGACTATGTTTCCTG	McClean *et al.*, 2008 (*B. rubrifaciens*)
Forward primer GSP2F	CATTACTGTTTCTCCTCGCTAATC	
Reverse primer GSP2R	GATGTAAATTAGCCATACACGGAATG	
Forward primer BrAF	ATGTACGCAGTCTCTATTTGG	McClean and Kuepfel, 2009; Thapa *et al.*, 2010 (*B. rubrifaciens*)
Reverse primer BrAR	CCATCAGCCTGAAATAACTCA	
Forward primer 2BrIF	CGGGATCCATGTTAGAAATATTCGATGTC	
Reverse primer 2BrIR	ATCAGCTGTCAAGCCTCTTCCTTTTTG	

Biosca and López,2012

PCR test
Brenneria rubrifaciens

- Two primer pairs:

i. BrAF; 5′- ATGTACGCAGTCTCTATTTGG corresponding to position 33 to 54 and BrAR; 5′- CCATCAGCCTGAAATAACTCA corresponding to position 548 to 569 of *B. rubrifaciens* asparagine synthetase gene (Genbank accession no. FJ205695), and

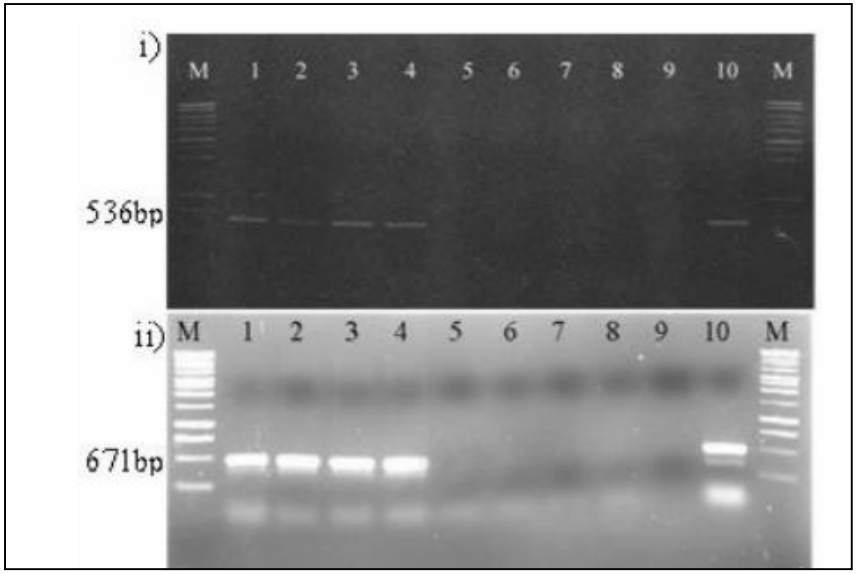
ii. 2BrIF; 5′-CGGGATCCATGTTAGAAATATTCGATGTC and 2BrIR; 5′ ATCAGCTGTCAAGCCTCTTCCTTTTTG (McClean and Kluepfel, 2009) designed from the autoinducer synthase gene involved in rubrifacine production were tested.

iii. Thus, we may expect the size of amplicons as 536 and 671bp to those of BrAF-R and 2BrIF-R, respectively.

Thapa *et al.*,2010

PCR test
Brenneria rubrifaciens

- Specific detection of *B. rubrifaciens* with primers
  i. BrAF/BrAR and
  ii. 2BrIF/2BrIR:
- Mixed bacterial cell:
- Lane 1 to 4, *B. rubrifaciens* LMG 5110 and (WT3, ATCC 15580, LMG 2712 and ATCC 15713, respectively);
- Lane 5, ATCC 15580; Lane 6, LMG 2712; Lane 7, WT3; Lane 8, ATCC 15713; Lane 9, water; Lane 10, chromosomal DNA LMG 5110. B). Lane M, size marker (1kb DNA ladder, Promega™).



Thapa *et al.*,2010

PCR detection and identification of plant pathogenic bacteria *Brenneria salicis*

- Es1A (5'-GCGGCGGACGGGTGAGTAAA-3') and
- Es4B (5'-CTAGCCTGTCAGTTTTGAATGCT-3').
- The expected 553-bp fragment was amplified only from *B. salicis* strains.

Genus *Brenneria*					
Species	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
B. salicis	**Es1A/Es4B** 16S rRNA gene	Conventional	Bacteria (boiled) and plant vascular fluid (DNA extraction)	Hauben *et al.*, 1998	

Palacio-Bielsa *et al.*,2009

PCR test

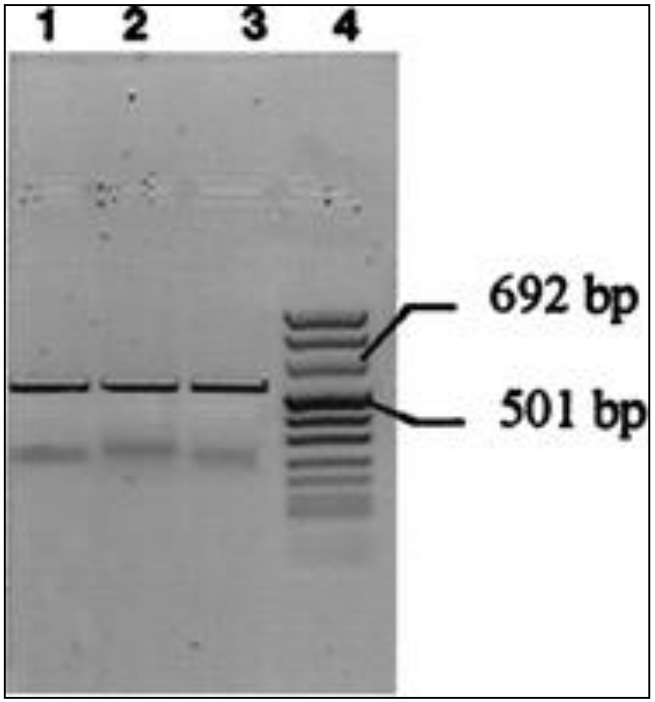
Species-specific detection of *Brenneria salicis*

- A PCR-based identification method for *B. salicis* was developed based on four specific base positions of the 16S ribosomal DNA (rDNA) sequence.
- PCR amplifications: PCR with primers:
- Es1A (5'-GCGGCGGACGGGTGAGTAAA-3') and
- Es4B (5'-CTAGCCTGTCAGTTTTGAATGCT-3') was performed in a Genius thermal cycler.
- The expected 553-bp fragment was amplified only from *B. salicis* strains.
- No DNA fragments were amplified from the 27 related phytobacterial strains.

PCR test

Species-specific detection of *Brenneria salicis*

- PCR product, formed with primers Es1a and Es4b, of vascular fluid of infected willow clones 89/036.73 (lane 1), 89/036.68 (lane 2), and 89/036.62 (lane 3).
- Lane 4, DNA molecular size marker VIII. (Boehringer Mannheim)



The expected 553-bp fragment was amplified only from *B. salicis* strains.

Pathogenicity test
Brenneria nigrifluens

- **Injection method:**
- A drop of bacterial suspension, with a concentration of approximately 10^8 cells ml^{-1}, was injected with a syringe into the bark of an English walnut (*Juglans regia*).
- The hole was then covered for 24 hours parafilm.
- Eight injections were made in the same tree, 4 with bacterial suspension and 4 with sterile water, as a control.
- The trees were kept under observation up to 5 months when the symptoms were observed and attempts were made to re-isolate the bacteria.
- After 5 months, drops of brownish liquid were noted at the injection sites. When the outer part of the bark was removed, damp, blacken areas were exposed, with a diameter of approximately 2.5 cm.
- There were no exudates or black areas in the bark at the control injection sites.

Saccardi *et al.*,1998

Pathogenicity test
Brenneria nigrifluens

- **Wounding method:**
- Bacterial isolates were tested for pathogenicity on 2-year-old Persian walnut seedlings, about 120 cm high.
- To prepare the inoculum, bacteria were grown onto NA at 27±1°C for 48 h, suspended in sterile deionized water and spectrophotometrically adjusted to 10^8 cfu ml^{-1}.
- 20 µl of the suspensions were placed in wounds (about 1 cm long) made in the bark of walnut stems with a scalpel.
- The wounds were protected with Parafilm.
- Sterile water, instead of bacterial suspension, was used for control plants.
- Plants were kept in a heated greenhouse at 22-28°C under natural light conditions and 70-85% RH.
- Three months after the inoculation, symptoms were recorded.

Moretti *et al.*,2007

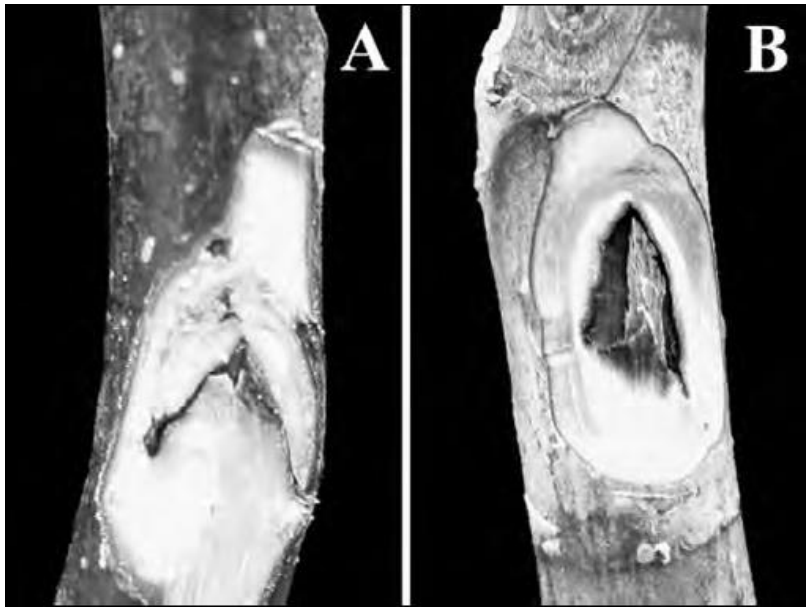
Pathogenicity test
Brenneria nigrifluens

- Pathogenicity test of the of *Brenneria nigrifluens* on Persian walnut plants.

A. Symptoms on control,

B. and inoculated plants 3 months after inoculation.

- Browning of the internal wood is visible under the bark tissue in correspondence of the site inoculated with the bacterial isolate.



Moretti *et al.*,2007

Pathogenicity test
Brenneria nigrifluens

- Immature walnut fruit inoculations:
- The test on immature fruits from adult walnut trees, slightly modified from Moretti and Buonaurio, 2010 by including a disinfection step, was used for assaying *B. nigrifluens* pathogenicity.
- Fruits should be briefly disinfected (1 min) with 70% ethanol and repeatedly washed with sterile water prior to inoculation.
- Then, *B. nigrifluens* (10^8 CFU ml^{-1}(48 from a 48 h culture grown on KB medium) was infiltrated into the mesocarp with a syringe either with needle (in an area about 1 cm in diameter) or without needle (three punctures per fruit, in areas about 0.5-0.8 cm in diameter).
- Inoculated fruits were incubated in plastic boxes for 8 days at 20°C, with a 12 h photoperiod. The test was considered positive when symptoms of necrosis and exudates (starting after 2 days) appear in inoculated sites.

Biosca and López,2012

Pathogenicity test
Brenneria rubrifaciens

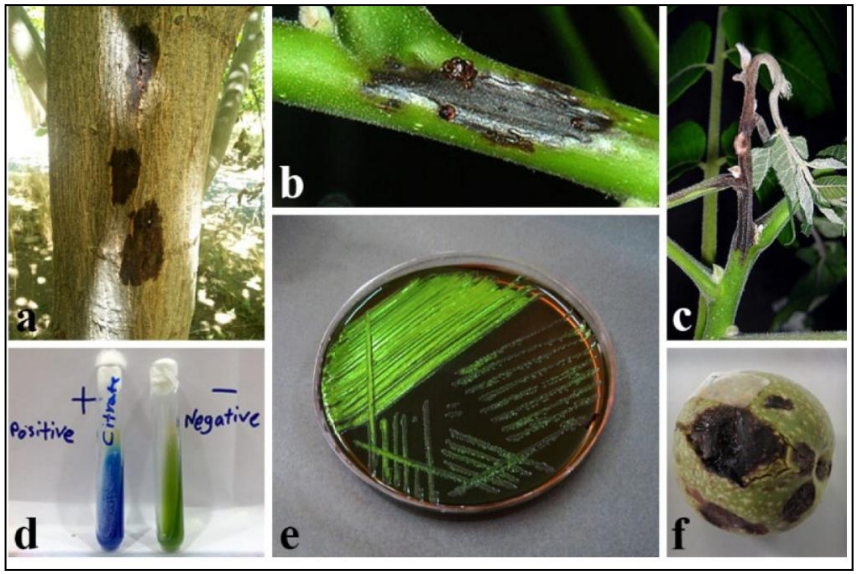
- According to Wilson *et al*.,1957 it is convenient to use walnut plants older than seven years for a better expression of symptoms.
- The test was carried out on Persian walnut plants by inoculating *B. rubrifaciens* suspensions at 10^8 CFU ml^{-1}(48 h culture grown on KB plates) in deep wounds made in the trunk with a knife at 40 and 80 cm from the crown.
- Inoculated plants should be maintained for 3 months at 22-28°C with 16 h light and high RH.
- If external symptoms of DBC were not observed, the bark was carefully removed to observe lesions in the inner part.
- At least, two months of incubation were required.

Biosca and López,2012

Pathogenicity test

The disease, phenotypic and pathogenicity symptoms of *B. rubrifaciens*

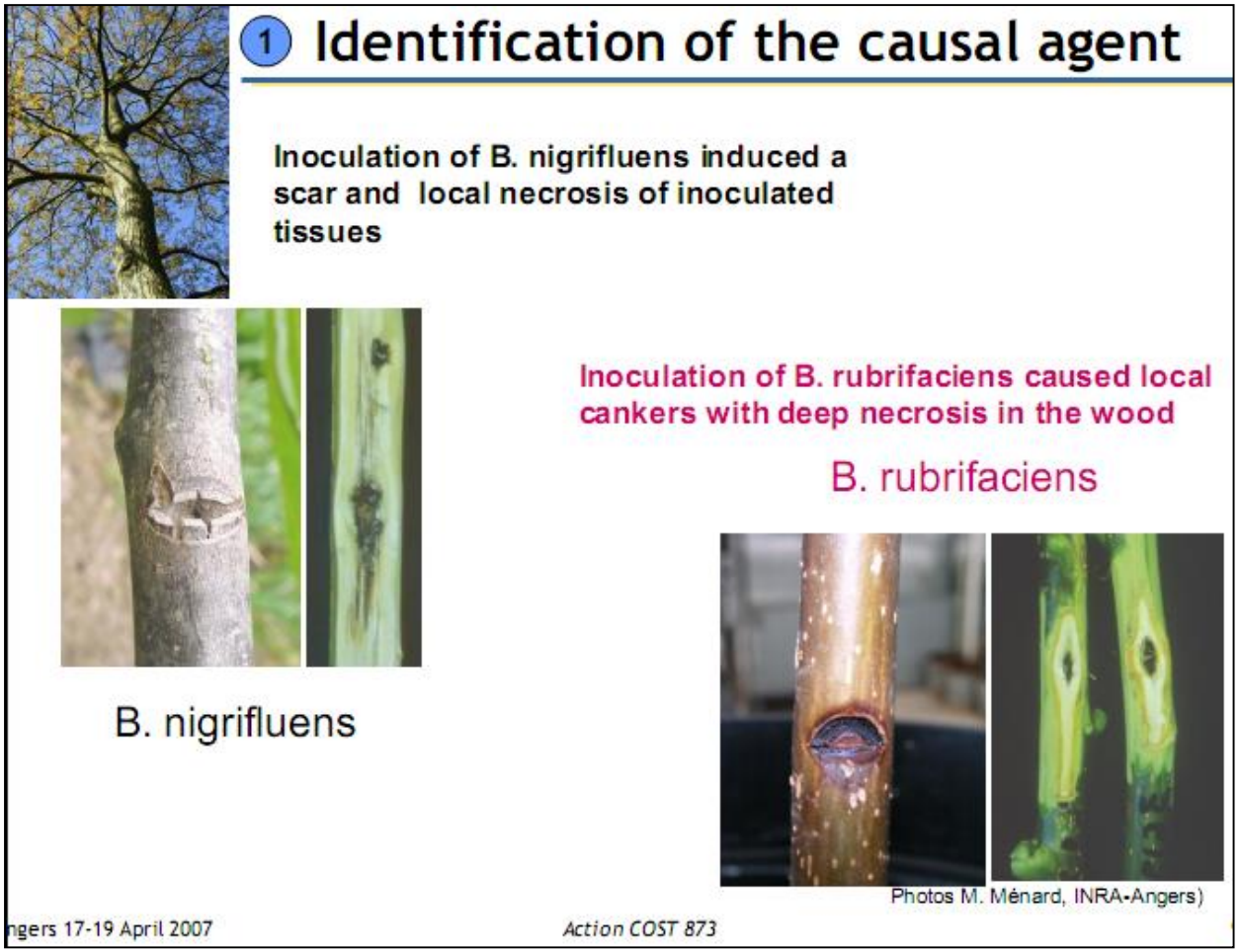
- a) The deep canker symptoms on walnut trunk,
- b, c) the sapling inoculated with *B. rubrifaciens* 10^8 cfu/ml suspension three months after contamination,
- d, e) the created symptoms in citrate test and growth on EMB(Eosin Methylene Blue) medium,
- f) raw walnut fruit inoculated with bacterial suspension 12 days after contamination with 10^8 cfu/ml concentration.



Amirsardari *et al.*,2017

Pathogenicity test

Symptom differentiation caused by *B. rubrifaciens* and *Brenneria nigrifluens*



Meyer *et al.*,2007

Pathogenicity test

Virulence Bioassay

Brenneria rubrifaciens

- Bacterial colonies used for walnut virulence assays were streaked from frozen stocks onto YDCA plates and grown for 4 days at 28°C.
- Walnut plants (cv Hartley) were transferred into individual magenta boxes (1 plant per magenta box) on walnut tissue culture media. All plants were wounded using a sterile scalpel (size 25). Mock treated plants were wounded with the scalpel only. Plants inoculated with *B. rubrifaciens* mutants and wild type were wounded with a scalpel covered with bacteria scrapped from a culture grown on YDCA. Negative control plants were not wounded. All plants were incubated in a Conviron growth chamber for 30-36 days at 25°C 40% humidity 16 hour light 8 hour dark regimen with a maximum light level of 1525 lux.

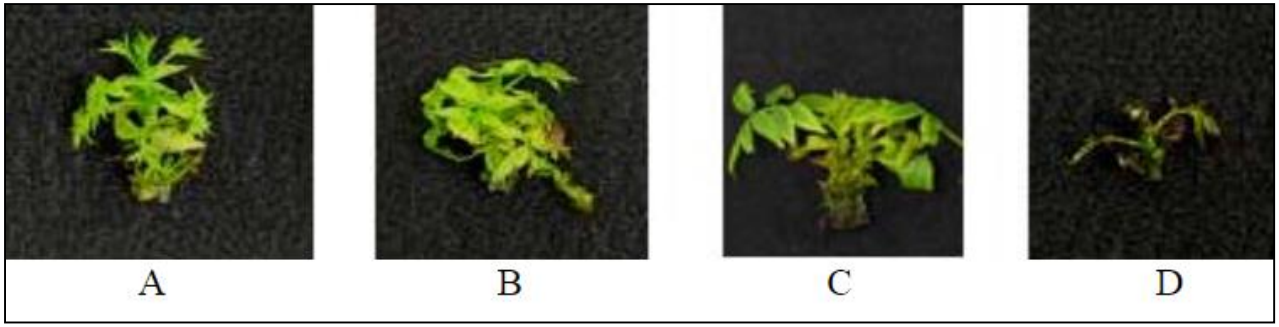
McClean and Kluepfel,2008

Pathogenicity test

Virulence Bioassay

Brenneria rubrifaciens

- Bioassay on Walnut Plants.
- Tissue cultured walnut plants were inoculated with *B. rubrifaciens* mutants. Plants were infected using a sterile scalpel containing A) no bacteria B) mutant Br-415 (rpoN-like mutant) C) mutant Br-212 (expI-like mutant), and D) wild type *B. rubrifaciens* 6D370.
- Photos were taken 1 month post inoculation.



McClean and Kluepfel,2008

Characteristics of:
The genus *Lonsdalea*

- *Brenneria quercina* (drippy nut of oak) was reclassified in a new genus, *Lonsdalea*.
- None of the bacterial species except *Lonsdalea* (*Brenneria*) *quercina* cause a bacterial soft rot.
- Oak strains of *B. quercina*, however, did not incite drippy pod disease on lupine (legume).

Lonsdalea quercina	
Lonsdalea quercina* subsp. *quercina	**Bark canker and drippy nut of oak (rotting of acorn)** ***Quercus agrifolia* and *Q. wislizenii***
Lonsdalea quercina* subsp. *iberica	**Bark canker of oak** ***Quercus agrifolia* and *Q. wislizenii***
Lonsdalea quercina* subsp. *britannica	**Acute oak decline** ***Quercus robur* and *Q. petrea***
Lonsdalea quercina* subsp. *populi	**Symptomatic bark tissue of poplar(Populus x euramericana) canker**

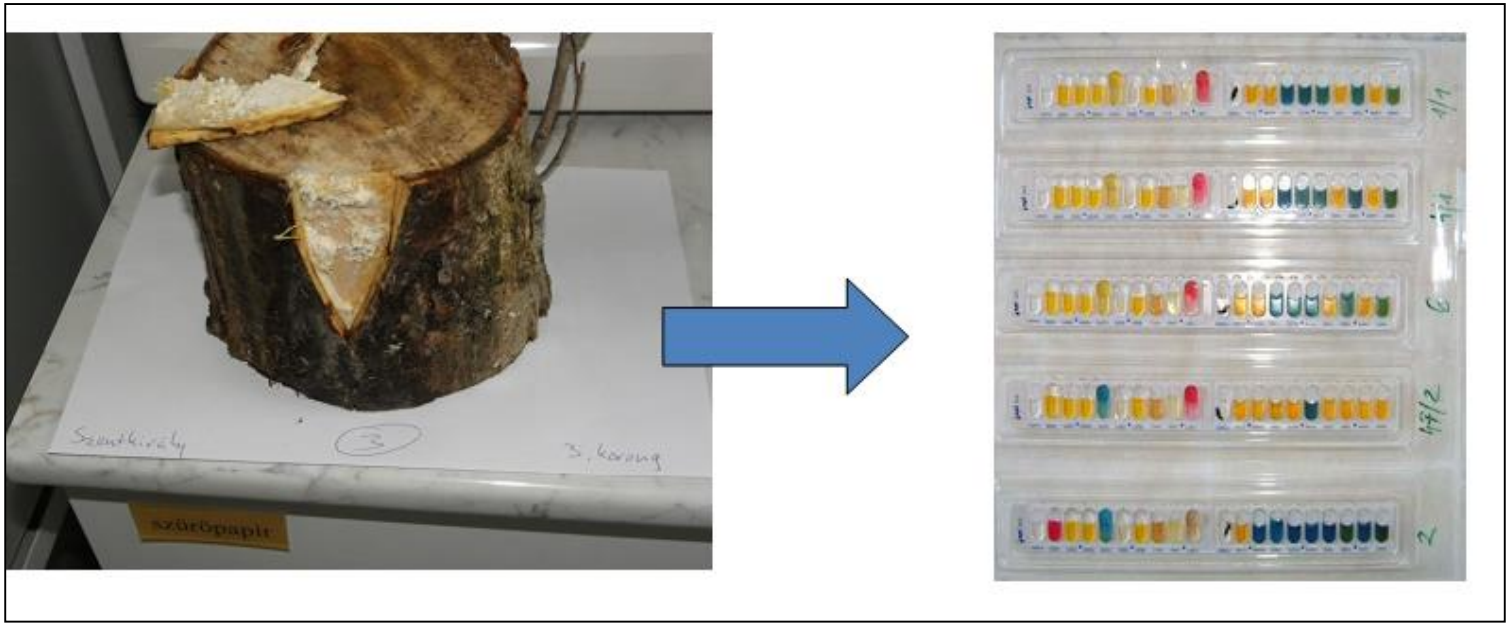
Koltay *et al.*,2013;Bull *et al.*,2014

Bark canker and drippy nut
Lonsdalea quercina subsp. *quercina*

- Trunk cankers with dark exudates staining the bark of a *Quercus ilex* (oak) tree seriously affected by *Lonsdalea quercina* subsp. *quercina*.
- The bacterium *Lonsdalea quercina* subsp. *quercina* exuding out of a kermes scale feeding site.

Sitz,2018;..

Isolation and identification with API kit *Lonsdalea quercina* subsp. *pouli*



Koltay *et al.*,2013

The genus *Lonsdalea*

Phenotypic characteristics that enable differentiation of the subgroups of the '*quercina* clade' from each other and from the '*Brenneria* clade', containing the remaining species of the genus *Brenneria*

- Taxa:

1. *Lonsdalea quercina* subsp. *quercina* comb. nov.;
2. *Lonsdalea quercina* subsp. *iberica* subsp. nov.;
3. *Lonsdalea quercina* subsp. *britannica* subsp. nov.

Characteristic	1	2	3
Citrate utilization	+	+	+
Voges-Proskauer	V	+	−
Acid from:*			
Glycerol	−	+	+
D-Arabinose	−	−	−
L-Arabinose	−	−	−
D-Xylose	−	−	−
D-Galactose	+	+	−
Amygdalin	−	−	−
D-Cellobiose	−	−	−
D-Trehalose	−	−	+
D-Turanose	+	+	+
Potassium gluconate	+	−	+

Brady *et al.*,2012

The genus *Lonsdalea*

Phenotypic characteristics differentiating the four novel species of the genus *Lonsdalea* Strains: 1, *Lonsdalea quercina* sp. nov. (n=3); 2, *Lonsdalea iberica* sp. nov.(n=3); 3, *Lonsdalea britannica* sp. nov.(n=3); 4, *Lonsdalea populi* (n=5); sp. nov. (n=5). All data are from the present study +, Positive; −, negative; v, variable, type strain is positive; w, weak; n, number of strains.

Characteristic	1	2	3	4
Voges-Proskauer	v	+	−	+
Acid from (API 50CH):				
Glycerol	−	+	+	+
d-Galactose	+	+	−	+
Trehalose	−	−	+	+
Turanose	+	+	+	−
Potassium gluconate	+	−	+	+
Raffinose	w	−	−	−
Carbon source utilization (Biolog GN2)				
Raffinose	−	+	−	−
Trehalose	−	+	+	+
Turanose	+	+	+	−
Pyruvic acid methyl-ester	−	−	−	+
Succinic acid mono-methyl-ester	−	−	−	+
Citric acid	+	+	+	−
Formic acid	−	+	+	−
α-Keto glutaric acid	−	+	+	+
d,l - Lactic acid	−	+	+	−
Succinic acid	−	+	+	+
l-Glutamic acid	−	−	+	v
l-Histidine	+	−	−	−
l-Leucine	+	−	−	−
l-Phenylalanine	+	−	−	−
l-Threonine	+	−	−	−

Brady *et al.*,2012

Pathogenicity test

Lonsdalea quercina subsp. *quercina*

- Mature acorns were rinsed, disinfected for 5 min with a solution of 0.5% sodium hypochloride and 3% ethanol (wt/vol), and rinsed again with sterile distilled water prior to inoculation.
- **First, acorns detached from holm oak** were punctured(pricked/perforated) with a needle, a drop of a bacterial suspension in PBS (10^9 CFU/ml) was placed in the wound, and the inoculated nut was incubated in a sterile moist chamber at 29°C.
- In a **second method**, disinfected acorns attached to **small branches** were immersed in a bacterial suspension (10^9 CFU/ml), placed in sterile 1% agar (wt/vol) in closed 500-ml bottles, and incubated at 25°C.
- Symptoms appearance was monitored for 2 weeks.
- Bacteria were reisolated on King's B medium.

Pathogenicity test
Lonsdalea quercina subsp. *quercina*

- **Trunk inoculations** were conducted on *Quercus ilex* and *Q. pyrenaica* trees grown in pots or identified in the forest.
- The group of potted trees consisted of 288, 3- to 5-year-old, *Q. ilex* and *Q. pyrenaica* specimens.
- The 72 oak trees tested in the forest were at least 20 years old.
- *Quercus ilex* and *Q. pyrenaica* trees were inoculated in the summer with one representative bacterial isolate from each host species.
- The bacterial suspension in PBS (10^9 CFU/ml) was injected into the wound (25 to 50 µl and 400 µl for young and old trees, respectively), and the wounds were sealed with plastic film.
- Plants in pots were maintained in the open air.
- The appearance of external and internal symptoms was recorded before and after removing the bark and measuring the length of the affected tissue 3 and 28 months after inoculation.

Pathogenicity test

Lonsdalea quercina subsp. *pouli*

- Second year old hybrid poplar(*Populus*) clones (Kopecky, Koltay and Pannónia) saplings (young trees) were wounded and infected with 10 µl 10^5CFU/ml concentration of bacteria suspension.

Koltay *et al.*,2013

Pathogenicity test
Lonsdalea quercina subsp. *pouli*

- Evaluation: 4 weeks after the treatment.
- Pathogenicity scale: 0– without symptoms, 1– necrosis only on the margins of the wound, 2– necrosis starting from the wound margins, but still less in area than the wound itself 3– large scale necrosis, tissue sap bleeding.

Koltay *et al.*,2013

Characteristics of:
The genus *Samsonia*

- DNA–DNA hybridization indicated that strains from erythrina belonged to a discrete genomospecies (89-100% hybridization) and had low levels of DNA relatedness (2-33% hybridization) with reference strains of phytopathogenic *Erwinia*, *Brenneria*, *Pectobacterium*, *Pantoea* and *Enterobacter* species.
- It is proposed that these strains are included in a new genus, *Samsonia*.
- The name *Samsonia erythrinae* is proposed for the new species.
- Causes bark necrotic lesions of *Erythrina* spp. (coral tress).
- The GMC content of the DNA of the type strain is 57.0 mol%.

Sutra *et al.*,2001

Characteristics of:
The genus *Samsonia*

- Gram-negative, oxidase-negative and catalase-positive.
- They are rod-shaped with rounded ends, and are motile by means of peritrichous flagella.
- The strains are β-galactosidase-positive, produce acetoin but not hydrogen sulphide, and do not possess arginine dihydrolase, lysine decarboxyase, ornithine decarboxylase, urease or tryptophan desaminase.
- Glucose is fermented without gas production.
- Nitrate is reduced.
- Strains hydrolyse aesculin but not gelatin, Tween 80, casein or lecithin.
- They do not utilize citrate and malonate as carbon source and do not produce indole from tryptophan.
- They were pathogenic when inoculated to erythrina cuttings.
- The type species is *Samsonia erythrinae*.

Sutra *et al.*,1999

Diseases caused by: *Samsonia*

Samsonia erythrinae	**Bark necrotic lesions of *Erythrina* spp. (coral tress)**

- This disease was characterized by:

1. Defoliation of the main branches of trees, and
2. Browning of large areas of the bark surface, which girdled the base of defoliated branches.

- Removal of the superficial layer of the bark revealed brown, water-soaked necrotic lesions of the deeper layers of the bark and of the cambium, exhaling a nasty smell.
- All these symptoms suggested that the causal agent of the disease was different from *Xanthomonas axonopodis* pv. *erythrinae* which caused exclusively a leaf spot disease on *erythrina* in India.

Biochemical characteristics that differentiate *Samsonia erythrinae* strains (phenon 4) from other plant pathogenic *Enterobacteriaceae*.

+, Positive; –, negative; numbers correspond to the percentage of positive strains.

Characteristic	Phenon no.*							Reference strains†															
	1	2	3	4	5	6	7	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Pectin hydrolysis	+	+	+	w	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Growth at 36 °C	+	+	66	+	75	+	–	+	+	–	–	+	+	+	–	+	+	–	–	+	–	–	–
Growth at 39 °C	80	33	–	+	–	33	–	+	–	–	–	+	+	+	–	+	–	–	–	–	–	–	–
Growth on Simmons' citrate	80	+	66	–	25	–	50	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–
Growth in 5% NaCl	80	33	+	+	25	33	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–
Gelatin hydrolysis	+	66	66	40	–	+	–	+	–	+	+	–	–	–	+	–	–	–	–	–	+	–	–
Lecithin hydrolysis	+	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Production of indole	+	+	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Utilization of malonate	+	+	–	–	+	–	+	+	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
Acid production from:																							
Arabinose	40	33	33	+	+	+	+	–	–	–	+	–	+	+	+	–	+	+	–	+	–	–	–
Lactose	60	–	+	–	+	+	+	–	–	–	–	+	+	–	–	–	–	–	–	+	–	–	–
Melibiose	+	–	+	–	+	+	+	+	–	–	–	–	–	+	–	+	–	–	+	+	+	–	–
Raffinose	+	–	+	–	–	+	+	–	–	–	–	+	–	–	–	+	–	–	+	+	–	–	–
Assimilation of:																							
D-Alanine	–	–	–	–	+	+	+	–	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–
L-Alanine	60	–	–	–	–	–	–	+	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–
L-(+)-Arabinose	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	+	+	–	+	–	–	–
D-(+)-Cellobiose	60	66	+	+	50	+	+	–	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–
cis-Aconitate	60	–	–	–	75	+	+	–	–	–	–	–	+	+	+	–	–	–	–	–	–	–	+
Aesculin	+	+	+	+	+	66	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	–
D-Galacturonate	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–
β-Gentobiose	–	33	+	–	25	+	+	+	+	+	–	+	+	+	+	–	+	–	–	–	+	–	–
D-Glucosamine	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+	–	–	–
D-Glucuronate	80	+	66	40	+	+	50	+	–	+	–	–	–	–	+	+	+	+	+	+	–	–	–
L-Glutamate	+	66	66	20	+	+	+	+	–	+	+	+	+	+	+	+	–	–	–	+	–	–	–
DL-Glycerate	80	33	+	+	50	66	50	+	–	+	–	–	–	+	–	+	–	–	+	–	–	–	–
L-Histidine	–	–	–	–	+	+	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
5-Keto-D-gluconate	–	33	–	–	25	+	+	+	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–
2-Keto-D-gluconate	20	33	–	–	+	+	+	–	+	–	–	–	+	+	+	–	–	–	–	–	–	–	–
2-Ketoglutarate	20	66	–	40	25	33	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
DL-Lactate	80	+	–	+	+	+	+	+	+	+	–	–	+	+	+	–	–	–	–	–	–	–	–
D-Lyxose	–	–	–	–	+	+	50	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D-Malate	+	+	–	–	+	66	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Maltose	–	–	–	–	+	+	+	–	–	–	–	–	+	+	+	–	–	+	–	–	–	–	–
Maltotriose	–	–	–	–	+	+	+	–	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–
meso-Tartrate	+	+	–	–	+	66	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
1-*O*-Methyl α-galactoside	60	33	+	–	–	+	+	+	–	–	–	–	–	+	–	+	–	–	–	+	–	–	–
1-*O*-Methyl β-galactoside	+	66	+	–	75	+	+	+	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–
Mucate	+	+	+	+	+	+	–	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–
myo-Inositol	+	+	+	+	+	+	+	–	+	+	+	–	–	–	+	–	–	–	+	–	+	–	+
L-Proline	–	–	–	–	+	+	+	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–
Procatechuate	–	–	–	–	–	+	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–
Quinate	–	–	–	–	25	+	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–
α-L-Rhamnose	40	+	+	+	+	33	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–
D-Saccharate	+	+	+	+	+	+	–	+	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–
Serine	+	+	+	60	+	+	+	+	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–
Trehalose	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–
trans-Aconitate	40	–	–	–	25	+	50	–	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–

Sutra *et al.*,2001

Characteristics of
The genus *Ewingella*

- The generic name honors the American bacteriologist, William Ewingle.
- Causes a browning disorder of the stipe (stalk) of mushrooms called internal stipe necrosis.
- Also it has been isolated from clinical specimens.
- A species of the *Enterobacteriaceae* since it is lipase, DNAase arginine dihydrolase and lysine and ornithine decarboxylase negative. citrate, esculin and VP positive.
- Facultative anaerobes.
- Motile by peritrichous flagella.
- It produces acid from D-glucose, but fails to produce acid from L-arabinose, melibiose, raffinose, D-sorbitol or sucrose.
- Type strain-*Ewingella americana*

Reyes *et al.*,2003;..

The characteristics of *Ewingella americana*

Biochemical and physiological characteristics of the strains of *E. americana* isolated according to origin

Test[a]	Cumulative % positive in mushroom strains		
	A. bisporus ($n=40$)	*L. edodes* ($n=42$)	*P. ostreatus* ($n=30$)
β-Galactosidase (ONPG)	100	100	85
Arginine dihydrolase	0	0	0
Lysine decarboxylase	0	0	0
Ornitine decarboxylase	0	0	0
Citrate utilization	100	100	100
H_2S production	0	0	0
Urease	0	0	0
Tryptophane deaminase	0	0	0
Indole production	0	0	0
Acetoin production	100	100	100
Gelatinase	0	0	0
Cytocrome-oxidase	0	0	0
NO_2 production	100	100	100
N_2 gas production	0	0	0
D-Glucose gas	0	0	0
Motility (36°C)	100	100	80
Fermentation/oxidation:			
Glucose	100	100	100
Lactose	70	93	0
Mannitol	100	100	100
Inositol	0	0	0
Sorbitol	0	0	0
Rhamnose	0	0	0
Sucrose	0	0	0
Melibiose	0	0	0
Amygdalin	100	100	100
Arabinose	0	0	0

[a]The tests were carried out using the API 20E system and conventional methods.

Reyes *et al.*,2003

Characteristics of
The genus *Gibbsiella*

- Isolated from oak trees displaying symptoms of extensive stem bleeding.
- In Britain, this disorder is called Acute Oak Decline (AOD).
- Gram-negative short rods, facultatively anaerobic, oxidase negative and catalase positive.
- Cells occur singly, in pairs or in groups of four, and possess very fine fimbriae but no flagella.
- Colonies are white to cream on nutrient agar, round, convex and smooth with entire margins.
- The phylogenetic and phenotypic data both demonstrated that the strains isolated from oak represented a novel genus and species within the family Enterobacteriaceae for which the name *Gibbsiella quercinecans* gen. nov., sp. nov.

Brady *et al.*,2010

Diseases caused by:
Gibbsiella spp.

Gibbsiella greigii	Oak decline in the USA
Gibbsiella quercinecans	Associated with Acute Oak Decline

Characteristics of
The genus *Gibbsiella*

- Phenotypic characteristics distinguishing *Gibbsiella quercinecans* from its closest phylogenetic neighbours. 1= ***Gibbsiella quercinecans*** (18 strains), 2= *Serratia marcescens* ssp. *marcescens* (LMG 2792T), 3 = *Serratia entomophila* (LMG 8456T), 4= *Serratia ficaria* (LMG 7881T), 5= *Serratia fonticola* (LMG 7882T), 6= *Serratia rubidaea* (LMG 5019T), 7=*Edwardsiella tarda* (LMG 2793T), = *Edwardsiella hoshinae* (LMG 7865T), and 9= *Edwardsiella ictaluri* (LMG 7860T).

Characteristic	1	2	3	4	5	6	7	8	9
β-Galactosidase	+	+	+	+	+	+	–	–	–
Lysine decarboxylase	–	+	–	–	+	+	+	+	+
Gelatinase	–	+	+	+	–	+	–	–	–
Acid from									
D-Sorbitol	+	+	–	+	+	–	–	–	–
D-melibiose	+	–	–	+	+	+	–	–	–
Erythritol	–	+	–	+	+	+	–	–	–
D-Arabinose	+	–	+	+	–	+	–	–	–
D-Adonitol	–	+	+	+	+	+	–	–	–
L-Sorbose	+	–	–	–	–	–	–	–	–
L-Rhamnose	+	–	–	+	+	–	–	–	–
D-Fucose	–	+	+	+	–	+	–	–	–
Potassium gluconate	+	–	–	–	+	–	+	+	+
Utilization of									
N-acetyl-galactosamine	–	+	–	–	+	+	+	+	+
Adonitol	–	+	+	+	+	+	–	–	–
D-Arabitol	+	–	+	+	+	+	–	–	–
Gentiobiose	+	+	+	+	+	+	–	–	–
Turanose	+	+	–	+	–	+	–	–	–
L-Alaninamide	–	+	+	+	+	+	–	–	–
D-Alanine	–	+	+	+	–	+	–	–	–
Glycyl-L-aspartic acid	–	+	+	+	+	+	+	+	+
Putrescine	–	+	+	+	+	+	–	–	–
2,3-Butanediol	+	+	–	–	–	–	–	–	–

Brady *et al.*,2010

Pectolytic enterobacteria

The pectolytic *Pectobacterium* and *Dickeya* species

- Plant pathologists have been studying the soft rot *Erwinia* (*Pectobacterium* and *Dickeya* species) for nearly 120 years and published thousands of pages on this pathogen.
- Because the soft rot ex. *Erwinia* are amenable to genetic manipulation and because they are wide spread in the environment, they serve as an important model for studying the ecology and evolution of enterobacterial pathogenesis.

Charkowski,2006

The current taxonomic position and nomenclature

The genus *Pectobacterium*

- The species and subspecies currently included in the pectolytic soft rot or *carotovora* group have now been placed in *Pectobacterium* and include:

1. *Pectobacterium carotovorum* subsp. *carotovorum*
2. *P. carotovorum* subsp. *odoriferum*
3. *P. atrosepticm*
4. *P. betavasculorum*
5. *P. wasabiae*
6. *P. cacticida*

Note: *P. carotovorum* subsp. *brasiliensis* does not conform to the Code. Also *Pectobacterium wasabiae* was transfer to *Pectobacterium parmentieri* sp. nov.

Diseases caused by *Pectobacterium* spp.

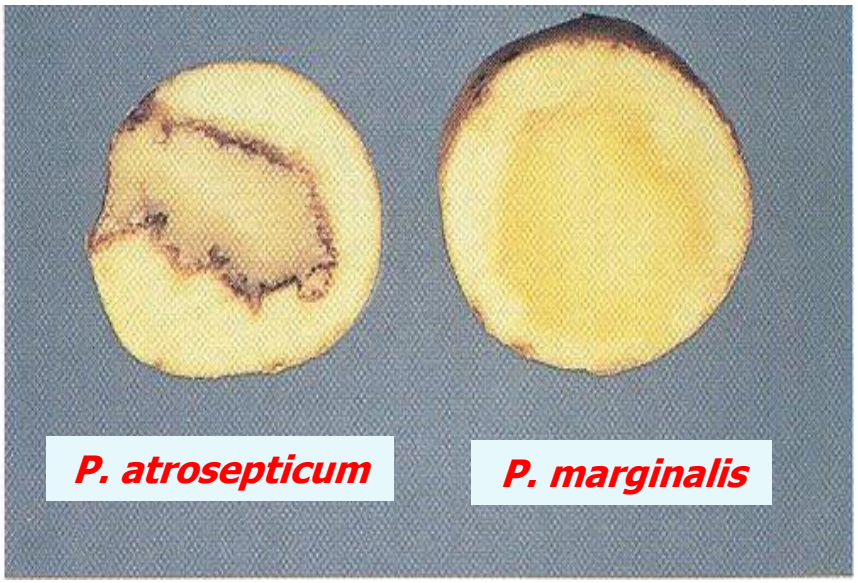
Pectobacterium atrosepticm	**Black leg disease on potato, tomato and chicory, sunflower,..**
P. betavasculorum	**Soft rot of beet**
P. cacticida	**Rotting of cactus**
P. carotovorum* subsp. *carotovorum	**Wide hosts: Rotting of potato, sunflower, sugar beet,…**
P. carotovorum* subsp. *odoriferum	**Soft rot of Endive and chicory**
P. wasabiae	**Rotting of Japanese horse-radish**

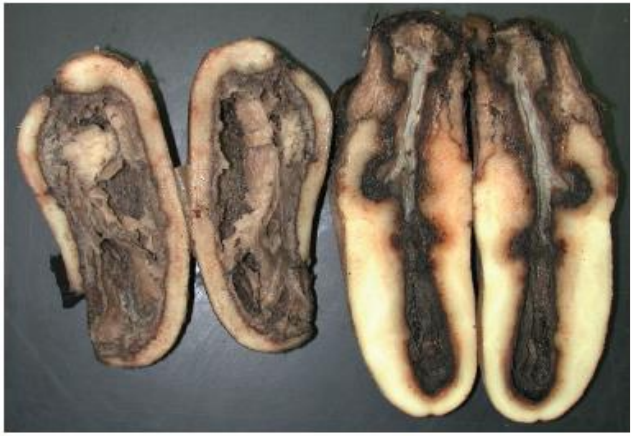
Blackleg on potato stems is historically associated to *Pectobacterium atrosepticum* (formerly *Erwinia carotovora subsp. atroseptica*). However, other species belonging to the genera *Pectobacterium* and *Dickeya* can also cause blackleg and soft rots on potato. Blackleg is caused by bacteria belonging to:

1. *Dickeya* spp. (Samson *et al.*,2005);
2. *P. atrosepticum* (Pba) (Gardan *et al.*,2003);
3. *P. carotovorum* subsp. *carotovorum* (Pcc) (De Haan *et al.*,2008) or
4. *P. c.* subsp. *brasiliensis* (Pcb) (Duarte *et al.*,2008). Not conformed by code.

For *Pectobacterium cypripedii* see *Pantoea cypripedii*.

Soft rot of potato tubers
Disease diagnosis





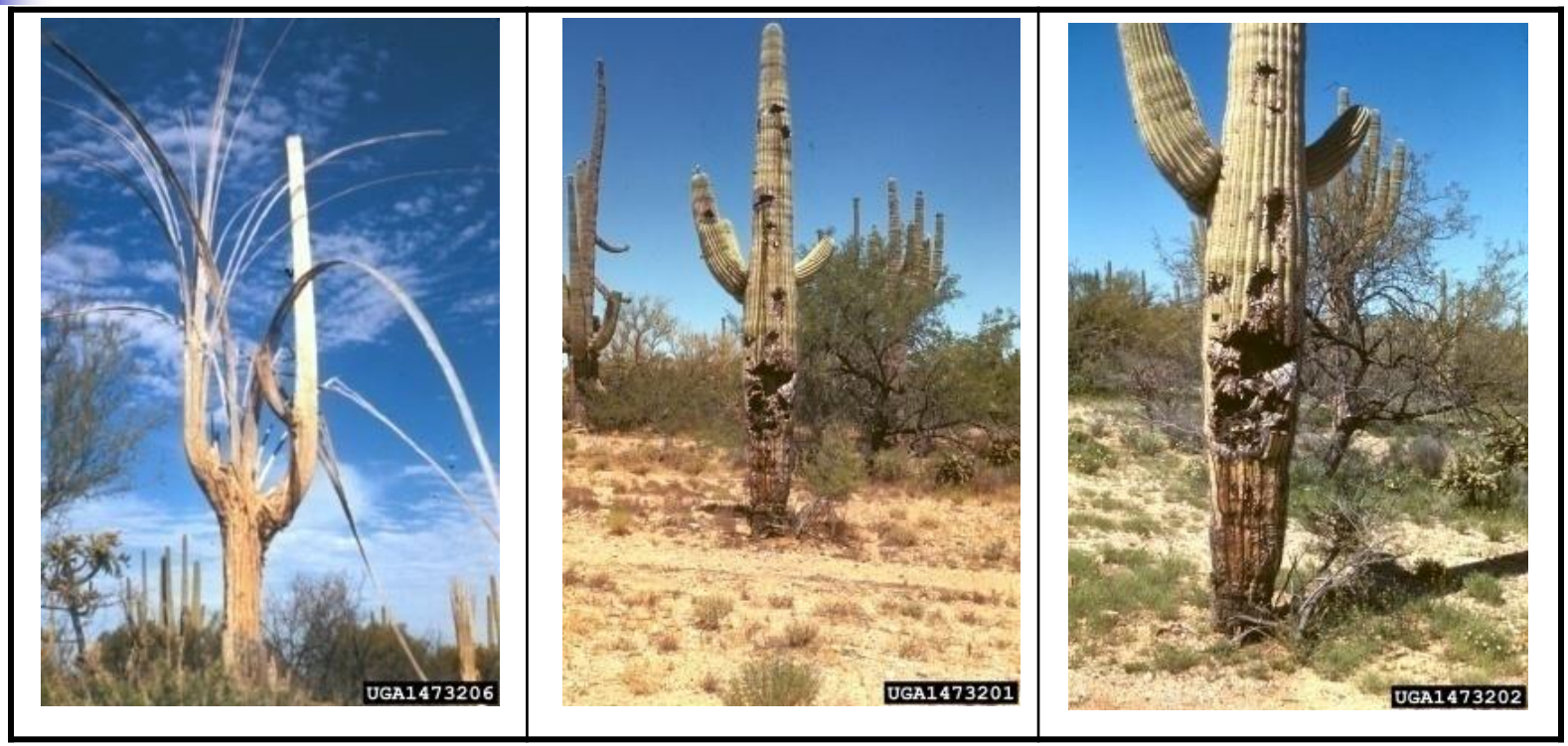
P.c.* subsp. *carotovorum

Olsen *et al.*,2006

P. atrosepticum (*Pa*): Primary cause of blackleg of potatoes.
P.c. subsp. *carotovorum* (*Pcc*): Widely distributed and most common.

Bacterial necrosis of giant cactus

Pectobacterium cacticida



USDA Forest Service,2009

Pectobacterium spp.

Pathogenesis of soft rot pathogens is temperature dependent

Different causal agents in different climates

- To date, only *P. carotovorum* subsp. *carotovorum* and *P. atrosepticum* have been reported to occur in the same field*.

1. *P. carotovorum* subsp. *carotovorum* is found worldwide
2. *P. atrosepticum* is found in cool climates;
3. *P. carotovorum* subsp. *brasiliensis* has been found only in Brazil, Israel, south Africa, and the United States.

- It is likely to have a wider distribution.
- Compared to the ecology and genetics of *P. carotovorum* subsp. *carotovorum* and *P. atrosepticum*, little is known about the ecology and genetics of *P. betavasculorum*, *P. wasabiae*, or *P. carotovorum* subsp. *brasiliensis*.

Kim *et al.*,2009;…

* *Pectobacterium-Dickeya* disease complex on potatoes was reported from South Africa. The three species in this complex are responsible for soft rot, aerial stem rot and blackleg of potatoes.

Blackleg disease

Different causal agents in different climates

- In temperate/moderate climates(average temperature between 0° and 20°C), *P. atrosepticum* was considered as the main causative agent of blackleg.
- *Dickeya* spp. was believed to be a major blackleg pathogen in tropical and subtropical regions.
- Some 'atypical' strains of *Dickeya* spp. with a relative low growth temperature maximum were also isolated from blackleg-diseased plants in temperate regions (Janse and Ruissen,1988).

Czajkowski *et al.*,2009;..

Both Pcc and Dch grow poorly and fail to induce soft rot of fresh produce at 10°C or below.
At 20°C or higher, Pcc is considered the most destructive soft-rotting pathogen of fruits and vegetables.

Blackleg disease

Different causal agents in different climates

- *Pectobacterium carotovorum* subsp. *carotovorum* is considered to play a minor role in potato blackleg in temperate zones, although it has already been proven that tuber infections with virulent Pcc strains can result in true blackleg (De Haan *et al.*,2008).
- To date, *P. c.* subsp. *brasiliensis* (Pcb) has only been found in subtropical regions (Duarte *et al.*,2008).

Czajkowski *et al.*,2009

Blackleg disease
Pectobacterium atrosepticum (Pa)

- *P. atrosepticum* is found in cool climates.
- Logan's medium (nutrient agar 28.0 g, yeast extract 5.0 g and glucose 5.0 g dissolved in distilled water 1000 ml) was also used for describing the morphology of *P. atrosepticum* on media (Fahy and Hayward 1983).

UC IPM, Photo by Jack Kelly Clark;..

Blackleg disease
P. carotovorum subsp. *brasiliensis* (Pbcb)

- Typical blackleg symptoms without chlorosis on potatoes infected with *P. carotovorum* subsp. *brasiliensis* (Pbcb) in a commercial field under wet, cool conditions.
- The occurrence of *P. carotovorum* subsp. *brasiliensis* in commercial and seed potato fields in Minnesota and North Dakota was reported by McNally *et al.*,2017.

van der Merwe *et al.*,2010

Blackleg disease
P. carotovorum subsp. *brasiliensis*

- The recently described organism *P. carotovorum* subsp. *brasiliensis* (Pcb) (Duarte *et al.*,2008) causing potato tuber and stem rot, is genetically distinct from previously described *Pectobacterium* taxa:
- Approximately 82% of its genes are shared with *P. atrosepticum*, and 84% of its genes are shared with *P. carotovorum* subsp. *carotovorum*.
- While 13% of its genes are found in neither *P. atrosepticum* nor *P. carotovorum* subsp. *carotovorum* (Kim *et al.*,2009).

Characteristics of:
The genus *Pectobacterium*

- All the pectolytic *Pectobacterium*/*Dickeya* species can be differentiated from one another on the basis of a limited number of tests:

1. Gram negative stain reaction;
2. Rod shape;
3. Facultative anaerobic metabolism;
4. Colony characteristics on CVP (Crystal violet pectate) medium;
5. Peritrichous flagellation;
6. Mostly positive to reducing substances from sucrose test;
7. Resistant to erythromycin.

Colony characteristics of pectolytic bacteria on CVP

- Isolation of pectoylytic bacteria on CVP(Crystal violet pectate) medium.
- Pattern of cavity formation by Pcc, Pa and Dch on CVP medium at different temperatures:
- Pa generally forms cavities at 27°C only;
- Pcc and Dch also do so up to temperatures of 33.5 and 37ºC, respectively

Dickeya chrysanthemi (Dch)
P. atrosepticum (Pa)
P. carotovorum subsp. *carotovorum* (Pcc)

Pérombelon & Hyman,1986

Differentiated characteristics of (ex. *Erwinia* soft rot group) *Pectobacterium/Dickeya* spp.

- Biochemical tests are currently the accepted standard for identification and taxonomy of the soft rot ex. erwinias, but on a routine level they are very time-consuming (taking up to 14 days) and, when carried out by nonspecialist laboratories, do not always provide a definitive identification.

Toth *et al.*,2001

Differentiated characteristics of (ex. *Erwinia* soft rot group) *Pectobacterium/Dickeya* spp.

- The indole test, distinguishes *Pectobacterium* from *Dickeya*.
- Indole is positive for *Dickeya* strains and all the *Dickeya* type strains whereas it is negative for all the other soft rot ex. erwinia spp.

Differentiated characteristics of *Pectobacterium/Dickeya* spp. (ex. *Erwinia* soft rot group)

Test	Ecc	Eca	Ecb	Eco	Ecw	Ech	Ect
Growth at 37°C	+	-	+	+	-	+	+
Reducing sugars from sucrose	-	+	+	+	-	-	-
Phosphatase activity	-[b]	-	-	-	-	+	V
Sensitivity to erythromycin	-	-	-	ND	-	+	-
Indole production	-	-	-	-	-	+	-
Acid produced from :							
sorbitol	-	-	-	+	ND	-	-
melibiose	+	+	-	+	-	+	-
citrate	+	+	-	+	+	+	ND
raffinose	+	+	-	ND	-	+	-
arabitol	-	-	-	+	ND	-	ND
lactose	+	+	+	+	+	+	-
Utilization of keto-methyl glucoside	-	+	+	+	-	-	-

+, 80% or more strains positive; V, between 21-79% of strains positive; -, 80% or more strains negative; ND, not determined.

[a] Ecc = *E. carotovora* subsp. *carotovora*
Eca = *E. carotovora* subsp. *atroseptica*
Ecb = *E. carotovora* subsp. *betavasculorum*
Eco = *E. carotovora* subsp. *odorifera*
Ecw = *E. carotovora* subsp. *wasabiae*
Ech = *E. chrysanthemi*
Ect = *E. cacticida*

[b] Some strains of *E. carotovora* subsp. *carotovora* may be weakly positive

Schaad *et al.*,2001

Tests to differentiate soft rot species *Pectobacterium* and *Dickeya*

Test	*E. rhapontici* (one strain)	*E. cypripedii* (two strains)	*E. carotovora* var. *carotovora* (77 strains)	*E. carotovora* var. *atroseptica* (16 strains)	*E.* spp. (sugarbeet) (three strains)	*E. chrysanthemi* (322 strains)
Pectate degradation	−*	−*	+	+	+	+
Potato soft rot	+	−*	+	+	+	+
Gluconate oxidation	+*	+*	−	−	−	−
Gelatin liquefaction	−*	−*	+	+	+	V
Sensitivity to erythromycin (15μg)	+	+	−*	−*	−*	+(99)
Sensitivity to penicillin G (two units)	−	−	+(90)	+(87)	−	V
KCN tolerance	−	+	+	+	−*	V
Phosphatase	+	−	−	−	−	+*
Gas from D-glucose	−	+	−(99)	−	−	+*
Growth at 36 C	−	+	+	−(87)	+	+
Growth at 39 C	−	−	−(97)	−(94)	−	V
Growth in 5% NaCl	+	+	+	+	+	+(75)
Blue pigment	−[b]	−	−	−	−	+(67)
Casein hydrolysis	−	−	+(93)	+(94)	−	+(62)
Acetoin production	+	−	+(92)	+	+	+
Indole	−	−	−(86)	−	−	V
DNase	−	−	−(91)	−(88)	−	−(96)
Lecithinase	−	−	−	−	−	V
Phenylalanine deaminase	+	+	−	−(62)	−	−(99)
Decarboxylase (L[+]arginine monohydrate)	−	−	−	−	−	V
Acid from:						
D(−) arabinose	−	−	−	−	−	V
D(+) xylose	−*	+	+	+	+	+(99)
D(+) lactose	+	−*	+	+	+	−(62)
D(+) maltose	+	+	−	+(69)	+	−
D(+) trehalose	+	+	+(90)	+	+	−*
D(+) melibiose	+	+	+(97)	+	−	V
D(+) raffinose	+	−	+(97)	+	+	V
D(+)melezitose	+*	−	−	−	−	−
starch	−	+(50)	−	−	−	−
inulin	−	−	−	−	+*	V
dextrin	+	+(50)	−	−(94)	−	−
ethanol	−	+	−(95)	−	+	+
dulcitol	+*	−	−	−	−	−
α-methyl-d-glucoside	+	−	−(97)	+	+	−
α-D-galacturonic acid	+	+	+	+	−*	+
palatinose	+	−	−(96)	+	+	−
i-inositol	+	+	+(97)	−(94)	+	+(70)
Utilization of:						
sodium citrate	−	+	+	+	−*	+(99)
sodium malonate	+	+(50)	−	−	−	+
sodium tartrate	−	+	−(99)	−	−	V

[a]Symbols: +, all strains positive; −, all strains negative; (%), percentage of strains + or −; *, may be useful as presumptive test; V, variable results, see Table 4 for details.
[b]Pink.

Dickey,1980

Tests to differentiate soft rot species *Pectobacterium* and *Dickeya*

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

Goszczynaska *et al.*,2000

Biochemical characteristics that differentiate reference strains of *Dickeya* (ex. *Pectobacterium*) *chrysanthemi* bvs 1-9 and type strains of *Pectobacterium carotovorum* subspecies.

The characteristics that differentiate *Dickeya* (ex. *Pectobacterium*) *chrysanthemi* biovars are in bold face.

The characteristics that differentiate *Pectobacterium carotovorum* subspecies are underlined.

Characteristic	*Pectobacterium chrysanthemi* biovars									*Pectobacterium carotovorum* subspecies*				
	1	2	3	4	5	6	7	8	9	*caro.*	*atro.*	*odori.*	*beta.*	*wasa.*
ADH (Moeller)	+	−	+	−	+	−	+	−	−	−	−	−	−	−
Growth at 39 °C	−	+	+	+	+	+	−	+	−	−	−	−	−	−
Growth at 36 °C	+	+	+	+	+	+	+	+	+	+	−	+	+	−
Growth on Simmons' citrate	−	+	+	+	+	+	+	+	+	−	+	+	−	+
Growth in 5% NaCl	+	+	+	−	+	−	−	+	−	+	+	+	+	+
Gelatin hydrolysis	+	−	+	−	+	+	+	+	+	+	−	+	−	+
Lecithin hydrolysis	+	+	+	+	+	+	+	+	+	−	−	−	−	−
Reducing substances from sucrose	−	−	−	−	−	−	−	−	−	−	+	+	+	−
Production of indole	+	+	+	+	+	+	+	+	+	−	−	−	−	−
Utilization of malonate	+	+	+	+	+	+	+	+	+	−	−	−	−	−
Utilization of inulin	+	−	−	−	+	−	+	−	+	−	−	−	+	−
Acid production from:														
D-Arabinose	−	+	+	+	−	−	−	+	−	−	−	+	−	−
Lactose	−	−	+	−	+	−	−	+	−	+	+	+	−	−
Melibiose	+	−	+	+	+	+	−	+	−	+	+	+	+	−
α-Methyl glucoside	−	−	−	−	−	−	+	−	−	−	+	+	+	−
Raffinose	+	−	+	+	+	+	−	+	−	+	+	+	+	−
Assimilation of:														
L-Alanine	−	−	+	+	+	+	−	−	−	−	−	−	−	−
D-(+)-Cellobiose	−	+	+	−	+	−	+	−	−	−	+	+	+	+
cis-Aconitate	−	−	+	−	+	−	−	+	−	−	−	−	−	−
trans-Aconitate	−	−	+	−	−	−	−	+	−	−	−	−	−	−
D-Galacturonate	+	+	+	+	+	+	+	+	+	+	+	+	−	+
β-Gentobiose	−	+	−	+	−	−	−	−	−	+	+	+	+	+
D-Glucuronate	+	+	+	+	+	−	+	+	+	+	+	−	−	+
L-Glutamate	+	+	+	+	+	+	+	+	−	+	−	+	+	−
DL-Glycerate	+	−	+	+	+	−	+	+	−	+	+	+	−	+
5-Keto-D-gluconate	−	+	−	+	−	−	−	−	−	−	−	−	−	−
2-Keto-D-gluconate	−	+	+	−	−	−	−	−	−	−	−	−	+	−
2-Ketoglutarate	−	+	−	−	+	−	+	−	−	−	−	−	−	−
DL-Lactate	+	+	+	+	+	−	+	+	+	−	−	−	+	+
Lactulose	−	−	−	−	−	−	−	−	−	+	+	+	−	−
D-Malate	+	+	+	+	−	+	+	+	+	−	−	−	−	+
meso-Tartrate	+	+	+	+	−	+	+	+	+	−	−	−	−	+
1-*O*-Methyl α-galactopyrannoside	+	−	−	+	+	+	+	−	−	+	+	+	−	−
1-*O*-Methyl β-galactopyrannoside	+	+	+	+	+	+	+	+	−	−	+	+	+	−
Palatinose	−	−	−	−	−	−	−	−	−	−	+	+	+	−
α-L-Rhamnose	+	+	+	+	+	−	+	−	+	+	+	+	+	+
L-(+)-Tartrate	−	+	+	+	−	−	−	+	−	−	−	−	−	−
D-(−)-Tartrate	+	+	−	+	−	−	+	−	+	−	−	−	−	−
Trehalose	−	−	−	−	−	−	−	−	−	+	+	+	+	+

* *caro.*, *carotovorum*; *atro.*, *atrosepticum*; *odori.*, *odoriferum*; *beta.*, *betavasculorum*; *wasa.*, *wasabiae*.

Sutra *et al.*,2001

Physiological and biochemical tests for differentiating the more common soft rot erwinias[1]

Test*	Ecc	Eca	Ecb	Eco	Ecw	Ech	Ecy	Erh
Cavity formation on CVP medium (27°C, 48 h)	+	+	+	+	+	+	-	-
Growth on NA at 37°C	+	-	+	+	-	+	+	d
Growth in 5% NaCl*	+	+	nd	nd	nd	-	d	+
Sensitivity to erythromycin*	-	-	-	nd	-	+	+	+
Production of reducing substances from sucrose**	-	+	+	+	-	-	-	d
Production of: indole**	-	-	-	-	-	+	-	-
Phosphatase**	-	-	-	nd	-	+	d	d
Acid production from:								
lactose**	+	+	d	+	+	-	-	+

Physiological and biochemical tests for differentiating the more common soft rot erwinias[1] Continued

Test*	Ecc	Eca	Ecb	Eco	Ecw	Ech	Ecy	Erh
maltose**	-	+	+	d	nd	-	+	+
α-methyl glucoside**	-	+	+	+	-	-	-	d
trehalose	+	+	+	nd	nd	-	nd	nd
cellobiose	+	+	-	+	nd	d	+	+
sorbitol	-	-	-	+	nd	-	+	+
inulin	-	-	+	-	-	d	nd	nd
raffinose	+	+	-	nd	-	+	nd	nd
palatinose*	-	+	+	+	nd	nd	nd	nd
melibiose	+	+	-	+	-	nd	nd	nd

Pérombelon & van der Wolf,2002

Physiological and biochemical tests for differentiating the more common soft rot erwinias[1] Continued

Test*	Ecc	Eca	Ecb	Eco	Ecw	Ech	Ecy	Erh
d-arabitol	-	-	-	+	nd	nd	nd	nd
Utilization of organic acids:								
citrate	+	+	-	+	+	+	nd	nd
malonate*†	-	-	-	-	nd	+	nd	nd

- [1] Adapted from Hyman (1995).
- d, 21-79 % of strains positive; nd, not done.
- Ecc, *E. c. carotovora* ; Eca, *E. c. atroseptica*; Ecb, *E. c. betavasculorum* ; Eco, *E. c. odorifera* ; Ecw,*E. c. wasabiae* ; Ech, *E. chrysanthemi* ; Ecy, *E. cypripedii* (now *Pantoea cypripedii*); Erh, *E. rhapontici.*
- * Details given in Appendix.
- *† Tests to differentiate Ecc, Eca and Ech (now *Dickeya*).

Pérombelon & van der Wolf,2002

Tests to differentiate

Pectobacterium carotovorum subsp. *brasiliensis* (Pbcb) and *Pectobacterium carotovorum* (Pc) isolates

Characteristic	JJ54 *Pbcb*	JJ74 *Pbcb*	JJ145 *Pbcb*	JJ147 *Pbcb*	JJ68 *Pbc*	*Pbcb*[b] (*n*=16)[c]	*Pbc*[b] (*n*=5)
Gram testing (3% KOH)	–[a]	–	–	–	–		
Hugh and Leifson`s oxidation/fermentation test	+	+	+	+	+		
α-Cyclodextrin	–	–	–	–	–	–	–
Dextrin	–	–	–	–	–	–	40[d]
Tween 40	–	–	–	–	–	–	20
Tween 80	–	–	–	–	–	-	60
N-Acetyl-D-Glucosamine	w+	w+	+	w+	+	–	–
L-Arabinose	+	w+	+	w+	+		
D-Arabitol	–	–	–	–	–	–	–
D-Cellobiose	+	w+	+	w+	+	+	+
D-Fructose	w+	w+	+	+	+		
D-Galactose	w+	w+	+	–	+		
Gentiobiose	+	–	+	w+	+	94	100
α-D-Glucose	+	w+	+	+	+		
m-Inositol	+	–	w+	w+	+		
Lactulose	–	–	–	–	+	6	–
Maltose	–	–	–	–	–	19	–
D-Melibiose	+	+	+	+	+	+	+
β-Methyl-D-Glucoside	+	w+	+	w+	+		

van der Merwe *et al.*,2010

Tests to differentiate

Pectobacterium carotovorum subsp. *brasiliensis* (Pbcb) and *Pectobacterium carotovorum* (Pc) isolates

Characteristic	JJ54 *Pbcb*	JJ74 *Pbcb*	JJ145 *Pbcb*	JJ147 *Pbcb*	JJ68 *Pbc*	*Pbcb*[b] (*n*=16)[c]	*Pbc*[b] (*n*=5)
D-Psicose	–	–	–	–	+	6	100
D-Raffinose	+	w+	+	w+	+	–	20
L-Rhamnose	+	+	+	w+	+		
D-Sorbitol	–	–	–	–	–	100	100
Sucrose	+	w+	+	+	+	50	80
D-Trehalose	+	–	+	–	+		
Pyruvic Acid Methyl Ester	w+	+	+	+	+		
Acetic Acid	–	–	–	–	–	12	80
Citric Acid	w+	–	+	w+	w–	75	80
Formic Acid	w+	+	+	w+	–	19	80
D-Galactonic Acid Lactone	w+	–	+	w+	–	–	–
D-Galacturonic Acid	+	–	w+	–	+	–	–
D-Gluconic Acid	w+	–	–	–	–	69	20
D-Glucosaminic Acid	–	–	–	–	–	–	–
D-Glucuronic Acid	–	–	–	–	–		
D,L-Lactic Acid	–	–	–	–	w+		
Malonic Acid	–	–	–	–	–	100	100
Succinic Acid	–	–	w+	–	+	–	–
Bromosuccinic Acid	w+	+	+	+	w+	31	100
Succinamic Acid	–	–	–	–	–	38	100

van der Merwe *et al.*,2010

Tests to differentiate

Pectobacterium carotovorum subsp. *brasiliensis* (Pbcb) and *Pectobacterium carotovorum* (Pc) isolates

Characteristic	JJ54 *Pbcb*	JJ74 *Pbcb*	JJ145 *Pbcb*	JJ147 *Pbcb*	JJ68 *Pbc*	*Pbcb*[b] (*n*=16)[c]	*Pbc*[b] (*n*=5)
L-Proline	–	–	–	–	–		
Inosine	–	–	w+	–	–		
Uridine	–	–	w+	–	–	–	60
Thymidine	–	–	w+	–	w+		
Glycerol	w+	w+	+	w+	+		
D,L,α-Glycerol Phosphate	–	w+	+	w+	+		
α-D-Glucose-1-Phosphate	–	–	–	–	–		
D-Glucose-6-Phosphate	+	+	+	w+	+		

[a] –, negative reaction; w–, weak negative; + , positive reaction; w+, weak positive

[b] Compared to *Pbcb* and *Pbc* strains from Duarte et al. (2004)

[c] Number of strains tested

[d] Percentage of strains showing a positive reaction

van der Merwe *et al.*,2010

Major pathogenicity factors
Pectobacterium spp.

- Pectobacteria are often described as brute-force pathogens because their virulence strategy relies on plant cell-wall degrading enzymes (PCWDE) which disrupt host cell integrity and thus promote rotting.
- The main virulence determinants of *Pectobacterium* are the pectolytic enzymes secreted through the type II secretion system.
- Although these enzymes are required for development of symptoms, many other virulence genes have been shown to contribute to *Pectobacterium* pathogenicity, including:

1. The type III secretion system (T3SS) genes,
2. Coronafacic acid (CFA), a component of coronatine encoded by the *cfa* gene cluster,
3. Type IV secretion system genes.

Kim *et al.*,2009; Põllumaa *et al.*,2012

Major pathogenicity factors

Differences in aggressiveness of *Pectobacterium* spp.

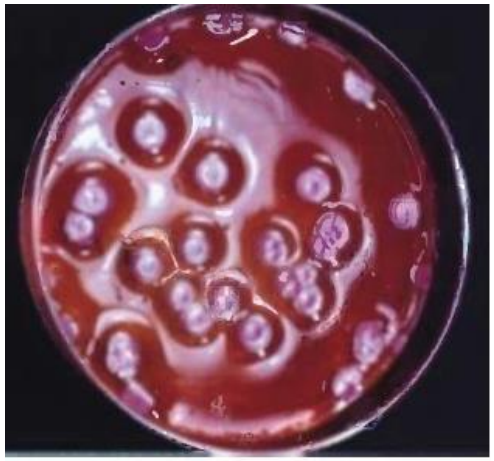
- Some *Pectobacterium* strains lacking the Type III secretion system,T3SS, isolated from diseased potato tubers) are still virulent in potato tubers and stems.
- There are also differences in aggressiveness among *P. carotovorum* ssp. *carotovorum* isolates originating from monocotyledonous or dicotyledonous plants.
- Isolates originating from monocots exhibited higher virulence towards the tested monocot plants than dicot isolates.

Yishay *et al.*,2008

Pectinase production
CVP-S2

- Pectin hydrolysis by pectinolytic enzymes of *Pectobacterium carotovorum* on the double-layer medium of Perombelon after 48 h of growth.
- The top layer is pure pectin and by hydrolysis the bacteria form pits in the surface.

CVP-S2 (Hyman et al., 2001)	500 ml
Tryptone	0.5 g
Tri-sodium citrate	2.5 g
$NaNO_3$	1.0 g
10% aqueous solution of $CaCl_2.H_2O$	5.1 ml
0.075% aqueous solution of crystal violet	1.0 ml
5 M NaOH	1.3 ml
Agar	2.0 g
Sodium polypectate	9.0 g
(Slendid type 440 or Genu pectin type X-914-02)	
Cold distilled water	500 ml

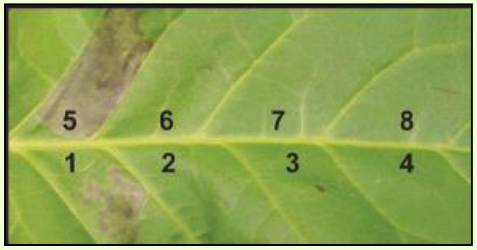


Janse,2006

HR assay

16 of 45 *Pectobacterium* isolates and *P. wasabiae* were unable to elicit an HR

- The bacterial cell suspensions a concentration of 10^8 CFU/ml were infiltrated into fully expanded leaves of 6- to 7-week-old *N. tabacum* L. cv. Xanthi NN with a needleless 1-ml syringe.
- Plants were examined for HR elicitation 24 h after infiltration.
- Sixteen of 45 *Pectobacterium* isolates and *P. wasabiae* SCRI488 were unable to elicit an HR on *N. tabacum* cv. Xanthi.
- Leaf panels 1 through 8 were infiltrated with *Pectobacterium* sp. strains WPP14(Pcc), WPP17(Pcb), WPP161(Pw), WPP163(Pcc), WPP165(Pcb), and WPP172 (Pw), *P. wasabiae* SCRI488 (isolated from *Wasabia japonica*), and a water control, respectively.



Kim *et al.*,2009

Pathogenicity test
On potato plants

- Typical blackleg symptoms on potato stems appeared 3-4 days after stem inoculation.
- Potato cv. Mondial was used to inoculate with *Pectobacterium carotovorum* subsp. *brasiliensis* strain JJ145.

van der Merwe *et al.*,2010

Tissue maceration

Potato tubers, carrots and onions slices

- **Vegetable slices inoculation method**
- Wash potato tubers, carrots, onions, and cut into slices about 7 mm (1/4 inch) thick.
- Put about 1/8 inch of sterile water in a Petri dish that contains two disks of sterile filter paper and add the vegetable slices.
- Make a slight cut in the center of each slice, and inoculate with a loopful of cells (ca. 10^6 CFU/ml) from the culture.
- Include a non-inoculated slice for each treatment used.
- Incubate at 20-27°C for 48 h and probe the tissue surrounding the inoculation site with sterile needle to determine whether decay and tissue maceration has occurred.

Note: Maceration ability dose not prove pathogenicity of the bacterium in a natural environment (Schaad *et al.*,2001).

Tissue maceration

Potato tuber soft rot

- Note: Tubers of some potato cultivars rot more rapidly and extensively than those of others.
- This is not a pathogenicity test as some non-pathogens may rot potato tubers under these conditions.
- It merely indicates the ability of the bacteria to produce large quantities of certain pectic enzymes.

Vacuum infiltration of seed tubers with soft rot bacteria simulate natural infection and to quantify the level of seed contamination (Pérombelon *et al.*,1987b).

Pérombelon & van der Wolf,2002

Tissue maceration

Whole potato tubers, carrots & onions

- **Whole vegetable inoculation method**
- Carefully wash the surface of whole potato tubers, carrots, and onions.
- Sterilize a teasing needle by dipping in 95% ethanol and flaming until red-hot.
- Let the needle cool (this is very important), dip into a culture of *Pectobacterium carotovorum* and then stab the potato 4-5 times with the needle.
- For controls, use the exact procedure, except dip the teasing needle into sterile water (instead of the bacterial culture) prior to stabbing the vegetables.
- Place inoculated vegetables onto a wet paper towel inside a plastic bag and seal tightly with a twist tie.
- Use a separate bag for each vegetable and separate bags for the controls (very important).

Carroll and Evans,2001

Host range studies
With different host and crop plants

- Different plants used for host range studies of ex. soft rot *Erwinia* spp., the causal agents of tip-over disease of banana.
- Clean, fresh, turgid, apparently healthy fruits and vegetables were selected, washed and surface sterilized with 1% sodium hypochlorite for 30 s.
- A bacterial suspension of $5x10^5$ cfu/ml was injected into surface sterilized fruits and vegetables with the help of a hypodermic syringe.
- After inoculation the fruits and vegetables were kept in a belljar lined with three layers of wet blotting paper which provides high humidity and incubated for 48 h at ambient temperature.
- Tomato, carrot, radish and cabbage took 3-4 days to produce visible symptoms whereas potato and mandarin produced visible symptoms within 4-6 days.

Snehalatharani and Khan,2010

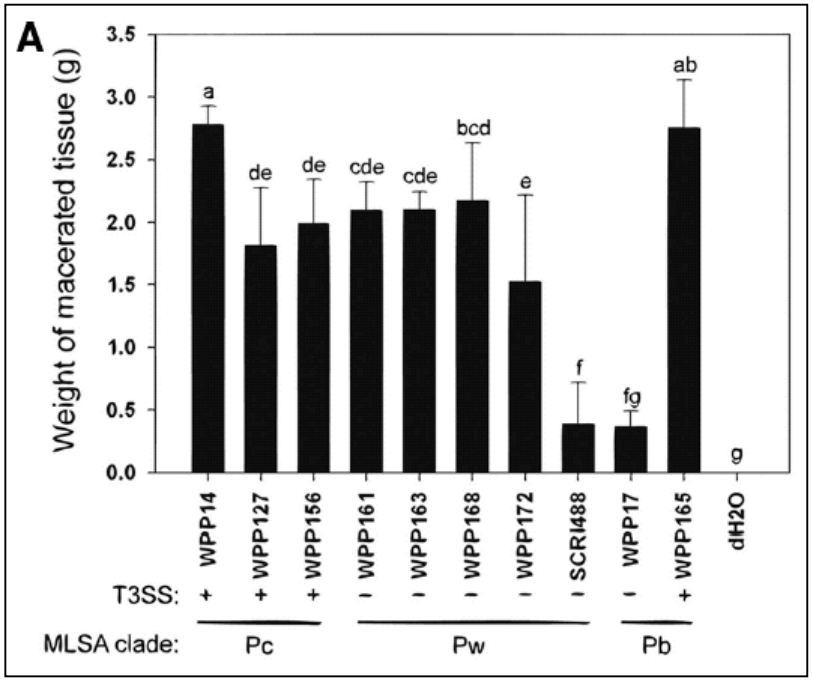
Virulence assays
With potato tubers

- Virulence assays (assessing the aggressiveness variability among *Pectobacterium* isolates) were performed with potato tubers and stems in the laboratory and field, respectively.
- The relative virulence of 10 *Pectobacterium* strains in potato tubers (cv. Atlantic) was determined by measuring the amount of macerated potato tissue.
- 10 to 12 potato tubers were inoculated by placing 10 µl of a 10^8-CFU/ml bacterial suspension into 1-cm-deep holes poked into the tubers with a pipette tip.
- The tubers were placed in plastic bags and incubated for 2 days at 28°C.
- After 2 days the inoculated tubers were cut open, and the macerated tissue was removed and weighed.

Kim *et al.*,2009

Virulence assays
With potato tubers

- The tuber maceration amounts obtained for *P. carotovorum* subsp. *brasiliensis* (*Pcb*) WPP17 and *P. wasabiae* SCRI488 were significantly lower, while *P. carotovorum* subsp. *carotovorum* WPP14 and *P. carotovorum* subsp. *brasiliensis* (*Pcb*) WPP165 macerated tuber tissue most efficiently.
- The abilities of rest of the strains to macerate potato tubers were intermediate.
- No correlation between the aggressiveness of tuber maceration and the presence of the T3SS was observed.



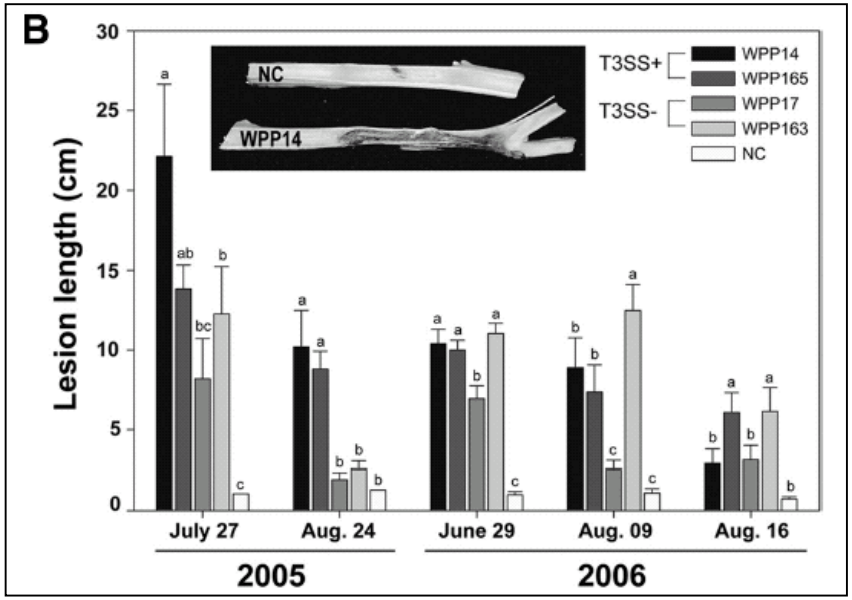
Kim *et al.*,2009

Virulence assays
With potato stems

- The three isolates of:

1. *P. carotovorum* subsp. *carotovorum*,
2. *P. carotovorum* subsp. *brasiliensis* (*Pcb*), and
3. *P. wasabiae* caused comparable levels of tuber and stem rot.

- It was also found T3SS is not required for *Pectobacterium* to cause soft rot disease in tubers or stems.



Kim *et al.*,2009

Virulence assays

On potato stems

Dickeya and *P. atrosepticum*

1. The optical density of the 15 newly isolated *Dickeya* strains and a virulent *P. atrosepticum* suspension was adjusted to an absorbance of 0.2 at 600 nm using a GeneQuant pro RNA/DNA Calculator.
2. Bacterial numbers in the suspensions were verified by dilution plating and counting of bacterial colonies.

- An OD value of 0.2 was equivalent to ~5×10^{8}-10^{9} cfu ml^{-1}.
- Potato tubers were inoculated with 100 µl of bacterial suspension and incubated for 4 days at 22°C in 10 moist boxes covered by plastic bags.
- To assess the amount of macerated tissue, tubers were weighed before and after removal of rotted tissue.

Laurila *et al.*,2008

Virulence assays

On tomato leaves

Dickeya and *P. atrosepticum*

- Spot symptoms on tomato leaves after inoculation with 10^8 cfu/ml.
- Only Japanese strain *Eca* 2962 (left) caused necrotic symptoms while no symptoms were observed with *Eca* 2967 and *Ecc* 436, respectively, 5 days after inoculation.

Mamdoh Ewis Esmael Ahmed,2001

Virulence assays

Rot severity/degree of resistance

- The amount of rotten tissue produced in each tuber was determined and the percentage of rotten tissue was calculated:
- Rot severity = (W1-W2)/W1x100

$$\frac{W1 - W2}{W1} \times 100$$

- Where,
- W1= Weight of the tissue (g) at time t_1 (before inoculation), and
- W2= Weight of tuber(g) after removal (washing away) of the macerated tissue at the time t_2.
- The difference W1-W2 gives the weight of macerated tissue.

Kelman and Dickey,1980

Pathogenicity test
On banana plants

- All bacterial suspensions prepared from fresh cultures of *Erwinia* spp., the causal agents of tip-over disease of banana were adjusted to 7 x 10^5 cfu/ml.
- Sixty day-old banana plants (variety G-9) were injected artificially into the pseudo-stem(near the base of the stem).
- The plants were kept openly in a glass house and studied for the development of typical tip-over symptoms.

(c) Severely infected banana plant.
(d) Diseased banana leaves.

Snehalatharani and Khan,2010

PCR test

DNA extraction from potato peel extract artificially contaminated by *Pcc* and *Pa*

- Potato tubers were peeled (ca. 1 g peel) with a knife hand peeler and crushed in a sterile mortar with 4 ml TE buffer pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and 0.1 ml of bacterial suspension (ca. 1.8 X 10^8 cfu/ml) was inoculated into the homogenate and serially diluted.
- Aliquots (100 µl) from the plant homogenate were centrifuged at 8.000 g for 10 min at 4 °C in Eppendorf tubes.
- Thenafter PCR protocol steps for DNA purification were applied.

Ahamd,2001

PCR test

Specific detection pectolytic *P. carotovorum*, except the *P. betavasculorum*

- **Primers Y1 and Y2**
- Pectate lyases are involved in soft-rot diseases and several *pel* genes (encoding pectate lyases) were sequenced.
- No homologous sequence was found in these data bases except for the *pel* sequences.
- Two oligonucleotides (24-mers) separated by 434 bp were selected as PCR primers, namely,
- Y1 (5'TFACCGGACGCCGAGCT GTGGCGT3'), and
- Y2 (5'CAGGAAGATGTCGTTATCGCGAGT3').
- **An amplified fragment of 434 bp was obtained from all:**
- *P. atrosepticum*, *P. carotovorum* subsp. *carotovorum*, *P. carotovora* subsp. *odorifera*, and *P. wasabiae* strains.
- These primers amplify a fragment of the *pelY* family pectate lyases from *Pectobacterium* species, with the exception of *P. betavasculorum*.
- No amplified fragment was observed with *D. chrysanthemi* and other *Erwinia* species.

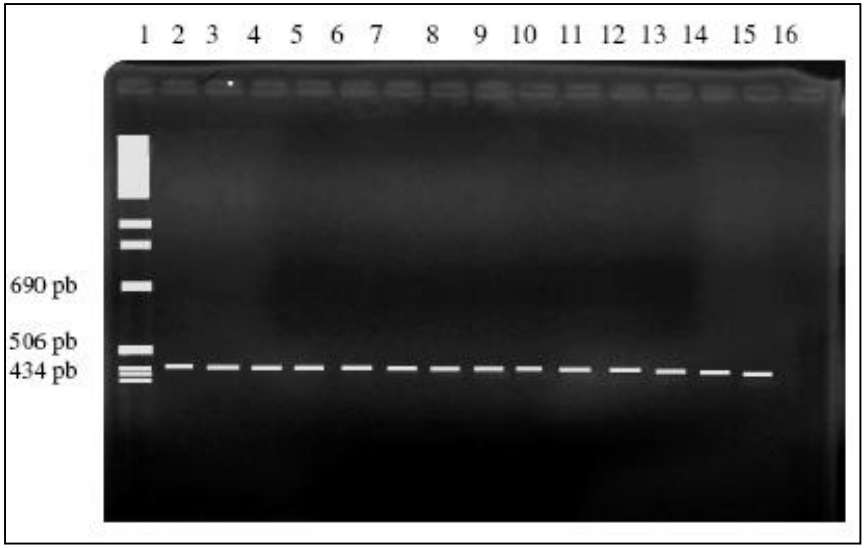
Darrasse *et al.*,1994

PCR test

Primers Y1 and Y2

Specific detection for great majority of the genus *Pectobacterium*

- An amplified fragment of 434 bp with primers Y1 and Y2 was obtained for the great majority of the genus *Pectobacterium*.
- lane 1, 1-kb molecular weight ladder.



Tumelero,2003

RAPD analysis

Two RAPD primers

P. carotovorum and pectolytic pseudomonads

- *P. carotovorum* and pectolytic pseudomonads are the most prevalent bacteria associated with soft rot of plants.
- A procedure based on random amplified polymorphic DNA (RAPD) analysis was developed to distinguish these bacteria rapidly and easily.
- Two RAPD primers were chosen based on cost considerations and on their capacity to discriminate between the bacterial strains.
- The combination of the two primers was sufficient for adequate distinction of *P. carotovorum* from pectolytic, fluorescent *Pseudomonas* spp.
- Furthermore, *P. carotovorum* subsps. *atrosepticaum* and *carotovorum* could also be distinguished from each other.

Parent *et al.*,1996

PCR test

Primers G1 and L1

ITS-PCR, in combination with ITS-RFLP for soft rot ex. erwinias

- The use of ITS-PCR, in combination with ITS-RFLP, for the rapid and accurate identification and differentiation of the soft rot ex. erwinias.
- **PCR amplification and restriction digestion of the ITS.**
- After the extraction of gDNA, the ITS was amplified using the primers:
- G1 (5′-GAAGTCGTAACAAGG-3′) and
- L1 (5′-CAAGGCATCCACCGT-3′) as described by Jensen *et al.*,1993.
- For each strain, 10 µl of the amplified product was digested with each of the restriction enzymes *Alu*I, *Cfo*I, *Hae*III, *Hha*I, *Hpa*II, *Mse*I, *Msp*I, *Mbo*I, *Rsa*I, *Sau*3aI, *Taq*I, and *Tha*I, as described by the manufacturer.
- Digested samples (10 µl) were electrophoresed through a 2% NuSieve GTG agarose gelin TBE buffer for 1.5 h and stained as described above.
- Gel images were digitized and band sizes analyzed by Gel Compar software.
- Fragment sizes were determined by comparison to a 1-kb Plus DNA molecular weight marker.

Toth *et al.*,2001

Primers for soft rot ex. *Erwinia* spp. *Pectobacterium* and *Dickeya*

Target	Name	Primer Sequence	Product size
Eca	ERWFOR ATROREV	5'-ACGCATGAAATCGGCCATGC-3' 5'-ATCGATATTTGATTGTC-3'	389
Eca	Y45 Y46	5'-TCACCGGACGCCGAACTGTGGCGT-3' 5'-TCGCCAACGTTCAGCAGAACAAGT-3'	438
Eca	ECA1f ECA2r	5'-CGGCATCATAAAAACACG-3' 5'-GCACACTTCATCCAGCGA-3'	690
Ech	ADE1 ADE2	5'-GATCAGAAAGCCCGCAGCCAGAT-3' 5'-CTGTGGCCGATCAGGATGGTTTTGTCGTGC-3'	420
Ech	ERWFOR CHRREV	5'-ACGCATGAAATCGGCCATGC-3' 5'-AGTGCTGCCGTACAGCACGT-3'	450

Schaad *et al.*,2001

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

Genus *Pectobacterium*

Genus *Pectobacterium*					
Species/subspecies	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
P. atrosepticum	**ECA1f/ECA2r** Chromosomal DNA (unknown)	Conventional	Bacteria, plant (DNA extraction) Tuber (immunomagnetic separation followed by alkaline lysis) Bacteria (boiled), enriched peel (DNA extraction) Bacteria, potato peel (enriched)	De Boer and Ward, 1995; van der Wolf *et al.*, 1996; Fraaije *et al.*, 1997; Hyman *et al.*, 1997	*Erwinia carotovora* subsp. *atroseptica*
P. atrosepticum *P. carotovorum* subsp. *carotovorum* *P. betavasculorum* *P. odoriferum* *P. wasabiae*	**Y1/Y2** *Y* family of pectate lyase (*pel*) genes	Conventional and RFLP	Bacteria (DNA extraction) Bacteria (boiled) after enrichment or immunomagnetic separation) Plant, soil and water (DNA extraction)	Darrasse *et al.*, 1994; Helias *et al.*, 1998	*E. carotovora* subsp. *atroseptica* *E. carotovora* subsp. *carotovora* *E. carotovora* subsp. *betavasculorum* *E. carotovora* subsp. *odorifera* *E. carotovora* subsp. *wasabiae* PCR reaction and restriction enzyme analysis do not clearly discriminate species.

Palacio-Bielsa *et al.*,2009

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

Genus *Pectobacterium*

P. atrosepticum	**ECA1f/ECA2r** Chromosomal DNA (unknown) **ECA4r** Contains ECA2r sequence (competitor template)	Conventional	Bacteria (boiled), potato peel (DNA extraction)	Hyman *et al.*, 1997	
P. atrosepticum	**PEAF/PEAR** *Rbs* family gene	Conventional	Bacteria, potato tubers (DNA extraction)	Park *et al.*, 2006	
P. atrosepticum	**ERWFOR/ ATROREV** **ERWFOR/CHRREV** **ERWFOR+ATROREV+ CHRREV** Metalloproteases coding genes	Conventional Multiplex	Bacteria (boiled), potato tubers (centrifugation and lysis buffer)	Smid *et al.*, 1995	*E. carotovora* subsp. *atroseptica* *Dickeya* sp. (*Erwinia chrysanthemi*) aso amplified. Lower specificity of multiplex PCR, undesirable band obtained with *P. carotovorum* subsp. *carotovorum*.
P. atrosepticum	**Dcd Forw/Dcd Rev** *pel*ADE gene fragments + **Pca For/Pca Rev** Chromosomal DNA (unknown)	Multiplex	Enriched potato tubers (microsphere immunoassay)	Peters *et al.*, 2007	*Dickeya dianthicola* also amplified.
P. carotovorum subsp. *carotovorum*	**EXPCCF/EXPCCR** Chromosomal DNA (unknown) **INPCCF/INPCCR** Nested to EXPCCF/EXPCCR	Competitive Nested	Bacteria (untreated), plant (DNA extraction)	Kang *et al.*, 2003	Amplification obtained with *P. carotovorum* subsp. *wasabiae* (distinction by restriction analysis).
P. atrosepticum, *P. carotovorum* subsp. *carotovorum*	**SR3F/SR1cR** 16S rRNA gene	Conventional and RFLP	Bacteria (untreated), microplant (enriched)	Toth *et al.*, 1999	*E. carotovora* subsp. *atroseptica* *E. carotovora* subsp. *carotovora* *Dickeya* sp. (*E. chrysanthemi*) also amplified. Amplification obtained for other genera. Banding patterns allow differentiation of *Pectobacterium* from other and restriction analysis improves discrimination.
P. atrosepticum, *P. carotovorum* subsp. *carotovorum*	**MpdEc-F/MpdEc-R** *mpd* gene	Real-time (iQ Supermix SBYR-Green)	Potato tubers (DNA extraction)	Atallah and Stevenson, 2006	*E. carotovora* subsp. *atroseptica* *P. wasabiae*, *P. betavasculorum*, as well as *Brenneria nigrifluens* and *B. quercina* also amplified. Primers for detection of four potato tubers pathogenic fungi are also described.

Palacio-Bielsa *et al.*,2009

Pathogenicity test

Pectobacterium carotovorum subsp.*carotovorum*; P. *atrosepticum* and *Pseudomonas marginalis* strains 7M& 8M

- **Pathogenicity on calla lily leaf petioles:**
- Observations performed within 3 days after dipping the bases of petiole segments into bacterial suspension of isolates.
- Total rotting was caused by *Pectobacterium carotovorum* subsp. *carotovorum*; P. *atrosepticum* and *Pseudomonas marginalis* strains 7M and 8M.
- The *Pectobacterium* spp. caused quick rooting (within 2–3 days) of artificially inoculated calla lily leaf petioles.

Fig. 3 Pathogenicity test of two bacterial isolates on calla lily cv. Treasure leaf petioles (7M, 8M *Pseudomonas marginalis*)

Mikiciński *et al.*,2009

Pathogenicity test

Pectobacterium carotovorum subsp.*carotovorum*

- **Pathogenicity on calla lily leaf petioles:**
- All investigated strains caused soft rot of calla leaf stalks, potato slices and aloe leaves, and induced hypersensitive reaction on tobacco.

Ivanović*et al.*,2009

Screening sugarbeet cultivars for resistance to isolates of *P. betavasculorum*

- *P. betavasculorum* suspensions were adjusted to OD 1.06 nm and diluted 10x to yield ~1 x 10^7 colony-forming units/ml (cfu/ml).
- For the Ecb isolate and sugar beet variety tests, two inoculation methods were used:

1. Bacterial suspensions were injected into petioles of the first two true leaves with a 26-gauge hypodermic needle.
2. 1 cm of leaf tips were sliced from the first two true leaves with a scalpel that had been dipped into bacterial suspensions.

- Plants were then transferred to a 28°C growth chamber and rated for disease development at 2 and 3 weeks post-inoculation using a 0 to 5 scale:
- **(0= no disease, 1= small black lesion, 2= wilted petiole, 3= systemic movement of pathogen evident as black streaking in non-inoculated parts of plant, 4= whole plant wilting, 5= dead).**

Blackened petioles of a young sugarbeet plant inoculated with *P. betavasculorum*, a wound pathogen.

Zidack *et al.*,2001

Diseases caused by: *Dickeya* spp.

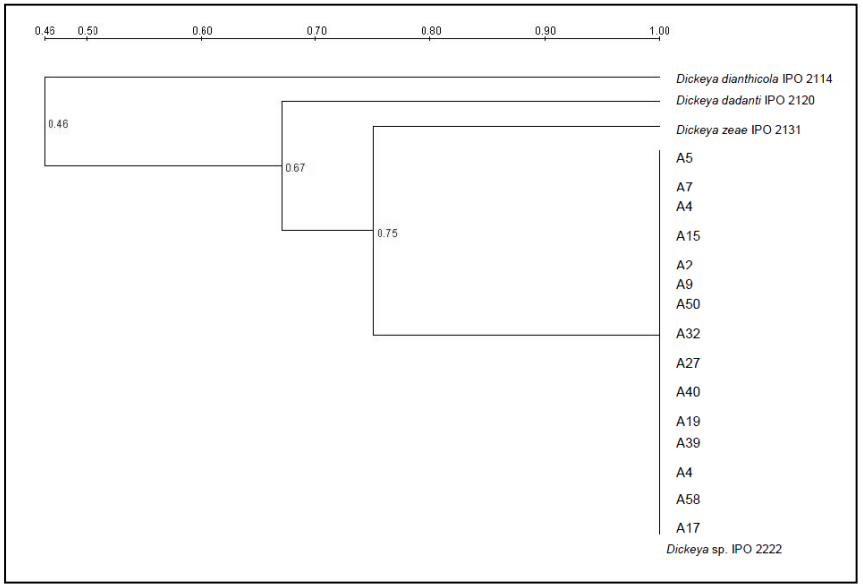
- The species in the genus *Dickeya* includes:

1. *D. chrysanthemi*
2. *D. dadantii*
3. *D. dianthicola*
4. *D. diffenbachiae*
5. *D. paradisiaca*
6. *D. zeae*

The new emerging strains of biovar 3, with the proposed name *D. solani* cause typical blackleg symptoms on potato.
D. solani strains were shown to cause more severe losses than *D. dianthicola*, *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum*.

Phylogenetic analysis of 16S rDNA sequences of *Dickeya* isolates

- Dendrograms showing the phylogenetic relationships between *Dickeya* isolates.
- The numbers adjacent to the nodes are the similarities calculated using the Neighbour-Joining method.
- The A-numbers are test isolates.
- *Dickeya dadantii* IPO2120,
- *Dickeya dianthicola* IPO2114,
- *Dickeya* sp. IPO2222 and
- *Dickyea zeae* IPO2131 were used as reference strains.



Czajkowski *et al.*,2009

Features of the soft rot ex. *Erwinia* genomes

Dickeya and *Pectobacterium*

- Both species have cirucular chromosomes of approximately the same size.
- No plasmids and relatively few insertion elements and transposons were found in either genome.
- Based on comparison with other sequenced enterobacteria, approximately 2300 of the *E. chrysanthemi* genes are likely to have been derived from the ancestor of this group.
- Only 500 additional genes beyond these 2300 are also found in *Erwinia carotovora* subsp. *atroseptica* and just over 400 of these genes are not found in animal associated enterobacterial.
- Thus the pool of soft rot *Erwinia* specific genes is not large.

Feature	*E. chrysanthemi*	*E. carotovora*
Genome size	4.9 Mb	5.06 Mb
Protein-coding genes	4,638	4,491
rRbosomal RNA-coding genes	22	25
tRNA-coding genes	75	76

Charkowski,2006

Characteristics of:
The genus *Dickeya*

- Gram negative, rod shaped.
- Facultative anaerobic metabolism.
- Colony characteristics on CVP (Crystal violet pectate) medium(*D. dianthicola* fails to grow on CVP).
- Peritrichous flagellation.
- Negative to reducing substances from sucrose test.
- Sensitive to erythromycin.

Transfer of *Pectobacterium* (*Erwinia*) *chrysanthemi to Dickeya* spp. according to Samson *et al.*,2005

New name	Old names	Hosts
Dickeya dianthicola	*Erwinia chrysanthemi* biovars 1, 7 and 9 *Erwinia chrysanthemi pv dianthicola* *Pectobacterium chrysanthemi pv dianthicola*	*Dianthus spp.*, potato, tomato, chicory, artichoke, *Dahlia* & *Kalanchoe.*
Dickeya dadantii	*Erwinia chrysanthemi* biovar 3 (some strains) *Pectobacterium chrysanthemi* biovar 3 (some strains)	*Pelargonium*, pineapple, potato, *Dianthus* spp., *Euphorbia*, *sweet potato*, banana, maize, *Philodendron* & *Saintpaulia.*
Dickeya zeae	*Erwinia chrysanthemi* biovar 8 and other strains of biovar 3 *Pectobacterium chrysanthemi* biovar 8 and other strains of biovar 3	Maize, potato, pineapple, banana, tobacco, rice, *Brachiaria*, & *Chrysanthemum*
Dickeya chrysanthemi bv. *chrysanthemi*	*Erwinia chrysanthemi* biovar 5 *Erwinia chrysanthemi* pv. *chrysanthemi* *Pectobacterium chrysanthemi pv. chrysanthemi*	*Chrysanthemum spp.*, chicory, tomato & sunflower
Dickeya chrysanthemi bv. *parthenii*	*Erwinia chrysanthemi* biovar 6 *Erwinia chrysanthemi* pv. *parthenii* *Pectobacterium* chrysanthemi pv. *Parthenii*	*Parthenium*, artichoke & *Philodendron.*
Dickeya paradisiaca	*Erwinia chrysanthemi* biovar 4 *Erwinia chrysanthemi* pv. *paradisiaca* *Erwinia paradisiaca* *Brenneria paradisiacal*	Banana & maize
Dickeya dieffenbachiae	*Erwinia chrysanthemi* biovar 2 *Erwinia chrysanthemi* pv. *dieffenbachiae* *Pectobacterium chrysanthemi pv. dieffenbachiae*	*Dieffenbachia*, tomato & banana

Elphinstone and Toth,2007

Diseases caused by *Dickeya* spp.

Dickeya chrysanthemi	**Wide host range:** **Soft rot of *chrysanthemum* spp.&carnation; potato bacterial stem and root rot; sugarcane bacterial mottle; etc.**
D. chrysanthemi* pv. *chrysanthemi	**Wide host range:** **Soft rot of *Chrysanthemum* spp., sunflower, chicory; tomato**
D. chrysanthemi* pv. *parthenii	**Soft rot of *Parthenium* spp.**
D. dadantii	
D. dadantii* subsp. *dadantii	**Wide host range:** **Soft rot diseases on many crops e.g. geranium, pineapple, carnation, poinsettia, sweet potato, banana, maize, *Philodendron*, African violet. Also has a secret life as an insect pathogen**
D. dianthicola	**Slow wilt of Dianthus; Dickeya Blackleg, chicory, carnation**
D. dadantii* subsp. *diffenbachiae	**Bacterial soft rot of Dieffenbachia, banana, tomato**
D. paradisiaca	**Rotting of roots of banana (Brown-black root rot),maize**
D. solani	**Typical blackleg symptoms on potato**
D. zeae	**Wide host range:** **Bacterial stalk rot of corn; Also causes diseases on pineapple, potato , banana, rice, chrysanthemum, tobacco**

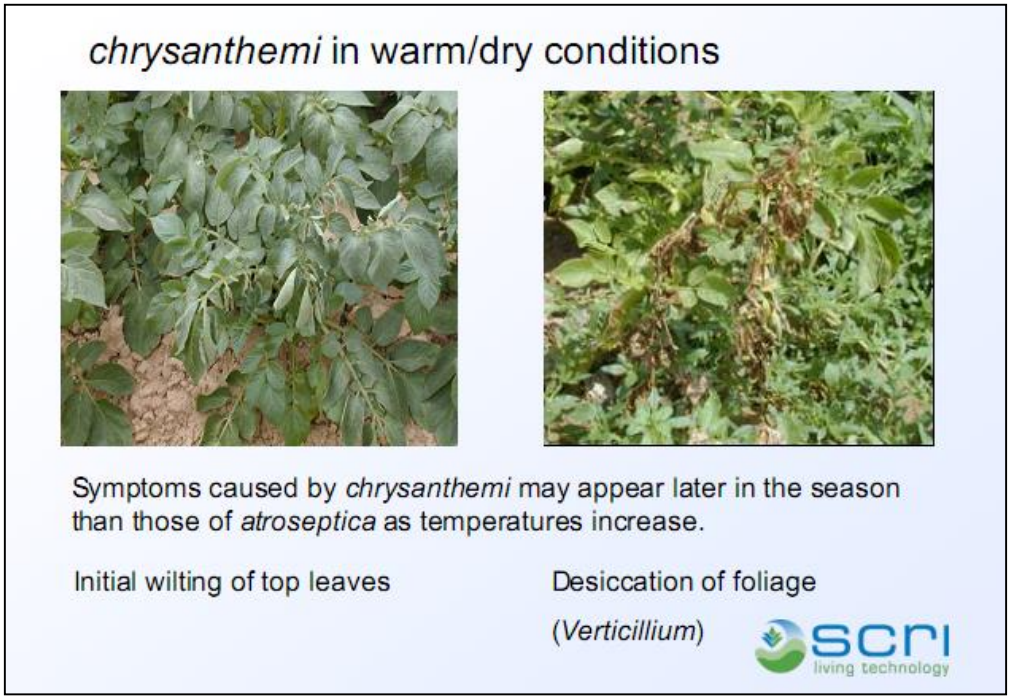
The genus *Dickeya*, their synonyms and main hosts; adapted from Samson *et al.*,2005

New name	Synonyms (including biovars)	Hosts
D. dianthicola	*Erwinia chrysanthemi* biovars 1, 7 and 9 *E. chrysanthemi* pv. *dianthicola* *Pectobacterium chrysanthemi* pv. *dianthicola*	*Dianthus* spp., potato, tomato, chicory, artichoke, *Dahlia, Kalanch*
D. dadantii	*E. chrysanthemi* biovar 3 (some strains) *P. chrysanthemi* biovar 3 (some strains)	*Pelargonium*, pineapple, potato, *Dianthus* spp., *Euphorbia*, sweet potato, banana, maize, *Philodendron, Saintpaulia*
D. zeae	*E. chrysanthemi* biovar 8 and other strains of biovar 3 *P. chrysanthemi* biovar 8 and other strains of biovar 3	Maize, potato, pineapple, banana, tobacco, rice, *Brachiaria*, *Chrysanthemum* spp.
D. chrysanthemi bv. *chrysanthemi*	*E. chrysanthemi* biovar 5 *E. chrysanthemi* pv. *chrysanthemi* *P. chrysanthemi* pv. *chrysanthemi*	*Chrysanthemum* spp., potato, chicory, tomato, sunflower
D. chrysanthemi bv. *parthenii*	*E. chrysanthemi* biovar 6 *E. chrysanthemi* pv. *parthenii* *P. chrysanthemi* pv. *parthenii*	*Parthenium*, artichoke, *Philodendron*
D. paradisiaca	*E. chrysanthemi* biovar 4 *E. chrysanthemi* pv. *paradisiaca* *E. paradisiaca* *Brenneria paradisiaca*	Banana, maize, potato
D. dieffenbachiae	*E. chrysanthemi* biovar 2 *E. chrysanthemi* pv. *dieffenbachiae* *P. chrysanthemi* pv. *dieffenbachiae*	*Dieffenbachia*, tomato, banana

Toth *et al.*,2011

Disease symptoms
D. chrysanthemi

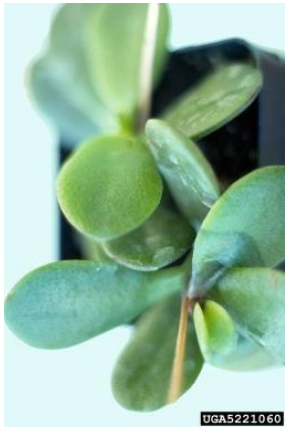
- *D. chrysanthemi* causes disease in warm/dry condition whereas *P. atrosepticum* incidence occurs at lower temperature (25°C).
- *chrysanthemi* versus *atroseptica* years.



Toth and Elphinstone,2011

Bacterial soft rot

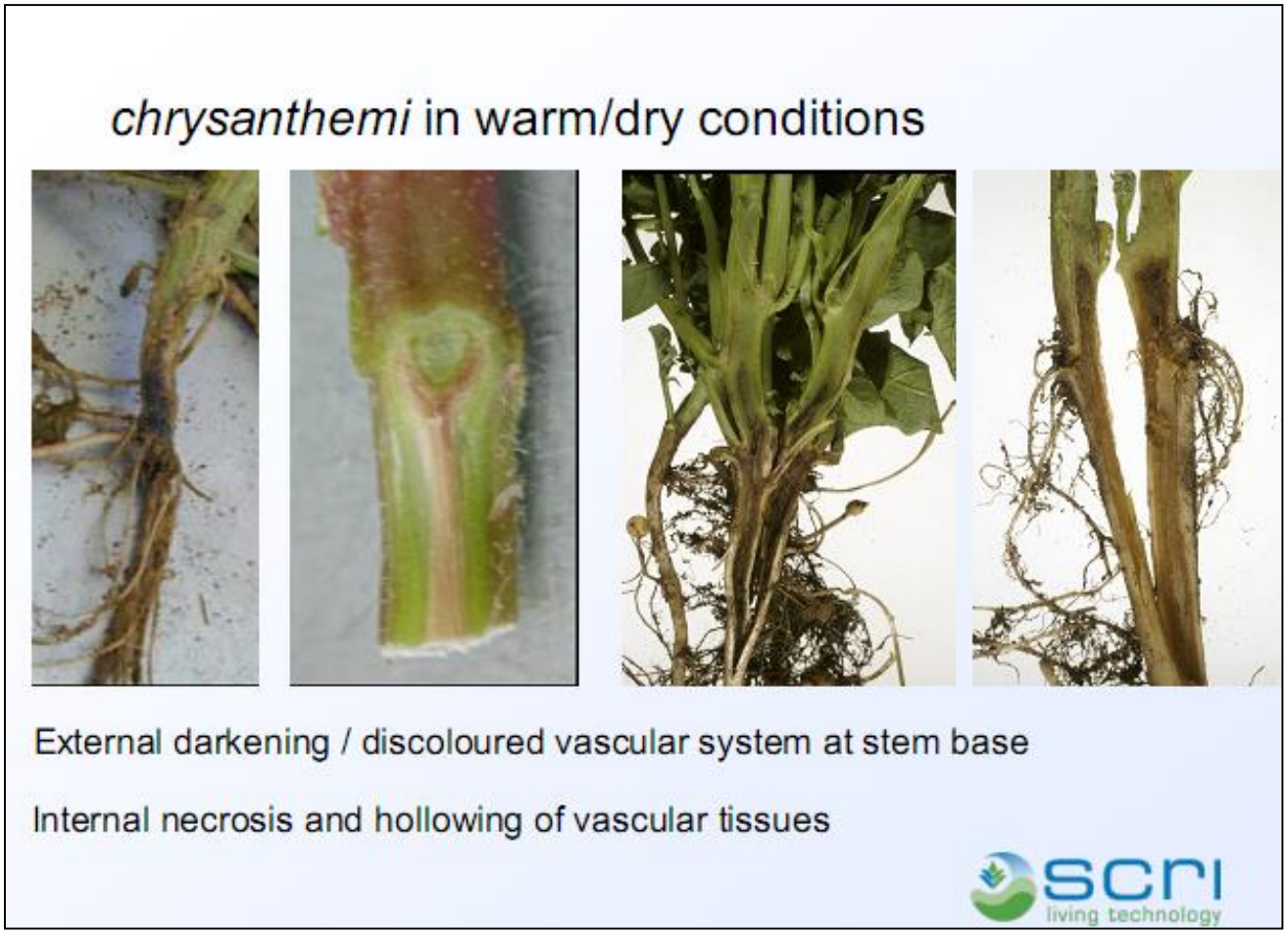
Dickeya chrysanthemi

		
Carnation(*Dianthus*)	Chrysanthemum	Philodendron
	USDA Forest Service,2008 	
Corn(*Zea mays*)	Dieffenbachia	Pygmyweed

Disease symptoms

Internal necrosis and hollowing of vascular tissues

Dickeya chrysanthemi



Toth and Elphinstone,2011

Dickeya chrysanthemi
Colony characteristics on PDA

- On PDA, young colonies are either circular, convex, smooth and entire, or sculptured with irregular margins, depending on the moisture content of the growth medium.
- After 4-5 days, both colony types resemble a fried egg, with a pinkish, round, raised centre and lobed periphery, which later becomes feathery or almost coralloid.

CABI and EPPO,1991

Dickeya dadantii

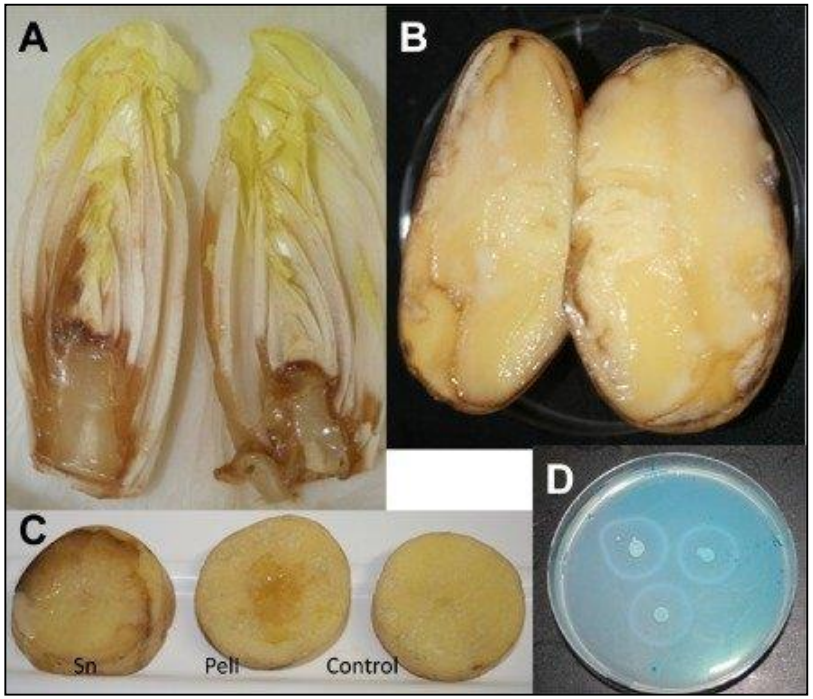
Colony characteristics on plates containing polygalacturonate

A. Symptoms caused by *D. dadantii* on chicory.

B. Soft-rot caused by *D. dadantii* on potato tubers.

C. Symptoms caused by culture supernatants (Sn) or purified pectate lyases (PelI) on potato discs.

D. Detection of pectate lyase-producing bacterial clones on plates containing polygalacturonate, after staining with a saturated solution of copper acetate.



Hugouvieux-Cotte-Pattat *et al.*,2014

Differentiated characteristics of *Pectobacterium*/ *Dickeya* spp.

Test	Ecc	Eca	Ecb	Eco	Ecw	Ech	Ect
Growth at 37°C	+	-	+	+	-	+	+
Reducing sugars from sucrose	-	+	+	+	-	-	-
Phosphatase activity	-[b]	-	-	-	-	+	V
Sensitivity to erythromycin	-	-	-	ND	-	+	-
Indole production	-	-	-	-	-	+	-
Acid produced from :							
sorbitol	-	-	-	+	ND	-	-
melibiose	+	+	-	+	-	+	-
citrate	+	+	-	+	+	+	ND
raffinose	+	+	-	ND	-	+	-
arabitol	-	-	-	+	ND	-	ND
lactose	+	+	+	+	+	+	-
Utilization of keto-methyl glucoside	-	+	+	+	-	-	-

+, 80% or more strains positive; V, between 21-79% of strains positive; -, 80% or more strains negative; ND, not determined.

[a] Ecc = *E. carotovora* subsp. *carotovora*
Eca = *E. carotovora* subsp. *atroseptica*
Ecb = *E. carotovora* subsp. *betavasculorum*
Eco = *E. carotovora* subsp. *odorifera*
Ecw = *E. carotovora* subsp. *wasabiae*
Ech = *E. chrysanthemi*
Ect = *E. cacticida*

[b] Some strains of *E. carotovora* subsp. *carotovora* may be weakly positive

Schaad *et al.*,2001

Tests to differentiate soft rot species
Dickeya strains on potato

Biochemical characterisation and identification of *Dickeya* strains isolated from potato plants and tubers

Biovar	1	2	3	3	3'	4	5	6	7
Species	*5*	*4*	*1*	*2*	*Not known*	*6*	*3*	*3*	*5*
Test									
(-)-D-Arabinse	-	V (57)	+	+	+	+	-	-	-
(-)-D-Winian	+	-	-	-	-	-	-	-	V (89)
Inulin (extract from chicory)	-	-	-	-	-	-	+	-	-
Inulin (extract from dahlia)	-	-	-	-	-	-	+	-	-
(+)-D-Melibiose	+	-	+	+	+	+	+	+	-
(+)-D-Raffinose									
5-Ketogluconate	-	-	-	-	-	+	-	-	-
Mannitol	+	+	+	+	+	-	+	+	+
β-Gentiobiose	-	+	-	V (17)	+w	+	-	-	-
(+)-L-Winian	-	-	V (46)	-	-	-	-	-	-
Dihydrolaza argininy	+	-	-	-	-	-	V (80)	-	V (89)
Growth in 39°C on NB	-	+	+	+	+w	+	+	+	-
Growth in 41°C on NB	-	-	+	-	-	-	+	+	-
Growth in 25°C on NB (control)	+	+	+	+	+	+	+	+	+

- Strains isolated in Netherlands, Finland, England, Poland from infected potato plants shared the same characteristics

Not known strain/unknown strain/strains are unknown.
3' indicates the presence of an unknown strain. Slawiak et al. 20029, Europ. J. Plant Pathol.

Tests to differentiate soft rot species *Pectobacterium* and *Dickeya*

Substrate	*E. carotovora* var. *carotovora*	*E. carotovora* var. *atroseptica*	Sugarbeet *Erwinia*	*E. chrysanthemi*	*E. cypripidii*[b]	*E. rhapontici*[b]
Citrate[c]	+	+	−	+	+	+
Galacturonate	+	+	−	+	+	+
α-Methyl glucoside	−	v[d]	+	−	−	−
Lactose	+	+	+	v	−	+
Malonate	−	−	−	+	v	+
Melezitose	−	−	−	−	−	+
Melibiose	+	+	−	v	v	+
m-Tartrate	−	−	−	+	+	−
Xylose	+	+	+	+	+	−
Growth at 36 C	+	−	+	+	+	−
Reducing compounds from sucrose	−	+	+	v	−	−

[a]Ten isolates of each species and the sugarbeet *Erwinia* were tested.
[b]Data from Bergey's Manual (11).
[c]Readings taken at 3 days.
[d]v = variable results; 21-79% of these strains were positive.

Thomson *et al.*,1977

Tests to differentiate soft rot species *Pectobacterium* and *Dickeya*

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

Goszczynaska *et al.*,2000

- Biochemical characteristics that differentiate reference strains of *Dickeya chrysanthemi* bvs 1–9 and type strains of *Pectobacterium carotovorum* subspecies.
- The characteristics that differentiate *Dickeya* (ex.*Pectobacterium*) *chrysanthemi* biovars are in **bold face**.
- The characteristics tha differentiate *Pectobacterium carotovorum* subspecies are **underlined**.

Characteristic	*Pectobacterium chrysanthemi* biovars									*Pectobacterium carotovorum* subspecies*				
	1	2	3	4	5	6	7	8	9	*caro.*	*atro.*	*odori.*	*beta.*	*wasa.*
ADH (Moeller)	+	−	+	−	+	−	+	−	−	−	−	−	−	−
Growth at 39 °C	−	+	+	+	+	+	−	+	−	−	−	−	−	−
Growth at 36 °C	+	+	+	+	+	+	+	+	+	+	−	+	+	−
Growth on Simmons' citrate	−	+	+	+	+	+	+	+	+	−	+	+	−	+
Growth in 5% NaCl	+	+	+	−	+	−	−	+	−	+	+	+	+	+
Gelatin hydrolysis	+	−	+	−	+	+	+	+	+	+	−	+	−	+
Lecithin hydrolysis	+	+	+	+	+	+	+	+	+	−	−	−	−	−
Reducing substances from sucrose	−	−	−	−	−	−	−	−	−	−	+	+	+	−
Production of indole	+	+	+	+	+	+	+	+	+	−	−	−	−	−
Utilization of malonate	+	+	+	+	+	+	+	+	+	−	−	−	−	−
Utilization of inulin	+	−	−	−	+	−	+	−	+	−	−	−	+	−
Acid production from:														
D-Arabinose	−	+	+	+	−	−	−	+	−	−	−	+	−	−
Lactose	−	−	+	−	+	−	−	+	−	+	+	+	−	−
Melibiose	+	−	+	+	+	+	−	+	−	+	+	+	+	−
α-Methyl glucoside	−	−	−	−	−	−	+	−	−	−	+	+	+	−
Raffinose	+	−	+	+	+	+	−	+	−	+	+	+	+	−
Assimilation of:														
L-Alanine	−	−	+	+	+	+	−	−	−	−	−	−	−	−
D-(+)-Cellobiose	−	+	+	−	+	−	+	−	−	−	+	+	+	+
***cis*-Aconitate**	−	−	+	−	+	−	−	+	−	−	−	−	−	−
***trans*-Aconitate**	−	−	+	−	−	−	−	+	−	−	−	−	−	−
D-Galacturonate	+	+	+	+	+	+	+	+	+	+	+	+	−	+
β-Gentobiose	−	+	−	+	−	−	−	−	−	+	+	+	+	+
D-Glucuronate	+	+	+	+	+	−	+	+	+	+	+	−	−	+
L-Glutamate	+	+	+	+	+	+	+	+	−	+	−	+	+	−
DL-Glycerate	+	−	+	+	+	−	+	+	−	+	+	+	−	+
5-Keto-D-gluconate	−	+	−	+	−	−	−	−	−	−	−	−	−	−
2-Keto-D-gluconate	−	+	+	−	−	−	−	−	−	−	−	−	+	−
2-Ketoglutarate	−	+	−	−	+	−	+	−	−	−	−	−	−	−
DL-Lactate	+	+	+	+	+	−	+	+	+	−	−	−	+	+
Lactulose	−	−	−	−	−	−	−	−	−	+	+	+	−	−
D-Malate	+	+	+	+	−	+	+	+	+	−	−	−	−	+
meso-Tartrate	+	+	+	+	−	+	+	+	+	−	−	−	−	+
1-*O*-Methyl α-galactopyrannoside	+	−	−	+	+	+	+	−	−	+	+	+	−	−
1-*O*-Methyl β-galactopyrannoside	+	+	+	+	+	+	+	+	−	−	+	+	+	−
Palatinose	−	−	−	−	−	−	−	−	−	−	+	+	+	−
α-L-Rhamnose	+	+	+	+	+	−	+	−	+	+	+	+	+	+
L-(+)-Tartrate	−	+	+	+	−	−	−	+	−	−	−	−	−	−
D-(−)-Tartrate	+	+	−	+	−	−	+	−	+	−	−	−	−	−
Trehalose	−	−	−	−	−	−	−	−	−	+	+	+	+	+

caro., *carotovorum*; *atro.*, *atrosepticum*; *odori.*, *odoriferum*; *beta.*, *betavasculorum*; *wasa.*, *wasabiae*.

Sutra *et al.*,2001

Serological analyses

Pcc ,Pa and *D. chrysanthemi*

- All these three bacterial soft rots are related serologically.
- Numerous serotypes have been identified for each organism (De Boer *et al.*,1979).
- Some antisera and ELISA kits are commercially available to detect *D. chrysanthemi*.
- The antibodies are generally directed against O-serogroup 1, recognizing only 68% of the strains (Samson *et al.*,1990).

Primers for soft rot ex. *Erwinia* spp.
DNA extraction
Preparation of bacterial cell lysates

- For uniplex or multiplex PCR assay either bacterial cell lysates or purified bacterial genomic DNA was used.
- **DNA extraction(preparation of bacterial cell lysates from colony):**
- Cells from a single bacterial colony growing on CVP, NA or LA using a sterile toothpick and resuspended in 500 µl of sterile double distilled water or 50 µl of 5 mM NaOH.
- Suspensions were boiled for 5 min at 95°C and immediately put on ice for 1-2 min and then centrifuged at 10000 *g* for 5 min.
- A 100 µL aliquot of the supernatant was transferred to a sterile tube and stored at -20°C until PCR testing.
- One or 2 µl of the cell lysate was used as a template in PCR.

Czajkowski *et al.*,2009;..

Primers for soft rot ex. *Erwinia* spp.

PCR assays amplifying 16S-23S rDNA ITS and ITS-RFLP *Pectobacterium carotovorum* ssp.

- **Primers yf1 and yr1 and G1 and L1**
- The 16S rDNA and ITS were amplified using the PCR conditions and primers:
- yf1 (5′-TGATGGAGGGGGATAACTACTGGA-3′) and
- yr1 (5′-CCCCTACGGTTACCTTGTTACGAC-3′),

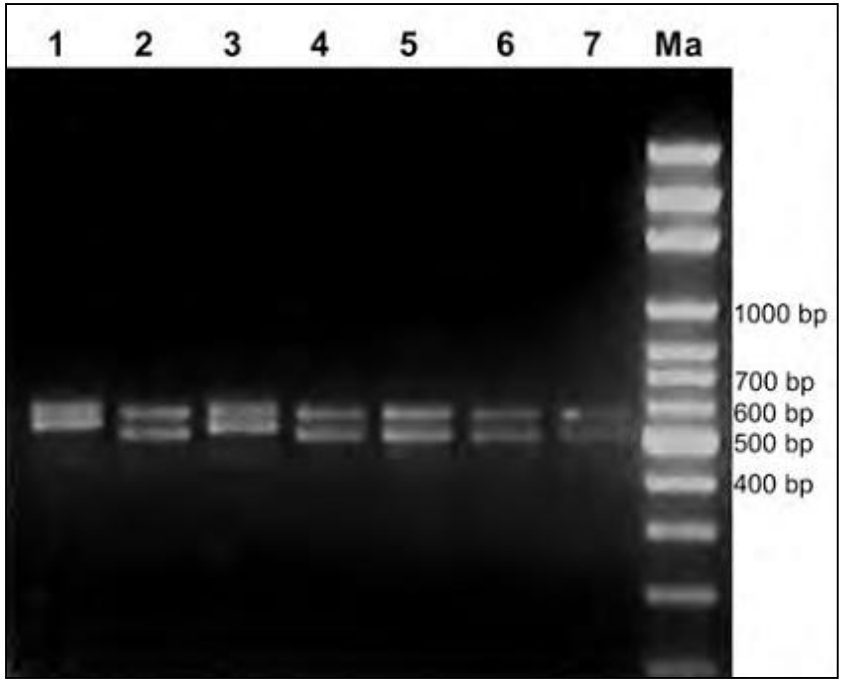
- G1 (5′GAAGTCGTAACAAGG-3′) and
- L1 (5′-CAAGGCATCCACCGT-3′) as described previously (Jensen *et al.*,1993; Toth *et al.*,2001; Yuan *et al.*,2004).

Zhu *et al.*,2010

Primers for soft rot ex. *Erwinia* spp.

PCR assays amplifying 16S-23S rDNA ITS and ITS-RFLP *Pectobacterium carotovorum* ssp.

- ITS patterns analysis:
- Lanes 1 to 7 in both panels (ITS and ITS-RFLP) comprise strains CA1, D4,BC1, Z1-2, Z2-1, Z2-2 and Z3-2.
- Most of the strains produced 540 bp and 575 bp fragments except D4, Z1-2, Z2-1, Z2-2 and Z3-2, whose PCR patterns were obviously different.



Zhu *et al.*,2010

Primers for soft rot ex. *Erwinia* spp.
Uniplex PCR assays
Virulent *P. c.* subsp*. carotovorum* and *P. atrosepticum*

- PCR detection of virulent *Pectobacterium c.* subsp*. carotovorum* was performed according to De Haan et al. (2008), using contig1R/contig1F primers:
- contig1F: 5’ CCTGCTGGCGTGGGGTATCG 3’,
- contig1R: 5’ TTGCGGAAGATGTCGTGAGTGCG3’) (De Haan et al.,2008).
- The expected fragment length of the amplicons was 500 bp.
- PCR detection of *P. atrosepticum* was performed according to Frechon et al. (1998), using Y45/Y46 primers:
- Y45: 5‘ TCACCGGACGCCGAACTGTGGCGT 3', and
- Y46: 5‘ TCGCCAACGTTCAGCAGAACAAGT 3') primers (Frechon et al.,1998).
- The expected fragment length of the amplicons was 439 bp.

Czajkowski *et al.*,2009

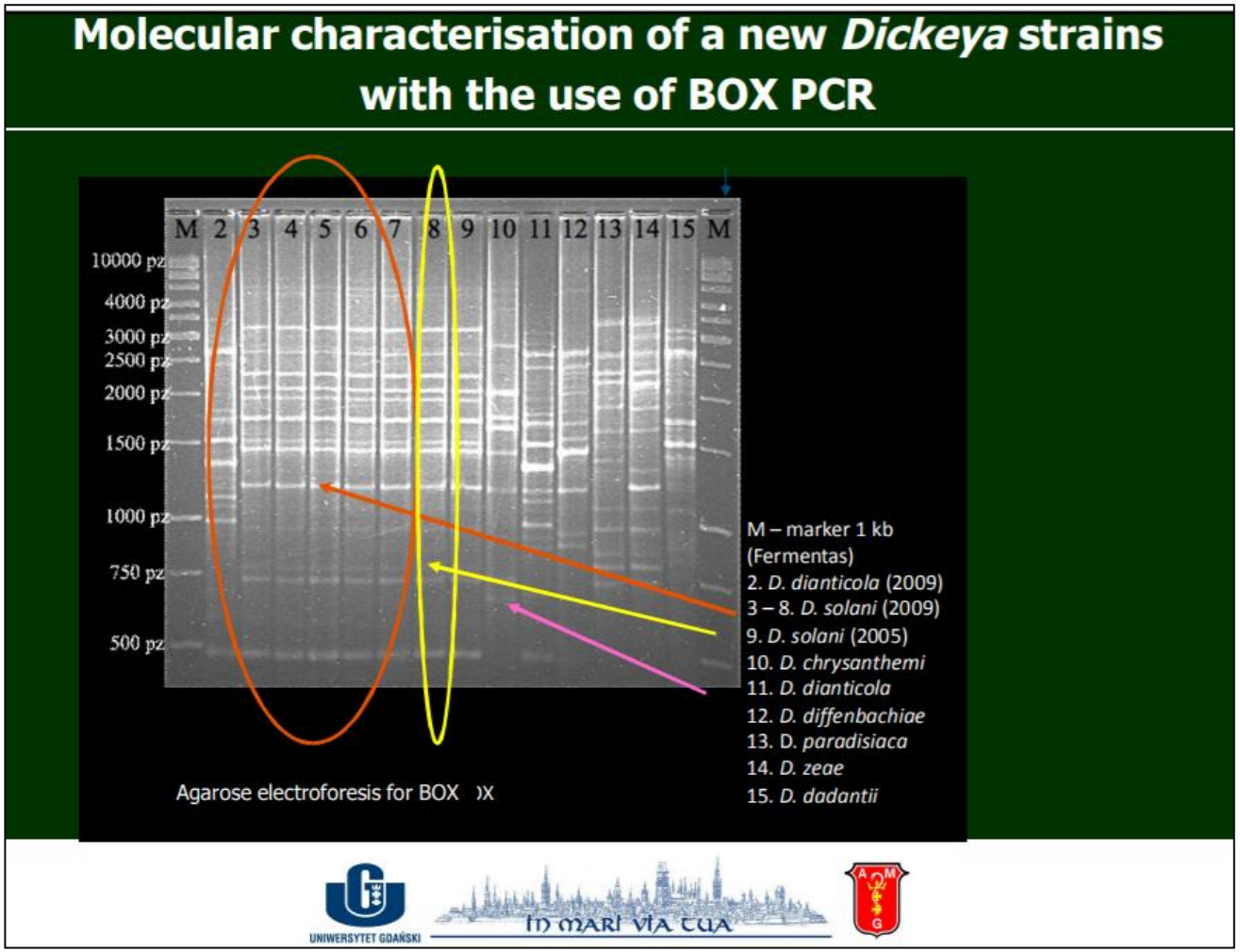
Primers for soft rot ex. *Erwinia* spp.
Uniplex PCR assays
Dickeya spp. and *Pectobacterium* spp.

- PCR detection of *Dickeya* spp. was performed according to Nassar et al. (1996), using ADE1/ADE2 Primers:
- ADE1: 5' GATCAGAAAGCCCGCAGCCAGAT 3',
- ADE2: 5'CTGTGGCCGATCAGGATGGTTTTGTCGTGC 3') (Nassar et al.,1996).
- The expected fragment length of the amplicons was 420 bp.
- PCR detection of *Pectobacterium* spp. was performed according to Darasse et al. (1994), using Y1/Y2 primers:
- Y1: 5‘ TTACCGGACGCCGAGCTGTGGCGT 3',
- Y2: 5‘ CAGGAAGATGTCGTTATCGCGAGT '3) (Darrasse et al.,1994).
- The expected fragment length of the amplicons was 434 bp.

Czajkowski *et al.*,2009

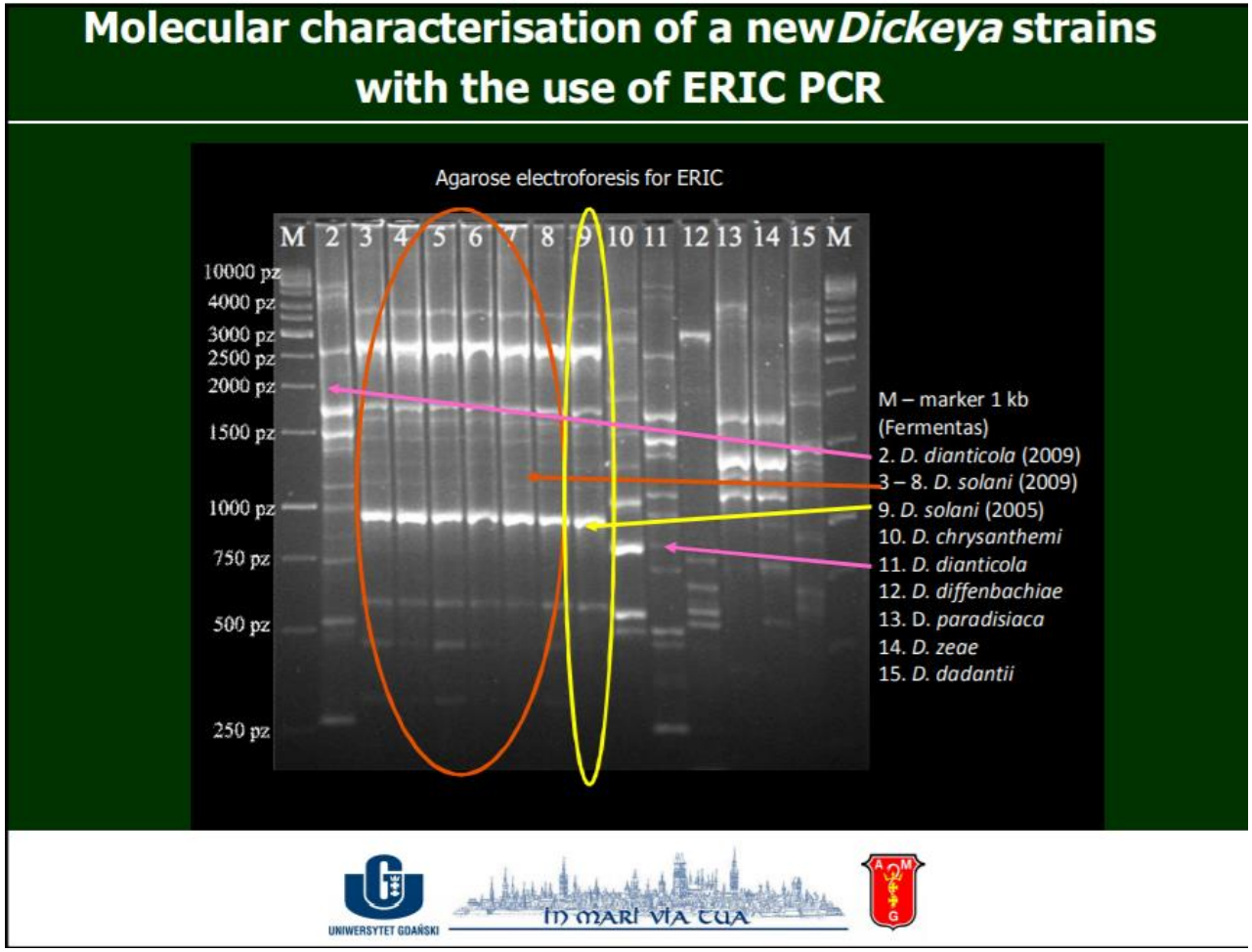
Primers for soft rot ex. *Erwinia* spp.

Molecular characterization of a new *Dickeya* strains with the use of BOX PCR



Primers for soft rot ex. *Erwinia* spp.

Molecular characterisation of a new *Dickeya* strains with the use of ERIC PCR



Primers for soft rot ex. *Erwinia* spp.
Multiplex PCR assays
Dickeya spp. and *Pectobacterium* spp.

- The multiplex PCR assay was developed on the basis of previously described three specific PCRs for detection of *Dickeya* genus (Dsp) with primers:
- Df (AGAGTCAAAAGCGTCTTG), and
- Dr (TTTCACCCACCGTCAGTC) (Laurila *et al.*,2010),
- *P. atrosepticum* (Pba) with primers:
- Y45 (TCACCGGACGCCGAACTGTGGCGT), and
- Y46 (TCGCCAACGTTCAGCAGAACAAGT)(Frechon *et al.*,1998)
- And *P. carotovorum* subsp. *carotovorum* (Pcc) together with *P. wasabiae* (Pwa) with primers:
- ExpccF (GAACTTCGCACCGCCGACCTTCTA), and
- ExpccR (GCCGTAATTGCCTACCTGCTTAAG) (Kang *et al.*,2003).

Potrykus *et al.*,2014

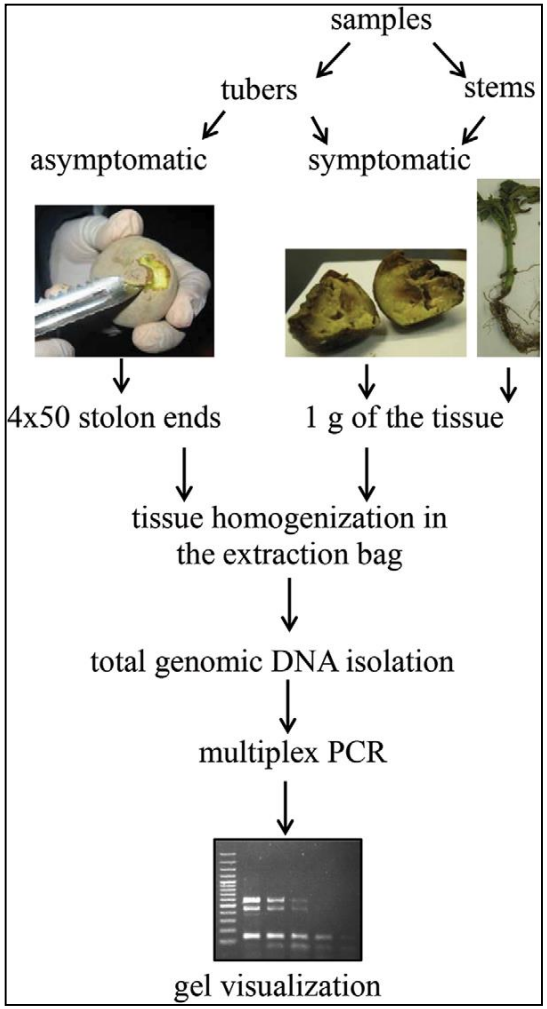
Primers for soft rot ex. *Erwinia* spp.
Multiplex PCR assays
Dickeya spp. and *Pectobacterium* spp.

- **Optimization procedure:**
- Extensive optimization steps were required to achieve proper functioning of implemented primer pairs in one PCR reaction and finally simultaneous detection of all desired groups of bacteria.
- The optimization procedure included establishing the concentration of magnesium chloride (from 2 to 3 mM), reaction buffer used for amplification(supplemented with 50mM KCl or with 20mM NH_4SO_4), the ratio between used primers (from 1:1:1 until the optimized one) and last but not least, the protocol for amplification.
- The use of a well-established positive control for each target group of bacteria in a multiplex assay for each series of tested material is crucial.

Potrykus *et al.*,2014

Primers for soft rot ex. *Erwinia* spp.
Multiplex PCR assays
Dickeya spp. and *Pectobacterium atrosepticum*

- Scheme representing fast and simple detection of Pcc/Pwa, Pba and Dsp in environmental samples in a single step using multiplex PCR reaction.
- It utilizes three pairs of primers and enables detection of three groups of pectinolytic bacteria frequently found in potato, namely: *P. atrosepticum*, *P. carotovorum* subsp. *carotovorum* together with *P. wasabiae* and *Dickeya* spp. in a multiplex PCR assay.



Potrykus *et al.*,2014

Primers for soft rot ex. *Erwinia* spp.
Multiplex PCR assays
Dickeya spp. and *Pectobacterium* spp.

- **Specificity of the multiplex polymerase chain reaction assay:**
- Three pairs of primers, each detecting a distinct group of bacteria namely

1. ExpccF/ExpccR detecting Pcc and Pwa (Kang *et al.*,2003),
2. Y45/Y46 detecting Pba, and
3. Df/Dr detecting Dsp (Laurila *et al.*,2010) were chosen for the development of the multiplex PCR.

- The primer set (EXPCCF/EXPCCR) amplified a single fragment of the expected size (0.55 kb) from genomic DNA of *Pectobacterium* strains.

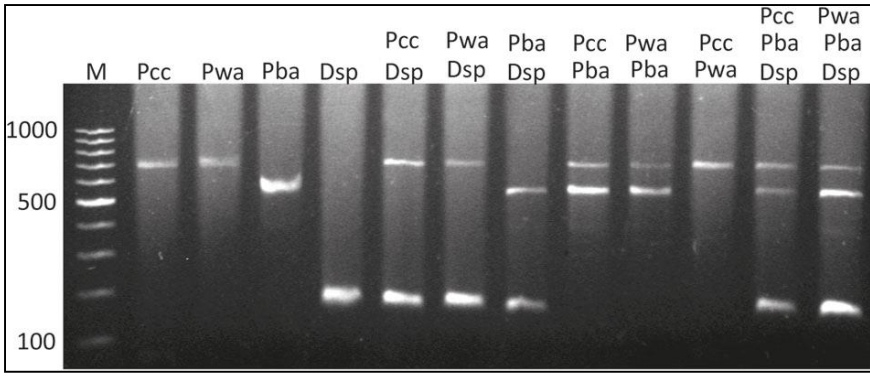
Potrykus *et al.*,2014

Primers for soft rot ex. *Erwinia* spp.
Multiplex PCR assays
Dickeya spp. and *Pectobacterium* spp.

- Multiplex PCR assay performed for simultaneous detection of major soft rot and blackleg pathogens:
- *P. carotovorum* subsp. *carotovorum*, *P. wasabiae*, *P. atrosepticum* and *Dickeya* spp.
- The size of the bands for each tested pathogen are:

1. 550 bp (Pcc/Pwa),
2. 420 bp (Pba),
3. 130 bp (Dsp).

- M – size marker 100 bp



Potrykus *et al.*,2014

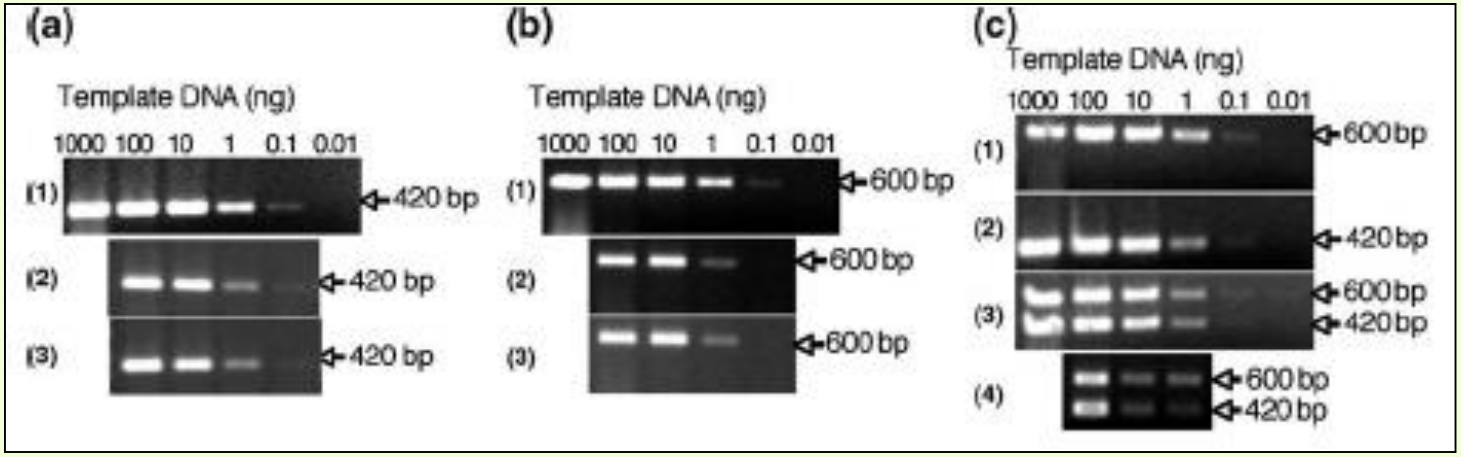
Primers for soft rot ex. *Erwinia* spp.
Uniplex vs. multiplex PCR assays
Dickeya spp. and *Pectobacterium atrosepticum*

- **Primers Y45/46** allowed amplification of a unique 420 bp fragment (Fig. 1a) from all of the 13 *P. atrosepticum* strains.
- **ADE primers** also led to specific PCR amplification of the 23 *Dickeya* tested.
- However, **the primer set Ech1/1'** located in the *pelI* ORF of *D. chrysanthemi* 2048T amplified a unique 600 bp fragment (Fig. 1b).
- Thereafter, we assayed **primers Ech1/1' and Y45/56** together in a single multiplex PCR procedure *in vitro*.
- As expected, amplification of *P. atrosepticum* 1526T and *D. chrysanthemi* 2048T DNA produced a 420 and a 600 bp PCR product, respectively (Fig. 1c).

Diallo *et al.*,2009

Primers for soft rot ex. *Erwinia* spp.
Uniplex vs. multiplex PCR assays
Dickeya spp. and *Pectobacterium atrosepticum*

- No amplification of *P. carotovorum* DNA was observed (data not shown).



Primers Y45/46
P. atrosepticum **amplified a unique 420 bp fragment**

Primers Ech1/1`
D. chrysanthemi **amplified a unique 600 bp fragment**

Multiplex PCR with primers Ech1/1' and Y45/46
P. atrosepticum **and** ***D. chrysanthemi*** **produced a 420 and a 600 bp PCR product, respectively.**

Diallo *et al.*,2009

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

Genus *Dickeya*

Genus *Dickeya*					
Species	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
Dickeya sp.	**ADE1/ADE2** *pel*ADE gen	Conventional	Bacteria (DNA extraction)	Nassar *et al.*, 1996	*Erwinia chrysanthemi* Restriction analysis results correlate with pathovar and biovar.
Dickeya sp.	**5A/5B** pT8-1, *idg* and *pecS* genes	Conventional	Not indicated	Chao *et al.*, 2006	*E. chrysanthemi*
Dickeya sp.	**PelZ-1-F/pelZ-1-S** *Zantedeschia aethiopica pelZ* gene (including an *AhdI* restriction site)	Conventional and RFLP	Bacteria (boiled)	Lee *et al.*, 2006	*E. chrysanthemi* Restriction analysis allows discrimination of *Z. aeothiopica* isolates from other hosts.
Dickeya sp. *Pectobacterium atrosepticum*	**ERWFOR/ATROREV** Metalloprotease genes (specific for *P. atrosepticum*) **ERWFOR+CHRREV +ATROREV** (Simultaneous detection of *Dickeya* spp. and *P. atrosepticum*)	Conventional Multiplex	Bacteria (boiled), potato tubers (centrifugation and lysis buffer)	Smid *et al.*, 1995	*E. chrysanthemi* *E. carotovora* subsp. *atroseptica* Specificity of multiplex PCR is lower than single assay, whereas an undesirable band can be also obtained with *P. carotovorum* subsp. *carotovorum*.
Dickeya dianthicola	**Dcd For/Dcd Rev** *pel*ADE gene + **Pca For/Pca Rev** Chromosomal DNA (unknown)	Multiplex	Enriched tubers extracts microsphere immunoassay (MIA)	Peters *et al.*, 2007	*P. atrosepticum* also amplified.
Dickeya sp. *Pectobacterium carotovorum* subsp. *carotovorum* *P. atrosepticum*	**SR3F/SR1cR** 16S rRNA gene	Conventional and RFLP	Purified isolate suspension or enriched microplant tissue (untreated)	Toth *et al.*, 1999	*E. chrysanthemi* *E. carotovora* subsp. *carotovora* *E. carotovora* subsp. *atroseptica* Other genera amplified also. Banding patterns allow differentiation of *Pectobacterium* and restriction analysis improves discrimination.

Palacio-Bielsa *et al.*,2009

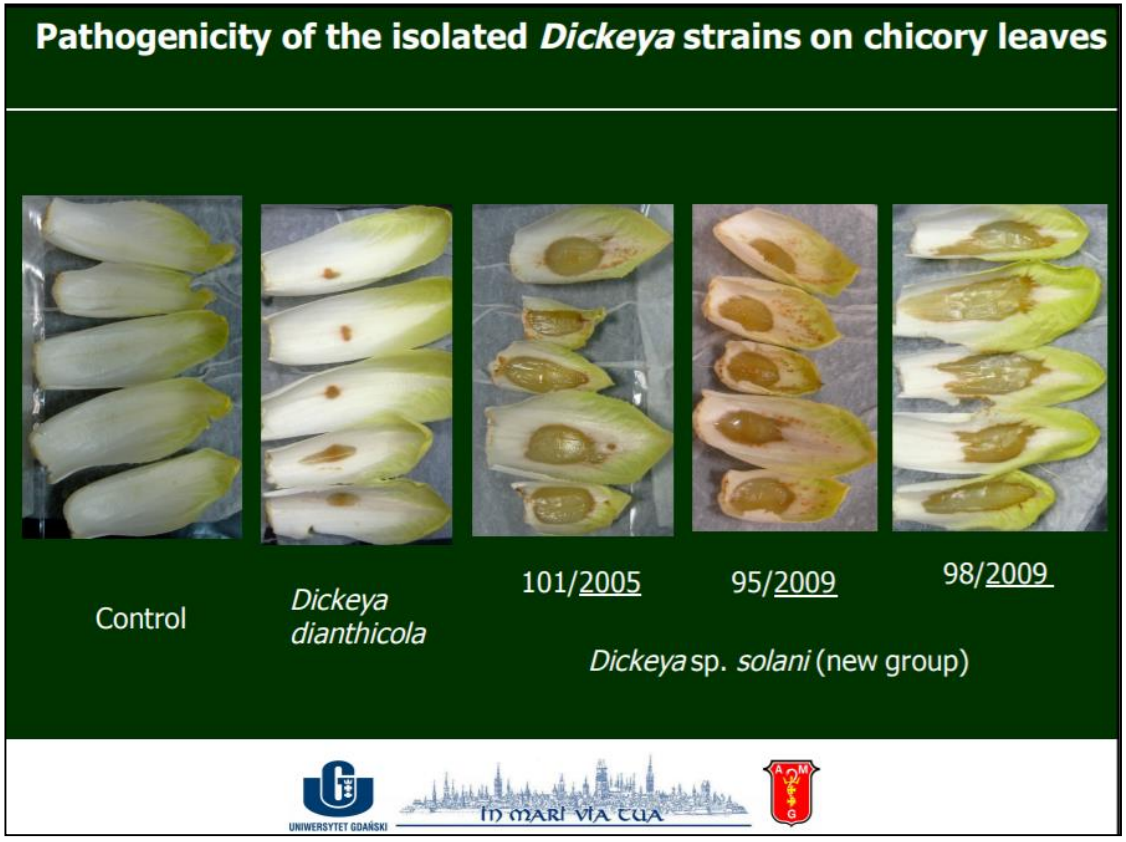
Pathogenicity test
Dickeya chrysanthemi

- Pathogenicity tests on potted *Saintpaulia ionantha* cv. Blue Rhapsody were performed as reported by Expert and Toussaint (1985) with modifications.
- Bacterial cells grown on LB agar medium for 24 h at 30°C were suspended in an NaCl solution at 9 g/liter to give an optical density at 600 nm of 0.6.
- The number of viable bacteria in each suspension was determined by serial dilution and plating.
- About 100 µl of the resulting suspension (approximately 6 x 10^7 bacteria) was inoculated to one leaf per plant.
- Twelve plants were tested for each bacterial strain.
- Progression of the symptoms was scored daily for 14 days.
- The assay was carried out in triplicate.

Reverchon *et al.*,2002

Pathogenicity test

Pathogenicity of the isolated *Dickeya* strains including *D. solani* on chicory leaves



D. solani cause typical blackleg symptoms on potato.
D. solani strains were shown to cause more severe losses than *D. dianthicola*, *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum*.

Pathogenicity and virulence

Dickeya zeae strains isolated from rice, banana and clivia rot plants show great virulence differentials

Inoculated plant			Inoculation amount, time	Diseased area (mm^2)					
Class	Species	Organ		EC1	MS2	MS3	JZL1	JZL2	JLZ7
Dicots	*Cucumis sativus*	Fruit	2 μL, 24 h	57.22 ± 7.70	107.97 ± 42.00	65.46 ± 7.88	0	0	0
	Benincasa hispida	Fruit	2 μL, 24 h	1140.57 ± 15.55	1336.50 ± 3.32	1252.61 ± 18.71	0	0	0
	Brassica pekinensis	Petiole	2 μL, 12 h	56.81 ± 3.14	49.83 ± 2.73	42.40 ± 3.17	0	0	0
	Raphanus sativus	Tuber	2 μL, 24 h	156.89 ± 2.60	106.72 ± 2.03	99.84 ± 9.76	0	0	0
	Daucus carota	Tuber	2 μL, 24 h	274.83 ± 18.74	266.70 ± 8.98	231.42 ± 14.11	0	0	0
	Solanum tuberosm	Tuber	2 μL, 24 h	173.33 ± 4.28	211.05 ± 20.54	171.45 ± 6.07	0	0	0
	Lycopersicon esculentum	Fruit	100 μL, 2 d	908.22 ± 5.95	904.75 ± 9.80	945.81 ± 13.77	0	0	0
	Solanum melongena	Fruit	100 μL, 2 d	66.52 ± 2.95	61.83 ± 1.67	86.72 ± 0.85	0	0	0
	Capsicum annuum	Fruit	2 μL, 24 h	101.79 ± 8.81	106.65 ± 10.76	191.42 ± 10.55	0	0	0
Monocots	*Oryza sativa*	Stem	200 μL, 7 d	720.62 ± 21.48	575.71 ± 29.53	539.35 ± 17.77	452.67 ± 19.53	413.01 ± 12.64	499.85 ± 12.09
	Musa sapientum	Stem	200 μL, 7 d	1110.83 ± 19.23	1358.89 ± 17.78	1201.88 ± 19.91	407.90 ± 12.48	377.89 ± 13.71	464.23 ± 11.06
	Clivia miniata	Leaf	200 μL, 24 h	1643.86 ± 6.94	2195.85 ± 7.06	1239.95 ± 7.55	1807.41 ± 9.52	1693.21 ± 20.27	1594.90 ± 14.66
	Allium cepa	Bulb	2 μL, 24 h	185.83 ± 21.68	194.27 ± 18.73	119.90 ± 11.29	0	0	0
	Zingiber officinale	Tuber	2 μL, 24 h	134.37 ± 14.17	86.92 ± 6.75	87.31 ± 12.23	47.44 ± 7.16	65.81 ± 6.11	81.33 ± 13.96
	Gladiolus gandavensis	Stem	200 μL, 7 d	254.16 ± 9.05	168.17 ± 9.23	171.59 ± 5.92	36.75 ± 1.62	36.38 ± 2.39	36.85 ± 3.05
	Colocasia esculenta	Tuber	2 μL, 5 d	461.847 ± 15.55	247.31 ± 15.31	117.34 ± 8.00	81.83 ± 6.00	40.46 ± 5.54	79.48 ± 3.91
	Alocasia macrorrhiza	Stem	200 μL, 7 d	2860.90 ± 65.15	2763.17 ± 30.46	2633.75 ± 35.94	1316.86 ± 17.97	1430.45 ± 32.58	1381.58 ± 15.23

Results showed that the JZL strains could not propagate in potato or cabbage tissues after inoculation for 12 h and 24 h, while EC1, MS2 and MS3 strains grew rapidly in potato in 12 h, and slowly in cabbage.

Hu *et al.*,2018

The current taxonomic position and nomenclature

The "herbicola" group

- The "herbicola" group of yellow pigmented strains which consists of epiphytic as well as plant pathogenic bacteria, has now been classified as *Pantoea* together with some species of the genus *Enterobacter*.
- The genus *Pantoea* forms a complex of more than 25 species, among which several cause diseases of several crop plants, including rice.

The current taxonomic position and nomenclature

The "herbicola" group

- The genus *Pantoea* described in 1989 was recently taxonomically classified as a member of the *Erwiniaceae* family.
- More than 25 species of this genus have been described and reported worldwide.
- Etymologically, the genus name *Pantoea* is derived from the Greek word 'Pantoios', which means "of all sorts or sources" and reflects the diverse geographical and ecological sources from which the bacteria have been isolated.
- Several species of the genus are qualified as versatile and ubiquitous bacteria because they have been isolated from many different ecological niches and hosts.
- Remarkably, some species have the ability to colonize and interact with members of both the plant and the animal Kingdom.

Kini *et al.*,2018

The current taxonomic position and nomenclature

The "herbicola" group

- The present genus *Pantoea* includes the phytopathogens:

1. *Pantoea allii*
2. *P. ananatis* subsp. *ananatis*
3. *P. ananatis* subsp. *uredovora*
4. *P. citrea* (now *Tatumella citrea*)
5. *P. stewartii* subsp.*stewartii*
6. *P. stewartii* subsp. *indologenes*
7. *P. agglomerans* pvs. *milletiae*, *gypsophilae* and *betae*.

Note: Other species in this genus represent non-pathogenic plant epiphytes and related soil bacteria, such as *P. agglomerans, P. punctata* and *P. terrea*. Transfer of *Pantoea citrea*, *Pantoea punctata* and *Pantoea terrea* to the genus *Tatumella* emend(Brady *et al.*,2009).

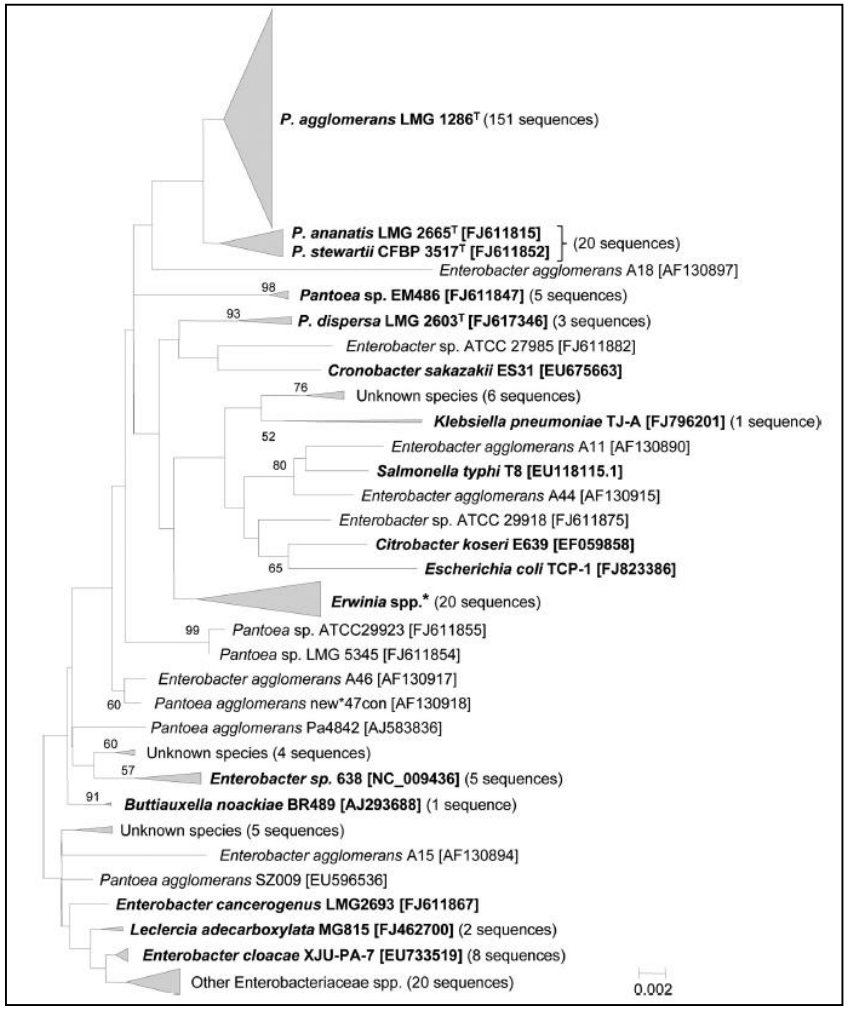
Diseases caused by *Pantoea* spp.

Some *Pantoea* spp. were reclassified in different genera

Pantoea agglomerans pv. *betae*	Root gall on table beet
Pantoea agglomerans pv. *gypsophilae*	Bacterial gall on Baby's breath (*Gypsophila paniculata*)
Pantoea agglomerans pv. *milletiae*	Gall formation on *Wisteria* species or *Milletia floribunda*
Pantoea allii	Centre rot of onion
Pantoea ananatis pv. *ananatis* (*ex. Erwinia ananas*)	Causes a range of diseases on a wide variety of agricultural crops, the most recent including rice, *Eucalyptus* (bacterial blight and die-back), maize(brown stalk rot, leaf spot) and sudan grass; Also Fruit let brown rot of pineapple.
Pantoea ananatis subsp. *uredovora*	A common plant pathogen of fruits and vegetables causing soft rot diseases; parasite on ustilago endospores
Pantoea stewartii subsp. *indologenes*	Leaf spot in foxtail and pearl millet and rot of pineapple
Pantoea stewartii subsp. *stewartii*	Stewart's bacterial wilt of sweet corn and maize
Pantoea citrea (now *Tatumella citrea*)	Associated with citrus fruit and causes pink disease of pineapple
Pantoea cypripedii (ex. *Pectobacterium cyrpripedii*)	Brown rot of cypripedium and other orchids
Pantoea dispersa	Associated with leaf spot of okra

Taxonomy of *P. agglomerans* isolates based on 16S rRNA gene sequences

- Taxonomy of putative *P. agglomerans* isolates based on 16S rRNA gene sequences retrieved from GenBank under the currently accepted species name or under the old basonyms *Enterobacter agglomerans* and *Erwinia herbicola*.
- Only bootstrap values greater than 50% are shown.
- The scale bar represents the number of base substitutions per site.



Rezzonico *et al.*,2010

Characteristics of:
The genus *Pantoea*

- Type species: *P. agglomerans*.
- Isolated from plant surfaces, seeds, soil, water, as well as animals (opportunistic human pathogen).
- *Pantoea* spp. share many characteristics with other members of the original genus *Erwinia*.
- They are Gram-negative, rod-shaped, facultatively anaerobic bacteria that are negative for nitrate reduction, oxidase negative and catalase positive.
- Most strains produce pili.
- Except for *P. stewartii* and *P. citrea*, they are motile by peritrichous flagella.
- Colonies of *P. citrea* and some strains of *P. agglomerans* are generally white (Schaad *et al.*,2001).
- Ice nucleation-active (INA) bacteria: *P. ananatis*, *P. agglomerans*, and *P. stewartii*.

Pantoea

Primarily plant pathogens

- The genus *Pantoea* currently includes seven species, the majority of which are either plant-pathogenic or plant-associated.
- The most notorious of the plant pathogenic species are:

1. *Pantoea stewartii* subsp. *stewartii*
2. *Pantoea stewartii* subsp. *indologenes*
3. *Pantoea agglomerans* - isolated from apical necrosis of walnut (Yang *et al.*,2011).
4. *Pantoea ananatis*
5. *Pantoea citrea*
6. *Pantoea cypripedii* (ex. *Pectobacterium cypripedii*)
7. *Pantoea allii*

- Although *Pantoea dispersa*, *Pantoea punctata* and *Pantoea terrea* have been isolated from the environment, they have not been found to be associated with diseases of plants.

Habitat and disease produced by members of the *Pantoea* group

Species	Disease name	Disease type	Natural host
P. agglomerans pv. agglomerans	Stem darkening	Secondary	Celery
P. agglomerans pv. betae	Root gall	Tumorigenesis	Table beets *Beta vulgaris* L.
P. agglomerans pv. gypsophilae	Bacterial gall	Tumorigenesis by indole-3-acetic acid production	Baby's breath *Gypsophila paniculata L.*
P. agglomerans pv. milliteae	Bacterial gall	Tumorigenesis	Japanese wisteria *Wisteria floribunda*
P. ananatis	Marbling	Soft rot	Pineapple, sugarcane, honey dew melon
P. citrea	Pink disease	Transluscent rot	Pineapple, mandarin orange
P. dispersa	Saprophyte	None	Edaphosphere resident, seeds
P. punctata	Brown spot	Delayed maceration	Mandarin orange
P. stewartii subsp. *indologenes*	Brown spot	Necrogenic	Graminae
P. stewartii subsp. *stewartii*	Stewart's wilt	Vascular wilt	Corn
P. terrea	Saprophyte	None	Soil inhabitant

Both *Pantoea agglomerans* pv. *betae* and *P. citrea* names are not found in Bull *et al.*,2010b comprehensive list. But four new *Pantoea* spp. namely *P. vagans*, *P. eucalypti*, *P. deley* and *P. anthophila* have been isolated from Eucalyptus (Brady *et al.*,2009).

The Prokaryotes (chapter 3.3.15),2006

Pantoea spp.
IAA production

- Fresh bacterial colonies were transferred in 10 ml of Nutrient Broth (NB) medium supplemented with L-tryptophan (3 g/l). Controls without L-tryptophan.
- Cultivation was performed in the dark at 30°C on a shaker (180 rpm) for 7 days.
- The bacterial cells were removed from the culture medium by centrifugation (8,000 rpm, 10 minutes).
- 200 µl of the supernatant was added to an ELISA plate, then 400 µl of the Salkowski reagent (49 ml of 35% perchloric acid +1 ml of 0.5 M $FeCl_3$) was added.
- The mixture was kept at room temperature for 35 minutes and then the absorbance at 490 nm was measured.
- The uninoculated tryptophan containing medium mixed with the Salkowski reagent was used in parallel as a blank.
- A standard curve was prepared from serial dilutions of IAA stock solution.
- The presented results are means of three independent cultivations.

Tsavkelova *et al.*, 2007; Bric *et al.*, 1991; Yu *et al.*, 2016

Pantoea spp.
IAA production


nylon 6,6 membrane

- Estimation of IAA was done by inoculation of 200 µl of bacterial suspension ($3x10^7$ cells ml^{-1}) in 10 ml Luria-Bertani amended with L-tryptophan (100 µg ml^{-1}).
- Each inoculated plate was overlaid with nylon 6,6 membrane (very resistant to mechanical, chemical and thermal stress).
- Plates are incubated until colonies reached 0.5 to 2mm in diameter.
- After an appropriate incubation period(46 h) the membrane was treated with salkowaski reagent (49 ml of 35% perchloric acid +1 ml of 0.5 M $FeCl_3$).
- Reaction was allowed to proceed until characteristic red halo colour within the membrane is developed.

Dastager *et al.*,2009;Cimmino *et al.*,2006; Sahasrabudhe,2011

Pantoea spp.
IAA production

- All regent incubations were carried out at room temperature.
- Bacteria producing IAA were identified by the formation of a characteristic red halo within the membrane immediately surrounding the colony.
- Known concentrations of IAA were also used to check the extent of red halo formed and also for the comparison for their ability to produce IAA.
- The IAA content in the culture suspension was estimated by the standard procedure (Gordon and Weber, 1951).

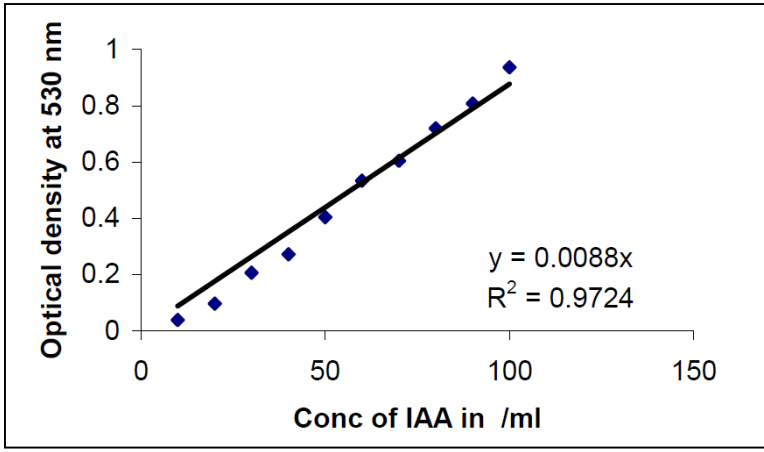
Screening on Nylon 6'6'membrane.

Dastager *et al.*,2009;Cimmino *et al.*, 2006; Sahasrabudhe,2011

Pantoea spp.

Preparation of standard graph of IAA

- Different IAA concentrations are prepared as aqueous solution of IAA ranging from 10 microgram/ml to 100 micrograms/ml.
- To each 1 ml of the standard, 2ml of 2% 0.5 M $FeCl_3$ in 35% perchloric acid i.e. Salkowaski reagent is added and readings are taken after 25 minutes at 530 nm by UV-Visible spectrophotometer SL Elico 159.
- Standard graph is prepared by plotting concentration of IAA in micrograms/ml Vs Optical Density at 530 nm.



Auxin quantification value was recorded by extrapolating calibration curve made by using IAA as standard (10-100µg/ml).

Sahasrabudhe,2011

Pantoea spp.
Confirmation of IAA by using TLC

- Selected 4 bacterial cultures were inoculated in YEMB amended with 5mM tryptophan. 1% inoculum of OD_{600} 1.0 was used for inoculation.
- The inoculated broth was incubated at 280C for 24 hrs. After 24 hrs of incubation, broth was centrifuged at 7000 rpm for 10 minutes.
- pH of broth brought to 3.0. 4:1 aliquots of liquid portion of centrifuged sample were extracted three times with ethyl acetate.
- The organic phase was concentrated to dryness and then diluted with 0.5 ml methanol.
- This solution along with the standard IAA was applied on silica gel G plate and TLC was run by using a solvent system chloroform: Ethyl acetate: Formic acid in 5:3:2 proportion and developed by using Salkowaski reagent.
- Red colour spots were developed. Rf value of the standard and IAA produced by the selected isolates was calculated.

Sahasrabudhe,2011

Bacterial gall formation
IAA and cytokinins production
Pantoea spp.

- Gall formation on *Gypsophila paniculata*
- Causal agents:

1. *Pantoea agglomerans* (ex. *Erwinia herbicola*) pv. *gypsophilae* (left labelled Ehg), and
2. *Pantoea agglomerans* (ex. *Erwinia herbicola*) pv. *betae* (right labelled Ehb):

- Illustrations kindly provided by I. Barash and S. Manulis.

Strange,2003

Stewart's wilt of corn

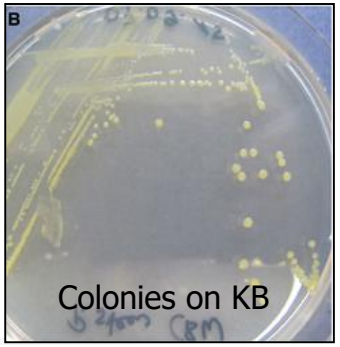
An endoglucanase is required for full virulence in sweet corn

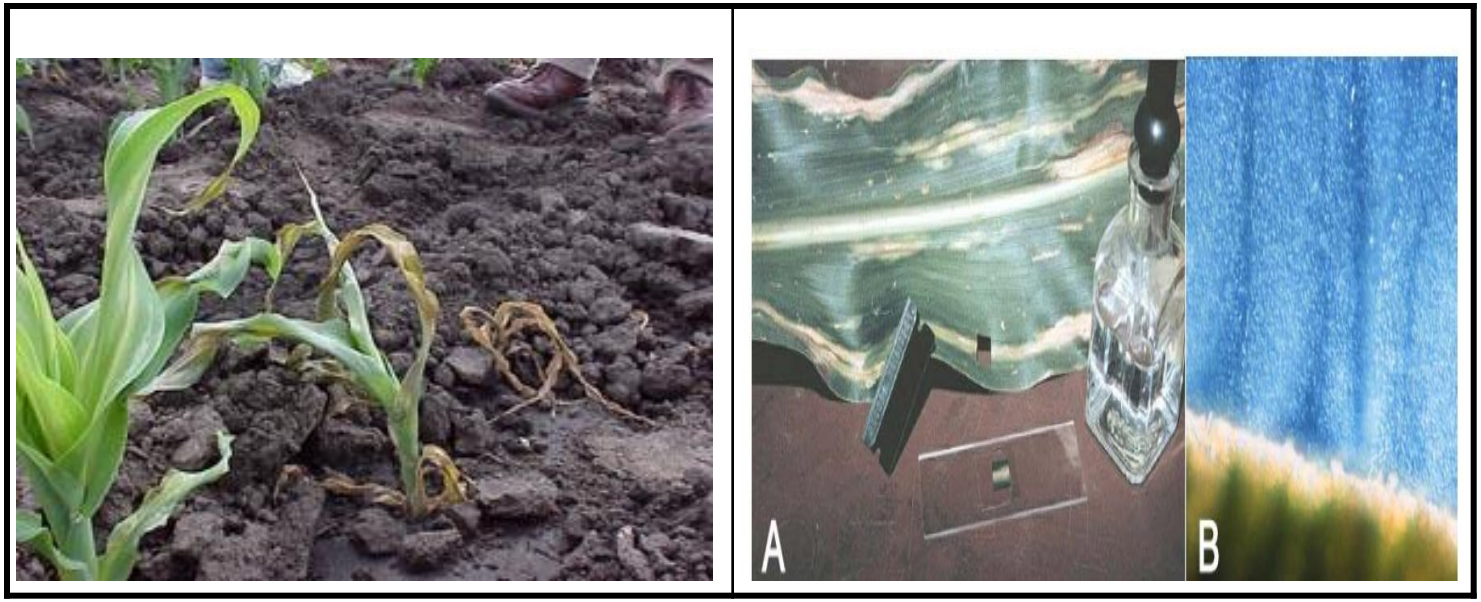
- *Pantoea stewartii* subsp. *stewartii* produces an Endoglucanase that is required for full virulence in sweet corn.
- The gene encodes an endoglucanase (EGase) was designated *engY*.
- EngY contributes to movement in the xylem and disease severity during the wilting phase of Stewart's wilt but is not required for water-soaked lesion formation.

Mohammadi *et al.*,2012

Stewart's wilt of corn

Bacterial symptom/streaming





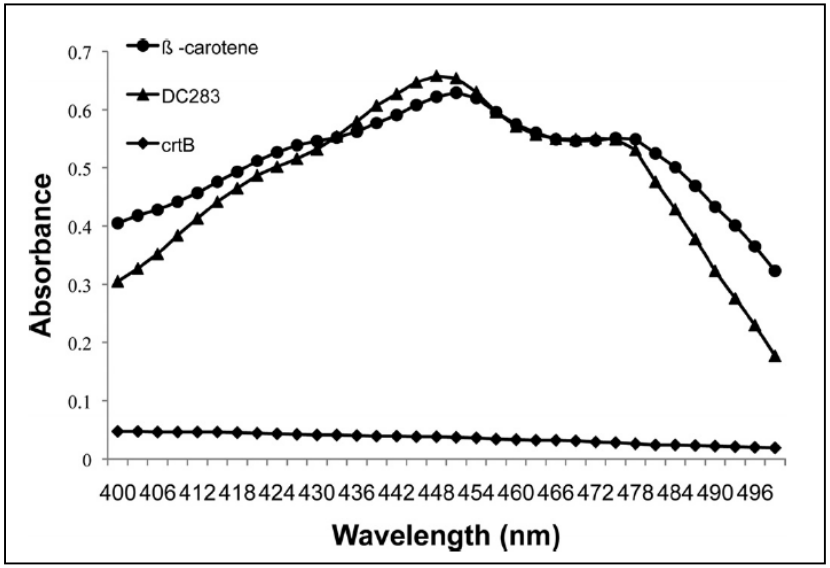
Strange,2003

Bacterial pigmentation

Carotenoids

Spectral analysis of the *P. stewartii* carotenoid pigment

- The methanole xtractable pigment from wild-type *P. stewartii* (DC283) has an absorption spectrum with a peak at 451 nm and a shoulder at 475 nm and with the maximum absorbance (max) at 450 nm, which was similar to that of the β-carotene standard.
- The *crtB*::*Mar2xT7* (*crtB*) mutant did not have detectable pigment in the methanol extract.



Mohammadi *et al.*,2012

Pink disease of pineapple

- Attempts at identifying the pathogen led to implicating several distinct bacteria as the causal agents of pink disease:

1. *Pantoea citrea* (now *Tatumella citrea*)
2. *Gluconobacter oxydans*
3. *Acetobacter aceti*
4. *Erwinia herbicola.*

Note: Some *Pantoea citrea* strains were reclassified as *Tatumella citrea* comb. nov. and others as *Tatumella morbirosei* sp. nov. The strains of both new species i.e. *Tatumella morbirosei* and *Tatumella ptyseos* are reported to cause pink disease in pineapple (Bull *et al.*,2012).

Kado,2003; Brady *et al.*,2010c

Pink disease of pineapple

Pantoea citrea (now *Tatumella citrea*)

	
Pink disease symptoms on pineapple fruit slices originating from a canned product. Healthy fruit (top), Diseased fruit (bottom).	**Example of an immature pineapple fruit bearing blossoms that are commonly visited by flying insects.**

Pink disease symptoms are difficult to observe in the field since outward symptoms are not apparent. Infections of the foliage are not usually found. Under severe invasion of the fruit by *P. citrea*, a translucent appearance of the sub-dermal fruit tissue occasionally can be observed.

Kado,2003

Pink disease of pineapple

Detection of gluconate dehydrogenase activity

2,5-diketogluconate production by *P. citrea*

- The disease is characterized by a distinct orange-brown color produced in fruit tissue after the heating process of canning.
- *Pantoea citrea* has enzymes that produce 2,5-diketogluconic acid, the compound responsible for the pigmentation of pineapple fruit affected by pink disease.
- Thus, discoloration appears to be due to production of 2,5-diketogluconate by *Pantoea citrea* (now *Tatumella citrea*).
- Only D-glucose in pineapple juice is oxidized into 2,5-diketogluconate via 2-ketogluconate dehydrogenase.
- No other sugar substrates lead to the formation of 2,5-diketogluconate.

Marin-Cevada *et al.*,2010;Kado,2003

Pink disease of pineapple

Conversion of glucose to 2,5-diketogluconate

Pantoea citrea (now *Tatumella citrea*)

- Pink disease color reaction in pineapple juice that promoted:

1. The growth of wild-type *P. citrea* (third tube from left) and
2. Mutant strains defective in their ability to convert glucose into 2,5-diketogluconate (tubes 4, 5, and 6 from left).

- Tubes 1 and 2 are:

1. Plain juice,
2. Juice containing heat-killed *P. citrea* as the inoculum.

orange-brown color produced in fruit tissue after the heating process of canning.

Kado,2003

Pink disease of pineapple

Detection of gluconate dehydrogenase activity

Assay for glucose dehydrogenase (GDB) activity in *Pantoea citrea*

- Bacteria are streaked on MGY agar(Mannitol L- 10g; glutamate,2g; K_2HP0_4 2g; $MgS0_4.7H_20$, 0.2g; Yeast extract 0.25g+20g glucose. Adjusted to pH 7.0 with 3 N NaOH).
- Colonies are overlaid with 4 ml of 0.5% agar containing a mixture of methylene blue (65 µg/ml) and eosin yellow (400 g/ml).
- Upon incubation for 1-5 min at 30°C, a purple halo around individual colonies indicates GDH activity due to the production of 2-gluconate from glucose.

Schaad *et al.*,2001

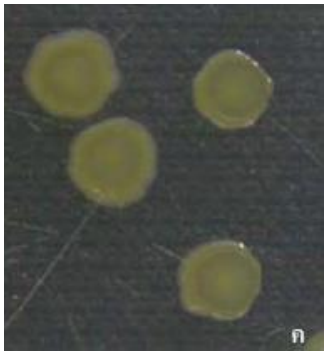
Pantoea

Differential characteristics from the other erwiniae

- The group is commonly differentiated from the other erwiniae by:

1. Production of a yellow pigment (*P. citrea* and a few *P. agglomerans* strains produce no yellow pigment);
2. They do not degrade pectate;
3. Do not require growth factors;
4. Do not produce urease, lysine decarboxylase or ornithine decarboxylase;
5. Do not produce H_2S on Triple Sugar Iron agar.

Colonies of *P. stewartii* subsp. *stewartii* LMG2715 on 3-day old NA medium. *P. stewartii* subsp. *stewartii* is a yellow, non-motile, non-sporing, Gram-negative rod. Colonies on nutrient-glucose agar are cream-yellow, lemon-yellow or orange-yellow and flat, raised or convex, respectively.



Comparative physiological and biochemical characteristics of *Pantoea* spp. versus *Erwinia* spp.

Characteristic	*Erwinia* spp.	*Pantoea* spp.	*Pectobacterium* spp.	*Brenneria* spp.	*Enterobacter* spp.
Gram-negative rods	+	+	+	+	+
Motile by peritrichous flagella	+	+/–	+	+	+
Facultative anaerobe	+	+	+	+	+
Colonies yellow or mauve	–	+	–	–	–
Percent genomic G+C content	49.8–54.1	55.1–60.6	50.5–56.1	50.1–56.1	52.0–54.0
Gluconate dehydrogenase	–	+	–	–	–
Cytochrome oxidase released	–	–	–	–	–
Catalase released	+	+	+	+	+
Indole production	–	+/–	–/+	–	–
Nitrate reduced to nitrite	+	+/–	+	–	+
H_2S produced from cysteine	+/–	–	+	+	–
Urease produced	–	–	–	–/+	–
Gas produced from glucose	–	–	+	–	+
Ornithine decarboxylase	–	–	–	–	+
Lipase	–	+	–	–	–
Acid from α-methylglucoside	+	–	–	–/+	–
Gelatin liquefaction	–/+	–	+	–	+
Acid from sorbitol	+	–	+	+	+
L-Malate utilized	+	+	+	+	+
DNase produced	–	+/–	–	–	–

Symbols: the +/– symbol indicates that a majority of species are positive; –/+ symbol indicates that a majority of species are negative.

The Prokaryotes (chapter 3.3.15),2006

Phenotypic characteristics of *Pantoea* species

Test[a]	*P. agglomerans*	*P. dispersa*	*P. citrea*	*P. punctata*	*P. terrea*	*P. ananas*	*P. stewartii* subsp. *stewartii*	*P. stewartii* subsp. *indologenes*
Yellow pigment	+	V	−	−	−	+	+	+
Motility	+	+	−	−	+	+	−	V
Growth, 41°C	−	+	−	−	−	V	−	V
Malonate	+	−	−	−	−	−	−	−
PDA	+	−	−	−	−	−	−	−
Indole	−	−	−	−	−	+	−	+
ONPG	+	+	+	−	−	+	+	+
Acid from								
Melibiose	−	V	+	+	+	+	+	+
Sorbitol	−	−	V	−	V	V	−	−
α-Methyl-D-mannoside	−	−	−	−	−	+	−	−

Symbols: see footnote in Table 4.
Abbreviations: PDA, phenylalanine deaminase; and ONPG, *o*-nitrophenyl-β-galactopyranoside.
[a]Reactions are at 30°C.
Adapted from Gavini et al. (1989), Kageyama et al. (1992) and Mergaert et al. (1993).

The Prokaryotes (chapter 3.3.1),2006

Phenotypic characteristics that distinguish *Pantoea allii* from its closest phylogenetic neighbours

- Species/subspecies:
- 1, *Pantoea allii*
- 2, *P. agglomerans* (n53);
- 3, *P. ananatis* (n54);
- 4, *P. anthophila* (n52);
- 5, *P. Brenneri* (n51);
- 6, *P. conspicua* (n51);
- 7, *P. deleyi* (n51);
- 8, *P. eucalypti* (n52);
- 9, *P. stewartii* subsp. *stewartii* (n51);
- 10, *P. stewartii* subsp. *indologenes* (n51);
- 11, *P. vagans* (n57).
- Data for reference taxa were taken from Brady *et al.*,2009 and Grimont & Grimont, 2005 (columns 2, 3, 9 and 10), Brady *et al.*,2009) (columns 4, 7, 8 and 11) and Brady *et al.*,2010b (columns 5 and 6).
- All data were generated under the same test conditions.
- +, 90–100% strains positive in 1–2 days;
- (+), 90–100% strains positive in 1–4 days;
- d, 11–89%strains positive in 1–4 days;
- (d), 11–89% strains positive in 3–4 days;
- -, negative.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Indole production (API 20E)	+	−	+	−	−	−	−	−	−	+	−
Acid from (API 50 CHB/E):											
Amygdalin	+	−	−	−	−	−	−	−	−	−	−
D-Fucose	−	d	d	−	+	−	+	d	−	+	+
Gentiobiose	+	+	d	−	−	−	+	−	−	+	−
Glycerol	+	(d)	+	+	d	−	+	d	−	d	d
Utilization of (Biotype 100):											
Adonitol	+	−	−	−	−	−	−	−	−	−	−
Gentiobiose	+	−	+	−	−	+	+	−	−	d	d
Quinate	+	−	(+)	d	−	−	−	−	−	+	−
L-Rhamnose	+	+	d	+	+	+	+	+	+	d	+
D-Sorbitol	+	−	d	−	−	−	−	−	−	−	−
L-Tartrate	−	−	−	−	d	+	+	+	−	−	+

Brady *et al.*,2011

Determinative characters of *Pantoea* spp.

Note: transfer of *Pantoea citrea* to the *atumella citrea*.

Test[a]	*P. agglomerans* (herbicola strains)	*P. ananas*	*P. citrea*	subsp. *stewartii*
Tobacco hypersensitivity	V[b]	-	+	+[b]
Yellow pigment	+	+	-	+
Taupe pigment on YDC	-	-	+	-
Growth at 37°C	+	+	+	-
H_2S from cysteine	+	V	+	-
Indole production	V	+	-	-
Nitrate reduction	+	V	+	-
Gelatin liquification	+	+	-	-
Production of 2,5 di-keto-D-gluconate	-	-	+	-
Motility	+	+	-	-
Utilization of:				
Citrate	+	+	-	-
Malonate	+	-	-	-
Tartrate	-	+	-	-
Acid production from[c]:				
Cellobiose	V	+	-	-
Glycerol	-	+	+	-
Lactose	V	+	+	-[d]
Maltose	+	+	+	-
Melibiose	-	+	+	+
Raffinose	V	+	-	+
Rhamnose	+	+	-	-
Salicin	V	+	-	-
Sucrose	+	+	-	+
meso-Inositol	-	+	-	-
Mannitol	+	+	-	+
Sorbitol	-	+	-	V
Arbutin	ND	ND	-	-

+, 80% or more strains positive; +[D], 80% or more strains delayed positive; V, between 21-79% of strains positive; -, 80% or more strains negative; ND, not determined.
Data from Cha et al. (2), Gavini et al. (7), Kageyama et al. (9), Mergaert et al. (12) and Wilson et al. (16).
[b]Saphrophytic strains of *P. agglomerans* do not give an HR on tobacco, but pvs. *gypsophilia* and *betae* do. Prior growth of *P. stewartii* and *P. agglomerans* pv. *gypsophilae* on IM medium is necessary.
[c]For acid production (see f, p. 47).
[d]*P. stewartii* subsp. *stewartii* cannot utilize lactose, but it is ß-galactosidase positive.

Schaad *et al.*,2001

Phenotypic characteristics of two *Pantoea* spp.

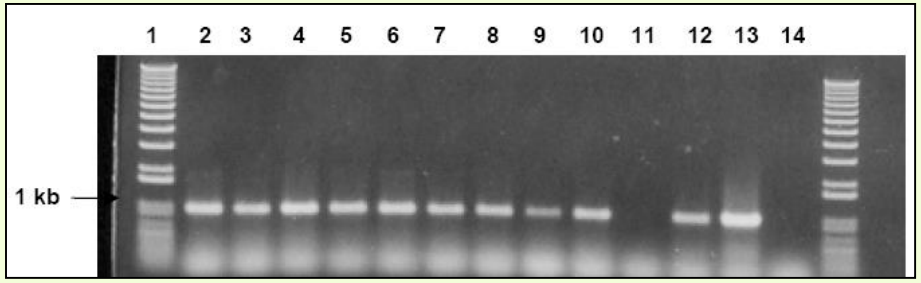
Morphological and biochemical tests for identification of *Pantoea stewartii* subsp. *stewartii* and distinction from similar species.

Tests	*Pantoea stewartii* subsp. *stewartii*	*Pantoea stewartii* subsp. *indologenes*	*Pantoea agglomerans*
Motility	Non-motile	Motile (or non-motile)	Motile
Gram staining	–	–	–
Kovac's oxidase test	–	–	–
Acetoin production	–	+	+
Indole production	–	+	–
Nitrate reduction	–	–	+
Aesculin hydrolysis	–	+	+
Growth on *cis*-aconitate	–	+	+
Acid production from			
Maltose	–	+	+
Arbutin	–	+	+
Salicin	–	+	+
Raffinose	+	+	–
Utilization of malonate	–	–	+

EPPO,2016

PCR analysis
Pantoea stewartii

- **PCR detection in a collection of *P. stewartii* strains of the abortive IS element preceding *hrpS*.**
- Primers ISR and SFBG flanking the 483-bp IS-like element in front of *hrpS* were used to amplify by "colony PCR" a ca. 1-kb product from a collection of *Pantoea stewartii* strains.
- Only strain SW4 tested negative and was later identified as *Pantoea ananas* (*Pantoea ananatis* pv. *ananatis*) by biochemical and nutritional assays.
- lane 1, 1-kb (+) molecular weight ladder;
- lane 2, *P. stewartii* strain SW2;
- lane 3, *P. stewartii* strain SW20;
- lane 4, *P.stewartii* strain DC102;
- lane 5, *P. stewartii* strain DC110;
- lane 6, *P. stewartii* strain DC119;
- lane 7, *P. stewartii* strain DC145;
- lane 8, *P. stewartii* strain DC146;
- lane 9, *P. stewartii* strain DC147;
- lane 10, *P. stewartii* strain DC162;
- lane 11, *P. ananas* strain SW4;
- lane 12, *P. stewartii* strain SW21; and
- lane 13, *P. stewartii* strain DC283.

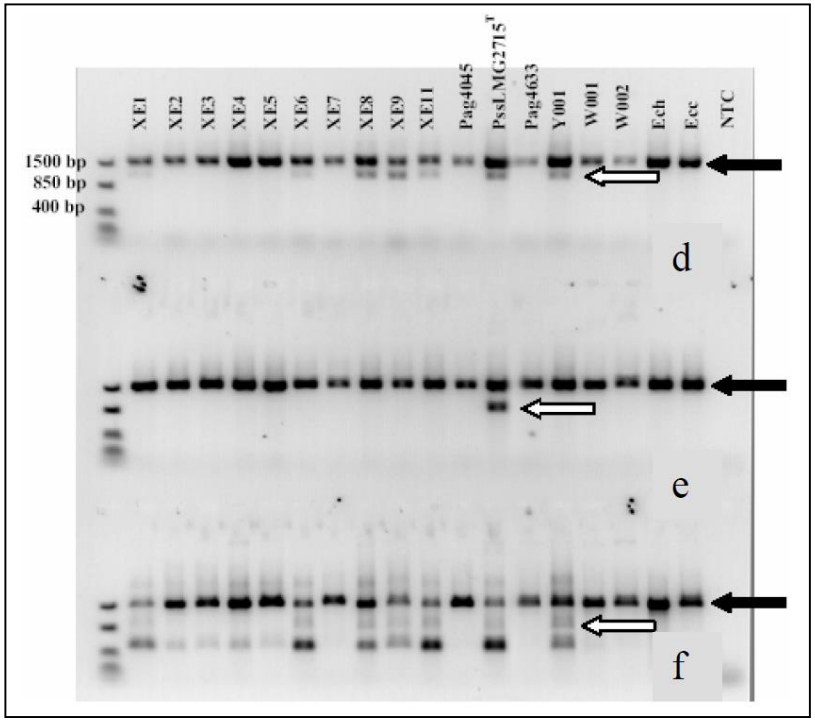


Merighi *et al.*,2003

PCR analysis

Pantoea stewartii

- DNA band of PCR product resulted from bacterial test, separated from maize kernel and *P. stewartii* subsp. *stewartii*.
- The product was synthesized by specify polymer per cpsDE gene of 1.1 kb
- (d) 0.9 kb of *hrpS* gene.
- (e) and 16s-23s rRNA/ITS of 0.9 kb size.
- (f) White arrow positions refer to genes' DNA bands, black arrow positions are DNA of genes of 16s rDNA of 1.5 kb used as control of PCR synthesis.



TAS 9031-2008; Juthathep, 2008

Sequences of oligonucleotide primer pairs used for PCR detection and identification of *Pantoea* spp.

Target	Name	Primer Sequence	Product size bp
P. stewartii 16S rRNA in ligase chain reaction	Es1 Es2 Es3 Es4	5'-GGCAGCGAACTTGGCAGAGATGCC-3' 5'-TTGGTGCCTTCGGGAACCGTGA-3' 5'-GCATCTCTGCCAAGTTCGCTGG-3' 5'-GGCACGGTTCCCGAAGGCACCAAG-3'	46
P. stewartii rRNA ITS	ESIR1 ESIR2	5'-CGAAGCGAGGACACACG-3' 5'-GCGCTTGCGTGTTATGAG-3'	290
	ESIR2 ES16	5'-GCGCTTGCGTGTTATGAG-3' 5'-GCGAACTTGGCAGAGAT-3'	920
P. agglomerans pv. *gypsophilae etzl*		5'-GCAAAAGAACGCGGCTGG-3' 5'-GGGTCTCTTGTTCCTGCC-3'	607
P. citrea glucose dehydrogenase B	*gdhB* Eco 1 *gdhB* Eco2	5'-GAAGCGAATTCCCCACTCGGAACATA-3' 5'-ATTCTGAATTCTGCCGCGAATCTATGG-3'	2703

Schaad *et al.*,2001

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

Genus *Pantoea*

Species/subspecies	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
P. agglomerans	**pagF/pagR** 16S rRNA gene	Conventional	Grape phylloxera (*Daktulosphaira vitifoliae*) (DNA extraction)	Vorwek *et al.*, 2007	
P. agglomerans pv. *gypsophilae*	**iaaH** Acetamine hydrolase gene **etZI** Cytokinin biosynthesis gene **etZII** Cytokinin biosynthesis gene	Conventional Nested	Bacteria (untreated), plant	Manulis *et al.*, 1998	*Erwinia herbicola* pv. *gypsophilae*
P. ananatis	**PanITS1/Gs4** ITS region	Immunomagnetic separation (IMS-PCR)	Bacteria (boiled), seed (IMS)	Walcott *et al.*, 2002	
P. stewartii subsp. *stewartii*	**16S-P5/16S-P3 (PCR)** 16S rRNA gene **Es1, Es2, Es3, Es4 (LCR)** 16S rRNA gene	PCR-coupled ligase reaction (LCR)	Bacteria, plant (DNA extraction and crude lysate)	Wilson *et al.*, 1994	*Erwinia stewartii*
P. stewartii subsp. *stewartii*	**ESIG1/ESIG2c** ITS region **ES16/ES1G2c** ITS region **HRP1d/HRP3r** *hrpS* region **CPSL1/CPSR2c** *cps*DE region	Conventional	Bacteria, plant (untreated)	Coplin and Majerczak, 2002	Faint bands obtained for *P. ananas* and *P. agglomerans* with ITS primers.
P. stewartii subsp. *stewartii*	**ES16/ES1G2c** 16S-23S rRNA/ITS region **HRP1d/HRP3r** *hrpS* ORF	Conventional	Bacteria (boiled or alkaline lysis)	Anon., 2006c	Rcommended in the EPPO protocol.
Pantoea agglomerans	**R 16-1/R 23-2R** 16S-23S rRNA/ITS region	Conventional	Bacteria (DNA extraction)	Kim and Song, 1996	*Erwinia herbicola* *Acidovorax avenae* (*Pseudomonas avenae*), *Burkholderia glumae* (*Pseudomonas glumae*), *Pseudomonas fuscovaginae*, *Pseudomonas syringae* pv. *syringae* and *Xanthomonas oryzae* (pathovars *oryzae* and *oryzicola*) also amplified and differentiated by primary and secondary fragments.

Palacio-Bielsa *et al.*,2009

Multiplex PCR
Total genomic DNA extraction

- Bacterial colonies were grown for 24 to 48 h on PSA plates containing 10 g peptone, 10 g sucrose, 16 g agar and 1 g glutamic acid per liter.
- Total genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega) according to the manufacturer's instructions.
- DNA quality and quantity were evaluated by agarose gel electrophoresis and spectrophotometry.

Kini *et al.*,2018

Multiplex PCR

A diagnostic multiplex PCR scheme for identification of plant-associated bacteria of the genus *Pantoea*

- Complete coding sequences of four housekeeping genes that have previously been used for multilocus sequence analyses (MLSA) of *Pantoea* species, *atpD*, *gyrB*, *infB*, and *rpoB*, were extracted and aligned.
- Sequence regions that were conserved in all strains of one species but were significantly different in the other two species were identified manually and chosen to design PCR primers.
- To allow multiplexing, we made sure that the amplicon sizes would be between 400 and 750 bp and different enough to be easily distinguishable from each other upon gel electrophoresis.

Kini *et al.*,2018

Multiplex PCR

List of PCR primers developed for the *Pantoea* mPCR along with the sequences of the GenBank accessions and the corresponding strains

Primer name	Target species	Sequence	Size (bp)	Strain
PANAG_infB_fwd	*P. agglomerans*	5'-GATGACGARGCCATGCTGC	730	*P. agglomerans*
PANAG_infB_rev		5'-TGTCCGGCGTGCCGGCTG		(CFBP 3615)
PANAN_gyrB_fwd	*P. ananatis*	5'-GATGACGARGCCATGCTGC	423	*P. ananatis*
PANAN_gyrB_rev		5'-GATCTTGCGGTATTCGCCAC		(ARC195)
PANST_rpoB_fwd	*P. stewartii*	5'-CACCGGTGAACTGATTATCG	539	*P. stewartii*
PANST_rpoB_rev		5'-GTCCTGAGGCATCAATGTGT		(ARC204)
PANsp_atpD_fwd	*Pantoea* sp.	5'-GAGGGTAACGACTTCTACCAC	330	*P. stewartii*
PANsp_atpD_rev		5'-CTGTACGGAGGTGATTGAAC		(ARC222)
				P. agglomerans
				(CFBP 3615)
				P. ananatis
				(ARC235)
16S_27F	Eubacteria	5'-AGAGTTTGATCMTGGCTCAG	920	Eubacteria
16S_907R		5'-CCGTCAATTCMTTTRAGTTT		

Kini *et al.*,2018

Multiplex PCR

Composition of the multiplex polymerase chain reaction

- The following amounts of bacteria or genomic DNA were used as templates for the PCR, corresponding to 10-fold serial dilutions: Lanes 1 to 12 10^6 CFU/mL, 10^5 CFU/mL, 10^4 CFU/mL, 10^3 CFU/mL, 10^2 CFU/mL, 10^1 CFU/mL, 10^0 CFU/mL, 10^{-1} CFU/mL, 10^{-2} CFU/ml, 10^{-3} CFU/ml, 10^{-4} CFU/mL and water; lanes 12 to 24, 50 ng, 5 ng, 0.5 ng, 50 pg, 5 pg, 0.5 pg, 50 fg, 5 fg, 0.5 fg, 50 ag, 5 ag and water; M, molecular size marker (1 kb DNA ladder, Promega).

PCR component	Volume per reaction (µL)		Final concentration
Type of template	Purified DNA	Bacterial cells	
Buffer (5x)	5.0	5.0	1x
dNTPs (2.5 mM each)	0.5	0.5	50 µM each
Oligonucleotides (10 µM)	0.4	0.4	0.16 µM each
Takara ExTaq™ (5 units/µL)	0.1	0.1	0.5 U
Template	2.0	5.0	
Sterile nanopure water (Promega)	13.4	10.4	
Total	25.0	25.0	

Kini *et al.*,2018

Multiplex PCR

Reaction parameters of the multiplex PCR thermocycler program

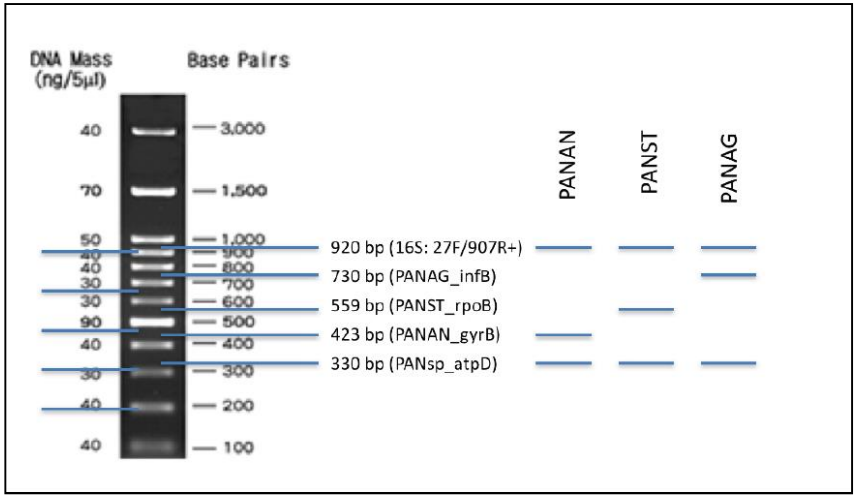
Step	Phase	Time	Temperature (°C)
1	Initial denaturation	3 min	94
2	Denaturation	30 sec	94
3	Annealing	30 sec	58
4	Extension	2 min	72
5	Cycling (steps 2-4)	30 cycles	
6	Final extension	10 min	72
7	Soak/hold	∞	4-10
8	End		

Kini *et al.*,2018

Multiplex PCR

Schematic representation of the multiplex PCR scheme

- Sizes of the five expected PCR amplicons are indicated in the middle and their expected migration in a 1.5% TBE agarose gel is shown on the left side.
- Diagnostic band patterns for the three plant-associated *Pantoea* species are shown on the right side.



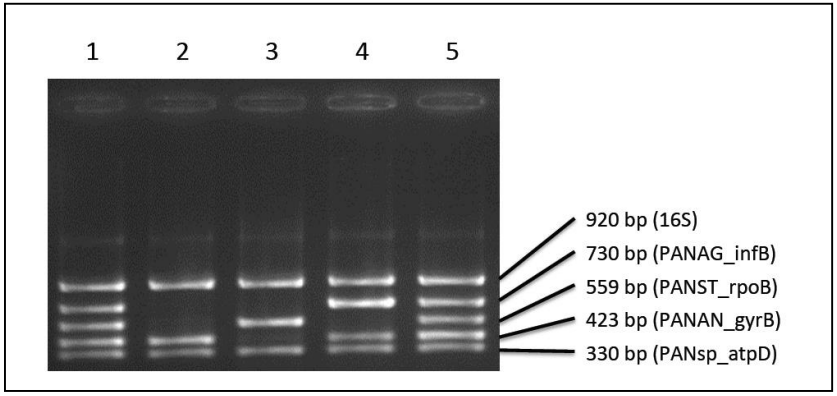
As a positive control for the PCR reaction, one primer pair was included that would amplify DNA from all bacteria belonging to the *Pantoea* genus, resulting in a smaller amplicon of less than 400 bp.

Kini *et al.*,2018

Multiplex PCR

A diagnostic multiplex PCR scheme for identification of plant-associated bacteria of the genus *Pantoea*

- Detection of three *Pantoea* species by multiplex PCR, using heated cell suspensions or genomic DNA as template.
- Three reference strains were used as representatives for the three *Pantoea* species, *P. ananatis* strain ARC60, *P. stewartii* strain ARC222, and *P. agglomerans* strain CFBP 3615.
- Lanes 1 & 5, pool of heated cells of the three *Pantoea* species; lane 2, *P. ananatis*; lane 3, *P. stewartii*; lane 4, pool of genomic DNA from *P. ananatis* and *P. agglomerans*.



Both genomic DNA and heat-inactivated bacteria were used. As a second control, a primer pair was included that targets the ribosomal 16S rRNA gene and leads to an amplicon that is larger than the four *Pantoea*-specific amplicons.

Kini *et al.*,2018

Pathogenicity test

Pantoea stewartii

- **HR test:**
- To test for HR elicitation on tobacco (*Nicotiana tabacum* L., cv. "White Burley") plants, *P. stewartii* strains were grown overnight in IM liquid medium, washed and resuspended in water at OD_{600}nm = 0.52 (ca. $1x10^9$ CFU ml^{-1}).
- Cells were infiltrated into tobacco leaves using four replicates per strain as previously described (Frederick *et al.*,2001) and necrosis was rated 24-48 h after infiltration.
- **Pathogenicity test:**
- The tests were performed by inoculating the whorls of 5-day-old sweet corn seedlings (*Zea mays* var*. saccharata*, cv. "Seneca Horizon") as previously described (Coplin *et al.*,1986).
- The plants were held in growth chambers at 29°C (photoperiod 16 h, 15000 lux, relative humidity 99%).
- After 3 days, disease severity was rated using a 0 to 3 scale:
- **(0= no symptoms, 1=scattered small lesions, 2= numerous lesions, and 3= extensive lesions that remained water-soaked with ooze forming on leaf surfaces).**

Merighi *et al.*,2003

In vitro pathogenicity tests

Pink disease assays

Pigmentation of juice and fruit slices

- The *in vitro* pathogenicity tests for pigmentation of pineapple juice and of fruit slices were performed as recommended by Coplin and Kado,2001 and Cha *et al.*,1997.

1. Fresh pineapple slices were heated for 10 min at 110°C to lower endophyte numbers and discard pink disease affected fruit, if present. Tested pineapple slices were then inoculated with approximately 10^6 CFU of *Tatumella ptyseos* (*ex. Pantoea citrea*) and incubated 48 h at 30°C.
2. Canned pineapple juice was inoculated with 10^6 bacterial cells and incubated for 72 h at 30°C.

- Thereafter, juice and slices were autocleaved for 5 min at 115°C.
- The production of a red or brownish pigment was considered indicative of pink disease.

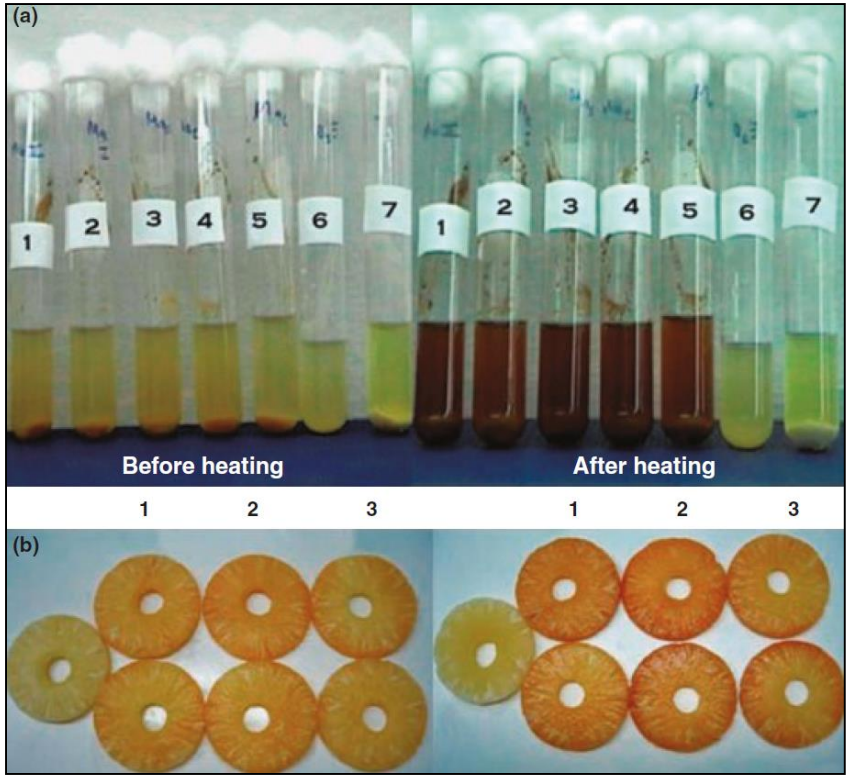
Marin-Cevada *et al.*,2010

In vitro pathogenicity tests

Pathogenicity test showing the diagnostic pink condition of infected pineapple

(a) Pineapple juice;(b) slices

- Numbers identify the inoculation isolates:
- 1, *Pantoea citrea* JCM 8882;
- 2, *Tatumella ptyseos* LMG 7888;
- 3, UAPS07001;
- 4, UAPS07002;
- 5, UAPS07007;
- 6, *P. agglomerans* JCM 1236;
- 7, UAPS07S008;
- 8, UAPS07005



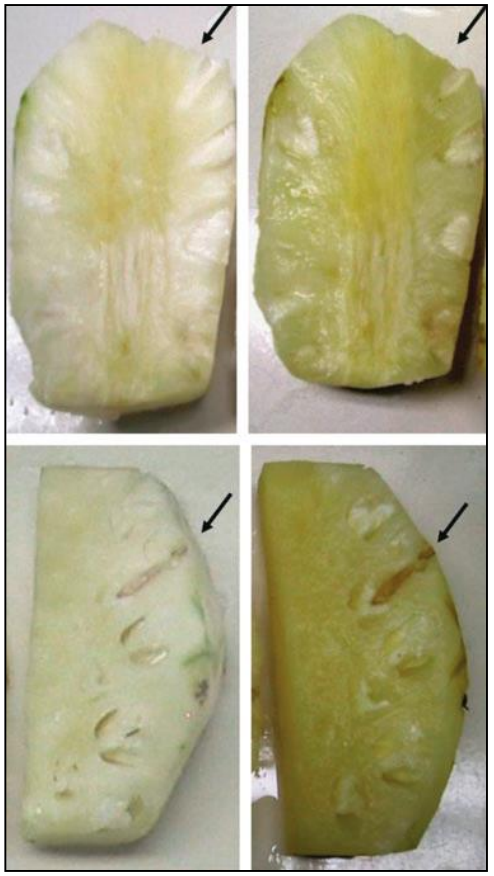
Marin-Cevada *et al.*,2010

In vitro pathogenicity tests

Pineapple fruit

Tatumella citrea (*ex. Pantoea citrea*)

- Washed bacterial cells re-suspended in 1% sucrose (ca. 10^6 CFU) were injected into fresh fruit and incubated for 7 days.
- Fruits were visually tested for deterioration of tissues and changes in pigmentation, before and after being autoclaved.
- Panels (a) and(b), controls infiltrated with 1% sucrose;
- Panels (c) and (d), strain UAPS07001;
- Panels (a) and (c), before autoclaving;
- Panels (b) and (d), after autoclaving.
- Arrows indicate inoculation site.



Marin-Cevada *et al.*,2010

Preservation

Maintenance and storage of bacterial cultures

- The working strains of *Pectobacterium* were maintained in sterile distilled water at room temperature in Eppendorf tubes;
- Some of the strains were maintained on YDC agar slants in test tubes and stored at 4 °C.
- For long time storage the strains were preserved as lyophilized.

Ahmad,2001

Preservation
Lyophilization

- *Erwinia* and its related genera can be stored indefinitely in the lyophilized state.
- Cells in exponential phase of growth are harvested by centrifugation and resuspended in 1% sterile solution of powdered milk (Carnation brand).
- An appropriate aliquot of the mixture is placed in an ampoule and is quickly frozen in liquid nitrogen or in ethanol containing chunks of solid CO_2.
- The frozen mixture is lyophilized under vacuum until it is completely desiccated.
- The ampoule is sealed in vacuum with a torch, appropriately labelled, and stored in a cold room at temperatures between 4 and 16°C.

Preservation

Freezing medium/glass beads

Dickeya and *Pectobacterium* spp.

1. Inoculate 10 ml of nutrient broth (NB) or Luria broth (LB) in a stoppered 150 ml conical flask with a loopful taken from a single colony of a pure fresh (<72 h) culture growing on NA or LBA and incubate in a shaking water bath at 27ºC for 18 h.
2. Mix 1 ml of the broth culture with 1 ml of a freezing medium, prepare 0.1 ml aliquots in 0.5 ml microcentrifuge tubes, flash freeze by dipping in liquid nitrogen for 2-3 min and store at -80°C.
3. Alternatively, coat small glass beads by dipping in the mixture of bacterial culture and freezing medium, drain, freeze and store at -80°C.

Pérombelon & van der Wolf,2002

Preservation

Freezing medium/glass beads

Dickeya and *Pectobacterium* spp.

4. When fresh cultures are required, thaw the frozen culture in the microcentrifuge tubes slowly and streak on to NA or LBA plates, or scatter some beads on to the plates, or streak-plate a loopful of bacterial suspension in water on to the plates.

- Incubate at 27°C for 48 h.
- **Glass beads:**
- ca. 1 mm diameter glass beads (any source); sterilize at 120°C for 20 min before use.

Pérombelon & van der Wolf,2002

Preservation

Freezing medium/glass beads

Dickeya and *Pectobacterium* spp.

- **Freezing medium**
- K_2HPO_4 12.6 g
- KH_2PO_4 3.6 g
- Sodium citrate 0.9 g
- $MgSO_4.7H_2O$ 0.18 g
- $(NH_4)_2SO_4$ 0.18 g
- Glycerol 88 g
- Water to 1000 ml

Pérombelon & van der Wolf,2002

Preservation

Dickeya and *Pectobacterium* spp.

- Evaluation of different preservation methods for maintaining the viability of soft rot ex. *Erwinia* spp. stored at room temperature.

Preservation method	Viability after 3-4 weeks			Viability after 6 months			Viability after 12 months			Viability after 16 months		
	Eca	*Ecc*	*Ech*	*Eca*	*Ecc*	*Ech*	*Eca*	*Ecc*	*Ech*	*Eca*	*Ecc*	*Ech*
Slants on YDC	-	-	-	-	-	-	-	-	-	-	-	-
Sterile water	++	++	++	++	++	++	++	++	++	++	++	++

++ = all cultures were viable

- = all cultures tested were dead

Mamdoh Ewis Esmael Ahmed,2001

Preservation

Dickeya and *Pectobacterium* spp.

- Evaluation of different preservation methods for maintaining the viability of *Erwinia* spp. stored at 4°C.

Preservation method	Viability after 3-4 weeks			Viability after 6 months			Viability after 12 months			Viability after 16 months		
	Eca	*Ecc*	*Ech*	*Eca*	*Ecc*	*Ech*	*Eca*	*Ecc*	*Ech*	*Eca*	*Ecc*	*Ech*
Slants on YDC	++	++	-	-	++	-	-	-	-	-	-	-
Sterile water	++	++	++	++	++	++	++	++	++	++	++	++

++ = all cultures were viable

- = all cultures tested were dead

Mamdoh Ewis Esmael Ahmed,2001

Identification of the bacterial pathogens *Pseudomonas*

Disease diagnosis and pathogen diagnostics

Domain: Bacteria
Phylum: Proteobacteria

- Class Gammaproteobacteria
  - Order *Pseudomonadales*
    - Family *Pseudomonadaceae*
      - Genus *Pseudomonas*
  - Order *Xanthomonadales*
    - Family *Xanthomonadaceae*
      - Genera *Xanthomonas*, *Xylella*

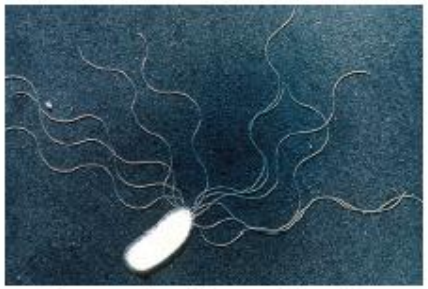
Phytopathogenic Pseudomonas sp.

Fluorescent

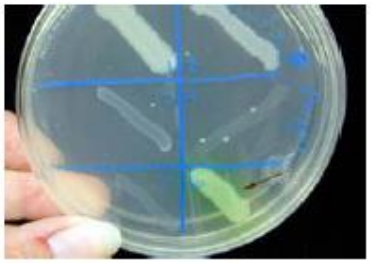
-RNA Grp I
-PHB (-)

Examples: *marginalis*
syringae

P. syringae pv. tomato
bacterial speck



P. marginalis, soft rotter



fluorescent bacterial growth
biology.uwsp.edu

Non-Fluorescent

- RNA Grp II
- PHB (+)

Examples: *cepacia, gladioli*

Now is *Burkholderia cepacia*
in Burkholderiaceae

Sour skin on onion, www.apsnet.org

http://www.apsnet.org/education/feature/BurkholderiaCepacia/
" Friend or Foe?"

General characteristics of the genus *Pseudomonas*

- Gram-negative,
- Rod-shaped, 0.5-0.8 μm x 1-3 μm.
- Strictly aerobic; the only anaerobic activities may be denitrification and arginine degradation to ornithine.
- Motile by polar flagella; some strains also produce lateral flagella.
- Oxidative, chemoorganotrophic metabolism.
- Catalase-positive, usually oxidase-positive.
- No organic growth factors are required.
- Diffusible and/or insoluble pigments may be produced.
- GC content of the DNA: 58-68 mol%.

Todar,2004

Pseudomonads
Fluorescent and nonfluorescent groups

- Phytopathogenic, fuorescent pseudomonads are clustered into the gamma-subclass of the *Proteobacteria* (Woese, 1987).
- They are divided into two main groups:

1. The oxidase-negative pseudomonads, which include:

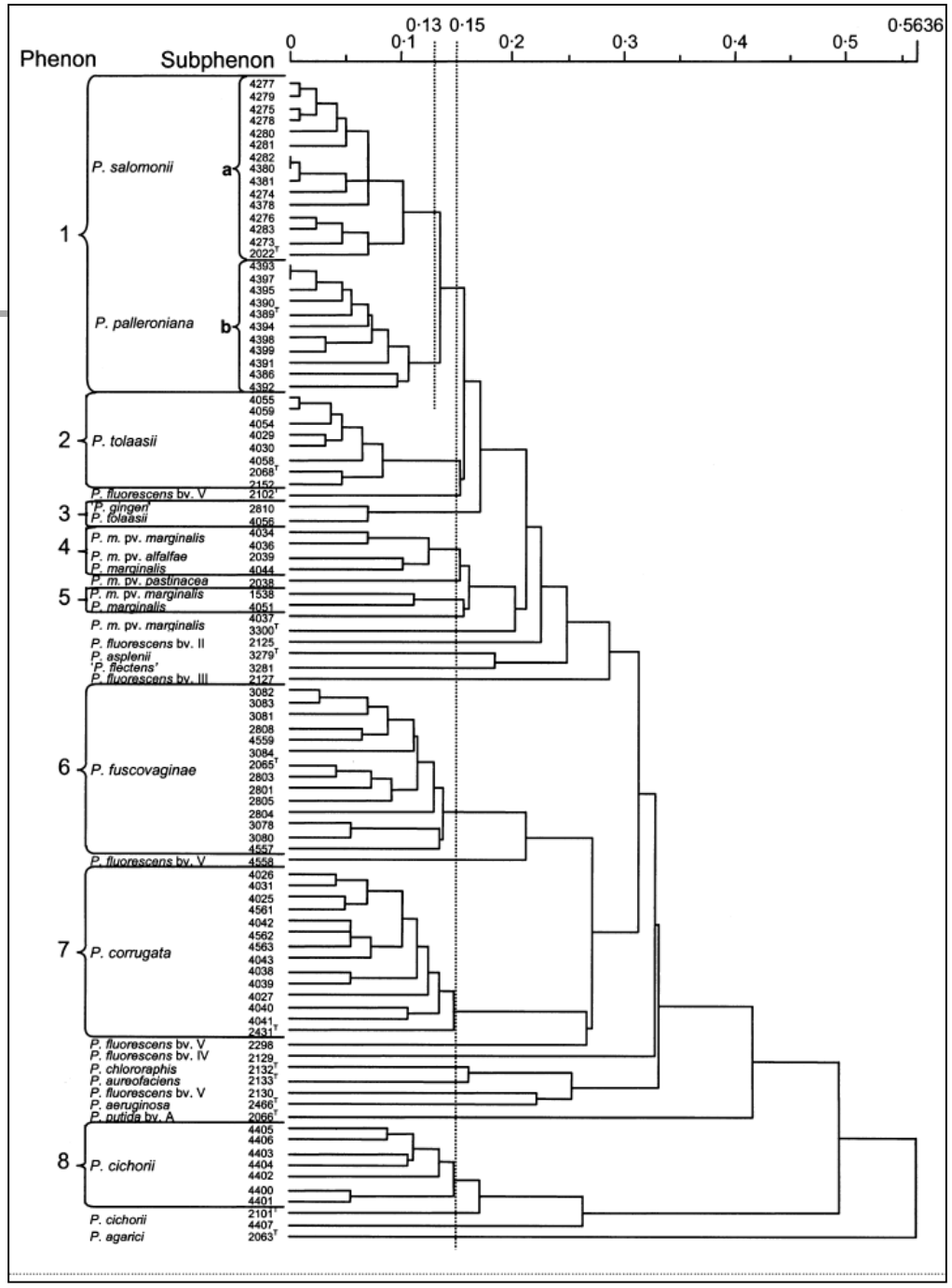
- *Pseudomonas syringae* pathovars and related bacteria, and

2. The oxidase-positive pseudomonads, including:

- *Pseudomonas agarici*,
- *Pseudomonas cichorii*,
- *Pseudomonas corrugata*,
- *Pseudomonas fuscovaginae*,
- *Pseudomonas tolaasii*,
- *Pseudomonas asplenii*,
- *Pseudomonas marginalis*.

Gardan *et al.*,2002

- Dendrogram of phenotypic distances of the Phytopathogenic fluorescent pseudomonads under study.
- Strain accession numbers are from the CFBP.



Gardan *et al.*,2002

Pseudomonads
Fluorescent and nonfluorescent groups

- The species with phenotypic description were distributed into two groups according to their physiological characteristics:

1. Group I including the species that do not require growth factors, and
2. Group II for those requiring growth factors.

- Group I was divided into two subgroups depending on the:

1. Fluorescent pseudomonads (absence of poly-β-hydroxybutyrate and arginine dihydrolase). e.g. *P. syringae*
2. Nonfluorescent pseudomonads (presence of poly-β-hydroxybutyrate and arginine dihydrolase). e.g. *P. corrugata*

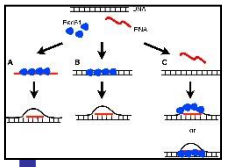
Peix *et al.*,2009;...

The genus *Pseudomonas*
Determination below genus level

- *Pesudomonas* comprises many different species including genomospecies still classified within *P. syringae*.
- Genus determination is largely based on 16S rDNA sequencing.
- Fatty acid analysis is also useful for genus determination.
- Species and many pathovars within them can be differentiated by:

1. Repetitive sequence PCR,
2. Traditional tests, and
3. Host tests.

Plant Bacteriology Manual,2008



Classification of pseudomonads

Based on rRNA and DNA homologies

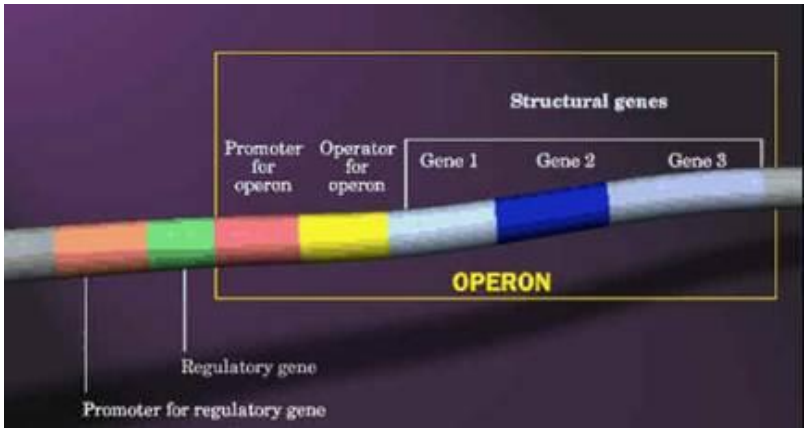
- According to Palleroni *et al.*,1973, Pseudomonads, including many plant pathogens, were split up into five rRNA homology groups (rRNA groups I to V), on the basis of rRNA-DNA hybridization.
- *P. syringae* pathovars corresponds to the rRNA group I.

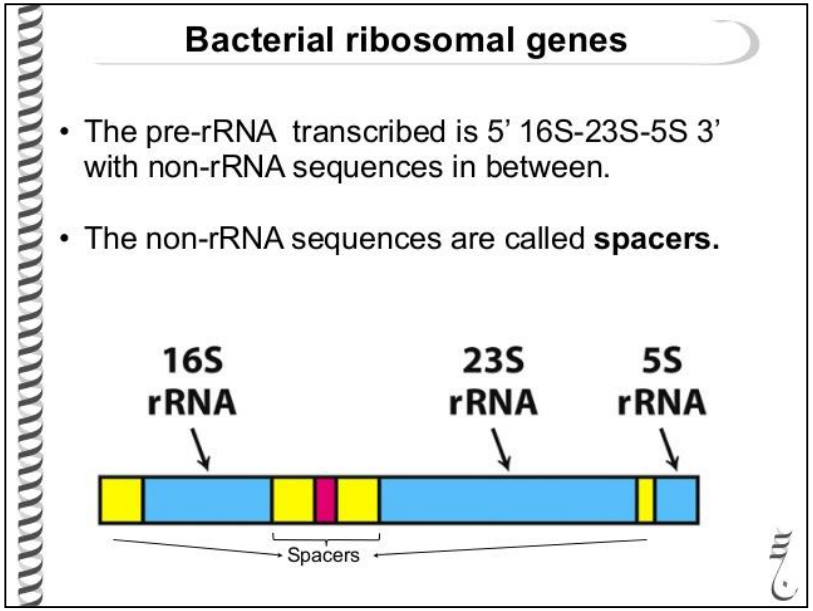
RNA similarity group	Constituent Species
I	*P. aeruginosa*, *P. fluorescens* (several biovars), *P. putida*, *P. chlororaphis*, *P. syringae* (many pathovars), *P. cichorii*, *P. stutzeri*, *P. mendocina*, *P. alcaligenes*, *P. pseudoalcaligenes*, *P. agarici*, *P. angulata*, *P. fragi*, *P. synxantha*, *P. taetrolens*, *P. mucidolens*, *P. oleovorans*, *P. resinovorans*
II	*P. cepacia*, *P. gladioli*, *P. caryophylli*, *P. pseudomallei*, *P. mallei*, *P. solanacearum*, *P. pickettii*, *P. pyrrocinia*, *P. andropogonis*
III	*P.* (*Comamonas*)*acidovorans*, *P.* (*Comamonas*) *testosteroni*, *P. saccharophila*, *P. facilis*, *P. delafieldii*, *P. alboprecipitans*, *P. palleronii*
IV	*P. diminuta*, *P. vesicularis*
V	*Xanthomonas* spp. including *X.* (*Pseudomonas*) *maltophilia*, *P. geniculata*, *P. gardneri*

See also Palleroni, Norberto J. (2010). "The Pseudomonas Story". Environmental Microbiology **12** (6): 1377-1383.

DNA Structure

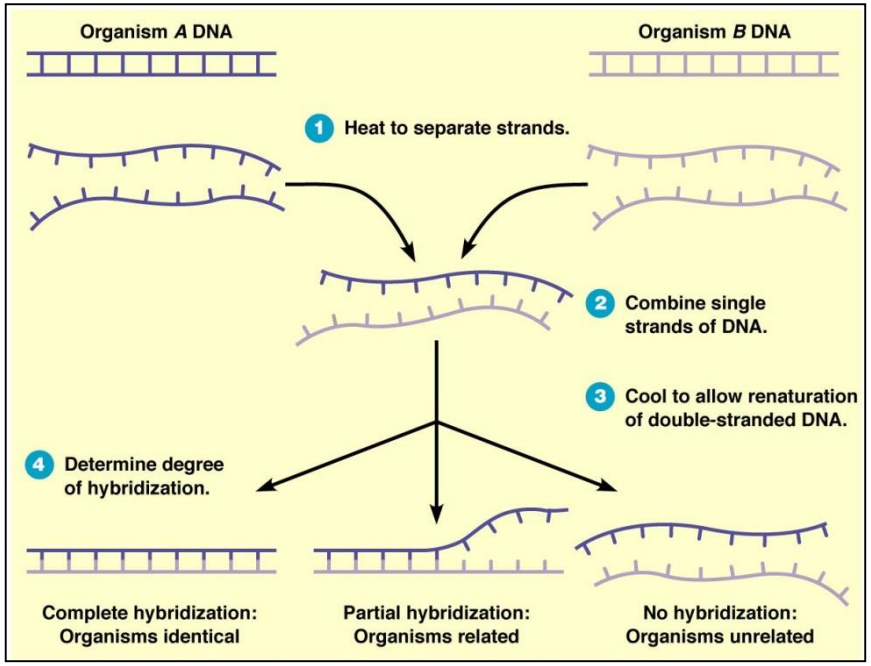
The rRNA-ITS Sequence





Nucleic acid hybridization
DNA/DNA hybridization

- In the 1970s: DNA-DNA hybridization was introduced.
- Isolates that showed >70% DNA homology were considered to belong to the same species.



Karlm,2004;..

Classification of fluorescent and non-fluorescent Pseudomonads

Based on rRNA and DNA homologies

The correlation plot of the two phylogenetic parameters DNA similarity and 16s rRNA homology

- *Pseudomonas* species, biovars and pathovars arranged according to:
  1. rRNA homology, and
  2. DNA homology.
- The RNA hybridization experiments demonstrate that the pseudomonads that we have studied fall into five RNA homology groups.
- The large (shaded) circles represent rRNA homology groups, within which DNA homologies groups are indicated by white circles. i.e. the species within each group have been differentiated on the basis of their DNA-DNA homologies.

Modified from Palleroni *et al.*,1973

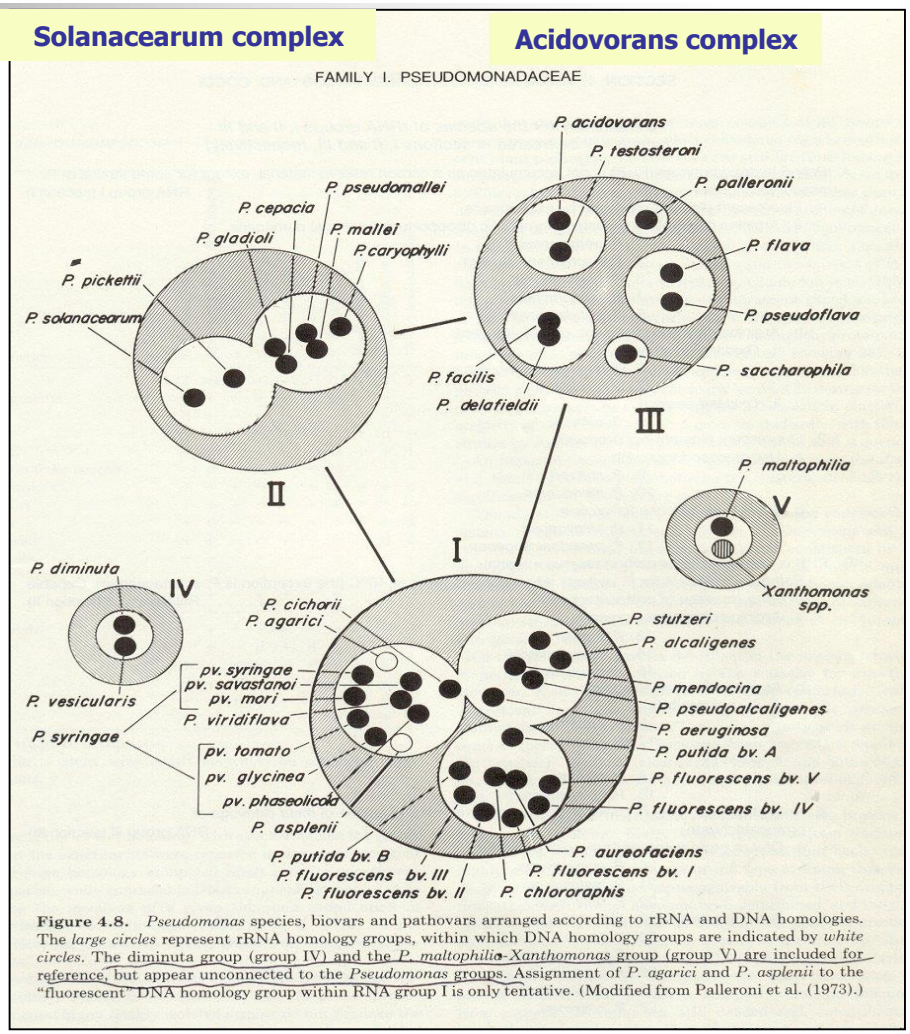
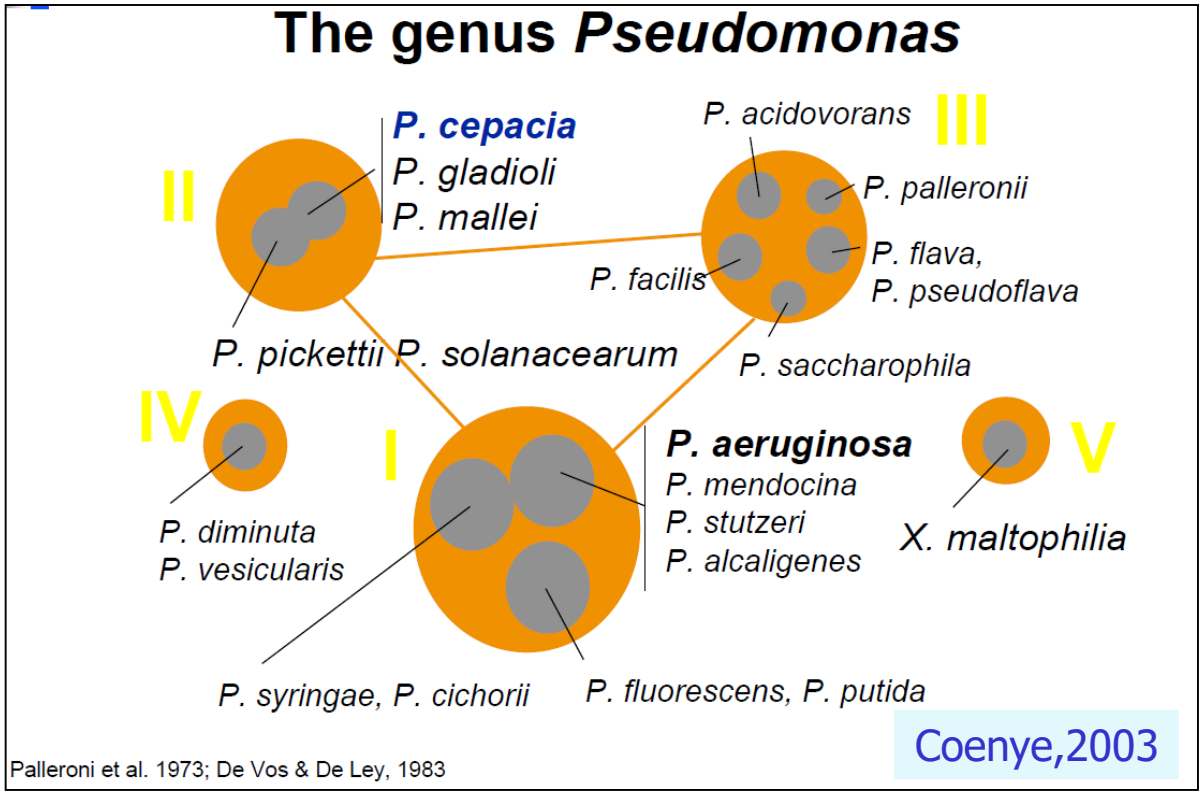


Figure 4.8. *Pseudomonas* species, biovars and pathovars arranged according to rRNA and DNA homologies. The *large circles* represent rRNA homology groups, within which DNA homology groups are indicated by *white circles*. The diminuta group (group IV) and the *P. maltophilia*-*Xanthomonas* group (group V) are included for reference, but appear unconnected to the *Pseudomonas* groups. Assignment of *P. agarici* and *P. asplenii* to the "fluorescent" DNA homology group within RNA group I is only tentative. (Modified from Palleroni et al. (1973).)

Characteristics used in polyphasic taxonomy



Pseudomonas solanacearum (now *Ralstonia solanacearum*), *Pseudomonas cepacia, gladioli* and *mallei*, transferred to *Burkholderia* and *Acidovorax*, previously designated as Pseudomonas rRNA homology group III.

The current taxonomic of *Pseudomonas*

- The true pseudomonads, including all of those producing fluorescent pigment, are contained in homology group I.
- The members of groups II, III, IV and V are moved into new or previously-existing genera.
- Most of those in homology group II to the genera *Burkholderia* and *Ralstonia*.
- Plant pathogens in homology group III are now assigned to the genus *Acidovorax*.

P. fragi is considered as the fluorescent subgroup of the rRNA group I, although *P. fragi* includes non-fluorescent strains. *P. fragi* is responsible for dairy spoilage and is grouped in *P. chlororaphis* group.

Classification of pseudomonads

Single locus sequence

Based on rRNA Gene Intergenic Spacer (IS) Sequences

- A potential target site for resolving intrageneric relationships of *Pseudomonas* may be found in the noncoding, intergenic spacer (IS) region between the 16S and 23S rRNA genes.
- This region is exposed to less evolutionary conservation than the rRNA genes.
- IS sequences from different strains of the same species did not exhibit sequence differences greater than 5% and, in most cases, were not more than 2% divergent.
- IS sequences of different species of the same genus exhibited significantly higher (25-20%) sequence dissimilarities.

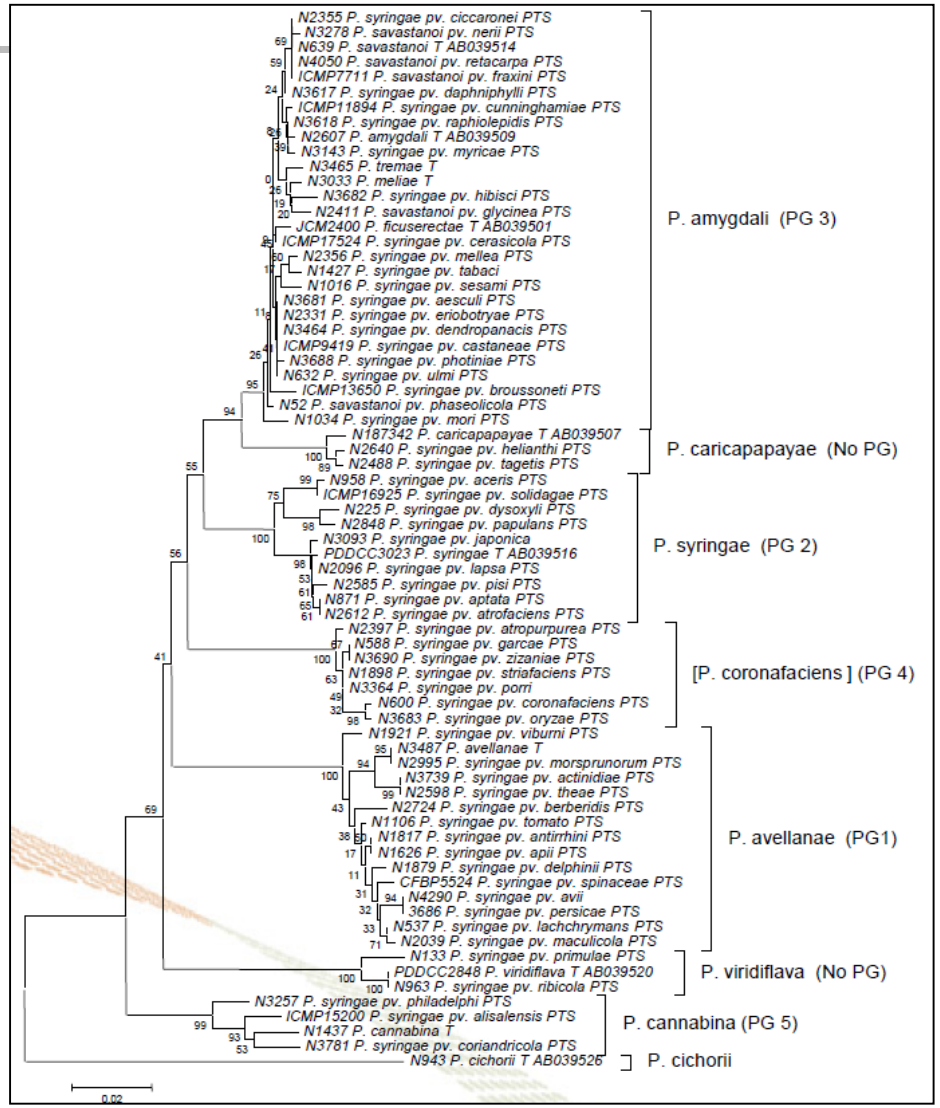
The Prokaryotes (chapter 3.3.23),2006

Classification of pseudomonads

Single locus sequence

Based on *rpoD* sequences

- Phylogenetic discrimination of *Pseudomonas syringae*-related pathogens.
- Reliability of use of a single locus.
- Hazelnut pathogens are found in Phylogroup 2 (two clades), and Phylogroup1 as single strain.
- Walnut pathogen is found as a single clade in phylogroup 2.
- Sweet chestnut pathogen in phylogroup 3.



Neil Parkinson

Classification of pseudomonads

Multilocus sequence typing

Based on multilocus sequence typing (MLST) analysis

- All procedures are the same as single locus sequences except in making the Master Mix instead of one primer pair (2 primers) it contains 5-7 primer pairs (10-14 primers).
- Although single locus sequences and DNA-DNA hybridization are informative and characterize the taxonomic structure of the bacteria, no clearer picture of the population structure of the species would be gained by focusing strictly on housekeeping genes.

Sarkar and Guttman,2004;…

Classification of pseudomonads

Based on multilocus sequence typing (MLST) analysis

- Housekeeping genes are components of the "core genome" and are less likely to undergo horizontal gene transfer.
- Housekeeping genes are particularly useful for:

1. clarifying clonal relationships among strains and
2. for assessing the importance of recombination in driving the evolution of clonal lineages.

Sarkar and Guttman,2004

DNA pools in the genomes of prokaryotes
Core and Flexible gene pool

- Recent comparative studies of bacterial genomes have found bacterial evolution to be a composite of forces acting on two largely independent yet intimately intertwined genomes:

1. The core and
2. Flexible genomes.

- The core genome consists of genes ubiquitously found among strains of a bacterial species.
- These genes typically encode proteins that are essential for the survival of the organism, such as housekeeping genes.

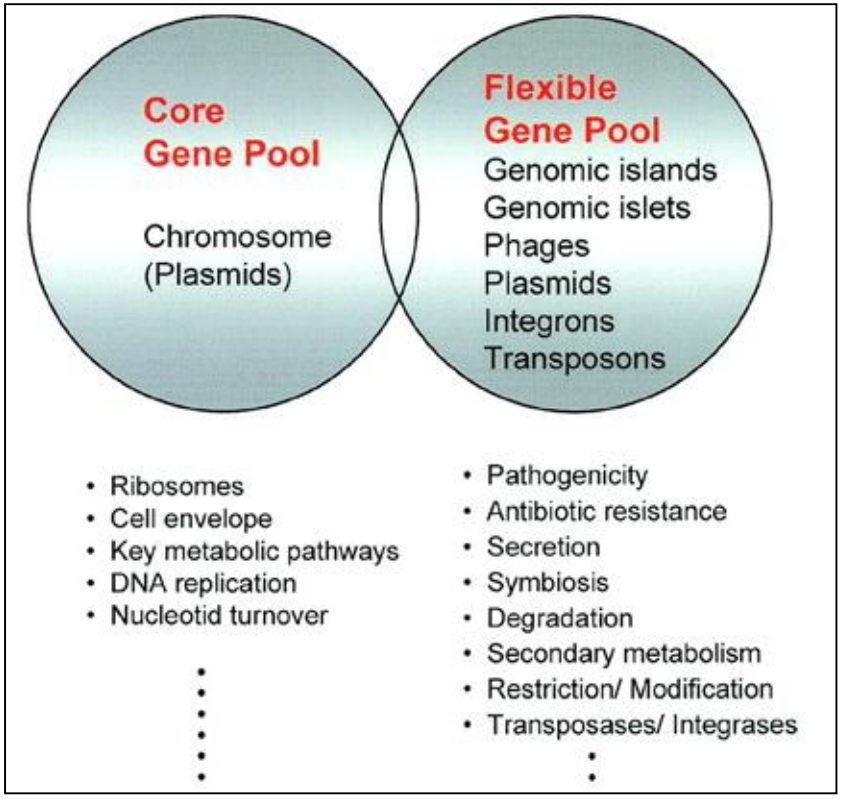
Sarkar and Guttman,2004

Prokaryotic genomes

DNA pools in the genomes of prokaryotes

Core and Flexible gene pools

- Proposed model of the DNA pools in the genomes of prokaryotes.
- Most of the horizontally transferred DNA is part of the "flexible" gene pool.
- Some functions encoded by the pools are given in the lower part of the diagram.
- Turnover metabolism is a dynamic process. The cell is continuously degrading and synthesizing molecules.



Model of the DNA pools in the genomes of prokaryotes. The DNA elements comprising the core as well as the flexible gene pools are presented in the circles. Functions encoded by the pools are given in the lower part of the diagram. Core genome: the genes shared by all members of a pre-defined group of bacteria or archaea. Flexible genome may also be referred to as the accessory, variable, dispensable, auxiliary, noncore, adaptive or distributed genome.

Hacker and Carniel, 2001; Scortichini,2005

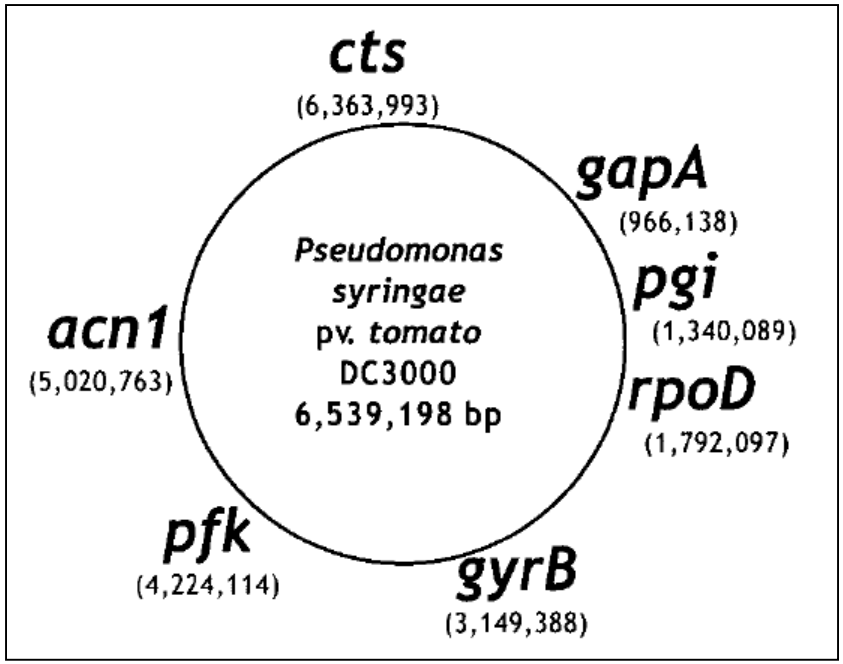
Core genome
Housekeeping (essential) genes

- Components of the core genome are generally less likely to undergo horizontal gene transfer, and they either evolve neutrally or are selectively constrained.
- The core genome can be thought of as the clonal backbone of the species, and its constituents can be used to track the evolutionary history of clonal lineages through time.
- Recent phylogenetic studies on the *P. syringae* complex based on 7/8 housekeeping gene.
- A housekeeping gene is a gene that codes for proteins needed all the time.
- Most investigators suggest that the presence of essential or housekeeping genes defines a chromosome.

Population structure and dynamics of the core genome of *P. syringae*

Based on multilocus sequence typing (MLST) analysis

- Schematic representation of the positions of the seven housekeeping genes used in this study, based on the sequenced *P. syringae* pv. *tomato* DC3000 genome (NCBI accession no. NC_004578).
- The position of each locus (in base pairs) is given below the gene name.



Sarkar and Guttman,2004

Core genome
Housekeeping (essential) genes

- Some of these genes are:

1. Ribosomal RNA genes e.g. 16S rRNA, metabolically essential genes (such as those encoding proteins involved in metabolism of amino acids),
2. Heat-shock proteins such as *groE*, *dnaK*, *gyrB* and *rpoD*.
3. *hrp* regulon appears to play a fundamental and important role in the overall life strategy of the bacterium. E.g. *hrpL* and *hrpS*

Flexible genome
Flexible or accessory genome

- Unlike the core genome, the flexible genome consists of genes that vary among strains within a species.
- These genes typically encode proteins that are responsible for adaptation to specific niches, hosts, or environments.
- The flexible genome may include:

1. Virulence-associated genes,
2. Resistance genes, and
3. Genes associated with mobile elements such as bacteriophage, plasmids, or transposons.

Sarkar and Guttman,2004

Flexible genome
Flexible or accessory genome

- By definition, the flexible genome evolves largely through horizontal genetic exchange (i.e., through gene acquisition and loss).
- Since horizontal transfer shuffles and effectively obscures evolutionary histories, the most reliable approach to characterizing bacterial diversity would focus strictly on the core genome.

Sarkar and Guttman,2004

Flexible genome

Flexible or accessory genome

- In *P. syringae*, as much as half of the genomic complement of this species should be considered to be part of the flexible genome.
- These genes typically encode proteins that are responsible for adaptation to specific niches and the many mobile elements that move in and out of genomes.
- The flexible genome largely evolves through horizontal genetic exchange. i.e. these are not transmitted vertically, but rather acquired and lost in a horizontal manner.

Genotype identification of bacteria

Molecular methods

MLST vs MLSA

- **Multilocus Sequence Typing (MLST):**
- MLST is usually applied to strains that belong to a well-defined species.
- **Multilocus Sequence Analysis (MLSA):**
- MLSA is more often used when species boundaries are not well known and MLSA data are used to improve species descriptions.

Classification of pseudomonads

Multiple loci typing(MLSA)

Strains are resolved to a high level of discrimination

- Sequence analysis using multiple loci (MLSA) has the advantage of providing greater phylogenetic information (evolutionary history is produced with greater certainty) compared to a single locus (benefits of single locus sequence analysis in diagnosis of pathogens).
- However, the use of MLSA in a diagnostic laboratory, at least as a first-level identification tool is costly, and increases the time needed to determine identification compared to a single locus analysis.

Parkinson and Elphinstone,2010

Classification of pseudomonads

Based on multilocus sequence typing (MLST) analysis

- A seven-locus analysis confirmed the reproducible recovery of five phylogroups (subgroups).
- Each phylogroup may contains many phylotype.
- In a study 53 phylotypes were grouped into 8 phylogroups.
- The procedure characterizes isolates of bacterial species using the DNA sequences of internal fragments of multiple (usually seven) housekeeping genes.
- Approximately 450-500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequencer.

Allele: One of a pair of genes at a single locus

Principle of MLST

Multilocus sequence typing (MLST) analysis

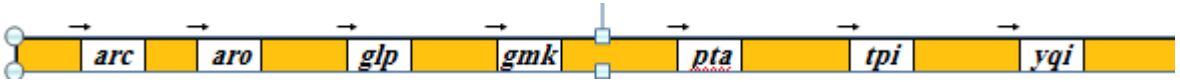
- MLST directly measures the DNA sequence variations in a set of housekeeping genes and characterizes strains by their unique allelic profiles.
- The principle of MLST is simple:
- The technique involves PCR amplification followed by DNA sequencing.
- Nucleotide differences between strains can be checked at a variable number of genes (generally seven) depending on the degree of discrimination desired.

Wikipedia,2011

Principle of MLST

Multilocus sequence typing (MLST) analysis

1. PCR: amplify fragments of house-keeping genes (7 x PCR reactions).



2. Sequencing: sequence both strands of PCR products.

- For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type (ST).

obtain 7-digit allelic profile → sequence type (ST)

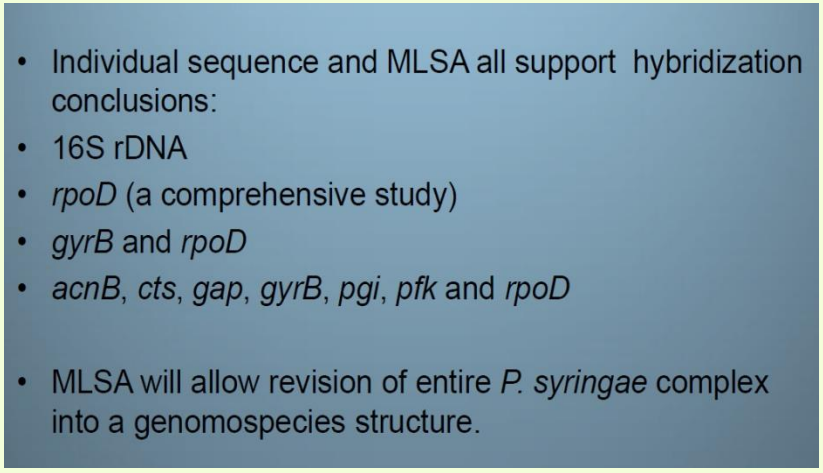
Allele: One of a pair of genes at a single locus

Classification of pseudomonads

Based on multilocus sequence typing (MLST) analysis

Sequence analysis

- Applying MLST technique on sequencing 6-8 selected housekeeping genes and identification of polymorphic nucleotide sites has been introduced for the characterization of *Pseudomonas* spp. Combination of the alleles at the different loci results in unique diploid sequence types that can be used to discriminate strains.



Young,2010;...

Classification of pseudomonads

Based on multilocus sequence typing (MLST) analysis

- We report here on the multilocus sequence analyses (MLST/MLSA) of the genus *Pseudomonas*.
- Four loci totaling 2046 bp of sequence were obtained from 367 Pseudomonads.
- The four housekeeping genes sequenced were:

1. *rpoD*, The "housekeeping" sigma factor, transcribes most genes in growing cells.
2. *gyrB*, encoding DNA gyrase B,
3. *gltA* (also known as *cts*), encoding citrate synthase,
4. *gapA*, encoding glyceraldehyde-3-phosphate dehydrogenase.

Guttman *et al.*,2008

The MLST primers

Used for DNA amplification and sequencing of the four loci

gapA, gltA, gyrB and ropD genes

Name	Primer sequence	Use[a]
gapA + 264	CCGGCSGARCTGCCSTGG	PCR
gapA-931	ASSCCCAYTCGTTGTCRTACCA	PCR
gapA + 312	TCGARTGCACSGGBCTSTTCACC	SEQ
gapA-874	GTGTGRTTGGCRTCGAARATCGA	SEQ
gltA + 174	GCCTCBTGCGAGTCGAAGATCACC	PCR only
gltA-1192	CTTGTAVGGRCYGGAGAGCATTTC	PCR
gltA + 513	CCTGRTBGCCAAGATGCCKAC	SEQ
gltA-1130	CGAAGATCACGGTGAACATGCTGG	SEQ
gyrB + 133	CTGCACCAYATGGTSTTCGAGG	PCR
gyrB-1124	CGNGCDGCRTCGAKCATCTTGC	PCR
gyrB + 271	TCBGCRGCVGARGTSATCATGAC	SEQ
gyrB-1022	TTGTCYTTGGTCTGSGAGCTGAA	SEQ
rpoD + 147	CAGGTGGAAGACATCATCCGCATG	PCR only
rpoD-1222	CCGATGTTGCCTTCCTGGATCAG	PCR
rpoD + 377	GRAATCGCCAARCGYATYGA	SEQ
rpoD-1078	CGGTTGATKTCCTTGATCTCGGC	SEQ

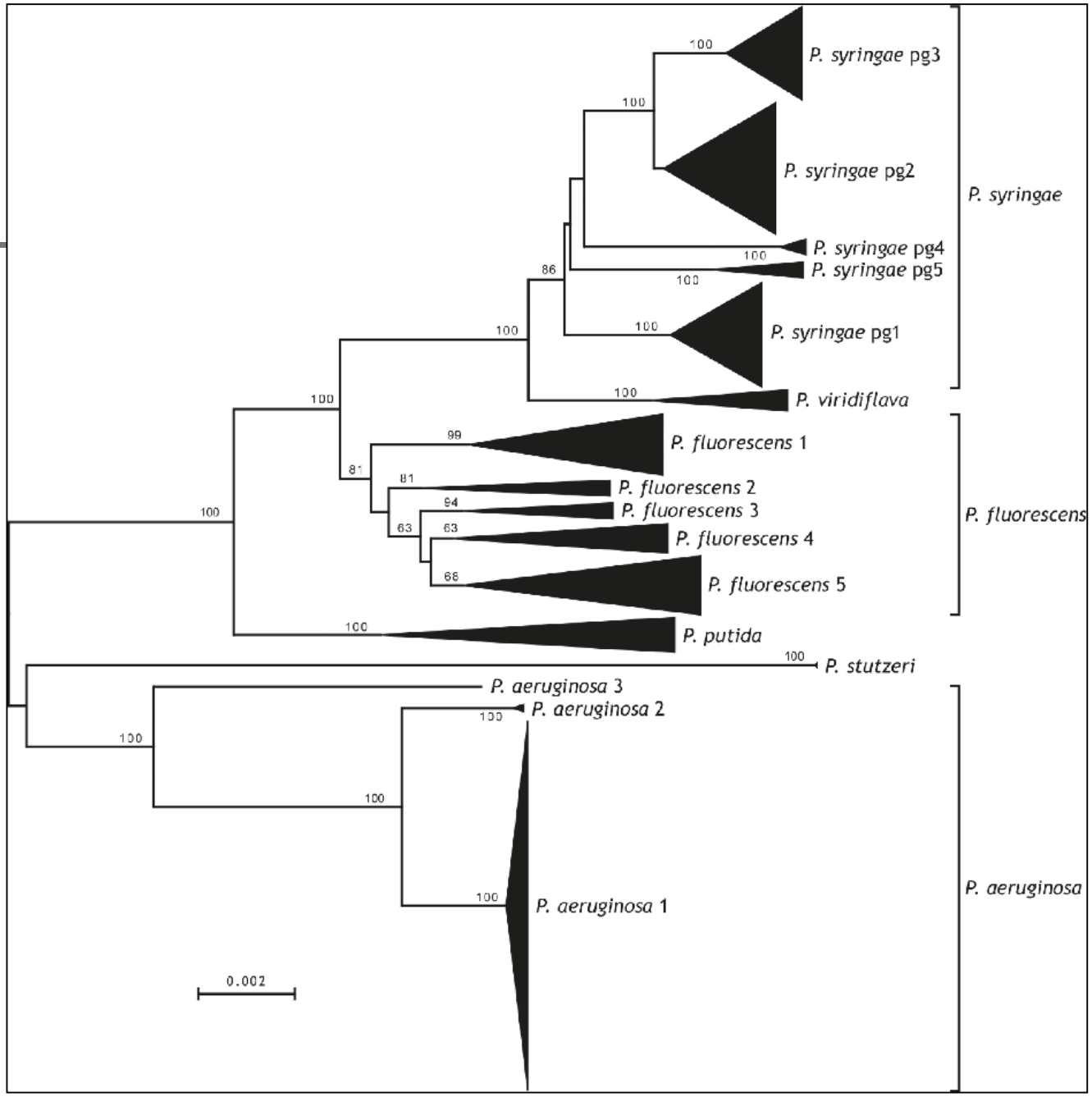
[a] Any primer labeled as 'PCR' can also be used for sequencing. Those labeled as 'PCR only' cannot be used for sequencing due to their distance from the region of interest

Guttman *et al.*,2008

Polyphasic approach

Based on Multilocus sequence typing(MLST) analysis

Pseudomonas



Guttman *et al.*,2008

Phylogenetic relationships between *P. syringae* pv. *actinidiae*

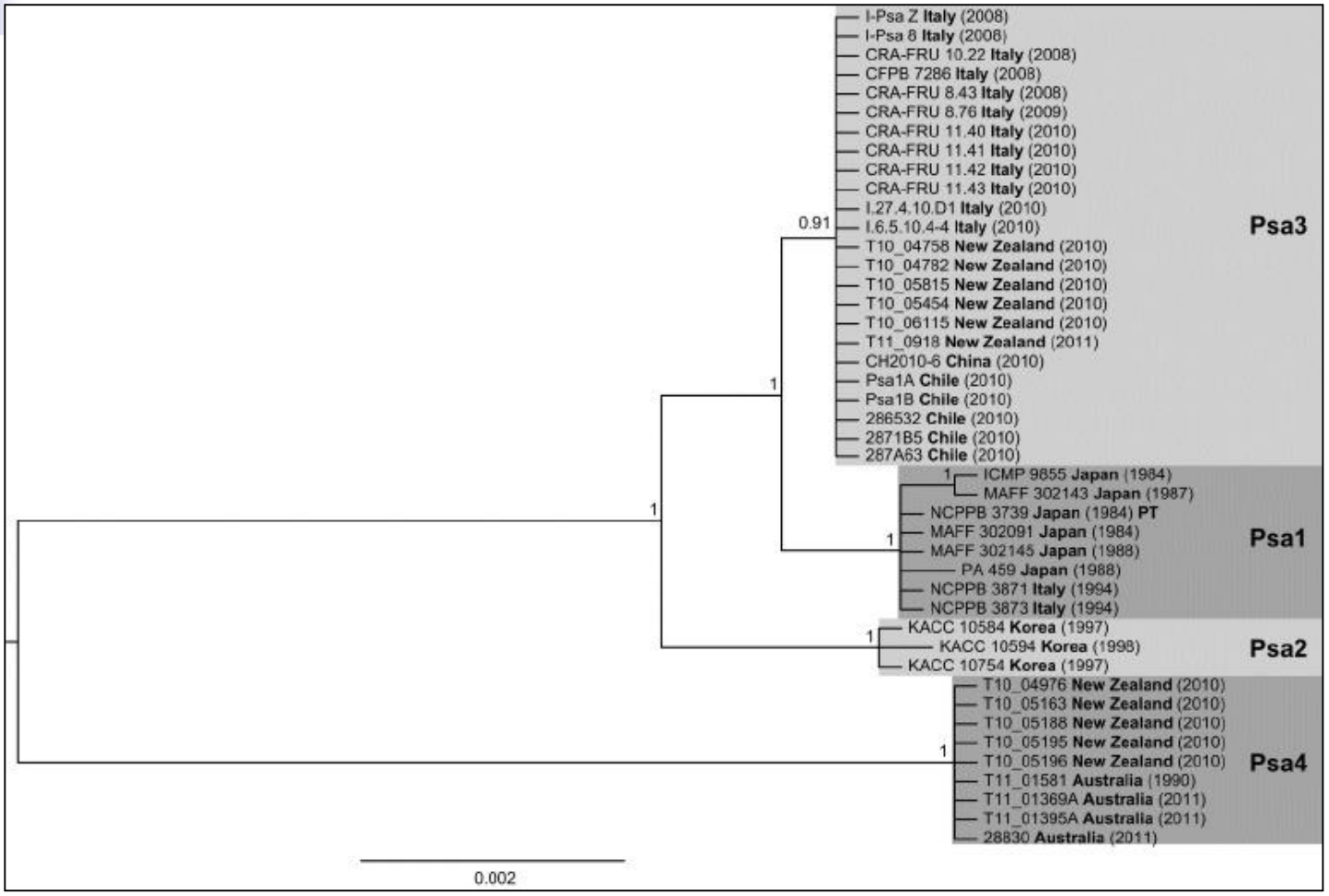
Based on MLST, TTSS effectors and toxins

- Multilocus sequence analysis (MLSA) of housekeeping, type III effector and phytotoxin genes was used to elucidate the phylogenetic relationships between *P. syringae* pv. *actinidiae* isolates worldwide.
- *P. syringae* pv. *actinidiae* (Psa) presents at least four Psa MLSA groups spreading worldwide.
- The highest losses of kiwi caused by MLSA Psa3 group of *P. syringae* pv. *actinidiae*.
- Psa3 is the strain causes the formation of cankers, production of exudates, and cane and shoot dieback in kiwi plantations.
- MLSA group Psa4 was detected in New Zealand and Australia.
- However, the MLSA group, Psa3 causes worldwide the highest losses.

Chapman *et al.*,2012

Phylogenetic relationships between *P. syringae* pv. *actinidiae*

Based on MLST, TTSS effectors and toxins



Chapman *et al.*,2012

Habitats

- **Foliar Pathogens:**
- The great majority of the foliar pathogens, approximately 50 or more when including pathovars and species, belong in habitats 1 and 2 (no soil phase or a transitory soil phase).
- Most of them are pathovars of *P. syringae*.
- **Soil-Borne Pathogens:**
- Examples of soil-borne bacteria are the fluorescent pathogens *P. tolaasii*, *P. marginalis* and *P. aeruginosa*.

Host specialization

- Many of the phytopathogenic pseudomonads have been characterized as having narrow host ranges.
- For example, most of the *P. syringae* pathovars were thought to attack only closely related species of plants.
- However, exceptions are known:

1. pv. *tabaci* is reported to cause a disease of both tobacco and soybean.
2. Some strains of pv. *phaseolicola* infect both mulberry and soybean.
3. pv. *tomato* has been isolated from diseased stone fruits.

Host specialization

- *P. s.* pv. *syringae* has a large host range and is a common epiphyte on many plants.
- *P. viridiflava*, *P. cichorii*, and *P. marginalis* appear to have broad host ranges.
- *P. cichorii* is a common lettuce pathogen but has been found on chicory, endive, clover, cauliflower, poppy, celery, tobacco and other plants.

Disease symptoms

Caused by phytopathogenic Pseudomonads

- Phytopathogenic pseudomonads are worldwide in distribution and cause diseases of most major groups of higher plants.
- Disease symptoms in plants range from:

1. Necrotic lesions, spots, cankers and blights,
2. Hyperplasias (galls, scabs),
3. tissue maceration (rots), and
4. Vascular infections (wilts).

- As mentioned earlier, some pseudomonads, for example, *P. aeruginosa* appear to infect both plants and animals.

Disease symptoms

Caused by Plant-Associated Pseudomonads

- Besides parasitic associations with plants, some pseudomonads exist in various other associations with plants.
- For example:
- some pseudomonads affect plant growth through their interactions with fungal plant pathogens or by their direct effect on the roots, possibly because of hormone production.
- These bacteria may colonize plant parts, and in some cases, they appear to damage the plant.
- Here, again, there is no clear demarcation between parasitism and saprophytism.

Todar,2004

Diseases caused by fluorescent Pseudomonads

Pseudomonas agarici	**Drippy gill of mushroom**
P. avellanae	**Bacterial canker and decline of hazelnut**
***P. cannabina* pv. *alisalensis* (ex. *P. syringae* pv. *alisalensis*)**	**Bacterial blight of crucifers**
P. cannabina* pv. *cannabina	**Leaf and stem rot of hemp (*Cannabis sativa*)**
P. cichorii	**Wide host range: necrotic lesions in eggplant; leaf spot on sunflower; rot in lettuce**
P. costantinii	**Brown blotch (spots)disease of mushroom**
P. fuscovaginae	**Sheath brown rot disease of rice, wheat and other grasses**
P. palleroniana	**Isolated from necrotic rice seeds and diseased leaf sheaths**
P. salomonii	**'*Cafe au lait*' disease on garlic plants**
P. tolaasii	**Brown blotch (spots on cap or stem or both) of mushroom**
P. tremae	**Gall of *Trema orientalis***
P. viridiflava	**Wide host range:** **Leaf spot of pumpkin, leaf rot of cauliflower, leaf blotch of passion fruit, stem rot of poppy, internal stem rot of tomato, panicle rot of grape,....**

Note that *P. syringae* (56), *P. savastanoi* (6) and *P. marginalis* (3) have multiple pathovars. Also *Pseudomonas cannabina* consists of two new pathovars namely pv. *alisalensis* and pv. *cannabina* (Bull *et al.*,2012).

Three more accepted fluorescent Pseudomonads

Pseudomonas beteli	**Bacterial leaf spot of betel**
Pseudomonas hibiscicola*	**Pathogenic to *Hibiscus Rosa-sinensis*; Leaf spot of apple blossom**
Pseudomonas flectens**	**Pod twist disease of beans (*Phaseolus vulgaris*)**

*Misnamed and should be transferred to the genera *Xanthomonas* or *Stenotrophomonas*.

****** *Pseudomonas flectens* was transferred to the family *Enterobacteriaceae* as *Phaseolibacter flectens* gen. nov., comb. nov. (Aizenberg-Gershtein *et al.*,2016)

Note *Pseudomonas veronii* was excluded from Bull *et al.*,2010b list.

These three names were appeared in Bull *et al.*,2010 comprehensive list

Important plant pathogenic fluorescent **Pseudomonads**

- *P. syringae*
- *P. viridiflava*
- *P. cichorii*
- *P. agarici*
- *P. marginalis*
- *P. tolaasii*

Important plant pathogenic fluorescent Pseudomonads

Host specificity is not known to exist

Pseudomonas cichorii

- *P. cichorii* causes disease on a wide range of vegetable, flowering ornamentals, and foliage plants.
- Symptoms may vary depending on the host and the infected part of the plant.

1. Water-soaked lesions (leaf spots;
2. Specific syndromes; e.g. 'Varnish spot of lettuce'
3. 'Bacterial blight' of celery;
4. 'Leaf rot of pepper';
5. A basal rot of geranium cuttings.

Aglaonema commutatum	coffee	*Monstera deliciosa*	*Pyracantha* sp
apple, paradise	cyclamen	oxslip	*Ruellia humilis*
Barleria cristata	daisy, Shasta	pear	rutabaga
broccoli	daisy, Transvaal	*pernettya*	*Schefflera arboricola*
cabbage	eggplant	*mucronata*	*Scindapsus* sp.
camellia	endive	*Philodendron panduraeforme*	*Spathiphyllum* sp.
carrot	escarole	poppy, arctic	tobaccos
cauliflower	gladiolus	poppy, cow	tomato
celery	hydrangea	poppy, oriental	*Viburnum* sp.
chicory	iris	potato	watermelon
chrysanthemum, florist's	larkspur	*Potentilla* sp.	wheat
	lettuce	pot marigold	
	magnolia, southern		

Bacterial canker and decline of European hazelnut

Pseudomonas avellanae

- Rapid wilting European hazelnut leaves in early spring caused by *Pseudomonas avellanae*.

The most common visual symptom is rapid wilting of the above ground shoots.

Scortichini,2002

Diseases caused by nonfluorescent Pseudomonads

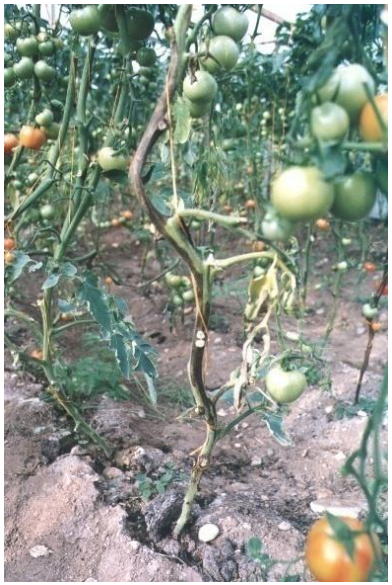
Pseudomonas amygdali	**Bacteriosis or Bacterial hyperplastic canker of almond**
Pseudomonas asplenii	**Bacterial blight of bird's nest fern (*Asplenum nidus*)**
Pseudomonas caricapapayae	**Leaf spot of papaya**
Pseudomonas cissicola	**Leaf spot of *Cissus japonicus***
P. corrugata	**Tomato pith necrosis**
***Pseudomonas ficuserectae* (*P. amygdali*)**	**Leaf spot in *Ficus erecta***
Pseudomonas mediterranea	**Tomato pith necrosis**
Pseudomonas meliae	**Leaf spot & blight of Neem**

Note: *Pseudomonas ficuserectae* is synonym of *P. amygdali* and the latter would take priority (Bull *et al.*,2010).

P. cichorii and *P. viridiflava* also cause pith necrosis of tomato.

Tomato pith necrosis

P. corrugata, P. viridiflava & *P. cichorii*



P. viridiflava - wide host range

P. corrugata - limited host range

P. cichorii - wide host range

Forgotten pathogen
Pseudomonas amygdali

***Pseudomonas amygdali* - Bacteriosis or Bacterial hyperplastic canker of almond**

- Reported from Greece and Turkey. Italy at risk
- Only on almond, perennial cankers
- Forgotten pathogen, produces t-zeatin and IAA, genes could be used for detection/idenitfication

www.atlasplantpathogenicbacteria.it

EU_COST 873 training course: Training in diagnostics of bacterial diseases of fruits, including quarantine pathogens of importance to the EU and Ukraine 2011-10-31 to 11-05 – lecture J.D.Janse

Janse,2011

COST 873 StoneFruitNutHealth

cost

Description of *P. mediterranea*
Causes tomato pith necrosis

- This species have the same general characteristics as *P. corrugata*.
- Colonies on YPGA are wrinkled or smooth.
- Yellow to brown pigments are frequently produced.
- Motile by means of multiple polar flagella.
- It is strictly aerobic,
- Non-fluorescent on King's B medium, oxidase-positive, does not produce levan, is not pectolytic, and reduces nitrates into nitrites.
- Most strains are arginine dihydrolase-positive after 15 days incubation, and few (two out of eight tested) produce hypersensitivity on tobacco leaves.
- Hydrolysis of Tween 80 and gelatin is variable.
- Acid is produced from sucrose and mannitol but not from erythritol and sorbitol.

Catara *et al.*,2002

Diseases caused by fluorescent *Pseudomonas marginalis* pathovars

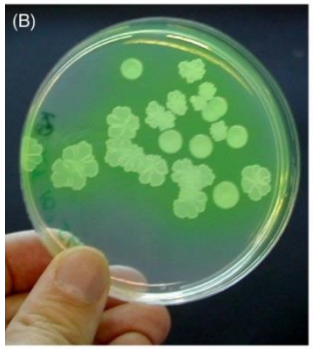
Pseudomonas marginalis	**Associated with soft rot diseases (miscellaneous host plants) & marginal leaf spot of lettuce**
Pseudomonas marginalis* pv. *alfalfae	**Brown rot of alfalfa or lucerne plants**
Pseudomonas marginalis* pv. *marginalis	**Brown rot of lettuce**
Pseudomonas marginalis* pv. *pastinacae	**Parsnip root rot**

Isolation
Pseudomonas species

- *Pseudomonas* species, in general, have simple nutritional requirements and are readily isolated from a variety of environments.
- In the laboratory they grow well in media containing:

1. some organic matter in solution, at neutral pH, and
2. at temperatures in the mesophilic range.

Pigmentation
On King's medium B(KB)



- Some *Pseudomonas* species produce diffusible yellow-green pigments that are short wavelength (254-360 nm) under which only the fluorescent pigments will fluoresce.
- A characteristic feature of all these species is the production of pyoverdine, a fluorescent yellow-green siderophore under iron-limiting conditions/iron-deficient media, such as King's medium B KB.
- Thus, KB (King *et al.*,1954) is the preferred medium for detection of fluorescein, a fluorescent green or blue, water-soluble, chloroform insoluble pteridine pigment (a yellow, crystalline bicyclic base $C_6H_4N_4$ compound).

Fluorescent pseudomonads
Fluoresce on KB medium

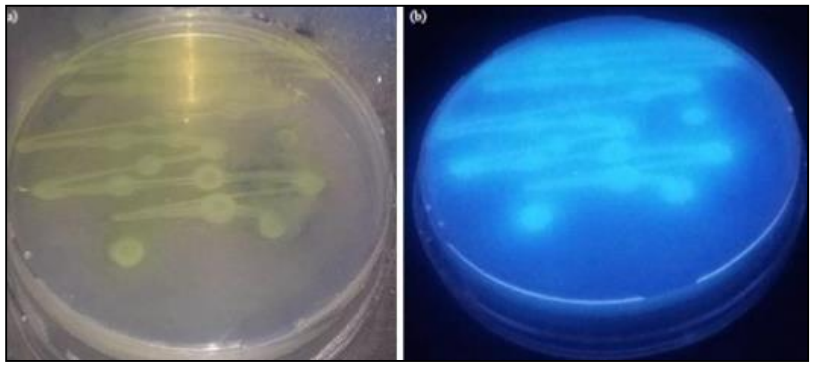
- After incubation the plates were exposed to UV light at for few seconds.

1. Fluorescent *Pseudomonas* fluoresce both:

✓ under short (254 nm), and

✓ long wave length (366 nm) ultraviolet light.

2. Nonfluorescent bacteria fluoresce just in short wavelength (254 nm) ultraviolet light.



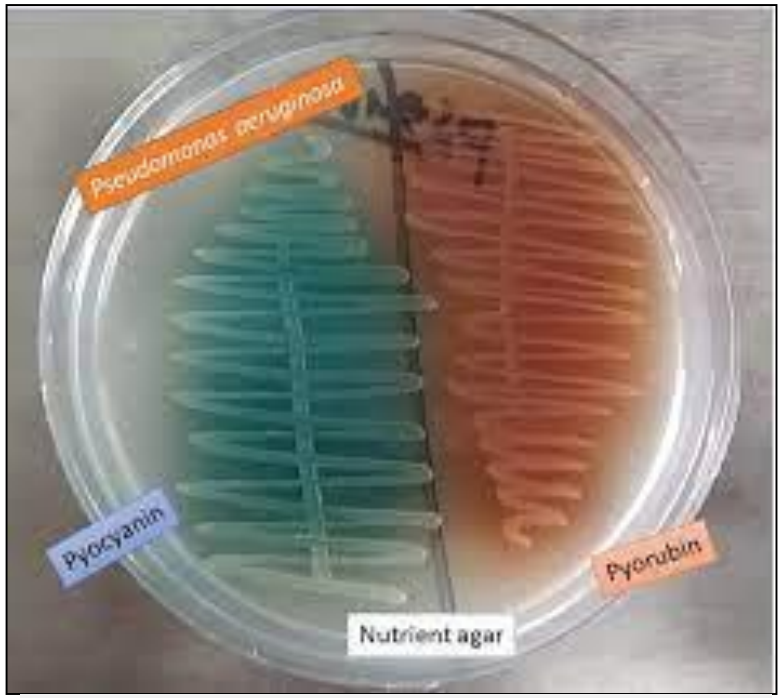
Fluoresce of *Pseudomonas aeruginosa* on a) pseudomonas cetrimide agar under light lamp, and b) under UV lamp.

Sajben *et al.*,2004;..

Characteristic of pigments in *P. aeruginosa*

- *Pseudomonas aeruginosa* produces a number of pigments which diffuse into surrounding medium and they are of following types:

1. Pyoverdin (Fluorescin, a greenish yellow);
2. Pyocyanin (bluish-green phenazine pigment);
3. Pyorubin (a reddish brown);
4. Pyomelanin (a brown to black pigment).



Non-fluorescent pigments also produced by *P. aeruginosa*: pyocyanin (left) and pyorubin (right) on nutrient agar.

Universe84a;..

King's Medium B

For common fluorescent pseudomonads

- King's Medium B for nonselective isolation and pigment production of *Pseudomonas* (King *et al.*,1954):
- Proteose peptone No. 3 (Difco) 20.0 g
- Glycerol 15.0 ml
- K_2HPO_4 (anhydrous) 1.5 g
- $MgSO_4.7H_4O$ 1.5 g
- Agar (Difco) 20.0 g
- Distilled water 1.0 liter
- Adjust pH to 7.2

Casamino-sucrose-gelatin medium
An alternative medium to KB
For some fluorescent pseudomonads fail to produce the pigment in KB

- CSGA or casamino-sucrose-gelatin medium is used to detect fluorescent pigmentation of some fluorescent pseudomonads such as *P. syringae* pvs. *actinidiae*, *morsprunorum* and *persicae*.
- Vitamin free casamino acids(Casaminic acid) 10 g
- Dipotassium phosphate 1 g
- Magnesium sulphate 1 g
- Sucrose 10 g
- Gelatin 30 g
- Agar (Difco) 20 g
- Distilled water 1 liter
- Adjust pH to 7.0

Luisetti *et al.*,1972; Lamichhane and Varvaro,2012

PGS agar

An alternative medium to KB

For fluorescent production/detection in almost all the pseudomonads, and in particular *P. syringae* pv. *actinidiae*

- The optimal composition of the medium per 100 ml was:
- Bacto peptone 1 g
- gelatin 2 g
- sucrose 2 g
- agar 1.5 g
- dipotassium hydrogen phosphate 0.1 g
- magnesium sulphate heptahydrate 0.1 g.
- Autoclave at 121°C for 15 min and left to cool to 55°C and then add 10% egg white, 0.002% conalbumin or 5µM tannic acid.
- Egg white was added by simple mixing; however, conalbumin (albumin found in chicken egg whites) or tannic acid are sensitive to heat, and were added by microfiltration (0.22 µm).
- Conalbumin and tannic acid are two chelating substances which render the medium iron-free.

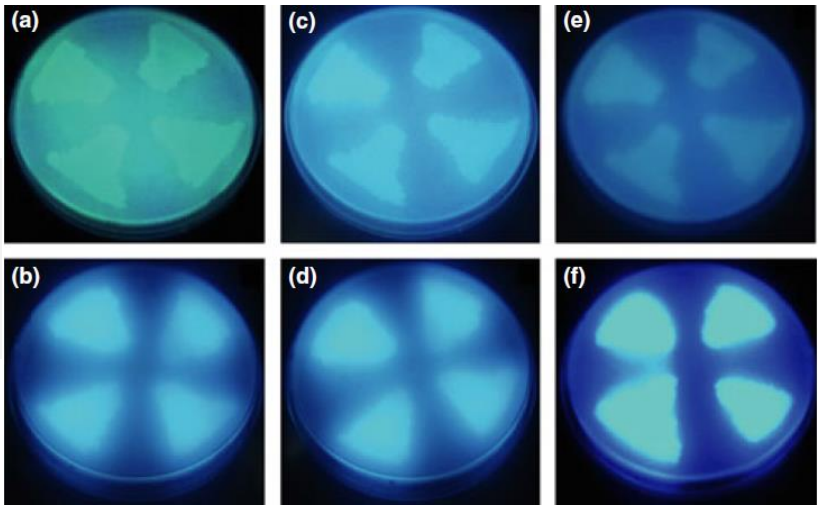
Lamichhane and Varvaro,2012

Three different media

Fluorescent production/detection in *P. syringae* pv. *actinidiae*

- Examples of pigment excretion by *Pseudomonas syringae* pv. *actinidiae* (Psa)on different media under ultraviolet light (365 nm) after 36 h of incubation at 26C.
- (a,b) King's medium B (KB) with and without a 0.002% conalbumin supplement;
- (c,d) CSGA medium with and without a 0.002% conalbumin supplement; and
- (e,f) PGS agar medium with and without a 0.002% conalbumin supplement.
- Clockwise from the upper left in each plate: strain Psa V (China); strain 36.43 (France); strain 18839 (New Zealand); and strain Psa 827 (Spain).

Note: Egg white, conalbumin and tannic acid, singly or in combination enhances fluorescent production.



Lamichhane and Varvaro,2012

King's Medium B plus KCB

P. syringae pv. *syringae* and *P. s.* pv. *phaseolicola*

- King's Medium B plus Cephalexin and Cycloheximide (KCB) (Mohan and Schaad,1987) for isolation of *P. syringae* pv. *syringae* and *P. s.* pv. *phaseolicola.*
- For KBC, the following ingredients are added to 900 ml of King's medium B (made with ingredients for a liter):

Boric acid, autoclaved 1.5% aqueous solution	100.0 ml
Cephalexin (stock solution of 10 mg/ml distilled water)	8.0 ml
Cycloheximide (stock solution of 100 mg/ml 75% methanol)	8.0 ml
Agar	16.0 g

The Prokaryotes (chapter 3.3.23),2006

Mushroom bacterial diseases
Isolation of bacterial agents

- Blotched outer layer tissues of affected mushrooms were recovered and suspended with vigorous shaking in sterile water.
- Single basidiocarp or a piece approximately 2.5 cm^2 can also surface sterilized in a 2.5% solution of sodium hypochlorite for 1 min and was then thoroughly rinsed in sterile distilled water(Besstete,1985).
- 50 µl of an appropriate serial dilution was spread on King's B agar medium and one of each of the phenotypically different colonies developing a fluorescent halo after 48 h of incubation at 25°C was further purified by streaking it on the same medium.
- Routine growth was in Luria-Bertani (LB) medium or King's B liquid medium.
- Strains were preserved by mixing overnight LB culture with 50% glycerol (1:1, vol/vol) and storage at -80°C.

Munsch *et al.*,2000

Mushroom bacterial diseases
Isolation of bacterial agents

- Gandy (1979) designed a rapid method to screen for pathogenic bacteria and to compare the intensities of damage.
- Caps of fresh sporophores were harvested and cut in blocks with the outer tissue peeled off.
- One drop of bacterial suspension was applied on the blocks.
- A rapid pitting of the mushroom blocks was observed within 10 min after a drop suspension was placed, long before browning.
- This effect was observed when the blocks were inoculated with *P. tolaasii* and any other brown blotch pathogens.
- Note that cap surface (pileus) was described as an important factor to improve the selectivity of the test when comparing the expression of symptoms.

Soler-Rivas *et al.*,2006

Bacterial diseases of mushrooms
Fluorescent *Pseudomonas* spp.

- The bacterial strains associated with cultivated mushrooms (*Agaricus bisporus*, *Pleurotus ostreatus* and *Psalliota edulis*) included:

1. *Pseudomonas aeruginosa* : Brown Blotch, Mummy Disease.
2. *Pseudomonas agarici* : Brown Blotch, Drippy Gill, Yellow Blotch. Affecting mushroom sporophores (fruiting bodies).
3. *Pseudomonas fluorescens* Biovar: Brown Blotch
4. *Pseudomonas fluorescens* Biotype G (=Biovar V): Bacterial Mummy Disease. Note Mummy disease caused by *Pseudomonas* spp.
5. *Pseudomonas gingeri* (not validly published): Bacterial Blotch, Ginger (yellow-brown) Blotch.
6. *Pseudomonas tolaasii* : Bacterial Spot/Pit/Brown Blotch/rotting of the mushroom tissues.

ENV/JM/MONO(2005)17;…

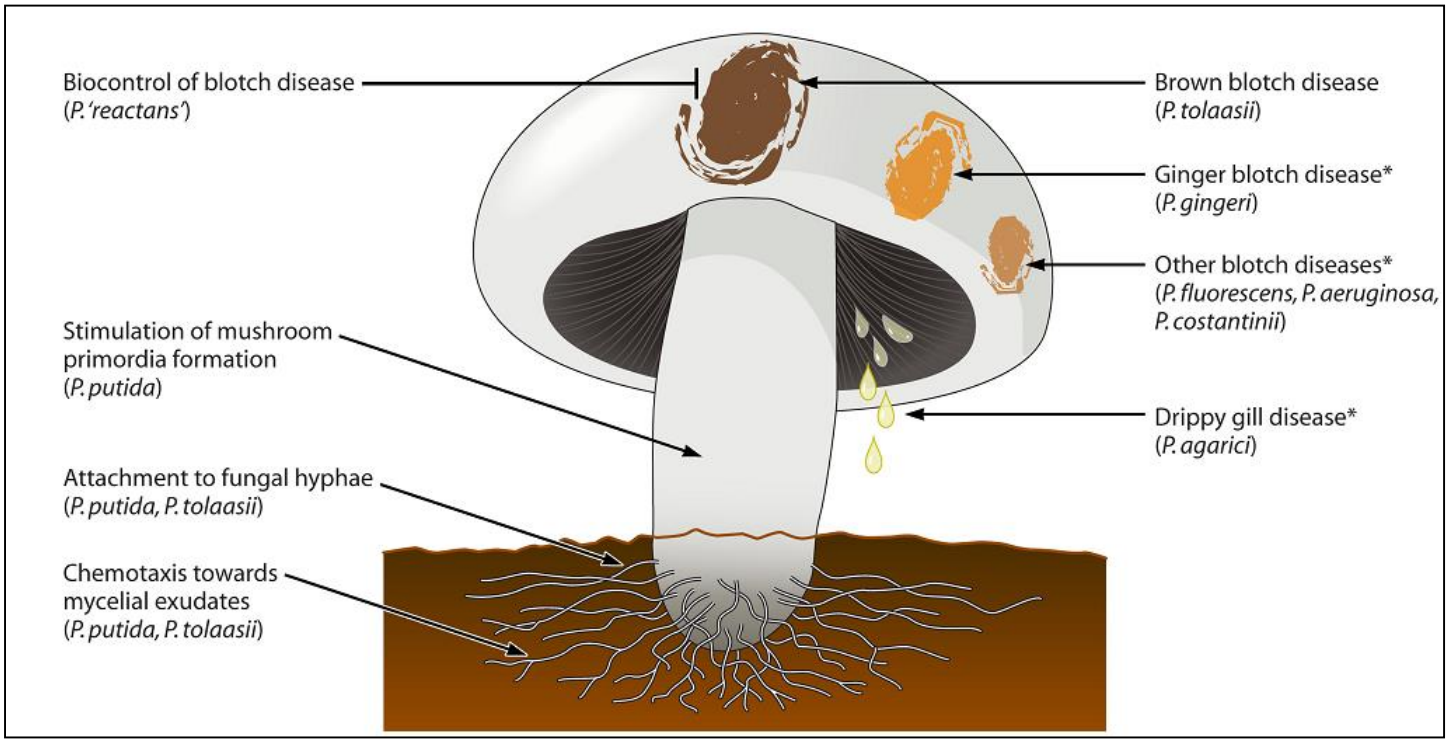
Bacterial diseases of mushrooms
Fluorescent *Pseudomonas* spp.

- The bacterial strains associated with cultivated mushrooms (*Agaricus bisporus*, *Pleurotus ostreatus* and *Psalliota edulis*) included:

7. *P. costantinii* : Typical brown blotch symptom.
8. *Pseudomonas reactans* (so called white line reacting organisms): Saprophytic fluorescent pseudomonads, including a diverse group of fluorescent pseudomonads referred to as *P. reactans*. Causes yellowing on oyster mushroom (*Pleurotus eryngii*).
9. Heterogenic fluorescent pseudomonads belonging to the *P. fluorescens* biovars I, II, III, or V seem to act as pathogens or as saprophytes in the bacterial community associated to cultivated mushrooms.

See also *Ewingella americana* causing agent of browning lesion and necrosis in the center of *A. bisporus* stipe.

Bacterial diseases of mushrooms
Pseudomonas spp.



Frey-Klett *et al.*,2011

Bacterial Blotch

Pit disease, bacterial spot, bacteriosis

Pseudomonas tolaasii

- *Pseudomonas tolaasii* is a soil bacteria cause brown blotches/spots) on the mushroom caps.
- It is known to produce a toxin called tolaasin (extracellular toxins), which is responsible for the brown blotches associated with the disease.
- It also demonstrates hemolytic activity, causing lysis of erythroctes.
- *P. tolaasii* has also been described as a saprophytic bacterium from pears and beans (*Phaseolus vulgaris*).

Janse,2006

Wikipedia,2008

Bacterial Blotch

Pit disease, bacterial spot, bacteriosis

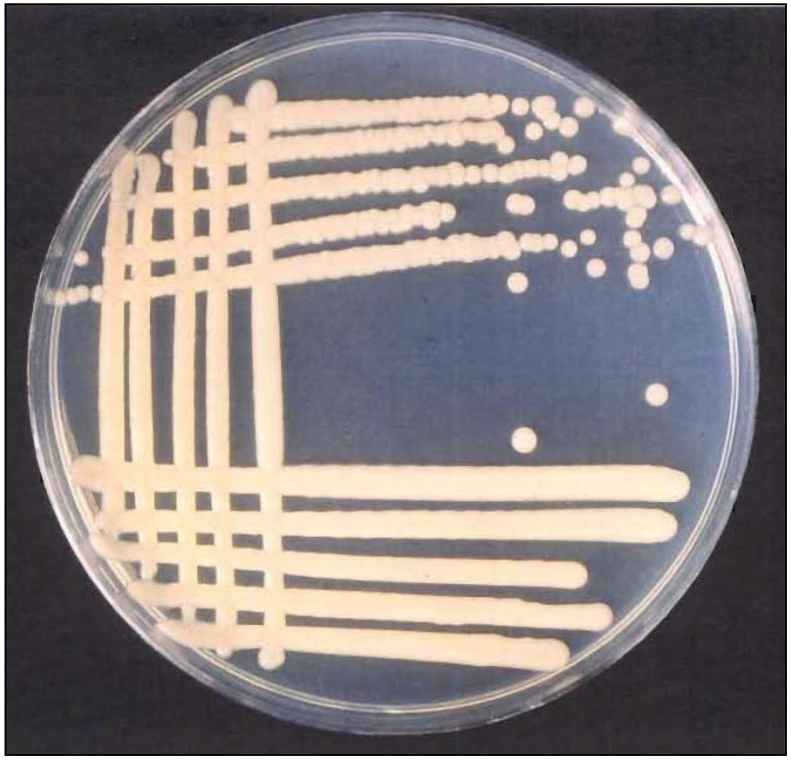
Pseudomonas tolaasii

- Pale yellow spots on the surface of the piles later it turns to yellow.
- Damage at storage and transit.
- Source of contamination may be soil or water.
- High humidity and watery conditions are favorable for disease.
- Vector: Tryoglyphid mite
- Lesions on tissue that are pale yellow initially, later become a golden yellow or rich chocolate brown.
- Discoloration is superficial (not more than 2 to 3 mm).
- Underlying tissue may appear to be water soaked and grey.
- Blotches appear in early button stage,
- Appear on any age - even on harvested refrigerated mushrooms.
- At favorable moisture conditions spots enlarge and coalesce, sometimes covering entire cap
- Mushroom stems can also be blemished similarly.
- Typical spotting is observed at or near the edge of mushroom caps wherever caps remain wet for a period of 4 to 6 hours or longer after water has been applied
- If very dry conditions occur after blotch has developed, infected caps may crack radially as the mushroom expands.

Drippy gill of mushroom

Pseudomonas agarici

- Drippy gill isolate PMS601 (Type Strain) streaked on KB agar.
- Note the lack of smooth to rough transformation in the colony morphology.
- Circular, two mm in diameter, domed, whitish, with a buttery consistency colonies were observed on nutritive agar after 5 days of incubation at 25°C.
- Pure cultures were maintained on slopes of nutrient agar plus 2% glycerol (NAG) at 4°C.



Gill,1994; Cantore and Iacobellis,2004

Mummy disease of mushroom
Pseudomonas spp.

- It is a serious mushroom bacterial disease caused by *Pseudomonas* spp. e.g.

1. *Pseudomonas aeruginosa*
2. *Pseudomonas fluorescens* Biotype G (=Biovar V).

- The characteristics of the diseased mushroom include the classic curved stems, tilted caps, profuse spawn growth at the base of mushrooms.
- Mummy-affected beds never recover and the mushrooms become mummified at the botton to an immature mushroom stage.

Penn State Lines

Mummy disease of mushroom
Pseudomonas spp.

- Fruit bodies have tilted caps,
- Early veil breaking,
- Base of the stem enlarged,,
- Curved stalk
- Tissue of the mushroom becomes:

1. Spongy,
2. Dry and Brown,
3. Mummified appearance.

- Rapid rate of spread through the bed, up to 30cm (12 inches) daily.
- Infected mushroom is tough and dry texture
- Gritty texture appeared when cut.
- Water-soaked appearance and cavities in the mushroom tissue

Bacterial disease of mushroom
Biotypes in fluorescent pseudomonads

- According to Stanier *et al.*,1966, fluorescent pseudomonads has the following biotypes:

1. Biotype A(I):Typical *Pseudomonas fluorescens*
2. Biotype B(II): *Pseudomonas marginalis* and *Pseudomonas tolaasii*
3. Biotype C(III):
4. Biotype D:*P. chlororaphis*
5. Biotype E:*P. aureofaciens*
6. Biotype F:*P. lemmonieri*
7. Biotype G: Miscellaneous strains

P. tolaasii was officially positioned into the taxonomic group of fluorescent *Pseudomonas* biotype II. Many other recent studies disagree with this classification and claim that it should be placed as separate species or into another group. However, its position in the systematic classification is still unclear because of the high heterogeneity of the group (Soler-Rivas *et al.*,2006).

Bacterial disease of mushroom

Biotypes in fluorescent pseudomonads

Biovars of *Pseudomonas fluorescens*

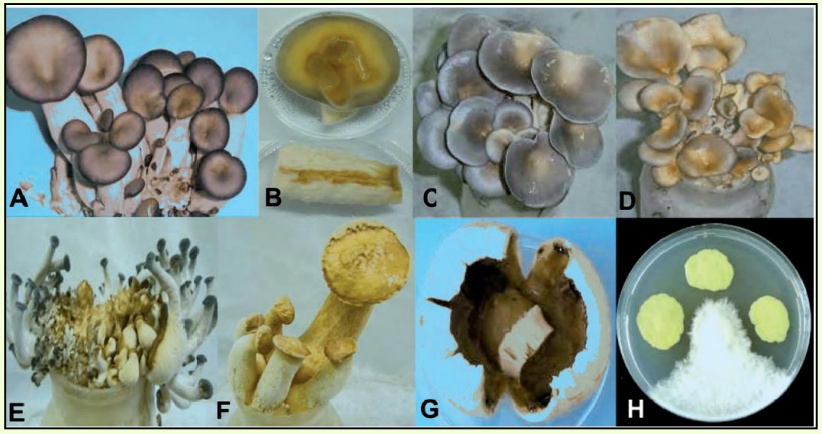
- Heterogenic fluorescent pseudomonads belonging to the *P. fluorescens* biovars I, II, III, or V seem to act as pathogens or as saprophytes in the bacterial community associated to cultivated mushrooms.
- *Pseudomonas fluorescens* has several biovars according to certain biochemical characters:

1. *Pseudomonas fluorescens* Biovar I: *P. palleronii* and *P. salomonii*
2. *Pseudomonas fluorescens* Biovar II: *Pseudomonas marginalis* and *Pseudomonas tolaasii*
3. *Pseudomonas fluorescens* Biovar III:
4. *Pseudomonas fluorescens* Biovar IV:
5. *Pseudomonas fluorescens* Biovar V(Biotype G):

Soft rot of mushroom
Burkholderia gladioli pv. *agaricicola*

- A. Typical natural symptoms of bacterial soft rot on mushroom as observed in a mushroom cultivation farm.
- B. Tissue soft rot of mushroom inoculated by dropping a bacterial suspension on the sporocarp.
- C, D, E, F. Symptoms of bacterial soft rot on oyster and king oyster mushroom inoculated by spraying.
- G. Tissue soft rot on button mushroom injected with *B. gladioli* pv. *agaricicola*.
- H. Inhibition of oyster mushroom mycelium by *B. gladioli* pv. *agaricicola*.

See also the genus *Burkholderia*



Lee *et al.*,2010

Postharvest bacteria

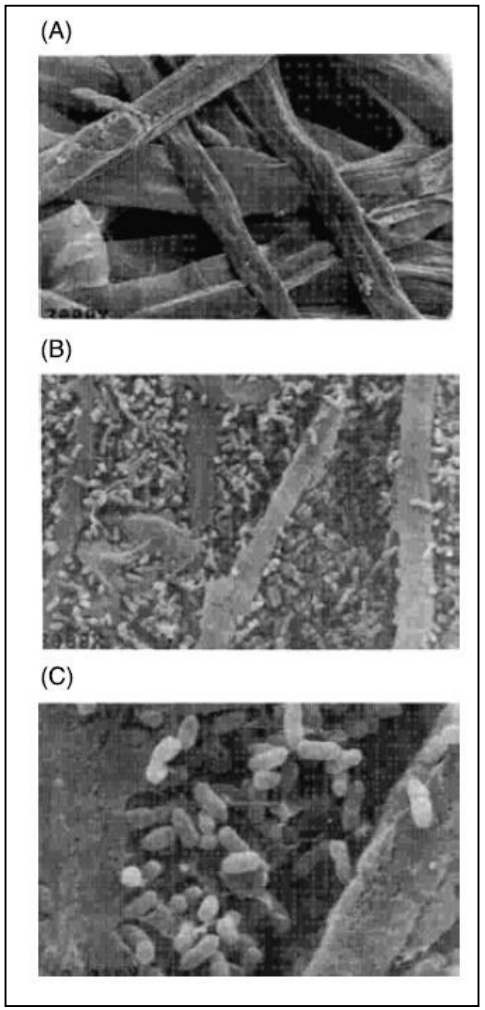
Pseudomonas spp. and *Flavobacterium* spp.

- *Pseudomonas* spp. and *Flavobacterium* spp. were the two main groups that predominated during agaricus mushroom postharvest storage.
- Scanning electron micrographs of mushroom cap surfaces:

A. Healthy tissue (X 3000);

B. Blotched tissue showing invading bacteria (X 3000)

C. (X 10,000).



Sapers *et al.*,2006

Description of mushroom bacterial agents
Drippy gills of mushroom
Pseudomonas agarici

- Green, fluorescent pigment produced (weak fluorescent).
- Aerobic, motile.
- Oxidase-positive
- Arginine dihydrolase, gelatin, aesculin, starch hydrolyses are negative.
- Levan negative
- Pectate liquefaction is negative.
- Does not produce acid from rhamnose, xylose, mannose, sucrose, maltose.
- Nitrate reduction and INA are negative.
- Assimilated mannitol but not trehalose.

Description of mushroom bacterial agents
Colony characteristics
Pseudomonas tolaasii

- *P. tolaasii* colonies were reported to present two different appearances when cultivated on King's B medium or *Pseudomonas* agar F.

1. The wild-type or smooth colony surface showed small, semi-mucoid, opaque and non-fluorescent colonies and was pathogenic for mushrooms.
2. The variant type or rough colony surface presented large, non-mucoid, translucent and fluorescent colonies, which were not pathogenic.
3. Except for its pathogenicity, *P. tolaasii* is difficult to distinguish from *P. fluorescens* (Janse *et al.*,1992).

Soler-Rivas *et al.*,2006;OECD,1997

Characteristics that differentiate *P. tolaasii*-like strains (phenon III) from the six other phena (numbered in Roman capitals)

- Phenon I comprised 10 strains clustered around *P. tolaasii*.
- Phenon II contained two *P. reactans* strains.
- Phenon III comprised all five *P. tolaasii* -like strains including *P. costantinii* and the type strain of *P. fluorescens* bv. 1, CFBP.
- Phenon IV contained *P. gingeri*.
- Phena V-VII comprised two strains each.

Characteristic	Phenon no.						
	I	II	III*	IV	V	VI	VII
Number of strains	11	2	5	2	2	2	2
Assimilation of:							
D-Xylitol	+	+	+	–	–	–	–
5-Keto-D-gluconate	+	–	+	–	–	–	+
Adonitol	+	+	+	–	–	–	–
D-Xylose	–	+	+	+	+	+	–
L-Arabinose	–	+	+	–	+	+	+
meso-Erythritol	+	+	+	+	–	–	–
Benzoate	–	d (1)	+	–	d (1)	–	+
Histamine	–	–	+	+	–	–	+
2-Keto-D-gluconate	+	+	+	–	+	–	+
D-Trehalose	d (8)	+	+	+	+	+	+
L-Tartrate	–	+	–	–	–	–	–
Sucrose	–	–	d (4)	–	+	–	+
Levan formation	–	–	–	–	+	–	+
Nitrate reduction	d (7)	–	–	d (1)	d (1)	–	d (1)

+, Positive reaction; -, negative reaction; d, strain-dependent reaction with numbers in parentheses indicating the number of strains showing a positive reaction.

Munsch *et al.*,2002

Description of mushroom bacterial agents
Bacterial soft rot of *Agaricus bitorquis*
P. tolaasii, P. agarici, P. gingeri,...

Characteristic	RR	*P.t.*	*P.g.*	*P.a.*	*P.f.*[a]	PMS-PV29[b]	P.m.[c]
Levan production	−	−	−	−	−	−	V
Oxidase reaction	−	+	+	+	+	+	+
Potato rot	+	−	−	−	NT	+	+
Arginine dihydrolase	+	+	+	−	+	−	+
Tobacco hypersensitivity	+	−	−	+	NT	−	−
Nitrate reduction	−	−	−	−	−	+	+
Gelatin hydrolysis	+	+	+	−	+	+	+
Poly-β-hydroxy butyrate	+	−	−	NT	−	−	−
H_2S production	−	−	−	+	+	+	NT
Fluorescence on King's B	−	+	V	+	+	+	+
Blotch test	−	+	+	−	−	−	−
White line	−	+	−	−	−	−	−

RR, mushroom soft-rot bacteria; *P.t.*, *P. tolaasii* NCPPB 2192; *P.g.*, *P. 'gingeri'* NCPPB 3146; *P.a.*, *P. agarici* ATCC 25942; *P.f.*, *P. fluorescens* 'mummy'; *P.m.*, *P. marginalis* NCPPB 667; PMS PV29, *Pseudomonas* sp. ICMP 9522.
[a] Data from Goor *et al.* (1986) and Betterley & Olson (1989).
[b] Data from Rainey & Cole (1988).
[c] Causes soft rot of a range of vegetables other than mushrooms.
NT, not tested; V, variable result.

Metabolism was respiratory, all used glucose oxidatively (O/F test).

Lincoln and Fermor,1991

Phenotypic features of Pseudomonas strains, mostly from cultivated mushrooms

Pseudomonas tolaasii, P. agarici

The percentage frequency of present features in phenons and unclustered strains is given(100=+,0= -).

Most strains are aerobes

Feature	*P. aeruginosa*	LMG 6652	*P. fluorescens* biovar I	NCPPB 2193	*P. tolaasii*	NCPPB 2413t1/t2	White line reacting organisms	NCPPB 2208	'Mummy disease' isolates	NCPPB 2304	*P. agarici*
Phenon . . .	I	–	–	–	IV	–	V	–	VI	–	VII
No. of strains . . .	3	1	1	1	21	2	12	1	2	1	8
Oxidative glucose metabolism (O/F)	100	100	100	100	100	100	100	100	100	100	75
H_2S production from cysteine	0	0	0	0	0	0	0	0	100	100	100
Arginine dihydrolase	100	100	100	100	100	100	100	100	100	0	0
Levan production from sucrose	0	0	100	0	0	0	0	0	0	0	0
Hydrolysis of:											
Casein	100	100	100	100	76	0	67	0	0	NT	0
Tween 80	100	100	100	100	100	100	92	100	100	100	100
Acid production from:											
Dulcitol	0	100	0	0	0	0	0	0	0	0	0
D-Arabinose	100	0	0	0	0	0	25	100	0	0	0
L-Rhamnose	0	0	0	0	0	0	100	0	0	0	0
D-Lactose	0	0	0	0	0	0	17	0	0	0	0
Sucrose	0	0	100	0	0	0	0	0	0	0	0
D-Trehalose	0	100	0	0	0	0	58	0	0	0	0
D-Fructose	33	0	0	100	29	100	25	100	100	0	63
Melibiose	100	100	0	0	0	0	58	100	0	0	0
Inulin	0	100	0	0	0	0	25	0	0	0	0
Adonitol	0	0	100	0	19	100	75	0	0	0	0
meso-Inositol	0	0	0	0	20	0	50	0	0	0	75
D-Xylose	100	100	100	100	90	100	100	100	100	100	0
D-Glucose	100	100	100	100	100	100	100	100	100	0	88
D-Ribose	100	100	100	100	100	100	100	100	100	0	100
D-Mannose	100	100	100	100	100	100	100	100	100	0	87
D-Mannitol	100	100	100	100	57	100	92	0	100	100	100
Litmus milk:											
Acid production	0	100	0	0	52	0	75	0	0	0	0
Reduction	0	0	0	0	29	50	17	0	0	0	0
Alkalinization	0	0	100	100	19	50	8	100	100	100	100
Coagulation	100	0	0	0	14	0	75	0	0	0	0
Peptonization	100	0	0	0	0	0	0	0	0	0	0
Alkalinization of:											
Sodium acetate	100	100	100	100	100	100	100	0	0	0	25
Sodium benzoate	100	0	0	0	0	0	8	0	0	0	0
Sodium formate	100	0	0	0	0	0	8	0	0	0	0
Sodium citrate	100	100	100	100	100	100	100	100	0	100	100
Sodium tartrate	0	0	0	0	0	0	25	0	100	0	0
D-Galacturonic acid	0	0	0	0	0	0	0	100	100	0	0
Propionic acid	100	100	0	0	57	100	8	0	0	0	0
Sensitivity to:											
Sodium nitrite (0·01%)	0	0	100	0	48	0	58	0	0	0	0
Potassium nitrite (0·01%)	0	0	0	100	57	50	8	0	0	0	0
Hexamethylenetetramine (0·01%)	0	0	0	0	0	0	0	0	0	100	0
Hexamethylenetetramine (0·2%)	100	0	100	100	71	100	25	100	100	100	100

Goor *et al.*,1986

Phenotypic features of Pseudomonas strains, mostly from cultivated mushrooms

Pseudomonas tolaasii, P. agarici

The percentage frequency of present features in phenons and unclustered strains is given (100= +,0= -).
Most strains are aerobes.

	P. aeruginosa	LMG 6652	*P. fluorescens* biovar II	'*P. gingeri*'	*P. fluorescens* biovar I	NCPPB 2193	*P. tolaasii*	NCPPB 2413t1/t2	White line reacting organisms	NCPPB 2208	'Mummy disease' isolates	NCPPB 2304	*P. agarici*
Growth in API 50AA on:													
Glycine	100	0	0	50	0	0	17	0	25	0	0	0	0
L-Norleucine	0	0	0	0	100	0	4	0	50	0	100	0	0
DL-Norvaline	25	0	0	0	0	0	22	0	19	0	0	0	0
DL-2-Aminobutyrate	0	0	33	25	0	0	48	50	50	0	0	0	0
L-Methionine	0	0	33	0	0	0	48	0	69	0	0	0	0
L-Tryptophan	100	0	33	0	100	100	100	100	81	0	0	0	0
Trigonelline	0	100	100	0	0	0	0	50	0	100	0	0	0
L-Ornithine	100	100	100	100	100	100	100	100	100	0	50	0	75
L-Lysine	100	100	100	100	100	100	87	100	100	0	100	100	13
L-Citrulline	0	0	67	100	0	100	83	100	100	0	0	0	0
DL-Kynurenine	50	0	0	0	0	0	70	50	56	0	0	0	0
DL-3-Aminobutyrate	50	0	67	75	0	0	22	50	6	0	0	0	0
DL-5-Aminovalerate	100	100	100	100	100	100	96	100	81	100	100	100	25
2-Aminobenzoate	100	0	0	0	0	100	87	100	69	0	0	0	0
Acetamide	100	0	0	0	0	0	0	0	0	0	0	0	0
Sarcosine	100	100	100	100	100	100	100	100	100	100	100	100	63
Butylamine	0	100	0	0	0	0	0	0	0	0	0	0	0
Amylamine	0	100	33	0	0	0	0	0	19	0	0	0	0
Ethanolamine	100	100	100	100	100	100	30	100	94	100	0	0	38
Histamine	100	100	0	100	0	100	0	100	0	0	0	0	0
Glucosamine	0	0	100	100	100	100	100	100	100	100	100	100	63
Colonies on YPGA medium:													
White	0	100	0	0	100	100	77	0	44	0	100	0	63
Cream	25	0	100	100	0	0	23	100	56	0	0	0	38
Yellow-brown	75	0	0	0	0	0	0	0	0	0	0	0	0
Yellow	0	0	0	0	0	0	0	0	0	100	0	100	0
Smooth	0	0	100	100	100	100	91	100	94	100	100	100	100
Irregular	100	100	67	100	0	100	59	0	56	100	100	0	25
Raised to convex	100	100	33	100	NT	100	95	100	81	100	100	100	100
Diameter <3 mm	50	0	100	100	100	100	100	100	100	0	100	100	100
Cells:													
Elongated	0	0	0	0	0	0	0	0	13	100	0	0	0
Filamentous	0	0	0	0	100	0	4	0	6	100	0	0	0
Diameter $\leqslant 2\,\mu m$	100	100	0	75	0	100	86	100	50	0	50	100	88
Sensitivity to:													
Chloramphenicol (30 µg)	0	0	0	0	0	0	0	0	19	0	50	0	0
Novobiocin (30 µg)	0	0	0	0	0	0	22	0	6	0	0	0	0
Fusidic acid (10 µg)	0	0	0	0	100	0	9	0	6	0	0	0	0
Gentamicin (10 µg)	0	0	0	50	0	0	0	0	0	0	0	0	88
Tobramycin (10 µg)	0	0	0	75	0	0	0	0	0	0	0	0	100
Neomycin (10 µg)	0	0	0	100	0	0	9	0	0	0	0	100	88
Streptomycin (10 µg)	0	0	67	50	0	0	9	0	13	0	0	100	100
Nalidixic acid (30 µg)	0	100	0	0	100	100	83	100	75	0	0	0	0
Kanamycin (30 µg)	0	100	100	100	100	100	74	50	81	100	100	100	100
Tetracycline (30 µg)	50	100	100	100	100	100	100	100	100	100	50	100	100
Fluorescence on:													
King A medium	100	0	0	0	0	0	0	0	6	0	0	0	0
King B medium	100	100	33	25	100	100	100	100	100	0	0	0	100

Goor *et al.*,1986

Description of mushroom bacterial agents

Phenotypic features of *Pseudomonas* strains, mostly from cultivated mushrooms

P. tolaasii, P. agarici, P. gingeri, P. reactans,..

	P. aeruginosa	LMG 6652	*P. fluorescens* biovar II	'*P. gingeri*'	*P. fluorescens* biovar I	NCPPB 2193	*P. tolaasii*	NCPPB 2413t1/t2	White line reacting organisms	NCPPB 2208	'Mummy disease' isolates	NCPPB 2304	*P. agarici*
Reduction of nitrate	100	100	100	0	0	0	0	0	0	0	0	0	0
Growth in the presence of NaCl:													
3%	100	100	100	100	100	100	100	100	81	100	100	100	0
4%	100	100	67	0	100	100	100	100	69	100	100	100	0
5%	100	100	0	0	0	100	4	0	0	0	100	0	0
6%	100	100	0	0	0	0	0	0	0	0	0	0	0
7%	100	0	0	0	0	0	0	0	0	0	0	0	0
Growth at:													
33°C	100	100	100	100	100	100	100	100	94	100	100	0	100
37°C	100	100	0	0	0	0	0	0	0	100	0	0	0
39°C	100	0	0	0	0	0	0	0	0	0	0	0	0
41°C	100	0	0	0	0	0	0	0	0	0	0	0	0

Goor *et al.*,1986

Description of mushroom bacterial agents

Phenotypic features of *Pseudomonas* strains, mostly from cultivated mushrooms

P. tolaasii, P. gingeri, P. reactans, P. fluorescens bvs. III and V

- Pathotype A: typical of *P. tolaasii* ; Pathotype B: typical of *P. gingeri* ; Pathotype C: typical of *P. reactans*
- [Y] By definition: *P. reactans*

	Percent positive strains[v] (number of strains tested[w])					
				Nonpathogenic		
	Pathotype[x]			White line-positive[y]	White line-negative	
Phenotypic property[u]	A (10)	B (10)	C (25)	Biovar V (14)	Biovar V (50)	Biovar III (50)
White line reaction[z]	100	0	100	100	0	0
Fluorescence						
Blue	80	60	28	36	38	52
Yellow-green	20	40	48	21	14	56
Weak	0	0	24	43	32	6
Mucoid	0	80	20	7	10	26
Oxidase, reaction with cells grown on nutrient agar + glucose	10	90	88	86	100	100
Lipase	100	100	96	57	62	20
Adonitol	100	100	88	100	84	88
L-arabinose	10	100	84	100	96	96
2-ketogluconate	100	100	100	88	96	96
Propylene glycol	0	70	54	56	60	94
L(+)tartrate	0	70	52	14	14	86
D-trehalose	50	100	100	100	86	100
L-rhamnose	0	60	68	86	80	80
D-sorbitol	100	100	100	79	96	98
Sucrose	0	60	52	36	28	38

[u] All strains positive for catalase, oxidase (on glucose-free medium), arginine dihydrolase, and gelatinase; and negative for levan, pectic enzymes, and growth at 41°C.
[v] Weak or delayed reactions considered positive.

Wells *et al.*,1996

Phenotypic and pathogenicity features of *Pseudomonas* strains, mostly from cultivated mushrooms

P. tolaasii, P. gingeri, P. reactans, P. fluorescens bvs. III and V

- Pathotype A: typical of *P. tolaasii* with dark lesion color;
- Pathotype B: typical of *P. gingeri* with brown/yellow lesion color;
- Pathotype C: typical of *P. reactans* with yellow lesion color.

	Pathogenicity			White line reaction[v] with:		Phenotype		
Strain designations[u]	Pathotype	Lesion color[w]	Tissue collapse[w]	ATCC 33618[x]	ATCC 14340[y]	Mucoid[z]	*P. fluorescens* biovar	Tentative identification
P2, P3a, P3b, Pf28, Pf29, Pf30, ATCC 33618	A	Dark	+	–	+	–	V	*P. tolaasii*
Pf31	B	Brown	+	–	–	+	V	*P.* 'gingeri'
Pf3, Pf11, Pf13	B	Yellow	+	–	–	+	III	*P.* 'gingeri'
Pf6, Pf14	B	Yellow	+	–	–	–	III	*P.* 'gingeri'
Pf2, Pf9	B	Yellow	+	–	–	+	V	*P.* 'gingeri'
Pf1, Pf8	C	Brown	–	+	–	–	III	*P.* 'reactans'
ATCC 14340	C	Brown	–	+	–	–	V	*P.* 'reactans'

Smear technique: lesion colors were determined on unwounded surfaces of fresh mushroom.
Puncture technique: Tissue collapse upon wound puncturing and directly into cap tissues.

Biotype G (Biovar V) now named as *Pseudomonas fluorescens*. *P. tolaasii* was officially positioned into the taxonomic group of fluorescent *Pseudomonas* biotype II.

Wells *et al.*,1996;.

Description of mushroom bacterial agents

Pseudomonas costantinii : Causes brown blotch disease on the cultivated mushroom *A. bisporus*

- Cells are Gram-negative straight rods, motile.
- Produces a fluorescent diffusible greenish pigment on KB medium.
- Several-days old colonies produce sectors which when subcultured prove to be non-pathogenic on mushroom sporophores(fruiting bodies).
- Produces tween esterase (on Tween 80), are oxidase-positive, arginine dihydrolyase-positive and hydrolysis gelatin.
- Does not hydrolysis aesculin and does not produce acid from L-tartrate.
- A typical white line in agar when streaked towards the 'P. reactans'.
- Nitrates are not reduced.
- Levan-sucrase-negative, non-pectinolytic and does not degrade polypectates at pH 5 or 8.
- Does not induce a hypersensitive reaction on tobacco leaves.

Munsch *et al.*,2002

Specific diagnostic traits of *P. tolaasii* and *P. reactans*

- Virulent strains of *P. reactans* produce in culture an extracellular substance called the White Line Inducing Principle (WLIP).
- WLIP is a lipodepsipeptide with a molecular mass of 1125 Da and presents structural similarities to tolaasin.
- WLIP has ability to interact with tolaasin I and form a white precipitates in the "white line" assay useful for the identification of *P. tolaasi*.
- On the other hand, virulent strains of *Pseudomonas tolaasii* produce lipodepsipeptides (LDPs) tolaasin I and II.
- Tolaasin I is considered the main virulence factor of *P. tolaasii* and responsible for the symptoms development on the mushrooms.

Andolfi *et al.*,2009;..

Pseudomonas tolaasii

Tolaasins production

Tolaasins I,II and A, B, C, D, and E

- *Pseudomonas tolaasii*, the causal organism of brown blotch disease of *Agaricus bisporus* and of the yellowing of *Pleurotus ostreatus*, was shown to produce LDP(lipopeptides) toxin in culture:
- Tolaasin I,
- Tolaasin II, and
- Five other minor metabolites, tolaasins A, B, C, D, and E.
- These extracellular necrosis inducing toxins were demonstrated to be important in the development of the disease (necrotic) symptoms.

Bassarello *et al.*,2004;..

Pseudomonas tolaasii

Tolaasins production

Tolaasins I,II and A, B, C, D, and E

- Tolaasin as a pore-forming peptide toxin is a 1985 Da non-host specific toxin form pores on the cellular membrane and destroy host cell structure.
- At high concentrations, tolaasin acts as a detergent that is able to directly dissolve eukaryotic membranes.
- The structural diversity of tolaasins is due to:

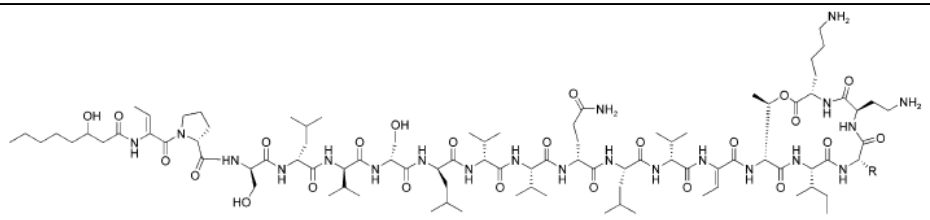
1. differences in the length and composition of the fatty acid tail, and
2. to variations in the number, type, and configuration of the amino acids in the peptide moiety.

Raaijmakers *et al.*,2006;..

Pseudomonas tolaasii

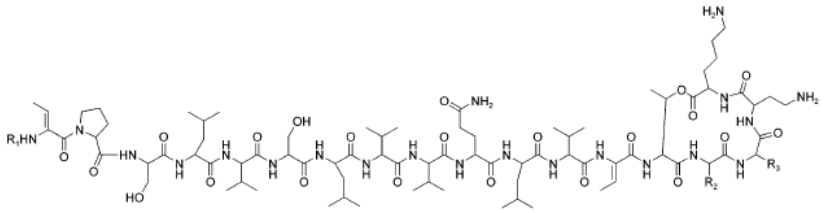
Tolaasins production

Tolaasins I,II and A, B, C, D, and E



Tolaasin I (**1**): R=CH_2CH_2OH

Tolaasin II (**2**): R=H

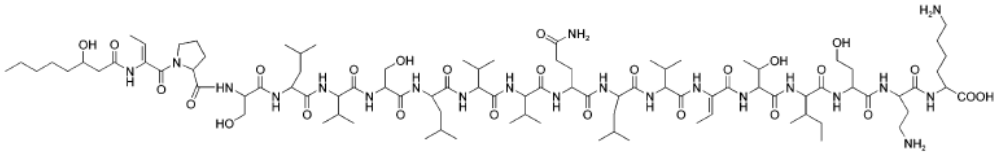


Tolaasin A (**3**): R_1=$HOOC(CH_2)_3CO$; R_2=$CH(CH_3)CH_2CH_3$; R_3=CH_2CH_2OH

Tolaasin B (**4**): R_1=$CH_3(CH_2)_4CH(OH)CH_2CO$; R_2=$CH(CH_3)_2$; R_3=CH_2CH_2OH

Tolaasin D (**6**): R_1=$CH_3(CH_2)_4CH(OH)CH_2CO$; R_2=$CH_2CH(CH_3)_2$; R_3=CH_2CH_2OH

Tolaaasin E (**7**): R_1=$CH_3(CH_2)_4CH(OH)CH_2CO$; R_2=$CH_2CH(CH_3)_2$; R_3=H



Tolaasin C (**5**)

Bassarello *et al.*,2004

Assay on tissue blocks and whole sporophores of *Agaricus bisporus*

- Brown lesions on tissue blocks of *Agaricus bisporus* (lower three blocks in each treatment), caused by deposition of 5 µl solutions containing:

A. 5.12 µg of WLIP, and

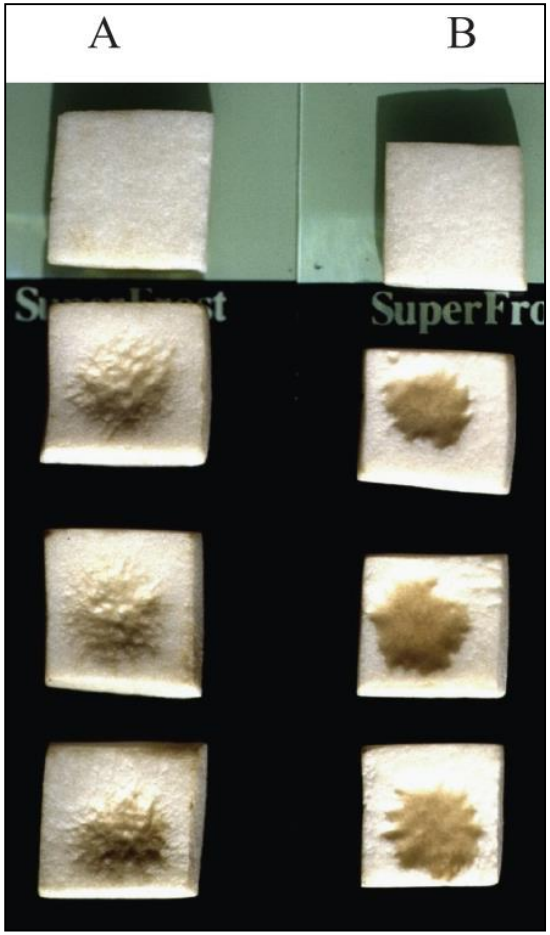
B. 0.64 µg of tolaasin I, respectively.

- On upper blocks, 5 µl of sterile water was deposited.
- Brown sunken lesions (pits) were observed with both A and B treatments.
- Application of 10 µg ml^{-1} caused pitting and 30 µg ml^{-1} of tolaasin caused browning and pitting after 16 h.

Andolfi *et al.*,2009;..

Assay on tissue blocks and whole sporophores of *Agaricus bisporus*

- Note that WLIP, the lipodepsipeptide of *P. reactans* is a potential inhibitor of the brown blotch symptoms caused by *P. tolaasii*.
- Applications of WLIP on mushroom caps before inoculation (with bacterial concentrations higher than the threshold) protected the mushroom against the bacterium and they did not show discolouration (Grewal and Hand,1992; Soler-Rivas *et al*.,2006).



Specific diagnostic traits of *P. tolaasii* and *P. reactans*

1. Hemolytic activity

- Red blood cells (RBCs) or erythrocytes, are the most common type of blood cell can be lysed by mushroom-associated pseudomonads such as:
- *P. tolaasii*, *P. costantinii*, *P. reactans* and *P. gingeri*.
- However, the minimal haemolytic quantity of tolaasin I, was four fold higher than that of WLIP.
- Blood Agar Base (BAB) medium was prepared according to the supplier's indications; 5% sterile defibrinated blood (from sheep or bovine) were aseptically added to the medium.
- All strains were streaked on BAB, and incubated at 25°C for 48 h.

Note: Rabbits blood can be substituted for sheep's blood for the growth of fastidious organisms (El Safey,2011).

Munsch & Alatossava,2002; Andolfi *et al.*,2009

Tolaasin

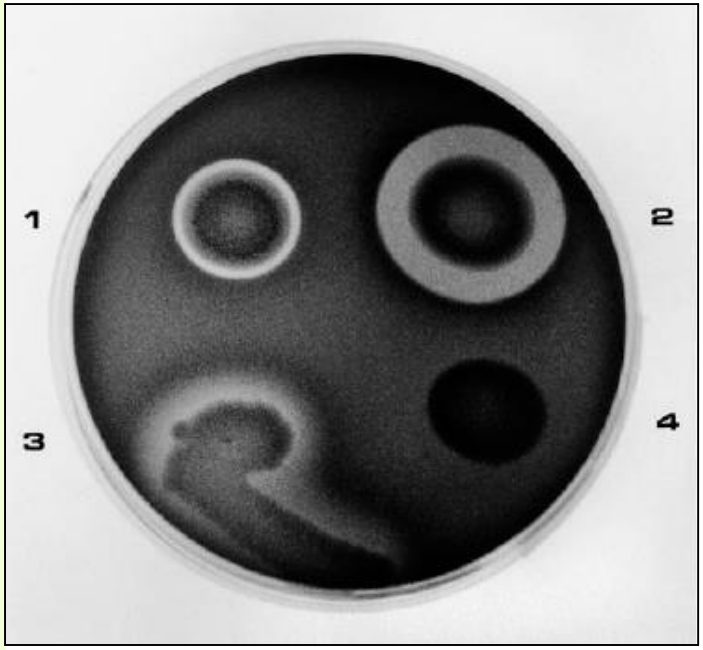
Hemolysis of erythrocytes

Red blood cells hemolysis assay

- Bovine erythrocytes lysis in Blood Agar Medium(BAB), induced by pseudomonads.
- 20 µl droplets of overnight cultures were laid on BAB plates, and were incubated for 48 h at 25°C.

1. *P. costantinii* PS 3aT
2. *P. reactans* LMG 5329
3. *P. gingeri* NCPPB 3146
4. *P. fluorescens* HAMBI 27 (a negative control with no hemolytic zones).

- Note: strong hemolysis was equally observed for *P. putida* RW10S2 cells grown on horse blood TSA agar.



Lysis of the blood cells and exhibit a colorless, transparent ring around the colonies.

Munsch & Alatossava,2002;..

Specific diagnostic trait of *P. tolaasii*
2. White line test

- *P. tolaasii* is easily distinguished from *P. fluorescens* strains or other fluorescent pseudomonads by two main features, which are:

1. The pathogenicity to mushrooms, and
2. The white line reaction.

- The white line in agar test was performed by inoculating pure cultures of the test isolates in a perpendicular line to a streak of a known reacting strain of *Pseudomonas fluorescens* on pseudomonas agar F (PAF) according to the method described by Wong and Preece,1979.

The interaction between the pathogenic form of *Pseudomonas tolaasii* and *Pseudomonas* "*reactans*" the heterogenic pseudomonads acting as white line reacting organisms (WLROs) gives rise a sharply white line (white precipitate) on King's B medium.

Specific diagnostic trait of *P. tolaasii*
2. White line test

- This interaction has been considered as highly specific.
- However, some other pseudomonads, associated with the cultivated mushroom *Agaricus bisporus*, e.g. *P. costantinii* or *P. gingeri* also yielded a white line precipitate when they were streaked towards *Pseudomonas tolaasii* LMG 2342T.
- *P. putida* RW10S2 strain RW10S2 generated a heavy white line 357 when confronting *P. tolaasii* (Rokni-Zadeh *et al.*,2012).
- Therefore, we propose that the white-line-in-agar (WLA) test should no longer be considered as an unequivocal diagnostic trait of *P. tolaasii*.

Munsch and Alatossava,2002

White-line-in-agar test
WLA test

- A reliable method to monitor *P. tolaasii* strains in mushroom crops.
- When the two pathogenic form of *P. tolaasii* and *Pseudomonas "reactans"* are streaked several millimeters apart, a white precipitate is recognized within 24 to 48 h incubation at 25°C.
- On King's B agar, bacterial patches of strains to be tested (3 µl of 1/10-diluted overnight LB cultures) were placed at a distance of 1 cm from each other, on a line located between lines of *P. tolaasii* LMG 2342^T (or *P. tolaasii* LMG 6641) and "*P. reactans*" LMG 5329 patches.
- No WLA was detected when another mushroom pathogen, *Pseudomonas agarici*, causal agent of drippy gill disease on *Agaricus bisporus* was tested against all studied strains including *P. tolaasii* and *P. reactans*.
- *P. fluorescens* CFBP 2102, which was included in this study as a negative control, never produced a WLA when confronted to the different tester strains.

Munsch *et al.*,2000; Munsch and Alatossava,2002

WLA(White-line-in-agar test)

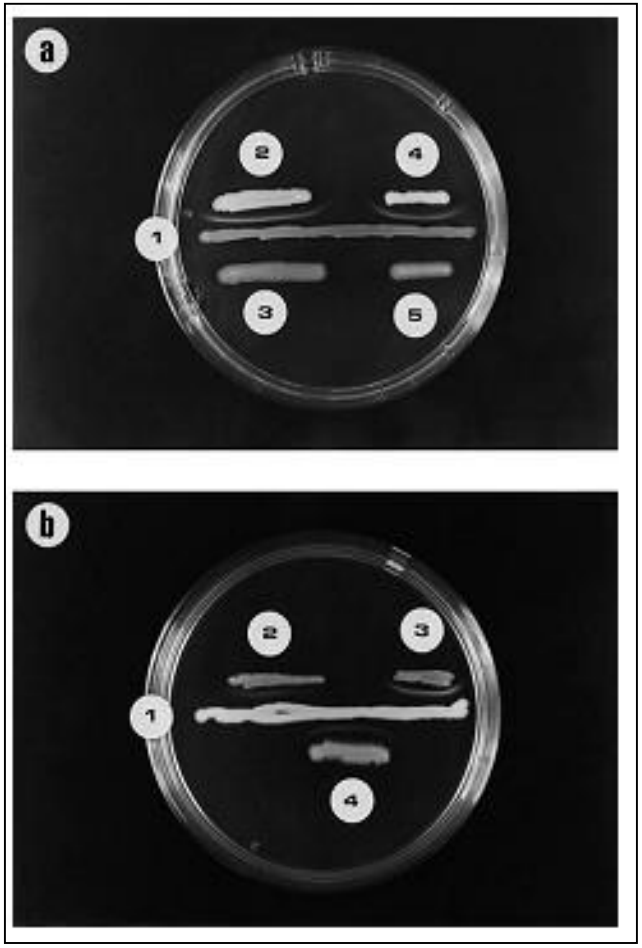
Diagnostic trait of *P. tolaasii*

- **a:**

1. *P. reactans* LMG 5329 used as tester/refernce strain;
2. *P. tolaasii* LMG 2342T;
3. Non-pathogenic form of *P. tolaasii* LMG 2342T;
4. *P. costantinii*
5. Non-pathogenic form of *P. costantinii*.

- **b:**

1. *P. tolaasii* LMG 2342;
2. *P. reactans* LMG 5329;
3. *P. gingeri* NCPPB 3146;
4. *P. aeruginosa* NCPPB 2195.



Munsch and Alatossava,2002

White-line-in-agar test

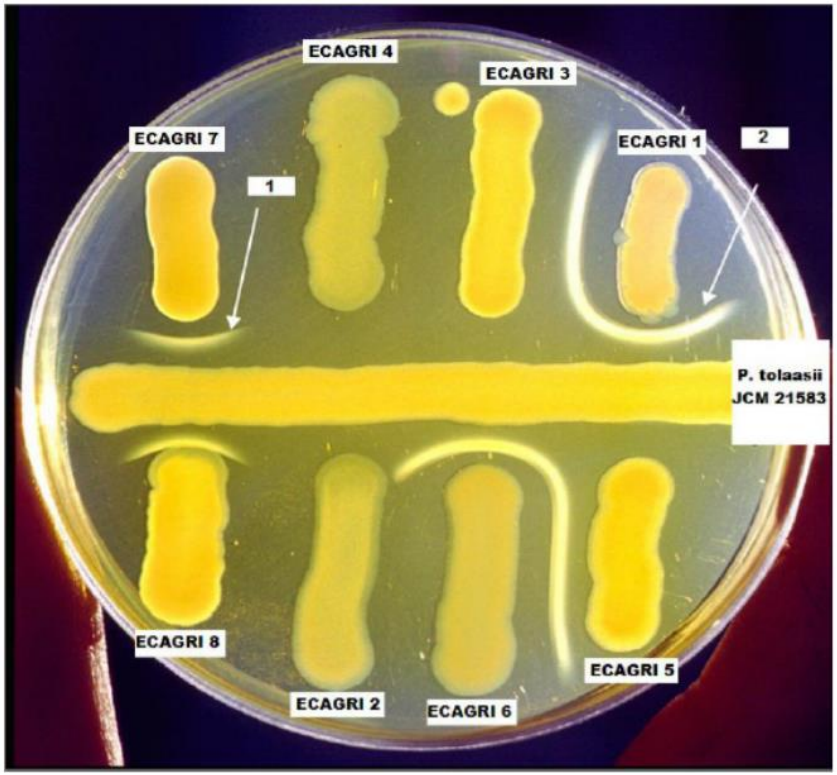
WLA test

- The identity of *P. tolaasii* strains can be confirmed by this test, in which test strains are cultured on solid media beside a strain of *P. reactans*.

White-line-in-agar test

WLA test

- White line assay (WLA) on King's B medium.
- he horizontal streak corresponds to *P. tolasii* reference strain (JCM21583) whereas the eight vertical bacterial colonies represent isolates proposed to be *P. "reactans*; 1 = weak white line reaction (WLA +) by *P. gingeri* (ECAGRI 7 and ECAGRI 8); 2 = strong white line reaction (WLA ++) by *P." reactans"* (ECAGRI 1 and ECAGRI 6).



Abou-Zeid,2012

Lipopeptide detection using thin layerchromatography (TLC)

Complementary test for WLA test

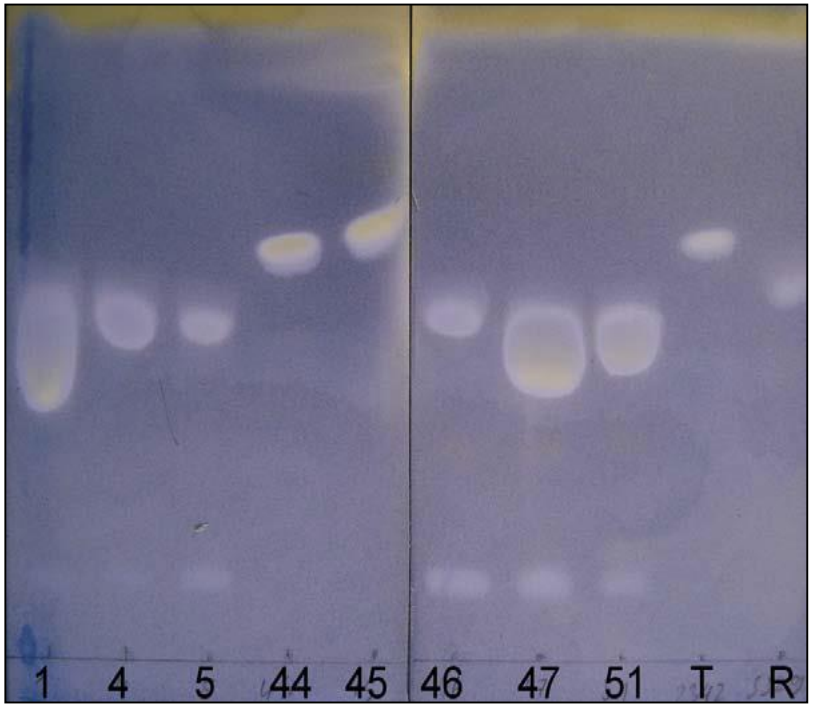
- To investigate the presence of the lipopeptides (complex lipopeptide production including tolaasin) in YE-P ferment broths which contained 20 g (wet weight) smashed oyster mushroom and 2 g yeast extract powder l^{-1}, we used 5 days old cultures incubated at 25°C on a rotary shaker at 160 rev/min.
- 4 µl of cell-free, ethyl acetate extracts were spotted onto silica gel plates.
- Metabolites were separated using a mobile phase of chloroform-methanol-NH_4OH (5 N) in a ratio of 80:25:4.
- Lipopeptides were visualized by staining with bromothymol blue (0.1% (wt vol^{-1}) in 10% (vol vol^{-1}) ethanol), followed by brief heating, according to the method, described by Berti *et al.*,2007.

Sajben *et al.*,2011

Lipopeptide detection using thin layerchromatography (TLC)

Complementary test for WLA test

- Complex lipopeptide production (including tolaasin) was examined with thin layer chromatography.
- Silica gel TLC separation of the ethyl acetate extractions, visualized with bromothymol blue staining, the white dots indicating a lipid-containing metabolites.
- The 1, 4, 5, 46, 47, 51 isolates shows similarity to the, R, that is *Ps.* "*reactans*" (LMG 5329) and these strains gives positive WLA reaction against *Ps. tolaasii* (LMG 2342T) also.
- The 44 and 45 are our *Ps. tolaasii* isolates, that shows similarity to T which is *Ps. tolaasii* (LMG 2342T) and gives positive WLA reaction against *Ps.* "*reactans*".



Sajben *et al.*,2011

The LOPAT determinative tests

LOPAT Tests(Lelliott *et al.*,1966)

- L, levan production;
- O, oxidase production;
- P, pectinolytic activity;
- A, arginine dihydrolase production;
- T, tobacco hypersensitivity.
- These tests are widely applied to differentiate the phytopathogenic fluorescent Pseudomonads.

LOPAT Test

After Lelliott *et al.*,1966

Test	Levan	Oxidase	Potato rot	Arginine dihydrolase	Tobacco hypersensitive reaction
P. syringae	+	-	-	-	+
P. viridiflava	+/-	-	+	-	+
P. cichorii	-	+	-	-	+
P. marginalis	+/-	+	+	+	-
P. fluorescens	+/-	-	-	+	-
P. tolaasii	-	+	-	+	+

LOPAT Test

After Lelliott *et al.*,1966

LOPAT group	Levan formation	Oxidase reaction	Pectolytic capability	Arginine dihydrolase	Tobacco hypersensitivity	Species
Ia	+	-	-	-	+	*P. syringae*
Ib	-	-	-	-	+	*P. syringae* pv. *savanastoi* *P. delphini*
II	-/+	-	+	-	+	*P. viridiflava*
III	-	+	-	-	+	*P. cichorii*
IVa	+	+	+	+	-	*P. marginalis*
IVb	-	+	+	+	-	*P. fluorescens*
Va	-	+	-	+	-	*P. tolaasii*; saprophytic pseudomonads
Vb	+	+	-	+	-	*P. fluorescens*; saprophytic pseudomonads

Starch is negative for *P. syringae*.

Bultreys and Kaluzna,2010

LOPAT Test
After Lelliott *et al.*,1966

Test	PHB[a]	Levan	Oxidase	Pectolytic activity	Arginine dihydrolase	fluorescent pigment	Growth at 37°C
P. syringae	-	+[b]	-	-	-	+	-
P. savastanoi	-	-	-	V[c]	-	+	+
P. viridiflava	-	-	-	+	-	+	-
P. cichorii	-	-	+	-	-	+	-
P. marginalis	-	+	+	+	+	+	-
P. corrugata	+	+	+	-	-	-	+
P. agarici	-	-	+	-	-	+	-

+, 80% or more strains positive; -, 80% or more strains negative
a Poly β hydroxybutyrate
b pathovars *delphinii*, *papulans* and *passiflorae* are negative
c between 21 – 79% of strains positive
d information based on Braun-Kiewnick and Sands,2001 and Smith *et al.*,1988.

Höfte and De Vos,2006

Grouping of green-fluorescent Pseudomonads by LOPAT scheme

Ia-Vb groups

Group	Presumptive (LOPAT) characters: Levan type colonies (6.2.17)	Oxidase reaction (6.6.2)	Potato rot (5.3.6) or pectate gel liquefaction	Arginine dihydrolase (6.4.3)	Tobacco hyper-sensitivity (5.2.1)	Confirmatory characters: 2-keto gluconate production (6.3.6)	Egg yolk reaction* (6.5.1)	Nitrate reduction (6.4.1)	Acid from sucrose (6.3.3)	Species
Ia	+	–	–	–	+	–	–	–	–	*P. syringae*
Ib	–	–	–	–	+	–	–		+	*P. s.* subsp. *savanastoi*; *P. delphini*
II			+		+	–	–	–	–	*P. viridiflava*
III		+			+	–	–	d	–	*P. cichorii*; *P. agarici*
IVa	+	+	+	+	–	+	+	+	+	*P. marginalis* (pectolytic *P. fluorescens*)
IVb	–	+	+	+	–	+	d	d	d	*P. fluorescens*
Va	–	+	–	+	–	–	+	–	–	*P. tolaasii*, and some saprophytic pseudomonads
Vb	+	+	–	+	–	+	d	d	d	*P. fluorescens* and some other saprophytic pseudomonads

d = result varies with isolate.

*Although Lelliott *et al.* (1966) originally used the lipolysis of margarine as one of the confirmatory characters, difficulties can arise due to delayed positives or delayed recognition of positives. As the egg yolk reaction correlated very closely with the margarine lipolysis test and there was generally no difficulty in interpretation of the results, the egg yolk can replace the lipase test as a confirmatory character.

Lelliott and Stead,1987

Differential LOPAT tests of *Pseudomonas* spp.

- **Growth on hypersucrose medium(levan test)**

A. *P . viridiflava* (negative control);

B. *P . syringae* pv. *syringae* (positive control);

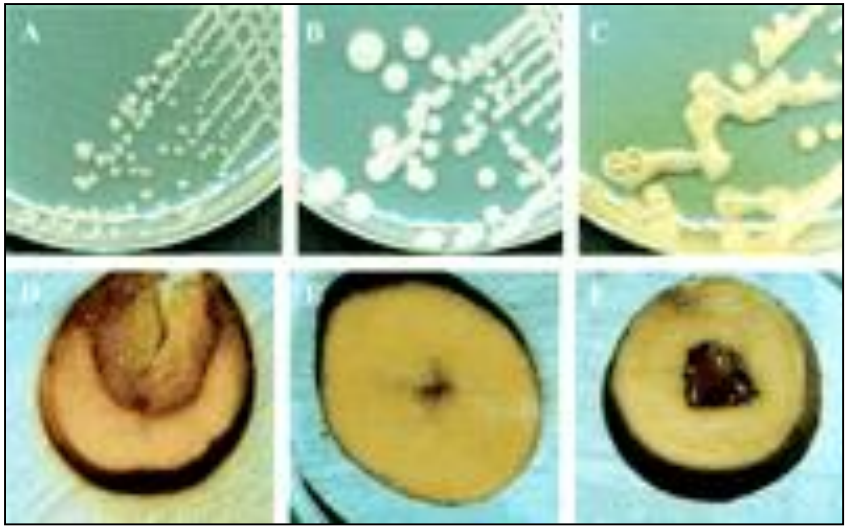
C. Atypical *P. viridiflava*

- **Pectinolytic activity on potato slices (D to F)**

D. *P . viridiflava* (positive control);

E. *P . syringae* pv. *syringae* CECT 4429 (negative control);

F. Atypical *P. viridiflava*.



González *et al.*,2003

Differential LOPAT tests of *Pseudomonas* spp.

- Differential LOPAT tests of *Pseudomonas* isolates:

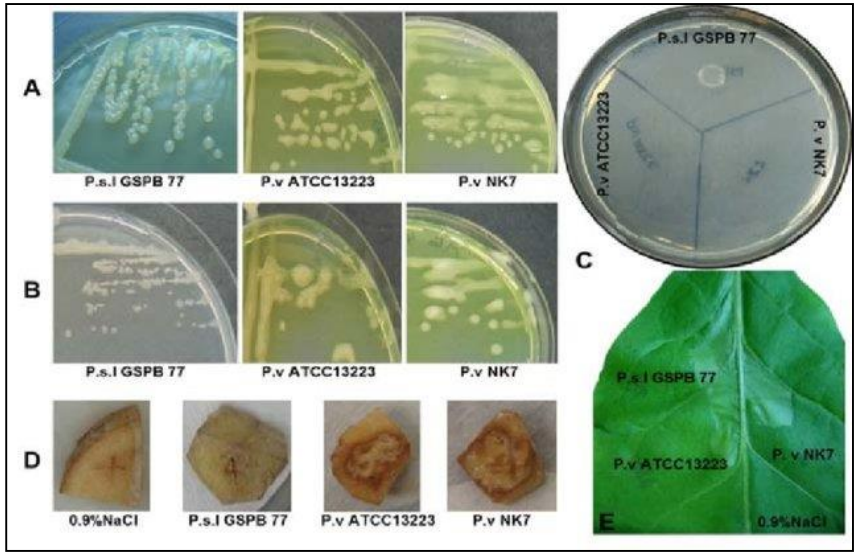
A. Growth on MG (Mannitol-Glutamate) medium supplemented with 5% sucrose(levan);

B. Growth on MG medium;

C. Cell free supernatant on MG medium supplemented with 5% sucrose;

D. Pectinolytic activity on potato slices and

E. hypersensitive response on tobacco plant.



Mannitol-Glutamate medium as minimal medium (10 g mannitol, 2 g L-Glutamic acid, 0.5 g KH_2PO_4, 0.2 g NaCl, 0.2 g $MgSO_4$x $7H_2O$, 15 g agar for 1 L, pH 7.0.

Al-Karablieh *et al.*,2017

Identification of plant-associated fluorescent *Pseudomonas* species

Suggested test sequence: 1 2 3 4 5 6

Carbon source utilization tests

Group	Species	Fluorescence on KBA[c]	Oxidase	Arginine dihydrolase	Levan	Potato rot	Tobacco hypersensitivity	Nitrate reduction	Gelatin hydrolysis	Glucose	β-Alanine	2-Ketogluconate	Trehalose	Sucrose	D(-) Tartrate	m-Tartrate	Sorbitol	Geraniol	Growth at 41C	Pyocyanin on KAA[c]	Polymer granules
	Pathogens																				
I.	P. syringae pathovars[d]	+	-	-	+[e]	-	+	-	f	+	-	-	-	+k	f	f	f	-	-	-	-
II.	P. viridiflava	+	-	-	-	+	+	-	+	+	-	-	-	-	+g	+	+	-	-	-	-
III.	P. cichorii	+	+	-	-	-	+	v	-	+	-	-	-	-	-g	+	+h	-	-	-	-
	P. agarici	+	+	-	-		-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
IV.	P. marginalis 1	+	+	+	+	+	-	+	+	+	+	+	+	+	v	-i	+	-	-	-	-
	(P. fluorescens biotype II)																				
V.	P. tolaasii	+	+	+	-	-	-j	-	+	+	+	+	v	-	-	+	+	-	-	-	-
	Saprophytes																				
	P. fluorescens biotype I	+	+	+	+			-	+	+	+	+	+	+	-	-	+	-	-	-	-
	" " " II	+	+	+	+			+	+	+	+	+	+	+	v	-	+	-	-	-	-
	" " " III	+	+	+	-			+	+	+	+	+	+	-	-	-	v	-	-	-	-
	" " " IV	+	+	+	+			+	+	+	+	+	+	+	-	-	+	-	-	-	-
	" " miscellaneous	+	+	+	-			-	+	+	+	+	+	v	-	v	v	-	-	-	-
	P. chlororaphis	+	+	+	+			-	+	+	+	+	+	+	-	-	-	-	-	-	-
	P. aureofaciens	+	+	+	+			v	+	+	+	+	+	v	-	-	-	-	-	-	-
	P. putida	+	+	+	-			-	-	+	+	+	-	-	v	v	-	-	-	-	-
	P. aeruginosa	+	+	+	-			+	+	+	+	+	-	-	-	-	-	+	+	+	-

Fahy and Persley,1983

Identification of plant-associated saprophytic fluorescent *Pseudomonas* species

Characteristic	*P. aeruginosa*	*P. fluorescens* biovar I	bv. II	bv. III	bv. IV	bv. V	*P. chlororaphis**	*P. putida* bv. A	bv. B
Diffusible non-fluorescent pigment	+ (blue-green)	-	-	-	-	-	-	-	-
Non-diffusible, non-fluorescent Pigment	-	-	-	-	+ (blue)	-	+ (orange, green)	-	-
Levan	-	+	+	-	+	-	+	-	-
Oxidase	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	+
Pectolytic Activity	ND	-	-	-	-	-	-	-	-
Tobacco HR	$+^D$	-	-	-	-	-	-	-	-
Growth @ 41 C	+	-	-	-	-	-	-	-	-
Growth @ 4 C	-	+	+	+	+	$+^D$	+	$+^D$	+
Nitrate to N_2	+	-	+	+	+	-	+	-	-
Gelatin liquefaction	+	+	+	+	+	-	+	-	-
Growth on:									
L-arabinose	-	+	+	V	+	$+^D$	-	V	+
D-galactose	-	+	+	V	+	V	V	-	V
Trehalose	-	+	+	V	+	V	+	-	-
Saccharate	-	+	+	V	+	V	+	+	+
Butyrate	+	-	V	V	+	V	+	+	+
Valerate	+	V	V	V	-	V	+	+	+
Azalate	+	-	-	V	-	-	-	-	-
Sorbitol	-	+	+	V	+	V	-	-	V
Meso-inositol	-	V	+	V	+	V	+	-	-
Adonitol	-	+	-	V	-	V	-	-	-
Propylene glycol	+	-	+	V	-	V	-	V	+
Ethanol	+	-	+	V	-	V	V	V	V
Geraniol	+	-	-	-	-	-	-	-	-
Testosterone	-	-	-	-	-	-	-	-	+
Phenyl acetate	-	-	-	V	-	V	V	v	+
Butylamine	-	-	-	-	-	V	-	+	+
Nicotinate	-	-	-	-	-	V	-	V	+
Trigonelline	-	V	V	V	-	V	-	V	+
Lecithinase	-	+	$+^D$	+	+	$+^D$	$+^D$	-	-

+, 80% or more strains positive; $+^D$, 80% or more strains delayed positive; V, between 21-79% of strains positive; -, 80% or more strains negative; ND, not determined.

* The species *P. chlororaphis* now includes *P. aureofaciens*, which was formerly considered to be a separate species (31).

[a] Modified after Hildebrand et al., 1988 (24) and Holt et al., 1994 (27).

Schaad *et al.*,2001

Identification of fluorescent *Pseudomonas* species

Characteristic	*P.marginalis*	*P. tolaasii*	*P. agarici*	*P. cichorii*	*P. viridiflava*	*P. savastanoi*	*P. syringae*	*P. fuscovaginae*	*P. corrugata*
Diffusable fluroescent pigment	+	+	+	+	+	+	+	+	-
Non-diffusible pigment	-	-	-	-	V (blue-green)	-	-	-	-
Levan	+	-	-	-	-	-	+[b]	-	-
Oxidase	+	+	+	+	-	-	-	+	+
Arginine dihydrolase	+	-	-	-	-	-	-	+	+
Pectolytic Activity	+	-	-	-	+	V	-	ND	-
Tobacco HR	V	-	+	+	+	+	+	-	ND
Growth at 37°C	-	-	-	-	-	+	-	-	+
Nitrate to N_2	-	-	-	-	-	+	-	ND	+
PHB[c]	-	-	-	-	-	-	-	-	+
Gelatin hydrolysis	ND	ND	ND	-	+	-	V	ND	+
Utilization of:						+			
2-ketogluconate	+	+	+	+	+	+	+	-	ND
Mannitol	+	+	+	+	+	-	V	ND	+
Geraniol	-	-	-	-	-	-	-	ND	ND
Benzoate	-	-	+	-	-	-	-	ND	-
Cellobiose	-	-	-	-	-	+	-	ND	-
Sorbitol	+	+	-	-	+	-	+	ND	-
Trehalose	+	V	-	-	-	+	-	ND	+
Sucrose	+	-	-	-	-	-	+	+	+
meso-tartrate	V	+	-	+	+	-	+	ND	ND
D(-)-tartrate	V	-	-	-	+	-	-	ND	V
D-arabinose	-	-	-	-	-	-	-	ND	+
L-rhamnose	V	ND	-	-	-	-	-	ND	-
D-aspartate	-	ND	ND	+	-	-	-	ND	ND
Ice nucleation	-	+	+	-	-	-	+	-	-
IAA production	-	-	-	-	-	+	-	-	-

+, 80% or more strains positive; -, 80% or more strains negative; V, between 21-79% of strains positive; ND, not determined.

[a] Modified after Sands et al., 1970 (57), Hildebrand et al., 1988 (24); Holt et al., 1994 (27); and Young & Triggs, 1994 (74).

[b] Pathovars *delphinii*, *populans*, and *passiflorae* are negative.

[c] Poly β hydroxybutyrate (PHB)

Pseudomonas corrugata is non-fluorescent sp.

Schaad *et al.*,2001

Identification of fluorescent *Pseudomonas* spp.
Carbon source utilization

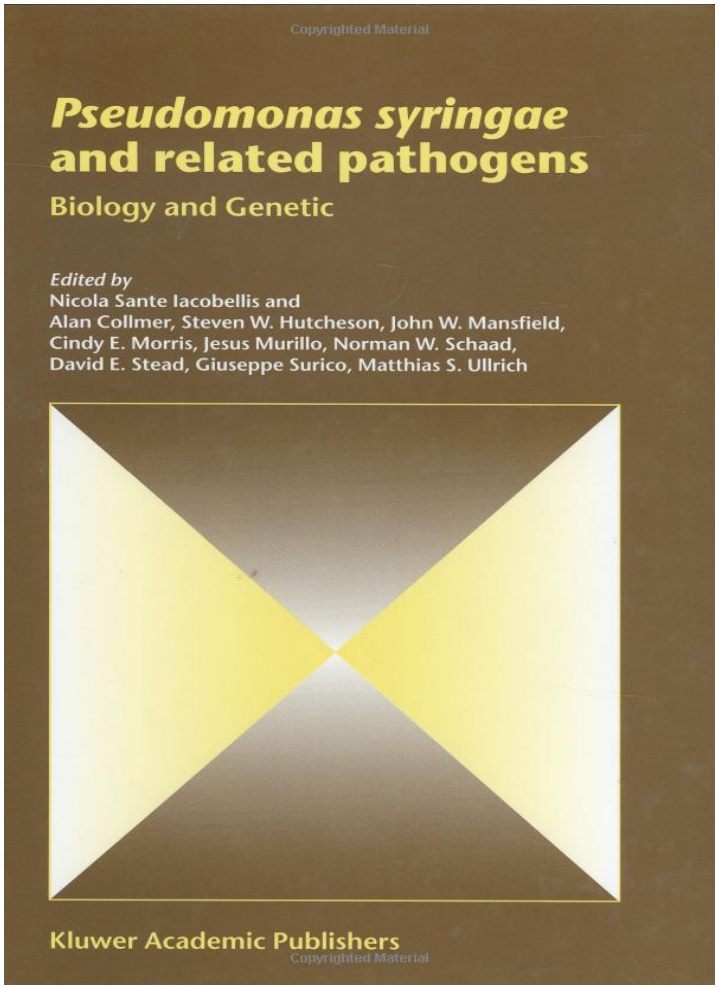
Utilisation of	*P. marginalis*	*P. tolaasii*	*P. agarici*	*P. cichorii*	*P. viridiflava*	*P. syringae* *P. savastanoi* pathovars
Mannitol	+	+	+	+	+	V
Benzoate	–	–	+	–	–	–
Cellobiose	–	–	–	–	–	–
Sorbitol	+	+	–	–	+	V
Trehalose	+	V	–	–	–	–
Sucrose	+	–	–	–	–	V
Meso-tatrate	V	+	–	+	+	V
D-tatrate	V	–	–	–	+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

V = variable reaction.

Goszczynaska *et al.*,2000

Pseudomonas syringae Pathovars and Related Pathogens

Biology and Genetics

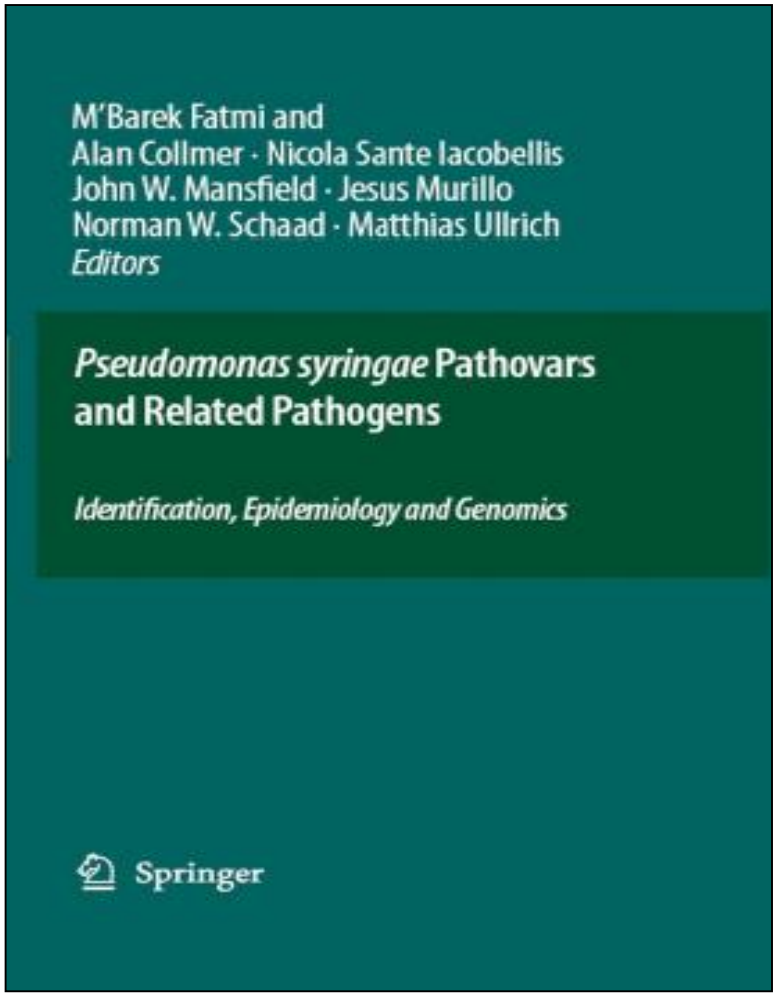


Product Details

- By N. S. Iacobellis, A. Collmer, S. W. Hutcheson, J. W. Mansfield, C. E. Morris, J. Murillo, N. W. Schaad, D. E. Stead, G. Surico, and M. S. Ullrich (Editors).
- Hardcover: 708 pages
- Kluwer Academic Publishers, The Netherlands.
- 2003.

Pseudomonas syringae Pathovars and Related Pathogens

Identification, Epidemiology and Genomics



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- Hardcover: 650 pages;
- Publisher: Springer; 1 edition;
- September 25, 2008.

Taxonomy of *P. syringae*

Genomospecies in *P. syringae* pathovars and related species

1. Based on DNA-DNA hybridization and ribotyping

- Gardan *et al.*,1999, studied a total of 48 pathovars of *P. syringae* and 8 related species by:

1. DNA-DNA hybridization and
2. Ribotyping and

- Proposed the delineation of 9 discrete genomospecies.

Group of strains that are phenotypically similar but genotypically different have been referred to as "genospecies", "genomospecies," or "genomic species".
It is a group of strains with high(ca 70% or greater) DNA-DNA hybridization values.

Taxonomy of *P. syringae*

Genomospecies in *P. syringae* pathovars and related species

Based on DNA-DNA hybridization and ribotyping

- Genomospecies 1: *P. syringae*, *P.s.* pv. *aptata*, *P.s.* pv. *lapsa*, *P.s.* pv. *papulans*, *P.s.* pv. *pisi*, *P.s.* pv. *atrofaciens*, *P.s.* pv. *aceris*, *P.s.* pv. *panici*, *P.s.* pv. *dysoxyli*, *P.s.* pv. *japonica*
- Genomospecies 2: *P. savastanoi*, *P. ficuserectae*, *P. meliae*, *P. amygdali*, *P.s.* pv. *phaseolicola*, *P. s.* pv. *ulmi*, *P.s.* pv. *mori*, *P. s.* pv.*lachrymans*, *P.s.* pv. *sesami*, *P.s.* pv. *tabaci*, *P.s.* pv. *morsprunorum*, *P.s.* pv. *glycinea*, *P.s.* pv. *ciccaronei*, *P. s.* pv. *eriobotryae*, *P.s.* pv. *mellea*, *P.s.* pv. *aesculi*, *P.s.* pv. *hibisci*, *P.s.* pv. *myricae*, *P.s.* pv. *photiniae*, *P.s.* pv. *dendropanacis*
- Genomospecies 3: *P. s.* pv. *tomato*, *P.s.* pv. *persicae*, *P.s.* pv. *antirrhini*, *P.s.* pv. *maculicola*, *P.s.* pv. *viburni*, *P.s.* pv. *berberidi*, *P.s.* pv. *apii*, *P.s.* pv. *delphinii*, *P.s.* pv. *passiflorae*, *P.s.* pv. *philadelphi*, *P.s.* pv. *ribicola*, *P.s.* pv. *primulae*
- Genomospecies 4: *P. coronafaciens*, *P.s.* pv. *porri*, *P.s.* pv. *garcae*, *P.s.* pv. *striafaciens*, *P.s.* pv. *atropurpurea*, *P. s.* pv. *oryzae*, *P. s.* pv. *zizaniae*
- Genomospecies 5: *P. tremae*
- Genomospecies 6: *P. viridiflava*
- Genomospecies 7: *P. s.* pv. *tagetis*, *P.s.* pv. *helianthi*
- Genomospecies 8: *P.s.* pv. *theae*, *P. avellanae*, *P. s.* pv. *actinidiae*
- Genomospecies 9: *P. cannabina*

Höfte & De Vos,2006

Taxonomy of *P. syringae*

Nomenspecies in *P. syringae* pathovars and related species

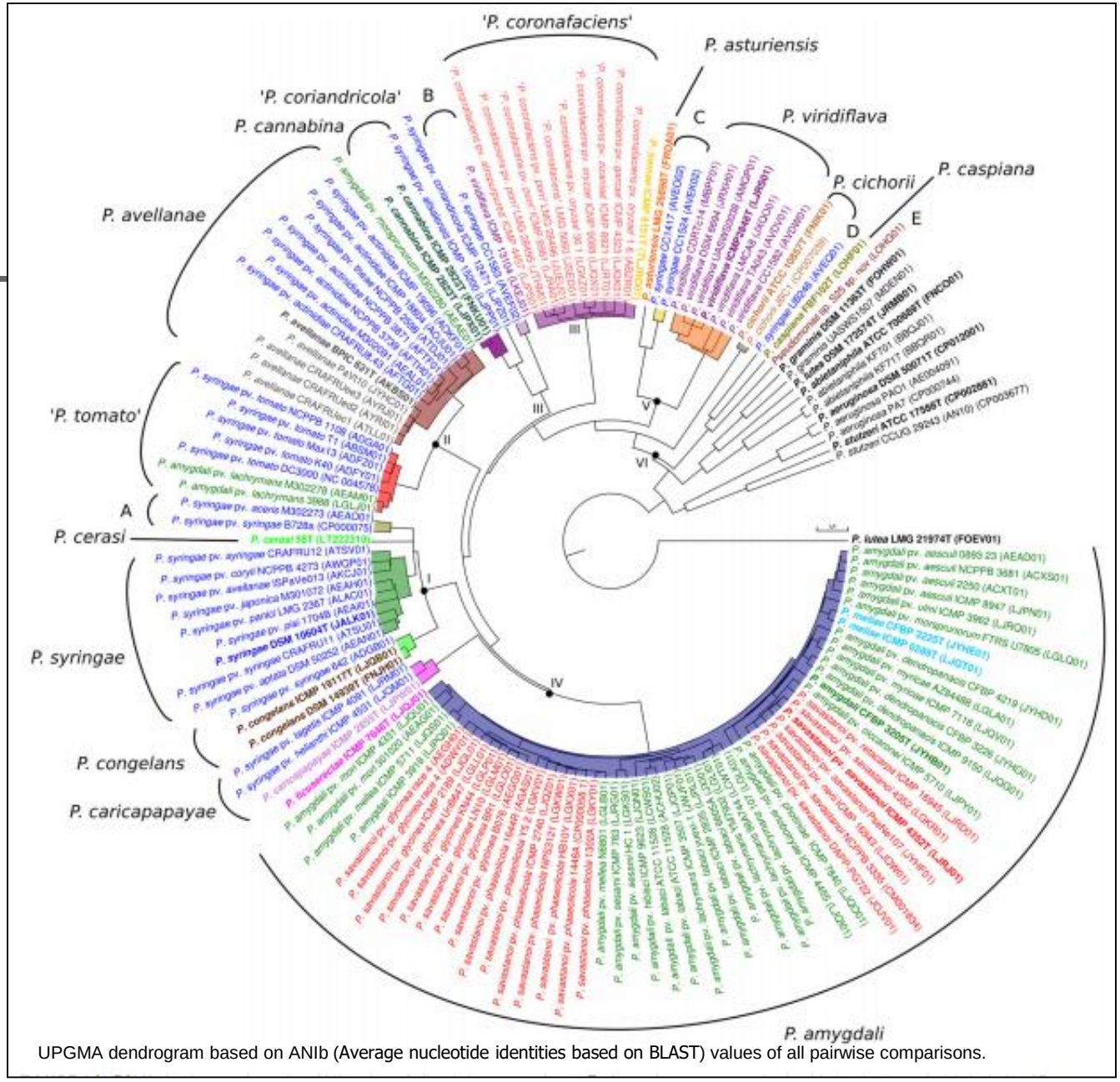
2. Based on complete genome sequences

- *Pseudomonas syringae* phylogenetic group includes 11 recognized nomenspecies (recognized species):

1. *P. amygdali*,
2. *P. asturiensis*,
3. *P. avellanae*,
4. *P. cannabina*,
5. *P. caricapapayae*,
6. *P. caspiana*,
7. *P. cerasi*,
8. *P. cichorii*,
9. *P. congelans*,
10. *P. syringae*, and
11. *P. viridiflava*.

Nomenspecies as a group that bears a binomial name.
Bacterial species can be grouped in different nomenspecies and genomospecies. E.g. based on partial (468 bp) rpoB gene sequences, *Acinetobacter* species isolated from humans were grouped in 10 nomenspecies and 4 genomospecies.

Gomila *et al.*,2017



UPGMA dendrogram based on ANIb (Average nucleotide identities based on BLAST) values of all pairwise comparisons.

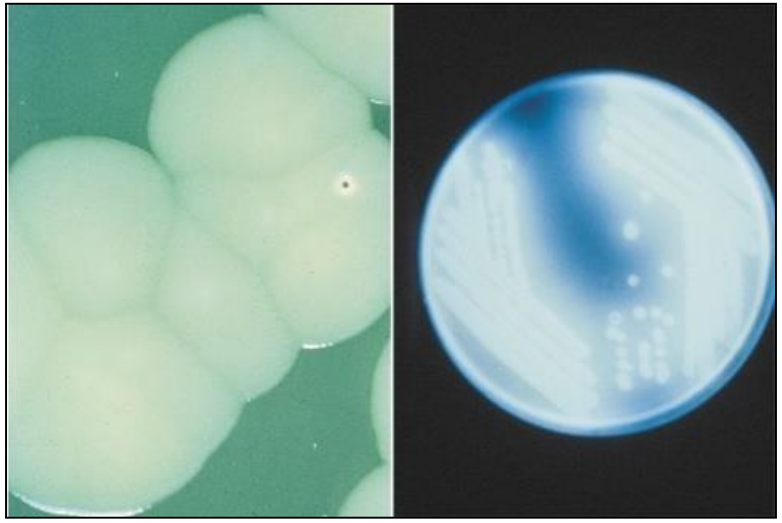
Gomila *et al.*,2017

The species *P. syringae*

- The proteobacterium ***Pseudomonas syringae*** van Hall 1902 is the causal agent of a variety of bacterial spot, speck, and blight diseases on a wide range of plant hosts, including(but not limited to):
- apples, beets, beans, cabbage, cucumbers, flowers, oats, olives, peas, tobacco, tomato, onion and rice.
- However, within the species, there is a great deal of specialization with respect to plants with which individual strains are likely to interact.
- The pathovar concept was introduced in order to distinguish among bacteria within the species that exhibit different pathogenic abilities.

The species *P. syringae*
A fluorescent pseudomonads

- Colonies of *Pseudomonas syringae* pv. *syringae* on King *et al.* medium B(KB), developed for the production and detection of specific fluorescent pigments (siderophore pyoverdin)
- The colonies are illuminated in:
- Visible light (left),
- Ultraviolet light (right).



All fluorescent pseudomonads fluoresce in short wavelength (254 nm) ultraviolet light. These also fluoresce in long wave length (366 nm) ultraviolet light.

Courtesy Schaad and Sands

The species *P. syringae*

Survival mechanisms

Rain Making Bacteria

- *P. syringae* like many other bacterial pathogens have developed diverse survival mechanisms.
- Usually it survives in association with the host plant and propagative material from the host plant.
- There is little evidence to suggest that these bacteria survive in soil.
- They may, however, survive in soil in association with residues of diseased plants, having some capacity to colonise root systems (both host and non-host plant).

Lindow (1984) to conclude that the effective size of a bacterial ice nucleus varied from 620 kDa (for a nucleus whose threshold was at -9°C) to 19000 kDa (for one with a threshold at -2°C).

The species *P. syringae*

Survival mechanisms

Rain Making Bacteria

- Most of the *P. syringae* group appears to have the capacity to survive as epiphytes on protected parts of healthy leaves, in the buds of the host, and even on non-host plants.
- Recently a comprehensive life cycle for *P. syringae* in agricultural and non-agricultural habitats was proved.
- The ice-nucleation activity of all strains from snow, unlike from other substrates, strongly suggests that *P. syringae* plays an active role in the water cycle as an ice nucleus in clouds (Sands *et al.*,2008).

The species *P. syringae*

Survival mechanisms

Rain Making Bacteria

- The remaining ice nucleation activity from these microbes can seed cloud condensation and formation, as observed in controlled atmospheric chambers.
- Acting as ice forming nuclei, these bacteria also catalyze ice crystal formation in clouds leading to precipitation.
- Consequently, ice active bacteria have been found to be more prevalent in rain than in surrounding air.

Lorv *et al.*,2014

Characteristics of the species

Pathogenic strains of *P. syringae*

- *P. syringae* is easily identified as:
- A Gram-negative strict aerobe in the subclass of the Proteobacteria which is rod-shaped, with polar flagella.
- With few exceptions (*morsprunorum*, *persicae* and *avii*) all pathovars produce fluorescent pigments.
- According to Lopez *et al.*,2010, some strains of *Psm* produce a fluorescent pigment on King's B under UV light.
- It is oxidase and arginine dihydrolase negative (phenotypes that distinguish it from most of the other fluorescent pseudomonads), and does not rot potato (which distinguishes it from *Pseudomonas viridiflava*).

Characteristics of the species
Syringomycin production

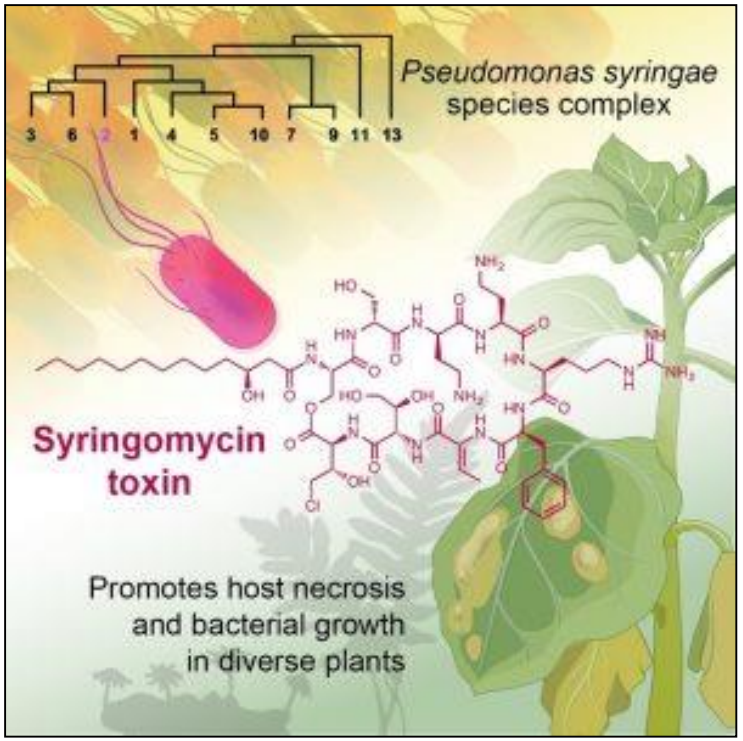
- Genetic determinants for the biosynthesis and secretion of the toxin syringomycin were found to be present in most strains of *P. syringae* pv. *syringae*, including many nonpathogenic strains of this species.
- Nonpathogenic strains of *P. syringae* may synthesize syringomycin in amounts that are insufficient to cause cell necrosis and disease but are high enough to trigger a low-level release of plant metabolites.

The antagonist bacterium, *Pseudomonas syringae* is a saprophytic strain which was originally isolated from an apple leaf and can be frequently isolated from apple fruit. *P. syringae* ESC-11 is sold under the name BioSave™ 110 and is recommended for the control of postharvest decays of pear and apple.

Lindow and Brandl,2003

Characteristics of the species
Syringomycin production

- The toxin syringomycin produced by the most widely infectious *P. syringae* strains, and compared its effect on both non-flowering and flowering plants.
- The toxin syringomycin likely interferes with cell membranes across each of the diverse plants.
- A necrotizing toxin enables *Pseudomonas syringae* infection across evolutionarily divergent plants appears in Cell Host and Microbes.

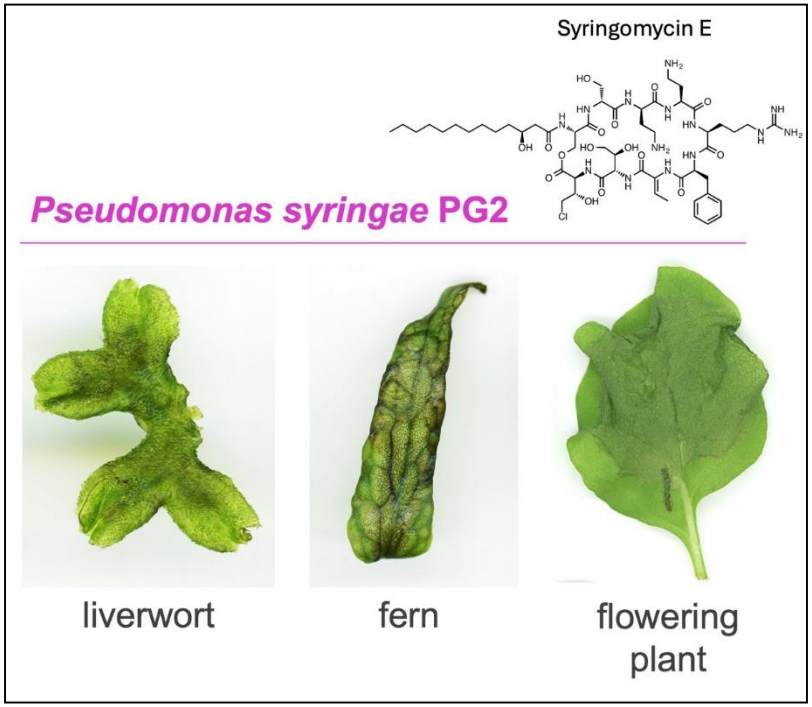


Evolutionary study reveals the toxic reach of disease-causing bacteria across the Plant Kingdom.

Characteristics of the species

Syringomycin production

- Three model plants showing disease symptoms after infection with *Pseudomonas syringae*.
- The toxin Sringomycin shown is critical to establish disease symptoms.



The pathovars of *P. syringae*
Host ranges

- Most of the pathovars exhibit narrow host ranges. e.g. *P. syringae* pv. *tabaci* or *P. s.* pv. *phaseolicola*.
- On the other hand, *P. s.* pv. *pisi* has a single host genus.
- The major exception is *P. syringae* pv. *syringae.*
- *P. syringae* pv. *syringae* infects more than 80 plant species.
- It is believed that structure and the pathogenic populations of this pathovar (pv. *syringae*) is complex and not well understood (Young,2010).

The pathovars of *P. syringae*

Host ranges

- From published data, it is not clear whether pv. *syringae* is a repository for strains that may in actuality have quite limited host ranges.
- *P. syringae* pv. *syringae* R32 have pili that function as adhesions anchoring the cell to the surface of plants, thereby enhancing epiphytic colonization (OECD,1997).
- A single plant species may serve as host for strains within two (or more) different pathovars, one of which is invariably pv. *syringae*.

Taxonomy of *P. syringae*

The *P. syringae* complex

Multilocus sequence analyses (MLSA): *gyrB* and *rpoD* genes

- Because 16S rDNA is so highly conserved, no discrimination within the group was achieved.
- A study of *Pseudomonas* by Yamamoto *et al.*,2000 using concatenated *gyrB* and *rpoD* sequences demonstrated the grouping of a few species of the *P. syringae* complex in accord with the work of Gardan *et al.*,1999.
- The genes *gyrB* and *rpoD* also gave a good support to genomospecies structure of the complex.

Group of strains that are phenotypically similar but genotypically different have been referred to as "genomospecies.

Young,2010

Taxonomy of Pseudomonads

Fluorescent Pseudomonads

The *P. syringae* complex

P. amygdali (non-fluorescent)
P. avellanae
P. cannabina
P. caricapapayae
P. ficuserectae
P. tremae
P. viridiflava
P. syringae
P. syr. pv. *aceris*
P. syr. pv. *actinidiae*
P. syr. pv. *aesculi*
P. syr. pv. *alisalensis*
P. syr. pv. *antirrhini*
P. syr. pv. *apii*
P. syr. pv. *aptata*
P. syr. pv. *atrofaciens*
P. syr. pv. *atropurpurea*
P. syr. pv. *berberidis*
P. syr. pv. *broussonetiae*
P. syr. pv. *castaneae*
P. syr. pv. *cerasicola*
P. syr. pv. *ciccaronei*
P. syr. pv. *coriandricola*
P. syr. pv. *coronafaciens*
P. syr. pv. *coryli*
P. syr. pv. *cunninghamiae*
P. syr. pv. *daphniphylli*
P. syr. pv. *delphinii*
P. syr. pv. *dendropanacis*
P. syr. pv. *eriobotryae*
P. syr. pv. *fraxini*
P. syr. pv. *garcae*
P. syr. pv. *glycinea*
P. syr. pv. *helianthi*
P. syr. pv. *hibisci*
P. syr. pv. *lachrymans*
P. syr. pv. *lapsa*
P. syr. pv. *maculicola*
P. syr. pv. *mellea*
P. syr. pv. *mori*
P. syr. pv. *morsprunorum*
P. syr. pv. *myricae*
P. syr. pv. *nerii*
P. syr. pv. *oryzae*
P. syr. pv. *papulans*
P. syr. pv. *passiflorae*
P. syr. pv. *persicae*
P. syr. pv. *phaseolicola*
P. syr. pv. *philadelphi*
P. syr. pv. *photiniae*
P. syr. pv. *pisi*
P. syr. pv. *porri*
P. syr. pv. *primulae*
P. syr. pv. *raphiolepidis*
P. syr. pv. *retacarpa*
P. syr. pv. *ribicola*
P. syr. pv. *savastanoi*
P. syr. pv. *sesami*
P. syr. pv. *solidagae*
P. syr. pv. *spinaceae*
P. syr. pv. *striafaciens*
P. syr. pv. *syringae*
P. syr. pv. *tabaci*
P. syr. pv. *tagetis*
P. syr. pv. *theae*
P. syr. pv. *tomato*
P. syr. pv. *ulmi*
P. syr. pv. *viburni*
P. syr. pv. *zizaniae*

Young,2010

Diseases caused by *P. syringae* pathovars

Pseudomonas syringae* pv. *aceris	**Leaf spot of maple (*Acer macrophyllum*)**
Pseudomonas syringae* pv. *actinidiae	**Bacterial canker of kiwifruit**
Pseudomonas syringae* pv. *aesculi	**Leaf blight of horse chestnut (*Aesculus indica*)**
Pseudomonas syringae* pv. *alliifistulosi	**Bacterial leaf spot of onions**
P. syringae* pv. *antirrhini	**Leaf spot & stem lesions on *Antirrhinum majus***
Pseudomonas syringae* pv. *apii	**Leaf spotting and necrosis of celery & leek**
Pseudomonas syringae* pv. *aptata	**Bacterial blight of beets (*Beta vulgaris*); Leaf spot of sunflower**
Pseudomonas syringae* pv. *atrofaciens	**Leaf spot, Basal glume rot of cereals (e.g. wheat glumes)**
Pseudomonas syringae* pv. *atropurpurea	**Halo blight of wheat, ryegrass and other plants of the family Gramineae**
Pseudomonas syringae* pv. *avii	**Bacterial canker of wild cherries (*Prunus avium*)**
Pseudomonas syringae* pv. *berberidis	**Leaf spotting of *Berberis* spp.**
Pseudomonas syringae* pv. *broussonetiae	**Bacterial blight of paper mulberry**
Pseudomonas syringae* pv. *castaneae	**Blight & canker of chestnut (*castanea crenata*). Forgotten disease**
Pseudomonas syringae* pv. *cerasicola	**Bacterial gall of cherry tree**

Diseases caused by *P. syringae* pathovars

Pseudomonas syringae* pv. *ciccaronei	**Leaf spot of carob tree (*Ceratonia siliqua*)**
Pseudomonas syringae* pv. *coriandricola	**Bacterial umbel blight and seed decay of coriander**
Pseudomonas syringae* pv. *coronafaciens	**Bacterial halo blight of cereals (oats)**
Pseudomonas syringae* pv. *coryli	**Bacterial twig dieback of hazelnut**
Pseudomonas syringae* pv. *cunninghamiae	**Bacterial needle blight of Chinese fir (*Cunninghamia lanceolate*)**
Pseudomonas syringae* pv. *daphniphylli	**Bacterial gall diseases of himeyuzuriha (*Daphniphyllum teijsmanni*)**
Pseudomonas syringae* pv. *delphinii	**Black leaf spot of *Delphinium* spp.**
Pseudomonas syringae* pv. *dendropanacis	**Bacterial gall of *Dendropanx trifidus***
Pseudomonas syringae* pv. *dysoxyli	**Leaf spot, shot hole of kohekohe tree (*Dysoxylum spectabile*)**
Pseudomonas syringae* pv. *eriobotryae	**Bud blight, twig canker Loquat trees (*Eriobotrya japonica*)**
Pseudomonas syringae* pv. *garcae	**Leaf spot& shoot dieback of coffee**
Pseudomonas syringae* pv. *helianthi	**Leaf spot of sunflower (*Helianthus* spp.)**
Pseudomonas syringae* pv. *hibisci	**Bacterial leaf spots of *Hibiscus japonica***

Diseases caused by *P. syringae* pathovars

Pseudomonas syringae* pv. *lachrymans	**Angular leaf and fruit spot of cucumber**
Pseudomonas syringae* pv. *lapsa	**Stalk rot of corn and sugarcane**
Pseudomonas syringae* pv. *maculicola	**Bacterial leaf spot of cauliflower, turnip (crucifers)**
Pseudomonas syringae* pv. *mellea	**Leaf spot of sunflower; Bacterial disease on tobacco plants**
Pseudomonas syringae* pv. *mori	**Leaf spot& blight of mulberry (*Morus* spp.)**
Pseudomonas syringae* pv. *morsprunorum	**Bacterial canker of sour cherry (*Prunes* spp.)**
Pseudomonas syringae* pv. *myricae	**Gall of *Myrica* trees (*Myricae rubra*)**
Pseudomonas syringae* pv. *oryzae	**Bacterial halo blight of rice**
Pseudomonas syringae* pv. *papulans	**Blister spot of apple (*Malus pumila*) &*Pyrus***
Pseudomonas syringae* pv. *passiflorae	**Bacterial grease-spot/necrotic spots of passion fruit tree (*Passiflora edulis*)**
Pseudomonas syringae* pv. *persicae	**Bacterial dieback of peach (*Prunus persicae*)**
Pseudomonas syringae* pv. *philadelphi	**Leaf spot of *Philadelphus* spp.**
Pseudomonas syringae* pv. *photiniae	**Bacterial leaf spot & blight of *Photinia glabra***
Pseudomonas syringae* pv. *pisi	**Bacterial blight of pea (*Pisum*)& *Vicia***

Diseases caused by *P. syringae* pathovars

Pseudomonas syringae* pv. *porri	**Leaf blight of leek and leaf spot of onion (*Allium* spp.)**
Pseudomonas syringae* pv. *primulae	**Leaf spot of *Primula* spp.**
Pseudomonas syringae* pv. *raphiolepidis	**Bacterial gall of sharinbai (*Raphiolepis umbellata*)**
Pseudomonas syringae* pv. *ribicola	**Leaf spot, defoliation of Golden Currant (*Ribes aureum*)**
Pseudomonas syringae* pv. *seasami	**Bacterial leaf spot of sesame plants**
Pseudomonas syringae* pv. *solidagae	**Bacterial leaf spot of tall goldenrod (*Solidago altissima*)**
Pseudomonas syringae* pv. *spinaceae	**Bacterial leaf spot of spinach**
Pseudomonas syringae* pv. *striafaciens	**Leaf striping of oat**
Pseudomonas syringae* pv. *syringae	**Wide host range:** **Leaf spot, blossom blight of lilac, citrus, wheat; brown spot of rice, canker of stone fruits,…**
Pseudomonas syringae* pv. *tabaci	**Wild fire of tobacco**
Pseudomonas syringae* pv. *tagetis	**Apical chlorosis in Canada thistle (*Cirsium arvense*) & sunflower**
Pseudomonas syringae* pv. *theae	**Shoot blight of tea**

Diseases caused by *P. syringae* pathovars

Pseudomonas syringae* pv. *tomato	**Leaf spot of tomato**
Pseudomonas syringae* pv. *ulmi	**Leaf and shoot blight of elm trees (*Ulmus* spp.)**
Pseudomonas syringae* pv. *viburni	**Leaf and stem spot of *Viburnum* spp.**
Pseudomonas syringae* pv. *zizaniae	**Bacterial leaf streak of cultivated wild rice (*Zizania palustris*)**

Pseudomonas syringae pv. *alisalensis* was renamed as *Pseudomonas cannabina* pv. *alisalensis*.

Updated according to Bull *et al*.,2010b and 2012 comprehensive bacterial name lists.

Multilocus sequence typing analysis of strains belonging to the *P. syringae* complex

A. The neighbor-joining tree was constructed with combined partial sequences of *rpoD* and *gyrB* housekeeping genes.
B. Exclusive representation of the phylogenetic group 2.

- Multilocus sequence typing analysis of strains belonging to the *P. syringae* complex.

A. The neighbor-joining tree was constructed with combined partial sequences of *rpoD* and *gyrB* housekeeping genes using MEGA 7 software. 150 strains belonging to the phylogenetic groups 1, 2, 3, 4, 5, 6, 7, and 11 of the *P. syringae* complex are depicted in the circular phylogenetic tree. **Marked in blue** are represented the strains belonging to the phylogenetic group 2, where the *P. syringae* pv. *syringae* strains isolated from mango are found.

B. Exclusive representation of the phylogenetic group 2. Three main groups are defined regarding the presence or not of the *mbo* genes necessary for mangotoxin production by *P. syringae* pv. *syringae* strains isolated from mango.

Gutiérrez-Barranquero,2019

Multilocus sequence typing analysis of strains belonging to the *P. syringae* complex

A. The neighbor-joining tree was constructed with combined partial sequences of *rpoD* and *gyrB* housekeeping genes.
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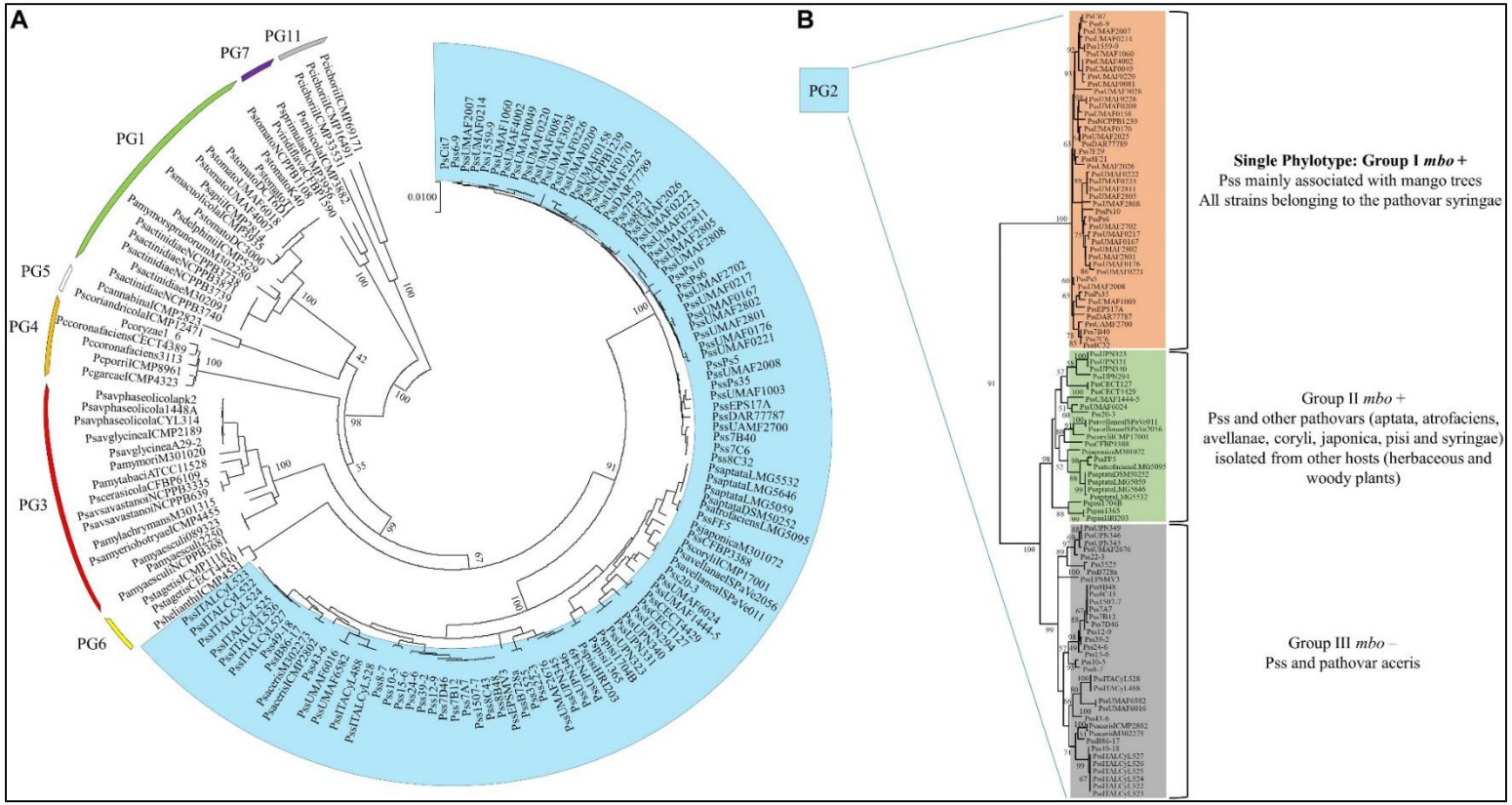


Gutiérrez-Barranquero,2019

Bacterial leaf spot of onions

Pseudomonas syringae pv. *alliifistulosi*

- *Pseudomonas syringae* pv. *alliifistulosi* pv. nov., the causal agent of bacterial leaf spot of onions (BLSO).
- This bacterium was first recorded in Japan by Goto (1972).
- It taxonomically investigated in details in 2012 and 2014.

Tsuji and Takikawa,2018;..

Canker of wild cherry

Pseudomonas syringae pv. *avii*

- Reported from France, 2003.
- Very much related to *Pseudomonas syringae* pv. *syringae* .
- Also pathogenic to cultivated cherry.

J. D. Janse

Canker/dieback on nectarine and peach *Pseudomonas syringae* pv. *persicae*

Pseudomonas syringae pv. persicae (EPPO Quarantine List A2) - Dieback/canker on nectarine and peach

- First observed in France 1967 and proved to be present also in New-Zealand in 1988 where also *Prunus salicina* (Japanese plum) was infected.
- Once isolated in the UK from *P. cerasifera*

Photo's : INRA Luisetti, France

Leaf blight of leek

Pseudomonas syringae pv. *porri*

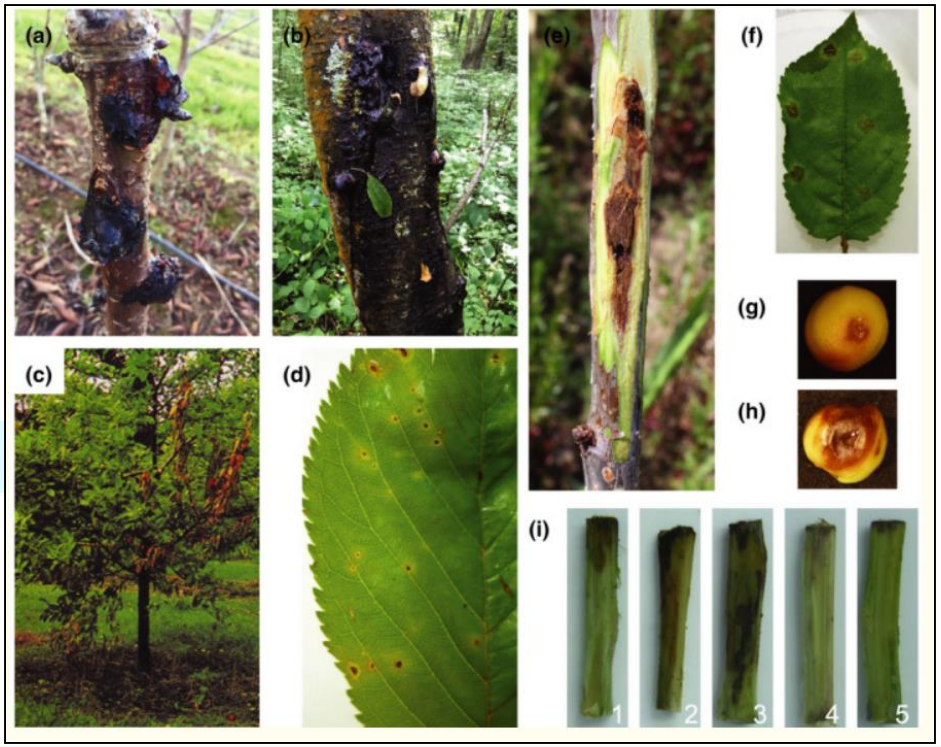
- Symptoms on leek (*Allium* sp.) flower stem.
- Left: Hypersensitive reaction induced by each of 25 pathovars of *Pseudomonas syringae*.
- Right: Typical water soaked lesions induced by *Pseudomonas syringae* pv. *porri*.
- Leek flower stems were inoculated by injecting a suspension of 10^8 bacteria per ml under the epidermis with a syringe.

Samson *et al.*,1998

Leaf blight of cherry

Pseudomonas syringae pv. *syringae*

Hulin *et al.*,2020



(a) Natural infection of cherry cv. Van, (b) Wild cherry infection in a forest in Kent, (c) Dieback of plum, (d) Leaf spots due to natural infection on cherry cv. Napoleon, (e) Artificial inoculation of cherry cv. Van with *Pseudomonas syringae* pv. *syringae* (Pss), picture taken 6 months after inoculation after stripping back the bark, (f) Leaf infiltrated with *P. syringae* pv. *morsprunorum* (Psm) showing symptoms after 7 days, (g) Immature cherry fruit inoculated with Psm, (h) Immature cherry fruit inoculated with Pss, (i) Cut shoot inoculation of Pss on cherry cv. Napoleon, (1–4) and a negative control (10 mM $MgCl_2$).

Apical chlorosis of Canada thistle

Pseudomonas syringae pv.*tagetis*

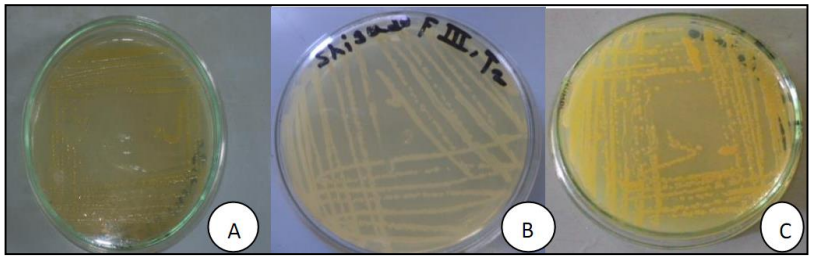
- The causal agent of this pathogen is a soil borne bacteria called *Pseudomonas syringae* pv. *tagetis* (Pst).
- It causes apical chlorosis (white or bleached-out in appearance) in:

1. Canada thistle (*Cirsium arvense*), and
2. Certain other composite weeds due to the production of tagetitoxin, a RNA polymerase III inhibitor that blocks chloroplast biogenesis.

Curran,2001

The bacterial blight of coffee

P. syringae pv. *garcae*



Growth of BBC pathogen on NA and King's B media.

Hinkosa *et al.*,2017

Diseases caused by *P. savastanoi* pathovars

P. savastanoi* pv. *savastanoi	**Olive knot (*Olea europaea*)**
P. savastanoi* pv. *fraxini	**Bacterial wart disease of ash tree (*Fraxinus excelsior*)**
P. sav.* pv. *glycinea	**Bacterial blight of soybean (*Glycine max*)**
P. sav.* pv. *mandevillae	**Leaf and stem spot of dipladenia (*Mandevilla* spp.)**
P. sav.* pv. *nerii	**Oleander (*Nerium oleander*) knot**
P. sav.* pv. *phaseolicola	**Halo blight of beans (*Phaseolus, Pisum &Vigna*)**
P. sav.* pv. *retacarpa	**Spanish broom (*Retama sphaerocarpa*) knot**

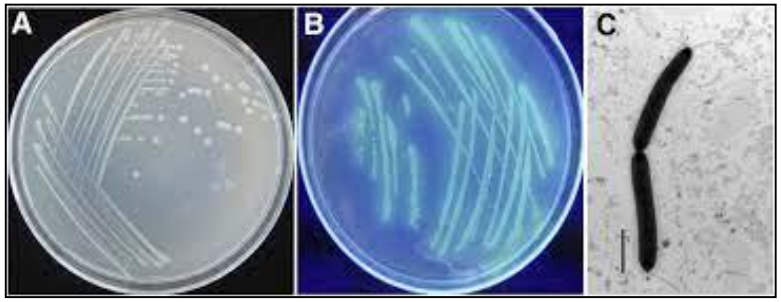
Disease caused by doubtful *P. savastanoi* pathovar

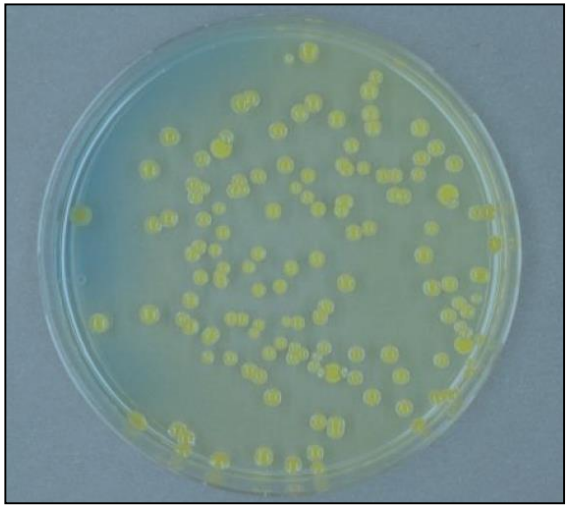
P. savastanoi* pv. *oleae	**Olive knot**

Note *P. savastanoi* pv. *oleae* was excluded from Bull *et al.*,2010 list.

Halo blight of bean
P. savastanoi pv. *phaseolicola*

- Isolation of *P. savastanoi* pv. *phaseolicola* by sectoring on KB medium showing white, creamy and flat colonies with fluorescent pigmentation.
- On semiselective modified sucrose peptone (MSP) plates after 4 d are circular, raised globose, glistening and light yellow, and the medium around the colony turns light yellow.





Kurowski and Remeeus,2008; Sun *et al.*,2017

Oleander gall
P. savastanoi pv. *nerii*

- Oleander gall, bacterial galls at pruning cuts, becomes established after introduction on plant material and pruning infected plants.
- *Pseudomonas* bacteria infections cause host cells to grow faster, creating woody galls on cut or wounded surfaces
- Control by pruning, but use 10% bleach solution to clean tools between cuts on infected plants.

Oleander gall is distinguished by hard brown galls at the floret or at pruning wounds.

Olsen,2008

Identification of *P. syringae* pathovars

- Due to their phenotypic and genomic heterogeneity, an integrated approach should be used to identify the isolates, selecting among the different techniques available, such as:

1. Biochemical and physiological tests,
2. Toxins, and siderophores production,
3. Fatty acids methyl-ester profiles (FAME),
4. Protein profiling analysis (SDS-PAGE),
5. Serological tests, rep-PCR profiles,
6. RFLP,
7. *gyrB* and *rpoD* sequences,
8. 16S rDNA sequences,
9. Multilocus sequence analysis (MLSA), and
10. others.

López *et al.*,2010

Identification of *P. syringae & P. savastanoi*
At species level

- Biolog and Biotype-100 systems can also be used to differentiate *Pseudomonas* species (Grimont *et al*., 1996).
- Other identification methods use a chemotaxonomic approach such as whole-cell fatty acid composition, which is useful for differentiation of major phylogenetic groups (Vancanneyt *et al*.,1996b) and SDS-PAGE of whole-cell proteins, which yield species-specific protein profiles (Vancanneyt *et al*.,1996a).
- These methods, however, do not give differentiation at the pathovar level.

Höfte & De Vos,2006

Identification of the pathovars of *P. syringae & P. savastanoi*

Pathovar differentiation methods

- Among the oxidase negative species, *Pseudomonas syringae* is economically the most important with more than 50 pathovars.
- Isolates of *P. syringae* and *P. savastanoi* are taxonomically subdivided into pathogenic varieties known as pathovars, based largely on their host of isolation.
- Pathovar identification is more complicated than species identification, since it relies on more tests and host specificity.
- Braun-Kiewnick and Sands (2001) have listed a series of tests that can be used to distinguish the most important pathovars of *P. syringae* with good accuracy.
- Identification, however, should always be confirmed by a pathogenicity test.

Höfte & De Vos,2006

Identification of the pathovars of *P. syringae & P. savastanoi*

Pathovar differentiation methods

- The methods used for pathovar determination are:

1. Nutritional, biochemical, physiological tests, and Nucleic acid-based tests;
2. Toxin bioassays can be helpful to differentiate toxin producing pathovars of *P. syringae*;
3. Indole acetic acid (IAA) is useful for identification of the gall producing pseudomonad *P. savastonoi*.

Höfte & De Vos,2006

Identification of the pathovars of *P. syringae & P. savastanoi*

Pathovar differentiation methods

- Antibody-based diagnostic kits and reagents are commercially available for various *P. syringae* pathovars including:
- pv. *glycinea*, pv. *lachrymans*, pv. *phaseolicola*, pv. *tomato*, pv. *pisi* and pv. *syringae*.
- Ice nucleation has been used as a trait to distinguish strains among some of the *P. syringae* pathovars.
- For example:

1. strains within pv. *syringae* frequently exhibit the ice phenotype, while
2. none of the strains tested thus far within pv. *tomato* or *morsprunorum* are ice nucleation active.

Identification of the most common pathovars of *P. syringae*

Characteristics	*tabaci*	*lachrymans*	*syringae*	*aptata*	*atrofaciens*	*dysoxyli*	*pisi*	*antirrhini*	*morsprunorum*	*delphinii*	*tomato*	*maculicola*	*eriobotryae*	*sesami*	*papulans*	*coronafaciens*	*striafaciens*	*garcae*	*oryzae*	*helianthi*	*mori*	*passiflorae*	*persicae*	*cannabina*	*phaseolicola*	*glycinea*
Ice nucleation	-	+	+	+	+	ND	+	-	-	-	-	-	-	+	+	+	+	ND	ND	ND	-	-	+	+^{D}	+^{D}	+^{D}
Levan	+	+	+	+	+	+^{D}	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+
Pectolysis[1]	4;8	4;8	-	-	-	ND	-	4	4	4	4	4	4	4;8	+	-	ND	ND	ND	ND	4	ND	4	4	4	4
β-glucosidase	+	+	+	+	+	ND	+^{D}	+	-	+	+	+	+	-	+	+	+	ND	ND	ND	-	+	-	-	-	-
Utilization of:																										
D-mannitol	+	+	+	+	+	+	+	+^{D}	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+^{D}	-	-	-	-	-
Insitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+
D-Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+^{D}	+	+	-	-	+^{D}
Trigonelline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-	+	+	+
D-quinate	+	+	+	+	+	+	+	+^{D}	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	-	+	+
Erythritol	+	+	+	+	+	+	+^{D}	-	+^{D}	+	-	-	+	+	+	+	+	+	-	+	-	+	-	-	-	-
L(+) tartrate	+	+	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	ND	+^{D}	-	-	-	-	-	-
D(-) - tartrate	-	-	+^{D}	+	ND	-	-	+	-	-	+	+	-	-	-	-	-	ND	-	ND	-	-	-	-	-	-
L-lactate	-	-	+	+	+	+	+^{D}	-	-	-	+^{D}	+^{D}	-	-	+^{D}	-	-	-	-	-	-	-	-	-	-	-
Anthranilate	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DL-homoserine	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Glutarate	+	+	+	+	+	+	ND	+	+	+	+	ND	ND	ND	-	ND	ND	+	+	+	-	ND	ND	+^{D}	-	+^{D}
DL-glycerate	+	+	+	+	+	+	ND	+	+	+	+	ND	ND	ND	+	ND	ND	+	+	+	-	ND	ND	-	-	-
Gelatin liquefaction	+	+	+	+	+	+	ND	+	-	+	+	ND	ND	ND	+^{D}	-	ND	+	+	-	-	ND	ND	-	-	-
Arbutin hydrolysis	+	+	+	+	+	+	ND	+	-	+	+	ND	ND	ND	+	ND	ND	+	+	-	+^{D}	ND	ND	-	-	-
Aesculin hydrolsis	+^{D}	+^{D}	+	+	+	+	ND	+^{D}	+^{D}	+	+	ND	ND	ND	+	ND	ND	+	-	-	+^{D}	ND	ND	-	-	-
Polygalacturonase	+^{D}	+	-	-	-	-	ND	-	-	-	-	ND	ND	ND	-	ND	ND	-	-	-	-	ND	ND	-	-	-
Pectate lyase	+^{D}	+^{D}	-	-	-	-	ND	-	-	+	-	ND	ND	ND	+	ND	ND	-	-	+	-	ND	ND	-	-	-
Toxin	+	-	+	+	+	-	ND	-	+	-	+	+	ND	ND	-	+	ND	+	-	-	-	ND	ND	+^{D}	+	+

[1]pH at which pitting on pectate gel occurs, 4=pH 4.6/ 8=pH 8.5.
+, 80% or more strains positive; +^{D}, 80% or more strains delayed positive; V, between 21-79% of strains positive; -, 80% of more strains negative; ND, not determined.
Modified after Hildebrand et al., 1988 (24) and Young & Triggs, 1994 (74).

Schaad *et al.*,2001

Tests to Differentiate *P. viridiflava* and some *P. syringae* pathovars

	Levan formed	Pectate gel[a]	Rutin glycosidase	β-Glucosidase	Adonitol	Anthranilate	Betaine	Erythritol	DL-Homoserine	Inositol	L-Lactate	Mannitol	Quinate	Sorbitol	D-Tartrate	L-Tartrate	Trigonelline
P. viridiflava	–	4.6–8.5	+	+	–	–	+	+	–	+	+	+	+	+	+	–	+
P. s. syringae	+	–	+	+	–	–	+	+	–	+	+	+	+	+	few +	–	+
P. s. antirrhini	+	4.6	+	+	–	–	+	–	+	+	–	+	+	+	+	–	+
P. s. aptata	+	–	+	+	–	–	+	+	–	+	+	+	+	+	slow	–	+
P. s. atrofaciens	+	NT	+	V	–	–	+	+	–	+	+	+	+	+	–	–	+
P. s. cannabina	W	4.6	NT	–	–	–	+	–	–	–	–	–	–	–	–	–	–
P. s. coronafaciens	+	–	+	+	–	–	+	+	–	+	–	+	+	+	–	–	–
P. s. delphinii	–	4.6	+	+	–	–	+	+	–	+	–	+	+	+	–	–	+
P. s. criobotryae	+	4.6	+	+	–	–	+	+	–	–	–	–	+	+	–	+	+
P. s. garcae	+	–	NT	+	–	–	+	+	–	+	–	+	+	+	–	–	–
P. s. glycinea	+	4.6	–	–	–	–	–	–	–	+	–	+	+	–	–	–	+
P. s. lachrymans	+	4.6–8.5	+	+	–	–	–	+	–	+	–	+	+	+	–	+	+
P. s. mori	+	4.6	+	–	few +	–	V	–	–	+	–	+	–	V	–	–	+
P. s. mori "var. *huszi*"	+	4.6	+	–	+	–	+	–	–	+	–	+	–	+	–	–	+
P. s. morsprunorum	+	4.6	+	–	–	+	+	slow	–	+	–	+	+	+	–	+	+
P. s. papulans	–	NT	+	+	–	–	+	+	–	+	+	+	+	+	–	–	–
P. s. passifloriae	–	NT	+	+	–	–	+	+	–	+	–	+	+	+	–	–	–
P. s. persicae	+	4.6	+	–	–	–	–	–	–	–	–	+	–	+	–	–	–
P. s. phaseolicola	+	4.6	+	–	–	–	V	–	–	–	–	–	+	–	–	–	+
P. s. pisi	+	–	+	V	–	–	+	V	+	+	V	+	+	+	–	–	+
P. s. ribicola	–	NT	+	–	–	–	slow	–	–	+	–	+	+	+	–	–	+
p. s. savastanoi	–	4.6	+	–	–	V	+	–	–	V	–	+	–	+	–	V	V
P. s. sesami	+	4.6–8.5	+	–	–	–	V	+	–	+	–	–	+	–	–	+	+
P. s. striafaciens	+	NT	+	+	–	–	+	–	–	+	–	+	–	+	–	–	–
P. s. tabaci	+	4.6–8.5	+	+	–	–	+	+	–	+	–	+	+	+	–	+	+
P. s. tomato	+	4.6	+	+	–	–	+	–	–	+	–	+	+	+	+	–	+

Symbols: +, product formed or substrate utilized; –, product not formed or substrate not utilized; V, variable results with different strains; W, weak positive.

[a]Indicates the pH at which pitting (sinking of colonies) occurs on pectate gels; –, no action on pectate; NT, not tested. For best results, the low pH pectate gels should be at pH 4.6 or lower (Burki, 1973).

From Hildebrand and Schroth (1972), Hildebrand and Caesar (1989), Hildebrand et al. (1988), and D. C. Hildebrand (unpublished observations).

The Prokaryotes (chapter 3.3.23),2006

Discrimination of the *P. syringae* pathovars *syringae*, *morsprunorum* race 1 and *morsprunorum* race 2 by the GATTa tests and other tests

- The so-called GATTa tests are specifically useful for discrimination *P. syringae* pv. *syringae* (*Pss*) and *P. syringae* pv. *morsprunorum* race 1 (*Psm* race 1) (Latorre and Jones,1979).
- But, the GATTa test responses of *Psm* race 2 and other *P. syringae* pathovars (>50) are largely variable or unknown and this could lead to confusions, as discussed by Gilbert *et al.*,2009.

Identifications at the pathovar level in *Pseudomonas syringae*
LOPAT and GATTa tests

Test	*Pseudomonas syringae*		
	pv. *syringae* KFB 0103	pv. *morsprunorum* KFB 0101	pv. *persicae* KFB 0102
Gram reaction	-	-	-
Fluorescence on King's medium B	+	+	-
Levan production (L)	+	+	+
Oxydase reaction (O)	-	-	-
Pectolytic capability (P)	-	-	-
Arginine dihydrolase (A)	-	-	-
Tobacco hypersensitivity (T)	+	+	+
Gelatine hydrolysis (G)	+	+	-
Aesculin hydrolysis (A)	+	+	-
Tyrosinase activity (T)	-	+	nt
Utilization of tartaric acid (Ta)	-	-	-
Acid production from:			
Inositol	+	+	-
Sorbitol	+	+	+
Erytritol	+	+	+
Ice nucleation	+	-	+
O/F test	O	O	O
Nutrient sucrose broth growth	yellow	white	nt
Vitality on NSA medium	7 days	4 days	7 days

Legend: + - positive reaction, – - negative reaction, nt - not tested, O - oxidative metabolism.

Gašić *et al.*,2012

Identifications at the pathovar level in *Pseudomonas syringae*
GATTa tests

- *P. syringae* pv. *morsprunorum* race 2 differed from the race 1 mainly by gelatinase activity and pathological characters.

	pv. *syringae*	pv. *morsprunorum* race 1	pv. *morsprunorum* race 2	pv. *avii*
Gelatin hydrolysis **G**	+	-	+	+
Aesculin hydrolysis **A**	+	-	+ / -	-
Tyrosinase activity **T**	-	+	+ / ± / -	NT
Use of tartric acid **Ta**	-	+	(+) / -	-
Use of lactic acid	+	-	-	?
Fluorescence on King B	+	+ / -	+ / -	-
Nutrient Sucrose Broth growth color	Y	W	W/YW	?

Alain Bultreys

Identifications at the pathovar level in *Pseudomonas syringae*

GATTa tests

Test	*syringae*	*morsprunorum* race 1	*morsprunorum* race 2[a]
Gelatin hydrolysis (G)	+	-	+
Aesculin hydrolysis (A)	+	-	-/+
Tyrosinase activity (T)	-	+	-/±/+
Utilization of tartaric acid (Ta)	-	+	-
Utilization of lactic acid	+	-	-/+
Nutrient sucrose broth growth	Yellow	White	Yellow/White
Anthranilate (an ester of anthranilic acid)	-	+	
Syringomycin production	+/-	-	-

[a] BOX-PCR and yersiniabactin tests are better suited for this pathogen (see text)

Bultreys and Kaluzna,2010

Tests to differentiated *P. syringae* and *P. morsprunorum*

Character	pv. morsprunorum	pv. syringae
5% sucrose nutrient broth	White growth	Yellow growth
Recovery from 5% nutrient agar after 6 days	-	+
Aesculin or arbutin hydrolysis	-	+
Gelatin liquefaction	-	+
Brown diffusible pigment on King's medium B	+/-	-
Green-fluorescent diffusible pigment on King's medium B	+/-	+

Lelliott and Stead,1987; Plant Bacteriology Manual,2008

Isolation
P. syringae pv. *persicae*

- Process the two subsamples separately.
- Triturate in 500 ml (spring) or 1,000 ml (autumn) sterile distilled water with a Waring blender.
- Decant.
- Dilute the supernatant by four serial tenfold steps in 0.01 M PBS pH 7.2.
- Plate 0.05 ml drops of supernatant and its dilutions on YGPA (Lelliott & Stead,1987) in triplicate.
- Incubate 3-4 days at 16-20°C.
- Colonies of *P. s. persicae* are usually grey, slightly bluish at the margin, translucent, or mucoid, 2 mm in diameter, with undulate margin.

OEPP/EPPO Bulletin,2005

Morphological characters of *Pseudomonas syringae* pv. *persicae* compared with pathovars *syringae* and *morsprunorum* on KB and NAS media

- *P. s. persicae* grows more slowly than the other two pathovars.
- Also it does not produce green fluorescent pigment on this medium.
- However this characteristics is not discriminative since nonfluorescent pv. *morsprunorum* strains were recorded.
- *P. s. persicae* produce less growth and small colonies on nutrient agar sucrose (NAS) medium after 72 h of incubation at 26°C.

Obradovic,2010

Morphological characters of *Pseudomonas syringae* pv. *persicae* compared with pathovars *syringae* and *morsprunorum* on KB and NAS media

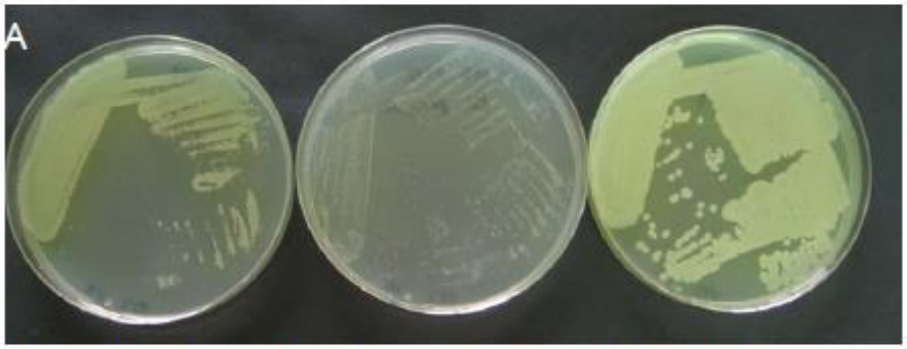


Figure 1. Growth on King's B medium. A - *P. s.* pv. *mors-prunorum*, B - *P. s* .pv. *persicae*, C - *P. s.* pv. *syringae*.

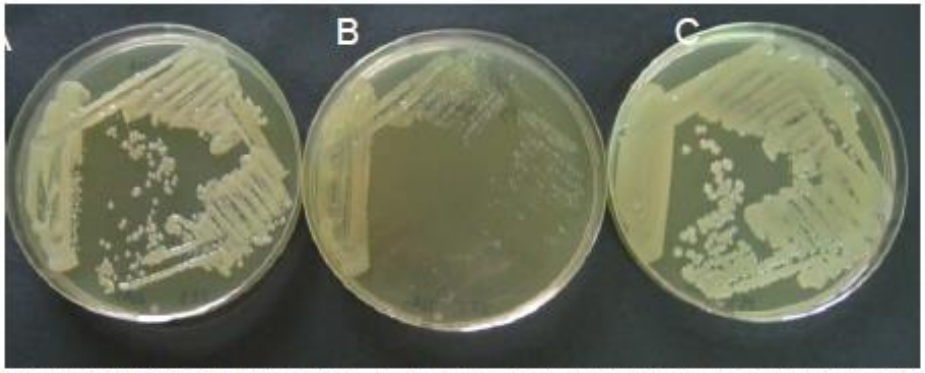


Figure 2. Levan production on NAS medium. A - *P. s.* pv. *mors-prunorum*, B - *P. s* .pv. *persicae*, C - *P. s.* pv. *syringae*.

Obradovic,2010

Casamino-sucrose-gelatin medium

Alternative culture medium for pseudomonads that do not produce fluorescent on KB medium

- Vitamin free casamino acids 1 g
- $MgSO_4.7H_2O$ 0.1 g
- Dipotassium phosphate 0.1 g
- Agar 2 g
- Sucrose 1 g
- Gelatin 3 g
- $D.H_2O$ 100 ml
- Autoclave for 15 min at 121°C, and pour into sterile Petri dishes.

Lelliott and Stead, 1987

Biochemical characters of *Pseudomonas syringae* pv. *persicae* in comparison with pathovars *syringae* and *morsprunorum*

- *P. s. persicae* belongs, like *P. s. syringae* (Pss) and *P. s. morsprunorum* (Psm), to LOPAT Group Ia of the determinative scheme of Lelliott *et al.*,1966.
- It grows significantly more slowly on King's B medium than the other two pathovars.
- All gave positive hypersensitivity test on tobacco.
- Host tests on peach have not been performed and the significance of this finding is not yet known.

Test[1]	pv. *syringae*	pv. *mors-prunorum*	pv. *persicae*
Fluorescence on King's B medium	+	+ or –	–
Fluorescence on CSGM[2]	+	+	+
Levan production	+	+	+
Gelatine hydrolysis	+	+	–
Aesculin hydrolysis	+		–
Acid production from:			
Inositol	+	+	–
Sorbitol	+	+	+
Erytritol	+ or –	+ or –	–
Utilisation of:			
DL lactate	+ or –	–	–
D(–) tartrate	+ or –	–	–
L(+) tartrate	–	+	–

[1]Fluorescence – appearance of green or blue pigment which diffuses into medium visible under UV-light; levan production – occurrence of mucoid colonies on sucrose-rich medium; gelatin hydrolysis – liquefaction of solid medium; aesculin hydrolysis – dark brown discoloration of the medium; remaining tests – yellow discoloration of medium. For preparation of media and performance of tests (see Lelliott & Stead , 1987; Fahy & Persley 1983; Schaad, 1988).

[2]Casamino-sucrose-gelatin medium (Lelliott & Stead, 1987).

Biochemical characters of *Pseudomonas syringae* pv. *persicae* in comparison with pathovars *syringae* and *morsprunorum* (based on EPPO document PM 7/43)

- Apart these morphological and biochemical characteristics, these pathovars can be efficiently differentiated based on toxin production (syringomycin, coronatine and persicomycin), ice nucleation activity, and severity symptoms on bean pods.

Test[1]	*Pseudomonas syringae*		
	pv. *syringae*	pv. *morsprunorum*	pv. *persicae*
Fluorescence on King's medium B	+	+ or -	-
Fluorescence on CSGM[2]	+	+	+
Levan production	+	+	+
Gelatine hydrolysis	+	+	-
Aesculin hydrolysis	+	+	-
Acid production from:			
Inositol	+	+	-
Sorbitol	+	+	+
Erytritol	+ or -	+ or -	-
Utilisation of:			
DL lactate	+ or -	-	-
D(–) tartrate	+ or -	-	-
L(+) tartrate	-	+	-
Ice nucleation	+	-	+

[1]Fluorescence – appearance of green or blue pigment which diffuses into medium visible under UV-light levan production – occurrence of mucoid colonies on sucrose-rich medium; gelatin hydrolysis – liquefaction of solid medium; aesculin hydrolysis – dark brown discoloration of the medium; remaining tests – yellow discoloration of medium. For preparation of media and performance of tests (see Lelliott & Stead , 1987; Schaad et al., 2001).
[2]Casamino-sucrose-gelatin medium (Lelliott & Stead, 1987).

Obradovic,2010

- **Bacteriological characteristics of three pathovars of *P. syringae*:**
- ***P.s.* pv. *syringae***
- ***P. s.* pv. *actinidiae***
- ***P. s.* pv. *morsprunorum*.**

Takikawa *et al.*,1989

Characteristic	Kiwifruit strains	*P. syringae* pv. *syringae*	*P. syringae* pv. *morsprunorum* Group C	*P. syringae* pv. *morsprunorum* Group B	*P. syringae* pv. *syringae*[a]	*P. syringae* pv. *morsprunorum*[a]
Fluorescent pigment						
on King's medium B	−	+	−	+	+	−
in Uschinsky's solution	−	+	−	+		
Gelatin liquefaction	−[b]	+[c]	−	−	+	−
Hydrolysis of						
Esculin	−	+	−	−	+	−
Arbutin	−	+	−	−	+	−
Casein	+	+[c]	−	−	+	−
Tween 80	+	+	−	+		
Tyrosinase	−	−	+	+	−	+
Urease	−	+	−	+		
Purple milk reaction	Kw	KD	Kw	K		
Maximum NaCl tolerance	3%[d]	4%	1%	3%	5%	3~5%
Maximum growth temperature	30 C	>35 C	32 C	32 C		
Growth in sucrose broth	white	yellow	white	white	yellow	white
Longevity on NSA (days)	>8	>8	<3	6~8	8~14	2~6
Utilization of						
D-Xylose	−[e]	+	−[e]	+		
L-Arabinose	(+)[f]	+	−	+	+	+
D-Ribose	+[f]	+	−	−		
Raffinose	+	+[g]	+[g]	−		
Inositol	+	+	−	+		
Erythritol	−	+	−	+		
DL-Tartrate	−	−	+	−	−	+
DL-Lactate	−	+	−	−	+	−
Malonate	+	+	−	+		
Acetate	(+)	+	+	+[h]		
n-Caprate	−	+	−	+		
Glutarate	(+)[f]	+	−[i]	+		
Saccharate	+	+	(+)	+		
L-Histidine	−	+	−[i]	+	+	+
L-Serine	(+)	+	−	+	+	+
L-Arginine	+	+	(+)	+	+	+
Betaine	+	+	−	−		
Trigonelline	−	+	−	−		
L-Leucine	(+)	+	−[i]	+	+	−
L-Tyrosine	+	−	(+)	+	−	+

+: positive; −: negative; (+): delayed positive after 2 to 3 weeks; K: alkaline reaction; D: digestion; w: weak reaction.

a) Data from Garrett *et al.* (1966).
b) Kiwifruit strains showed delayed and weak liquefaction after 3 to 6 weeks.
c) Strain W 7837 negative.
d) Strain Kw 21 could grow in 2% but not in 3% NaCl.
e) Acid production was observed without any visible growth.
f) Strain Kw 22 negative.
g) Strains W 7844 and U 7805 negative.
h) Strain W 8107 negative.
i) Strain U 7805 positive.

Tests to differentiated

P. s. pv. *syringae* from *P. s.* pv. *actinidiae*

- Carbohydrate utilization profiles of the isolates from kiwifruit in the present study compared with those of reference strains of:

1. *Pseudomonas syringae* pv. *actinidiae*, and
2. *P. syringae* pv. *syringae*.

	Kiwifruit isolates	*P.s.* pv. *actinidiae*	*P.s.* pv. *syringae*
Glycerol	+	+	+
Erythritol	–	–	+
D-Arabinose	–	–	–
L-Arabinose	+	+	+
Ribose	+	+	+
D-Xylose	+	+	+
L-Xylose	–	–	–
Adonitol	–	–	–
β-Methylxyloside	–	–	–
Galactose	+	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
L-Sorbose	–	–	–
Rhamnose	–	–	–
Dulcitol	–	–	–
Inositol	+	+	+
Mannitol	+	+	+
Sorbitol	+	+	+
α-Methyl-D-mannoside	–	–	–
α-Methyl-D-glucosamine	–	–	–
N-Acetyl glucosamine	–	–	–
Amygdalin	–	–	–
Arbutin	+	–	–
Esculin	+	–	+
Salicin	–	–	–
Cellobiose	–	–	–
Maltose	–	–	–
Lactose	–	–	–
Melibiose	–	–	–
Saccharose	+	+	+
Trehalose	–	–	–
Inulin	–	–	–
Melezitose	–	–	–
D-Raffinose	–	+	–
Starch	–	–	–
Glycogen	–	–	–
Xylitol	–	–	–
β-Gentiobiose	–	–	–
D-Turanose	–	–	–
D-Lyxose	–	–	–
D-Tagatose	–	–	–
D-Fucose	+	–	+(–)*
L-Fucose	–	–	–
D-Arabitol	+	–	–(+)**
L-Arabitol	–	–	–
Gluconate	–	+	+(–)*
2-Keto-gluconate	–	–	–
5-Keto-gluconate	–	–	–

* *P.s.* pv. *syringae* from sweet pepper (*C. annuum*);
** *P.s.* pv. *syringae* from laurel (*L. nobilis*)

Scortichini,1994

Tests to differentiated *P. avellanae* from *P.s.* pvs. *corylii*, *syringae* and *morsprunorum*

Pseudomonas avellenae - *Canker or decline of hazelnut*

Biochemical tests

Test	*P avellanae*	*P.s pv coryli*	*Pss*	*Pmorsprun*
Ice nucleation	-	V	+	-
Utilization of adonitol	-	-	+	-
sorbitol	-	+	+	+
erythritol	-	-	+	V
L+tartrate	-	-	-	+
L-lactate	-	-	+	-
Gelatine liquefaction	-	-	+	-
Arbutin hydrolysis	-	+	+	-
Aesculin hydrolysis	-	+	+	V
syrB gene	-	-	+	-

EU_COST 873 training course: Training in diagnostics of bacterial diseases of fruits, including quarantine pathogens of importance to the EU and Ukraine 2011-10-31 to 11-05 – lecture J.D.Janse

Janse,2011

COST 873 StoneFruitNutHealth

cost

Biochemical and nutritional tests differentiating *P. avellanae*, *P.s.* pv.*theae* and *P.s.* pv. *actinidiae* strains

- There are controversial reports on fluorescent pigment of *P. s.* pv. *actinidiae* strains.
- Vanneste *et al.*, 2001 reported Psc strains showed the same weak fluorescence as the strains of *P. syringae* pv. *actinidiae* recently isolated from Italy.
- Balestra *et al.*,2009 reported the Italian isolates of *P. s.* pv. *actinidiae* produce fluorescent pigment on KB medium.
- Here, Scortichini and Prospero,2002, showed that the strains from South Korea are positive on CSGA mdium.

	P. avellanae	*P.s.* pv. *theae*	*P.s.* pv. *actinidiae*
Fluorescence on KB	+[a]	−	−
Fluorescence on CSGA	+[b]	+	−[d]
Growth on NA	−	+	+[e]
Gelatin liquifaction	−	+	−
Casein hydrolisis	−	+	+
Arbutin hydrolisis	−	−	−[f]
Esculin hydrolisis	−	−	−[f]
Tyrosinase	−	−[c]	−
Tween 80	−[b]	−	−
Utilization of			
Arabinose	+	+	−
DL-tartaric acid	+	−	−
D-xylose	+	+	−
L-arginine	−	+	+
L-tyrosine	−	−	+
Trigonelline	−	+	−

[a]The fluorescence disappears after several tranfers on media especially with strains from central Italy.
[b]The strains from central Italy are negative.
[c]One strain studied by Takikawa et al. (1988) is positive.
[d]The strains from South Korea are positive.
[e]The strains from South Korea are negative.
[f]The strains from central Italy are positive.

Scortichini and Prospero,2002

Tests to differentiated

Some *P. 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.

Goszczynaska *et al.*,2000

Ethylene production

Test to differentiate some *P. syringae* and *P. savastanoi* pathovars

- Of six *P. syringae* pv. *pisi* strains, five produced ethylene.
- All strains of *P. s.* pv. *glycinea* isolated from soybean plants of various regions and all strains of *P. s.* pv. *phaseolicola* isolated from kudzu produced ethylene.
- However, strains of *P. s .* pv. *phaseolicola* isolated from beans and all other tested pathovars failed to produce detectable amounts of ethylene.

***P. syringae* pathovars tested for their ability to produce ethylene.**

Pathovar	No. of tested strains	Ethylene production (10^{-8} nl h^{-1} $cell^{-1}$)
aptata	4	0
atrofaciens	3	0
atropurpurea	4	0
cannabina	1	0
coronafaciens	3	0
glycinea	50	5–100
lachrymans	3	0
maculicola	3	0
mori	1	0
morsprunorum	4	0
phaseolicola (from kudzu)	4	30–70
phaseolicola (from bean)	8	0
persicae	1	0
pisi	5	1–3
pisi	1	0
primulae	1	0
savastanoi	3	0
striafaciens	1	0
syringae	5	0
tabaci	4	0
tagetis	1	0
tomato	7	0

Weingart and Völksch,1997

Tests to differentiated

P. s. pv. *maculicola* from *P. s.* pv. *alisalensis*

Host range of *P. s.* pv. *maculicola* and *P. syringae* pv. *Alisalensis on crucifers in CA*

	Pseudomonas syringae				
Experimental host	pv. *alisalensis*	pv. *maculicola*	pv. *tomato*	pv. *coronafaciens*	pv. *syringae*
Broccoli raab	+	-	-	-	-
Broccoli	+	+	-	-	-
Broccolini	+	+	-	-	-
Cauliflower	+	+	-	-	-
Tomato	+	+/-	+	-	-
Calif. brome	+	-	-	+	-
Corn	-	-	-	-	-
Oat	+	-	-	+	-
Rye	-	-	-	+	-
Timothy	+	-	-	-	-

Dr. Carolee Bull, USDA-ARS

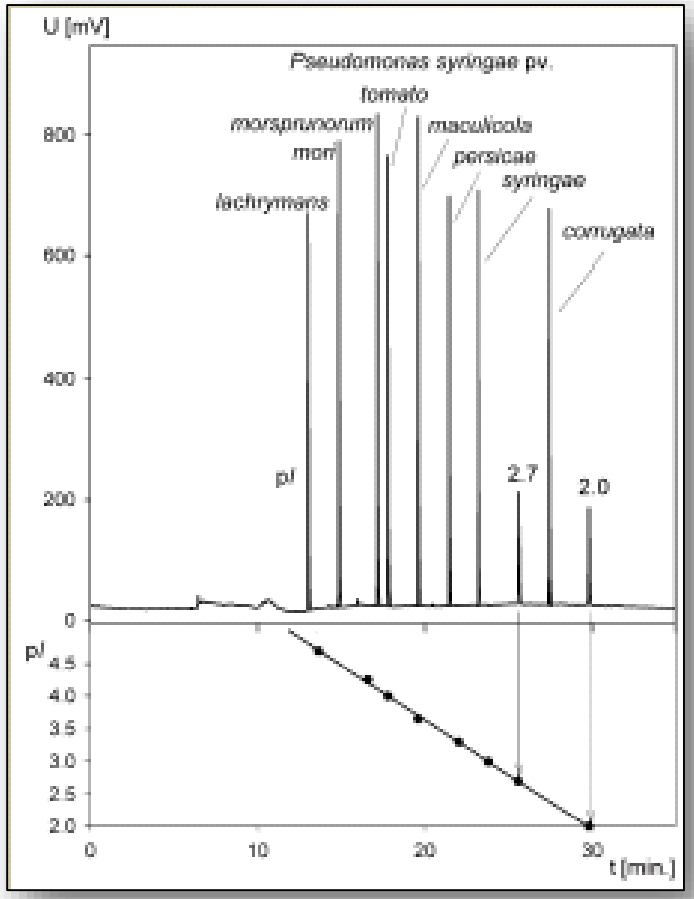
Ethylene and cytokinin production
P. savastanoi pv. *savastanoi*

- Bacterial galls on oleander stem and leaves caused by *Pseudomonas savastanoi* pv. *Savastanoi* (ex. *Pseudomonas syringae* pv. *savastanoi*).
- *P. savastanoi* pv. *savastanoi* also produce cytokinin.

Discrimination of phytopathogenic *Pseudomonas* pathovars

Capillary isoelectric focusing (CIEF)

- Commonly used chemical and biochemical diagnostic techniques such as BIOLOG™ and fatty acid analysis are not sufficiently discriminating all pathovars in the genus *Pseudomonas* (*P. syringae* pathovars).
- Free flow capillary isoelectric focusing (CIEF) was based on the movement of molecules in an electric field.
- It is fast and offers many advantages in terms of labor saving per test and reproducibility.
- See also protein analysis section.



Horký *et al.*,2009

Discrimination of phytopathogenic *Pseudomonas* pathovars
Capillary isoelectric focusing (CIEF)

General methods
Methods that can be used for most or all pathogens

Detection and Identification (continued)

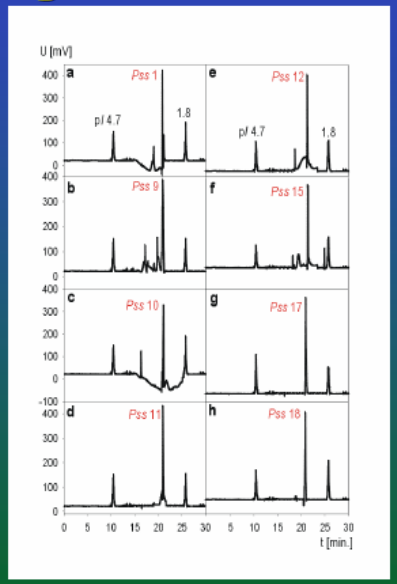
- **Free flow capillary iso-electric focussing (electrophoresis)**
  new method, very fast (30 min)

  Separates charged bacteria specifically in a capillary

  used in CZ lab, Jaroslav Horky

Horká et al. (2007) Separation of plant pathogens from different hosts and tissues by capillary electromigration techniques, **Anal. Chem. 79: 9539-9546.**

Horká et al. (2009) Free flow and capillary isoelectric focusing of bacteria from the tomatoes plant tissues. **J. Chromatogr. A 1216: 1019-1024.**



Courtesy J. Horky, CZ

Capillary isoelectric focusing (CIEF) with UV/Vis detection

Capillary Isoelectric Focusing (pH gradient 2-5)

Strain,isolate	Percentage of probability by GC identification	CIEF - pI
Pseudomonas syringae* pv. *syringae		
CCM 2870	*Pseudomonas syringae* pv. *syringae* 71,7 %	3,1
CCM 2114	*Pseudomonas syringae* 94,5 %	3,1
CCM 2868	*Pseudomonas syringae* pv. *syringae* 73,0 %, 73,7 %	3,1
PPBOL 1048	*Pseudomonas syringae* pv. *syringae* 84,0 %	4,8
PPBOL 1049	No result	3,1
PPBOL 1050	*Pseudomonas syringae* pv. *syringae* 84,0 %	3,1
PPBOL 1054	Not identified	3,1
PPBOL 1055	Not identified	3,1
B 32/08	*Pseudomonas syringae* pv. *syringae* 92,0 %	3,1
Pseudomonas syringae* pv. *lachrymans		
CFBP 1644	*Pseudomonas syringae* 94,2 %	4,8
CFBP 6465	*Pseudomonas syringae* pv. *syringae* 75,0 %	4,8
CFBP 6462	*Pseudomonas syringae* pv. *syringae* 93,9 %	4,8
Pseudomonas syringae* pv. *maculicola		
LMG 5071	*Pseudomonas syringae* pv. *maculicola* 63,9 %	3,7
Pseudomonas syringae* pv. *mori		
IVIA 10003.1a	*Pseudomonas viridiflava* 80,3 %, *Pseudomonas syringae* pv. *syringae* 74,6 %	4,5
CFBP 1642	*Pseudomonas viridiflava* 94,1 %, *Pseudomonas syringae* pv. *syringae* 88,7 %	4,5
B 141/08	*Pseudomonas viridiflava* 92,8 %, *Pseudomonas syringae* pv. *syringae* 92,7 %	4,5
B 104/08	*Pseudomonas syringae* 95,5 %, *Pseudomonas syringae* pv. *morsprunorum* 95,5 %	4,5
B 46/04	*Pseudomonas syringae* pv. *syringae* 92,9 %	4,5
Pseudomonas syringae* pv. *tomato		
IVIA 1733.3	*Pseudomonas syringae pv. tomato* 74,7 %	4,0
CFBP 1326	*Pseudomonas syringae* pv. *syringae* 92,9 %, *Pseudomonas viridiflava* 84,3 %	4,0

Continue:		
CCM 7018	*Pseudomonas viridiflava* 91,2 %, *Pseudomonas syringae* 91,1 %	4,0
CCM 7019	*Pseudomonas viridiflava* 93,6 %, *Pseudomonas syringae* 92,5 %	4,0
CFBP 2212	*Pseudomonas syringae pv. tomato* 86,1 %	4,0
CFBP 5422	*Pseudomonas syringae* 93,2 %, *Pseudomonas syringae* pv. *syringae* 89,1 %	4,0
CFBP 2546	*Pseudomonas syringae* 90,1 %, *Pseudomonas syringae pv. tomato* 66,2 %	4,0
B 106/08	*Pseudomonas syringae* 92,9 %, *Pseudomonas syringae* pv. *syringae* 92,9 %	4,0
Pseudomonas syringae* pv. *persicae		
LMG 5184	*Pseudomonas viridiflava* 93,1 %, *Pseudomonas syringae* pv. *syringae* 90,3 %	3,4
LMG 5078	*Pseudomonas syringae* 35,9 %	3,4
LMG 5566	*Pseudomonas syringae* 34,2 %	3,4
LMG 5568	*Pseudomonas syringae* 41,0 %	3,4
LMG 5569	*Pseudomonas syringae* 37,0 %	3,4
Pseudomonas corrugata		
IVIA 614.5.3	*Pseudomonas syringae* 86,3 %	2,4
CFBP 5324	*Pseudomonas putida* 82,0 %	2,4
CFBP 4901	*Pseudomonas putida* 78,0 %, *Pseudomonas corrugata* 40,3 %	2,4
CFBP 5465	*Pseudomonas corrugata* 66,4 %	2,4
CFBP 6663	*Pseudomonas corrugata* 63,1 %, 61,8 %	2,4
Pseudomonas syringae* pv. *morsprunorum		
CCM 2859	*Pseudomonas syringae* pv. *mori* 94,6 %	4,1
CCM 2534	*Pseudomonas syringae pv. morsprunorum* 96,3 %, 96,9 %	4,1
CCM 2860	*Pseudomonas syringae, Pseudomonas syringae* pv. *mori* 95,7 %	4,1

Horký *et al.*,2009

Psedoumonas syringae

With variable virulence and resistance phenotypes

- Individual strains of the plant pathogenic bacterium *Pseudomonas syringae* vary in their ability to produce:

1. Toxins, enzymes
2. Phytohormones,
3. Resist antimicrobial compounds,
4. Nucleate ice.

- These phenotypes with similar evolutionary origin enhance virulence.

Application to Biotechnology: These microbes hold a number of special characteristics and produce a wide variety of potentially useful compounds. For example, they show a high resistance to copper and antibiotics. In fact, they encode genes that bestow resistance to cationic antimicrobial peptides and antibiotics. *Pseudomonas syringae* is also a microbe of great interest for its ice nucleation properties. By eliminating the genes that were responsible for the production of this protein, a significant amount of frost damage could be prevented on cash crops.

Hwang *et al.*,2005;MicrobeWiki,2011

Toxin production

- Several pathovars of *P. syringae* produce phytotoxins.

1. Phytotoxins produced by fungal pathogens are host-specific;
2. Phytotoxins produced by *Pseudomonas* spp. are non-specific, and active against plants and microorganisms (antimetabolite toxins).

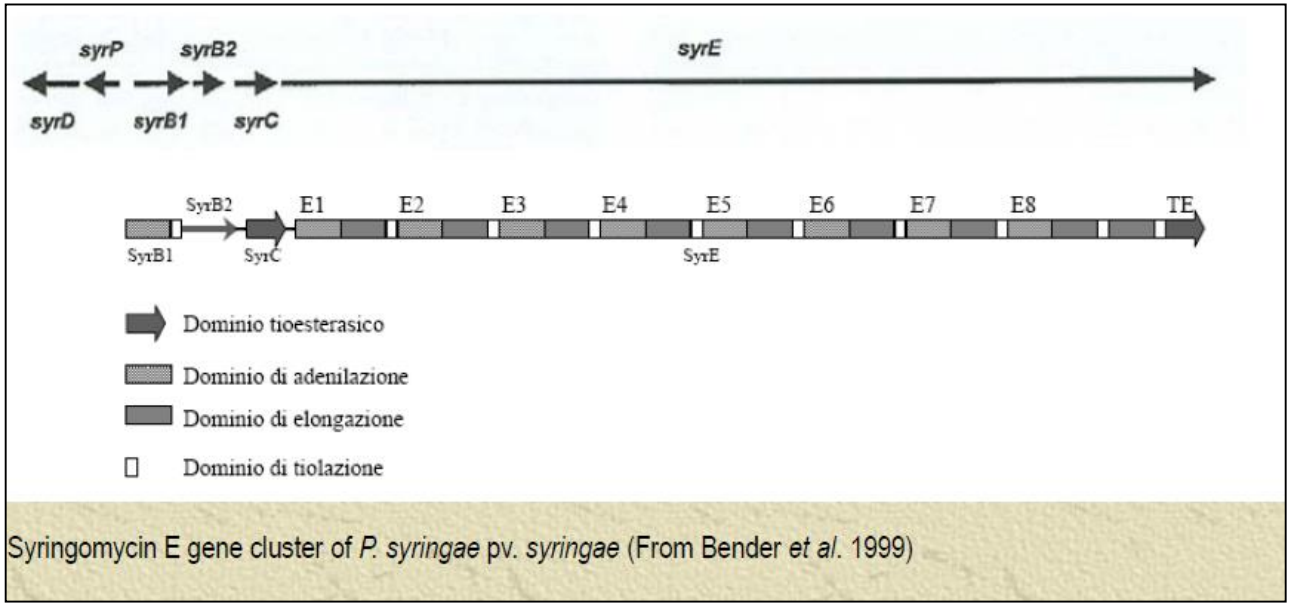
- Genes involved in toxin production have been used produce as diagnostic probes.
- For example,
- A *tox* gene fragment from *P. phaseolicola* has proven useful as a diagnostic probe (Schaad *et al.*,1989) and similar possibilities exist for other pathovars.

The Prokaryotes (chapter 3.3.23),2006;..

Toxin production

Pseudomonas syringae

- The genes encoding toxins are:
- usually located on the chromosome, but
- sometimes they reside on a plasmid(i.e. coronatine).



Syringomycin E gene cluster of *P. syringae* pv. *syringae* (From Bender *et al.* 1999)

M. Stefani

Toxin production

Host specialization

Pseudomonas syringae

- Some strains of *P. syringae* produce phytotoxins or exotoxins (coronatine, tagetitoxin, phaseolotoxin and persicomycin) with plant tissues as the only target.
- These host-specific toxins are generally produced by specific strains of *P. syringae* (referred to as pathovars) that can cause disease on a limited number of plant species including the species sensitive to the toxin.
- Strain 31a has a very limited host range suggesting that none of these toxins are produced.

Lagriffoul *et al.*,2010

Some toxins produced by phytopathogenic *Pseudomonas* spp.

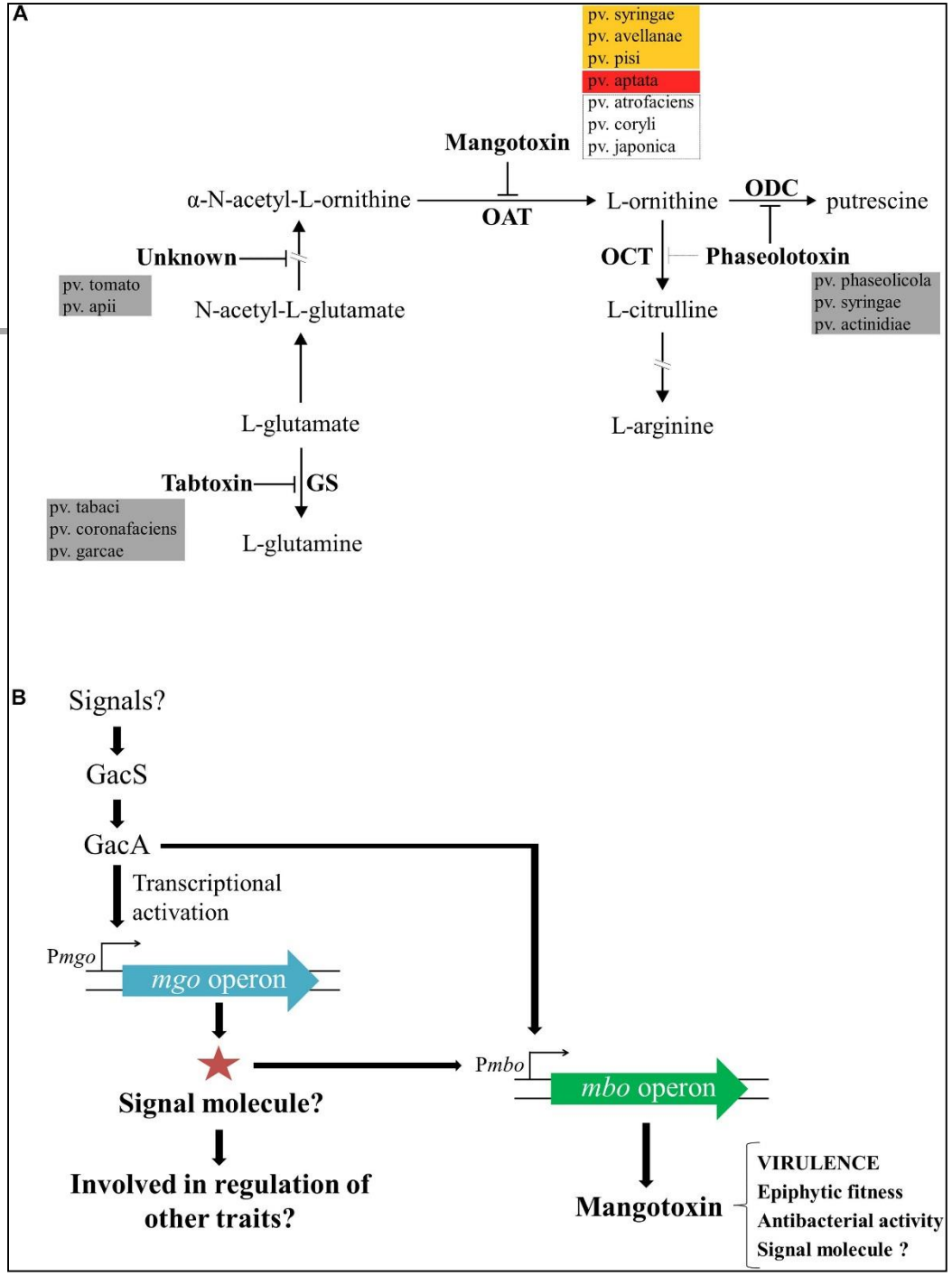
Pseudomonads	Toxins, EPS/hormones	Mechanism or site of action	Host plant(s)
1. *P. syringae*			
pv. *atropurpurea*	coronatine		Italian rye grass
pv. *coronafaciens*	tabtoxin-β-lactam	glutamine synthetase	oat
pv. *garcae*	tabtoxin-β-lactam	glutamine synthetase	coffee
pv. *glycinea*	coronatine/ polysaccharide		soybean
pv. *lachrymans*	extracellular polysaccharides		cucumber
pv. *maculicola*	coronatine		crucifers
pv. *morsprunorum*	coronatine		sour cherry
pv. *phaseolicola*	phaseolotoxin	ornithine transcarbamoylase	bean, kudzu
pv. *savasatoni*	IAA & cytokinins	plant growth regulators	olive, oleander
pv. *syringae*	syringomycins syringopeptins syringotoxins	plasma membrane	peach, maize
pv. *tabaci*	tabtoxin-b-lactam	glutamine synthase	tobacco
pv. *tagetis*	tagetitoxin	chloroplastic RNA polymerase	marigold
pv. *tomato*	coronatine		tomato
2. *P. tolaasii*	tolaasin	plasma membrane	mushroom

OECD,1997

Enzymatic target of mangotoxin and its biosynthesis regulation.

- ✓ Mangotoxin is the most recent antimetabolite toxin discovered and was first described to be mainly produced by Pss strains isolated from mango trees.
- ✓ GacS/GacA two-component regulatory system regulates directly or indirectly the transcription of the *mgo* operon.

Gutiérrez-Barranquero,2019



Toxin production
Bacterial toxins (phytotoxins)

- The phytotoxins produced by *Pseudomonas syringae* pathovars generally induce:

1. Chlorosis (tabtoxin, coronatine, and phaseolotoxin,), or
2. Necrosis (syringomycin, syringopeptin and syringostatins).

Bender *et al.*,1999;..

Toxin production

Chlorosis-inducing toxins

1. Chlorosis-inducing dipeptides:

- Examples: Tabtoxin, coronatine, phaseolotoxin, tagetitoxin.
- Produced by *Pseudomonas syringae* pv. *tabaci*, *P. s.* pv. *coronofaciens*, *P. s.* pv. *phaseolicola*, *P.s.* pv. *tagetis*, and some other pathovars.
- Phaseolotoxin has also been implicated in increased pathogen growth and spread *in planta*.

Chlorosis (yellowing of the leaf tissue typically as a result of chloroplast disruption or inhibition of its formation).
An experiment in a plant is "*in planta*"

Toxin production
Necrosis-inducing toxins

2. Cyclic lipopeptides (CLPs)

- Examples: syringomycins, syringotoxins and syringostatins, causing necrosis, produced by *P. s.* pv. *syringae*.
- Syringomycin is representative of the cyclic lipodepsinonapeptide class of phytotoxins, which are composed of:

1. A polar peptide head, and
2. A dydrophobic 3-hydroxy fatty acid tail.

- Also tolaasin, produced by *P. tolaasii* that attacks the mushroom *Agaricus bisporus*, belongs to necrosis group.
- The necrosis inducing toxins form ion channels in the cell membrane, causing leakage of cells.

Syringomycin and syringopeptin
Necrosis-inducing toxins

2. Cyclic lipopeptides (CLPs)

- *Pseudomonas syringae* pv. *syringae* produces two lipopeptide toxins, syringomycin and syringopeptin, which form pores in plasma membranes.
- The syringomycin (*syr*) and the syringopeptin (*syp*) gene clusters constitute a genomic island that encompasses almost 2% of the bacterial genome.

Toxin production
Multiple toxins production

- *P. syringae* strains are very unlikely to produce more than one toxin (e.g. the tomato pathogen *Pseudomonas syringae* pv. *tomato* strain(Pto KN10), which produced:

1. Tabtoxin,
2. Phaseolotoxin, and
3. Coronatine.

- This might be due a cost associated with the production of multiple toxins.

Toxin production

Tabtoxin determination using *E. coli*

MG vs. MG-glutamine plates

- Production of tabtoxin was determined by an agar plate diffusion test with *E. coli* N99 as the indicator strain.
- *E. coli* N99 was grown overnight in LB medium at 37°C and harvested by centrifugation.
- The pellet was washed and resuspended in 10 ml of sterile 0.9% NaCl at an optical density at 600 nm (OD_{600}) of 0.2.
- Two milliliters of 0.7% molten mineral salts-glucose (MG) agar (maintained at 45°C) was mixed with 2 ml of *E. coli* and poured onto MG agar plates.
- MG-glutamine plates were made by overlaying the *E. coli* MG soft agar mixture with 17 µmol of glutamine.
- Next, 10 µl of an overnight culture of *P. syringae* grown in MG medium was spotted onto the MG-*E. coli* and MG-glutamine-*E. coli* plates, followed by incubation at room temperature for 48 h.
- Strains were scored as positive for tabtoxin when there was a zone of inhibition surrounding the *P. syringae* colonies on the MG plates but not surrounding the corresponding colonies on the MG-glutamine plates.

Hwang *et al.*,2005

Toxin production

Phaseolotoxin determination using *E. coli*

- Phaseolotoxin production was determined by using a method modified from Staskawicz and Panopoulos,1979.
- *E. coli* N99 was grown in Davis minimal medium (Atlas and Parks,1993) for 48 h at 37°C.
- A 2-ml portion of culture was mixed with 2 ml of 2% molten agar in water and overlaid on Davis minimal medium plates.
- *P. syringae* strains were grown in minimal A medium for 48 h at 30°C, and 10μl of the *P. syringae* culture was spotted onto the *E. coli* test plates.
- The presence of phaseolotoxin was characterized by a zone of inhibition surrounding the *P. syringae* colonies after 24 h.

Hwang *et al.*,2005

Toxin production

1. ***Rhodotorula pilimanae*** **for both syringomycin/syringopeptin determination.**
2. ***Geotrichum candidum, B. subtilis*** **and** ***Rhodotorula pilimanae*** **for syringomycin determination.**
3. ***B. megaterium*** **for syringopeptins determination**

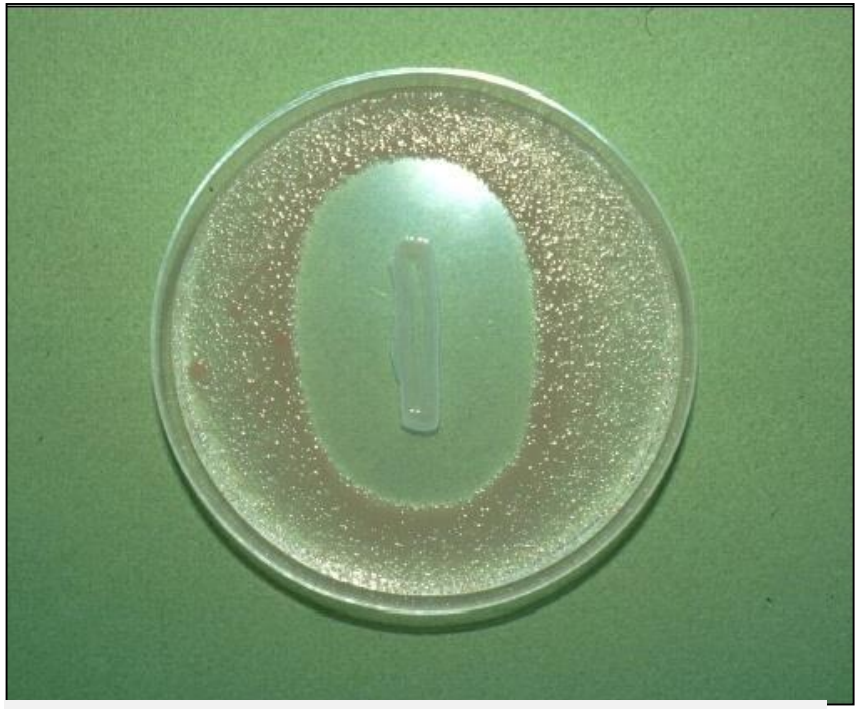
- Syringomycin (lipodepsipeptide) production was assayed by using a general method for detecting lipodepsipeptides (Bultreys and Gheysen,1999).
- Twenty microliters of a *P. syringae* overnight culture was spotted onto potato dextrose agar, followed by incubation for 48 h at 30°C.
- Subsequently, the plates were sprayed with an overnight culture of the pigmented yeast *Rhodotorula pilimanae* as an indicator for syringomycins/syringopeptins production (Bultreys and Gheysen, 1999; Schaad *et al.*,2001) and incubated for 24 h at room temperature.
- The presence of lipodepsipeptide was characterized by the development of a zone of inhibition surrounding (due to the antifungal activity of the toxin) the *P. syringae* colonies.

Hwang *et al.*,2005

Test for production of toxins

Syringomycin/syringopeptin determination using *Rhodotorula pilimanae*

- Detection of toxic lipodepsipeptide production(necrosis-inducing toxins) on the improved culture medium peptone-glucose-NaCl (PGNaClA) by using the very toxin-sensitive pigmented yeast *Rhodotorula pilimanae* (budding yeast).
- More reliable than the PDA test.



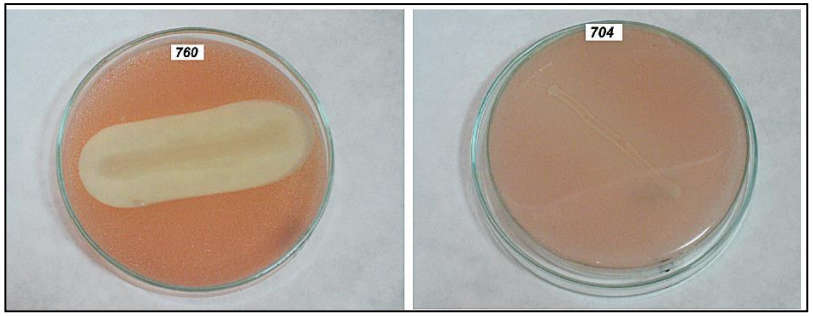
Center, spot inoculation of *P. syringae*.

Alain Bultreys

Test for production of toxins

Syringomycin production bioassay by *Rhodotorula pilimanae* to differentiate Pss from Psm

- Syringomycin production by *Pseudomonas syringae* pv.*syringae* (760) caused the largest inhibition zone of *Rhodotorula pilimanae* growth (Left).
- None of our *Pseudomonas syringae* pv. *morsprunorum* strains in both races 1 and 2 could retard *Rhodotorula pilimanae* growth.
- This is because lack of syringomycin production in *Psm* strains.



Right, *Rhodotorula pilimanae*, as a control.

Kałużna and Sobiczewski,2009

Test for production of toxin

Bioassay for syringomycin production on PDA medium

- Inhibition zone of the:

A. Budding yeast *Saccharomyces cerevisiae* and

B. A pigmented yeast *Rhodotorula pilimanae* growth on PDA medium caused by *Pseudomonas s.* pv. *syringae* strain KFB 0103.

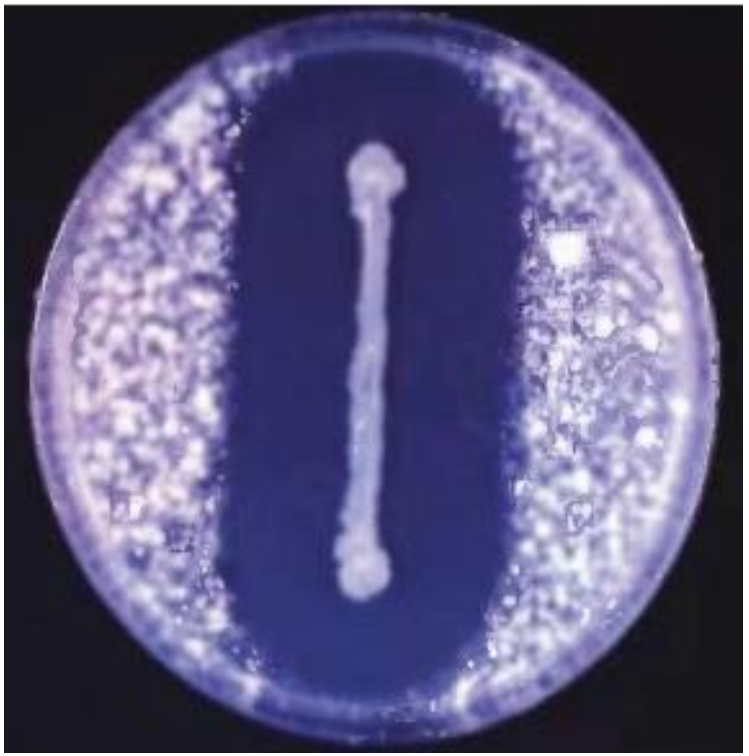
Right, *Rhodotorula pilimanae* growth in brown colour was inhibited by Pss.

Gašić *et al.*,2012

Test for production of toxins

Syringomycin determination using *Geotrichum candidum*

- In the middle of PDA medium, a strain of *P. syringae* pv. *syringae* has been streaked and grown for some days.
- Thereafter the medium was sprayed with a suspension of the fungus *Geotrichum candidum*.
- Where the toxin syringomycin has diffused in the agar the fungus is inhibited.



Janse,2006

Toxin production

Coronatine determination using potato tuber discs

- No bioassay with toxin-sensitive microbes is available.
- Coronatine is a polyketide molecules produced by strains of *P. s.* pvs. *tomato*, *atropurpurea*, *morsprunorum*, *maculicola* and *P.s.* pv. *glycinea*.
- Coronatine is detected by the induction of hypertrophies on potato tuber discs.
- Fifty microliters of an overnight *P. syringae* culture was added to 1 ml of Hoitink and Sinden medium (HSC), and this was followed by incubation on a 250-rpm rotary shaker at 20°C for 4 days.
- One milliliter of this bacterial suspension was centrifuged at 2,000 x *g* for 10 min at room temperature, and 20 µl of the bacterial supernatant was spotted onto the potato tuber disk prepared as described in Volksch *et al.*,1989.
- The presence of coronatine was characterized by a hypertrophic response (formation of outgrowth) on the potato disks.

Hwang *et al.*,2005; Schaad *et al.*,2001

Toxin production

Coronatine determination using potato tuber discs

- Bacterial supernatant was spotted onto the potato tuber disk.
- Visualization of coronatine production on a potato slide: induction of excrescences.

Coronatin is produced by *P. syrinagae* pvs. *lycinea*, *maculicola*, *morsprunorum* and *tomato*.

Toxin production
Persicomycins determination

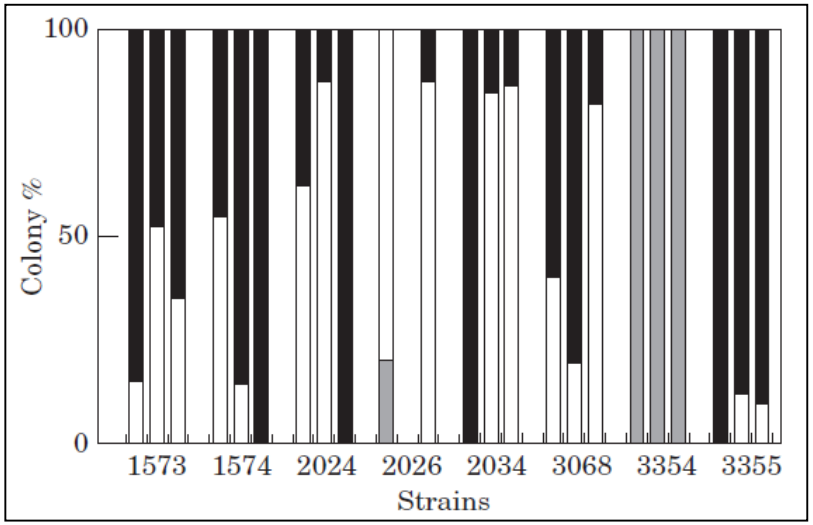
- *Pseudomonas syringae* pv. *persicae* is a phytopathogenic bacterium responsible for a dieback disease to peach, nectarine, and Japanase plum.
- Several toxic substances called persicomycins are synthesized by *Pseudomonas syringae* pv. *persicae*.
- It is now an accepted fact that *P. s.* pv. *persicae* is heterogeneous in toxigenesis i.e. a variable necrotic capability of strains on the peach tree host/variable biocidal activities (persicomycin production) of each and every individual colonies.
- Three classes of colonies were defined:
- Class 1 with no detectable production,
- Class 2 with a low production and
- Class 3 characterized by a larger production.
- It appears that the level of persicomycin production varied from cell to cell whatever the strain and the assay.

Barzic,1999

Toxin production

Toxin production capability of colonies

- Tests were realized with 10 µl of suspension of each colony.
- Data is from three separate assays with 40 colonies each.
- Colonies were divided into three classes:
- Class 1, colonies with no detectable production;
- Class 2, colonies giving a production scored as the mean growth inhibition ≤5 mm from the edge of the colony;
- Class 3, colonies giving a production scored as the mean growth inhibition >5 mm from the edge of the colony.



Barzic,1999

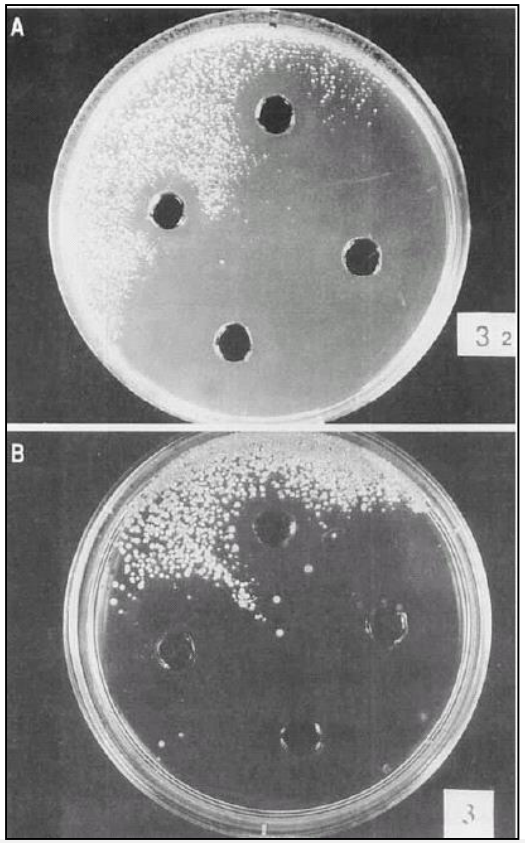
Toxin production

Persicomycin determination

In vitro study

- Both plant cells damage (necrosis of peach tree tissues) as well as *in vitro* antibiosis against microorganisms such as *Bacillus thuringiensis* (bacterial indicator) is due to antibiotic property of persicomycins.
- Antibiosis was displayed by persicomycin extracted from:

A. Culture extracts and
B. Necrotic peach tree tissues.



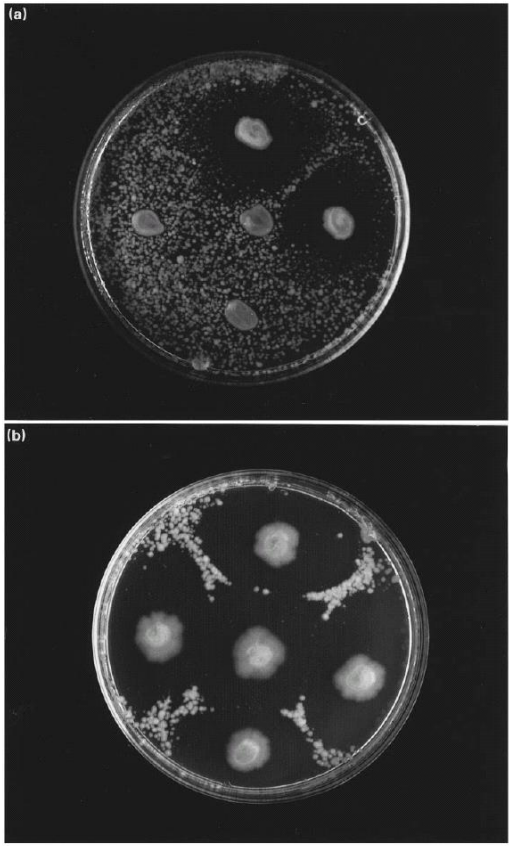
Effects of persicomycins on *in vitro* growth of *Bacillus thuringiensis.*

Barzic and Guittet,1996

Toxin production

Persicomycin determination using *Bacillus thuringiensis*

- Toxigenicity among colonies of *Pseudomonas syringae* pv. *persicae* obtained from strain 3354 (a) and strain 3355 (b) shown by antibiosis to *Bacillus thuringiensis* on LPGA medium.
- All *P.s.* pv. *persicae* isolates from a range of geographical origins have been shown to produce persicomycins *in vitro*.
- *P.s.* pv. *persicae* can synthesize its necrosis inducing toxins, and freezing temperatures, e.g. -5°C, that can occur with frosts did not seem to affect bacterial toxigenesis.



Barzic,1999

Enzymatic activity of *P. syringae* strains

- Enzymatic activity were detected in cell free culture filtrates as previously described
- The cell-wall degradation enzymes:

1. *N*-acetyl-β-D-glucosaminidase (NAGase),
2. β-glucosidase,
3. cellobiohydrolase,
4. Cellulase, and
5. Protease

- detected in culture filtrates of the majority of *P. syringae* strains while only a few strains showed chitinase and glucanase activities.

Cirvilleri *et al.*,2008

Enzymatic activity of *P. syringae* strains

		Production of[a]						
	% of strains	NAGase	β-gluc	cellbio	pro	cell	chi	glu
Group								
A	36	–	–	–	+	+	–	–
	21	+	+	+	+	+	–	–
	29	+	+	–	+	+	–	–
	14	+	–	–	+	+	–	–
B	19	+	+	+	+	–	–	–
	56	+	–	+	+	+	–	–
	25	–	–	+	–	–	–	–

[a]NAGase = *N*-acetyl-β-d-glucosaminidase; β-gluc = β-glucosidase; cellbio = cellobiohydrolase; pro = protease; cell = endocellulase; gluc = β-glucanase; chi = chitinase; glu = glucanase

Cirvilleri *et al.*,2008

Phytohormones production

Auxin production

- In addition to phytotoxins, phytohormones represent another group of low-molecular-weight substances which play a role in pathogenesis by *Pseudomonas* particularly *Pseudomonas syringae*.
- A useful conceptual distinction between toxins and phytohormones is that the latter are also produced by the host.
- *P.s.* pv. *syringae* synthesize exceptionally high amounts of IAA both in the presence and absence of exogenous tryptophan.

Phytohormones
Auxin production

- There is definitive evidence for the involvement of IAA in the interaction between *P. s.* pv. *savastanoi* (Psv) and two of its hosts.
- This pathogen synthesizes the hormone from tryptophan using genes *iaaM* and *iaaH* to incite hyperplasias on oleander and olive.
- Tryptophan monooxygenase and indoleacetamide hydrolase are the products of the *iaaM* and *iaaH* genes, respectively.
- They carry the *iaaM* and *iaaH* genes on plasmids (pIAA) rather than on the chromosome.
- *P. syringae* pv. *myricae* also induce proliferation of plant tissues and also harbor the *iaaM/iaaH* genes.

Phytohormones
Ethylene production

- Ethylene production has been demonstrated in various pathovars of *P. syringae*, including pvs. *glycinea*, *pisi*, *cannabina* and *sesami*.
- The *efe* gene encoding the ethylene-forming enzyme appears to be plasmid encoded (Watanabe *et al*.,1998).

Höfte & De Vos,2006

Siderophore

Useful in identification of some pvs. of *Pseudomonas syringae*

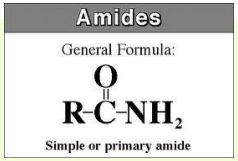
Crude Siderophore crystals

- The siderophore pyoverdin, produced by all fluorescent strains of *P. syringae*, is a character used for diagnosis (Bultreys *et al*.,2003).
- Among stone fruit pathogens, the siderophore yersiniabactin is produced by the pathovars *morsprunorum* race 2, *avii*, and *persicae* and, generally, by genomospecies 3, 7 and 8 of *P. syringae* (Bultreys *et al*.,2006).
- Pyoverdin is not involved directly in pathogenicity of *P. syringae* pv. *syringae* but is a virulence factor for *P. syringae* pv.*tabaci*.
- The *irp1* gene encoding a polyketide synthase/peptide synthetase involved in yersiniabactin synthesis is used in diagnostics.

Bultreys and Kaluzna,2010

The siderophore pyoverdine

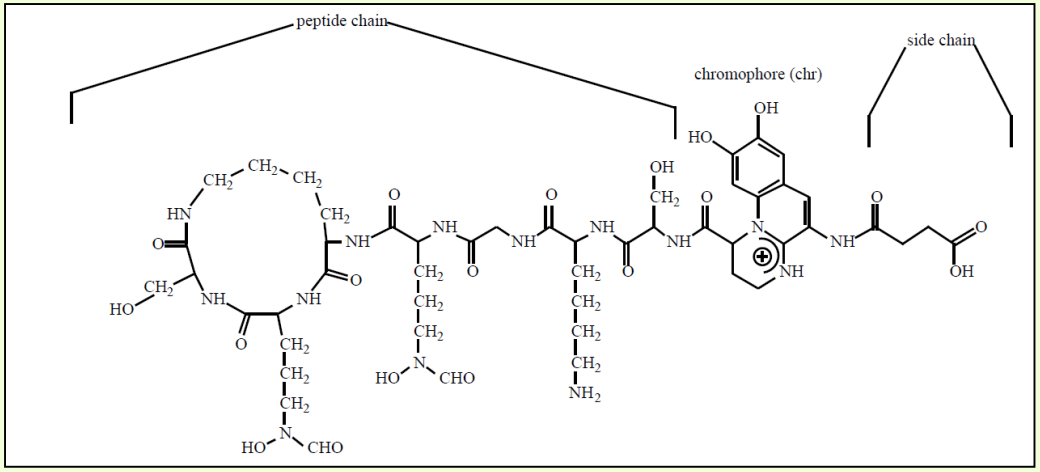
Pyoverdine from *P. fluorescens*



- A pyoverdin is made up of:

1. A constant quinoline chromophore,
2. A variable peptide chain of 6-12 amino acids, and
3. A side chain consisting of a dicarboxylic acid (amide).

- The peptide chain is strain specific and variable among strains and species.
- About 50 peptide chains are known, but 106 are predicted.



Fuchs *et al.*,2001; Bultreys and Gheysen,2008

Detection of siderophores

The chromeazurol (CAS) agar assay

Chrome azurol S reagent

- The chelator-iron (III) complex tints the agar (Chrome Azurol S(CAS)-agar plate) with a rich blue background.
- The color change from blue to orange halo around the colony of *Pseudomonas putida* indicates the excretion of siderophore and its dimension approximates the amount of siderophore produced.

For details see the plant bacterial disease management files.

Jankiewicz & Kuzawińska,2009

Pyoverdin Tests

Visual tests

- The fluorescent pseudomonads were obtained by isolation onto King medium B agar (King *et al.*,1954) to detect the pyoverdin-related fluorescence.
- At the same time, the GASN solid and liquid media were used for siderophores production prepared and stored at 4°C for several months.

1. Petri dishes assay
2. Glass tubes assay

Bultreys and Gheysen,2008

Pyoverdin Tests

Visual tests

- Siderophores were produced by growing cultures in/on GASN (glucose asparagines) solid/liquid medium in Petri dishes as described (Bultreys & Gheysen,2000; Bultreys *et al*.,2006b).
- GASN medium: For siderophores production, bacterial strains were cultivated at 22°C in GASN medium(2 g/L L-asparagine, 7 g/L glucose, 0.96 g/L Na_2HPO_4, 0.44 g/L KH_2PO_4, and 0.2 g/L $MgSO_4.7H_2O$, pH 7.0).

Bultreys and Gheysen,2008

Pyoverdin Tests

Glass tubes assay

With culture filtration

- Glass tubes contain 10 ml of GASN liquid medium.
- After incubation for 3 days, the liquid fraction was collected, iron was added (36 µl of an $FeCl_3$ solution (1 M) and the tube directly divided in two parts.
- The pH was adjusted to 3.0 and 7.0 using an NaOH or HCl solution.
- The pH was determined with an indicator paper.
- A strain was judged as an atypical pyoverdin producer by a color change.
- These colors could easily be observed by using one control.

Bultreys and Gheysen,2008

Pyoverdin Tests

Visual tests

Changes in pH during siderophore production

- Bultreys and Gheysen (2000) have reported marked changes in pH during the production of siderophores by *Pseudomonas* strains, grown in different media.
- In a medium containing asparagine, glucose and salts (GASN medium),

1. the pH decreased from 7 to 4.6 after one day,
2. increased to 6.6 on the second day, and
3. rose to 7.5 on the third day.

- The increase in pH resulted in sharp increase in siderophore concentration.

Gaonkar and Bhosle,2013

Pyoverdin Tests

Visual tests

Changes in pH during siderophore production

- Some authors (Villegas *et al*., 2002) have noted insignificant pH shift (from 7 to 7.5) despite a significant increase in siderophore production by *Pseudomonas aeruginosa* PSS when cultured in a succinate medium with sodium succinate as the carbon and energy source.
- pH also determines the type of siderophore produced by the microorganisms.
- For example, yersiniabactin and salmochelin are the dominant siderophores produced by E. coli under neutral to alkaline conditions, whereas,
- production of enterobactin and aerobactin increased under more acidic conditions (Sandy and Butler,2009).

Gaonkar and Bhosle,2013

Pyoverdin Tests

Glass tubes assay

Without culture filtration

- Visual test for strains producing an atypical or a typical pyoverdin.
- The culture medium was not filtrated and the pH was measured with indicator paper.
- The clearer and different color of the chelated atypical pyoverdins near pH 7 is evident.
- On the other hand the color of the typical pyoverdin most generally remains constant.
- These colors could easily be observed by using one control.

Bultreys and Gheysen,2008

Pyoverdin Tests

Glass tubes assay

Without culture filtration

- Atypical pyoverdins show a grayish-brown color near pH 3 and a yellow-orange color near pH 7.
- On the other hand, it allows differentiation from the atypical pyoverdin of *P. fuscovaginae* and *P. asplenii*, which produce the same pyoverdin and could be synonymous: this atypical pyoverdin appears similar at pH 7 but shows still a net orange color near pH 3.
- Also, the visual test enables distinction from all the other fluorescent species producing typical pyoverdins, which, most generally, show a constant brown color at both pH 3 and 7.

Bultreys and Gheysen,2008

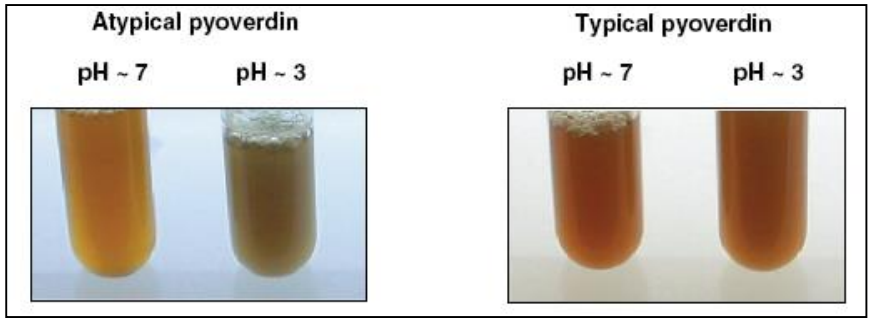
Pyoverdin Tests

Glass tubes assay

Atypical pyoverdins vs. typical pyoverdins

- The atypical pyoverdins of *P. syringae* and *P. cichorii* show a grayish-brown color near pH 3 and a yellow-orange color near pH 7.
- Whereas, the typical pyoverdin of *P. fuscovaginae* and *P. asplenii*, appears similar at pH 7 (remains constant) but shows still a net orange color near pH 3.

Typical pyoverdins, which, most generally, show a constant brown color at both pH 3 and 7



Atypical pyoverdin: Grayish-brown color near pH 3 and a yellow-orange color near pH 7.

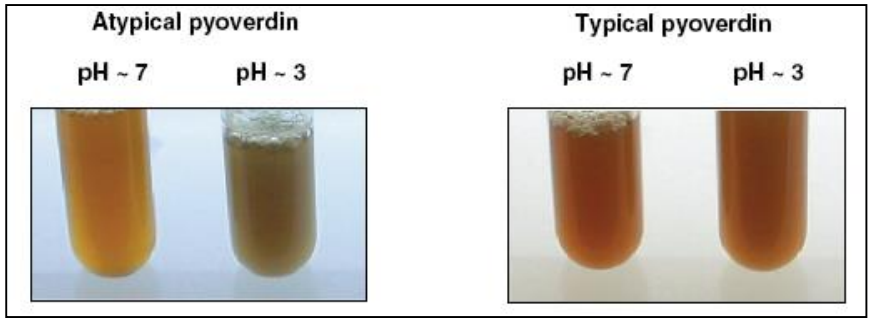
Atypical pyoverdin: Net orange color near pH 3.

Bultreys and Gheysen,2008

Pyoverdin Tests

Glass tubes assay

- The atypical pyoverdins of *P. syringae* and *P. cichorii* show a grayish-brown color near pH 3 and a yellow-orange color near pH 7.
- Whereas, the typical pyoverdin of *P. fuscovaginae* and *P. asplenii*, appears similar at pH 7 (remains constant) but shows still a net orange color near pH 3.



Atypical pyoverdin: Grayish-brown color near pH 3 and a yellow-orange color near pH 7.

Atypical pyoverdin: Net orange color near pH 3.

Bultreys and Gheysen,2008

Detection of siderophores

Spectrophotochemical detection of siderophore production

The test was performed after culture filtration

- Each strain was grown for 3 days in one petri dish containing 10 ml of GASN liquid medium and one block of GASN agar medium.
- Then 36 µl of an $FeCl_3$ solution (1 M) was added, and the cultures were shaken for 20 min, centrifuged (20 min, 10,000 × *g*), and filtered (culture filtration).
- The pH was modified between pH 3.0 and 7.0, and the colors of the solutions were noted.
- After being possibly diluted three times, when the siderophores were too concentrated to be directly analyzed, the culture media equilibrated at pH 7.0 were analyzed with UV-VIS spectrophotometer, starting from 700 to 360 nm.
- Liquid GASN medium was used as a control.

Bultreys *et al.*,2001

Pyoverdin Tests

Spectrophotometrical test

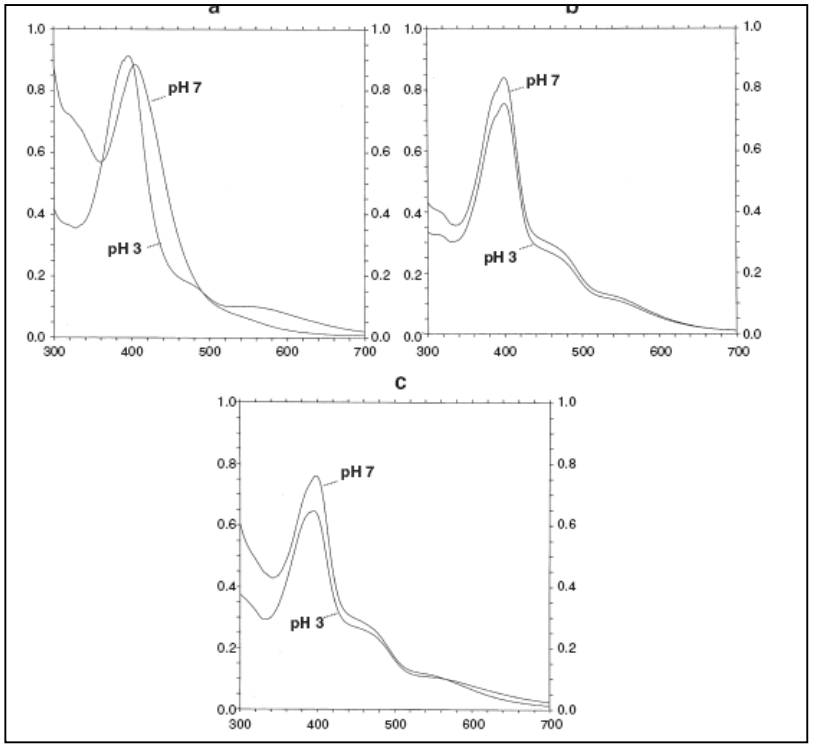
The test was performed after culture filtration

a. A strain producing an atypical pyoverdin: the spectral changes between pH 3 and 7 are evident and no charge transfer bands are observed near 470 and 550 nm at pH 7.

b. A strain producing a typical pyoverdin whose color didn't change with pH: no spectral changes; the charge transfer bands near 470 and 550 nm are observable at both pH.

c. A strain producing a typical pyoverdin whose color slightly varied with pH: a conformational change is clear from the crossing near 570 nm of the spectra at different pH and from the faster extinction at pH 7, but the molecule at both pH has the spectrum of a typical pyoverdin.

- In the spectrophotometric test, the spectra at pH 7 are compared and strains B and C would then clearly be classified among typical pyoverdin producers, and strain A among atypical pyoverdin producers.



The y-axis is absorbance; the x-axis is wavelength.

Bultreys and Gheysen,2008

Pyoverdin production

A complementary test to differentiate *P. syringae* from *P. viridiflava*, *P. chichorii* and other *Pseudomonas* spp.

- Differentiation of some *Pseudomonas* spp.
- On the basis of pyroverdin production, oxidase and potato rot.

	Pyoverdin	Oxidase	Potato rot
P. syringae	Pa	-	-
P. viridiflava	Pa	-	+
P.cichorii	Pa	+	-
Other *Pseudomonas* spp.	Pt	+/-	+/-

- = negative; + = positive; +/- = variable;
Pa = atypical pyoverdin; Pt = typical pyoverdin, see Bultreys et al., 2003

Janse,2010

Resist antimicrobial compounds
Copper and antibiotic resistance

- Pseudomonads in general have a reputation for being highly resistant to antimicrobial compounds and *P. syringae* is no exception.
- Antimicrobials such as copper and streptomycin have been used for decades to control *P. syringae* infections of crop plants.
- *P. syringae* strains also come into contact with medically important antibiotics, and their associated resistance genes, that spread in the natural environment.

Resist antimicrobial compounds
Copper resistance

- Copper resistance was determined by using the method of Cazorla *et al.*,2002.
- First, 50 µl of a *P. syringae* culture (OD_{600} = 0.5) was mixed with 50 µl of mannitol-glutamic-acid yeast extract medium containing $CuSO_4$ at the following final concentrations: 0, 0.5, 0.8, 1.0, 1.5, 2.0, 3.0, or 3.5 mM in 96-well microtiter plates.
- The plates were shaken at 30°C, and the OD_{600} of the mixture was taken immediately after inoculation and at 48 h postinoculation using a Tecan GENios microplate reader.
- Bacterial growth from the two time points was compared, and the MIC was determined.
- MIC is defined as the point where the OD of the bacterial culture at 48 h was the same or less than it was at 0 h.
- Strains with MICs of ≤0.8 mM $CuSO_4$ were scored as copper sensitive.
- 75% of our strains (72 out of 95) were resistant to copper.

Hwang *et al.*,2005

Resist antimicrobial compounds
Antibiotic resistance

- *P. syringae* strains were streaked onto KB plates containing:
- Ampicillin (100 µg/ml),
- Chloramphenicol (25 µg/ml),
- Kanamycin (50 µg/ml),
- Rifampin (50 µg/ml),
- Streptomycin (100 µg/ml), or
- Tetracycline (15 µg/ml).
- Bacterial growth was checked after 24 h and 48 h.

Hwang *et al.*,2005

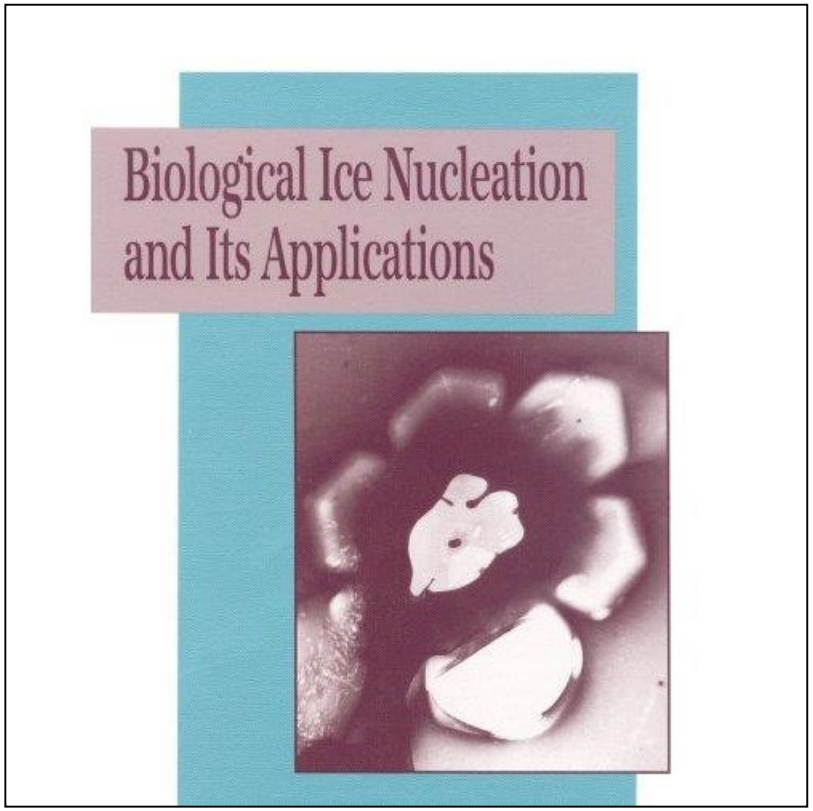
Biological Ice Nucleation and Its Applications

Bacteria

- **Biological Ice Nucleation and Its Applications**
- Richard E. Lee, Gareth J. Warren, L. V. Gusta.
- Publisher: Amer. Phytopathological Society; 1 edition (May 15, 1995).
- 370 pp.

Product Description

- A select group of bacteria plays a key role in the phenomenon of ice nucleation; their actions having an impact on the frost sensitivity of plants, the winter survival of certain insects, and even on weather systems.
- This is the first book to integrate the ice nucleation research of plant physiologists, crop scientists, microbiologists, biochemists, bacteriologists, entomologists, and food scientists worldwide.



Steven E. Lindow

Professor Ph.D. Plant Pathology University of Wisconsin

Ice lab

- Dr. Steven E. Lindow has been pioneer in research demonstrating the role of epiphytic bacteria in ice nucleation and resultant frost damage to plants as well as the genetic control of ice nucleation in bacteria and the feasibility of biological and chemical control of frost injury.
- icelab@socrates.berkeley.edu

Ice nucleation bacteria

Ice weapons

- Despite broad distribution of Ice-nucleating bacteria (INBs):
- On plants
- Leaf mulch,
- Soils,

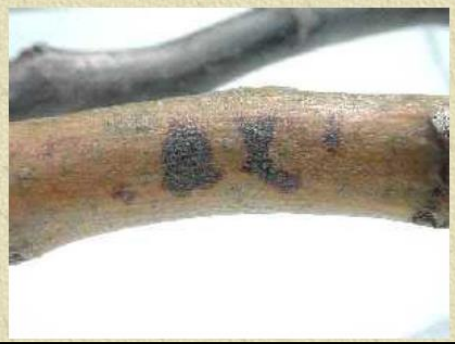
- The number of known INB species is relatively small.
- These ice crystals grow and pierce plant cells creating more wounds, through which the bacteria can enter the plant.

BSPP;..

Symptoms of frost injuries

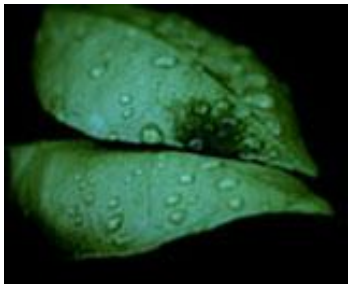
Ice nucleation activities for *P. syringae* pvs.

Below: Damage on a branch caused by an ice-nucleating *Pseudomonas syringae pv. syringae*
(Photo: N. Carter)



M. Stefani

Frost damages

Misshapen berries resulting from blooms which are partially damaged by frost.	
Frost injury on strawberry leaves.	
Ice formation in citrus leaf (Steven E Lindow,2009).	

Ice nucleation organisms
Bacteria, pollen, plankton and lichens

- The first-identified Ice+ bacterium was *Pseudomonas syringae* (Maki *et al.*,1974; Arny *et al.*,1976) and the activity was later identified in additional Pseudomonadaceae, Xanthomonadaceae (Kim *et al.*,1987) and Enterobacteriaceae (Lindow et al.,1978), which are all members of the Gammaproteobacteria.
- A small number of studies also reported INA in bacteria outside the Gammaproteobacteria (Ponder *et al.*,2005; Mortazavi *et al.*,2008).

L. R. Maki, E. L. Galyan, M. M. Chang-Chien, D. R. Caldwell, Ice nucleation induced by *Pseudomonas syringae*. Appl. Microbiol. 28, 456 (1974).

Failor *et al.*,2017

Ice nucleation organisms
Bacteria, pollen, plankton and lichens

- Fifty years ago, bacteria were identified as remarkable ice nucleators that enable water freezing close to 0°C (Renzer *et al.*,2024).
- Although Ice$^+$ bacteria have been well known to be the most active IN at warm temperatures since the 1970s (Maki *et al.*,1974), information about their diversity and the conditions associated with their deposition with precipitation is limited.

Failor *et al.*,2017

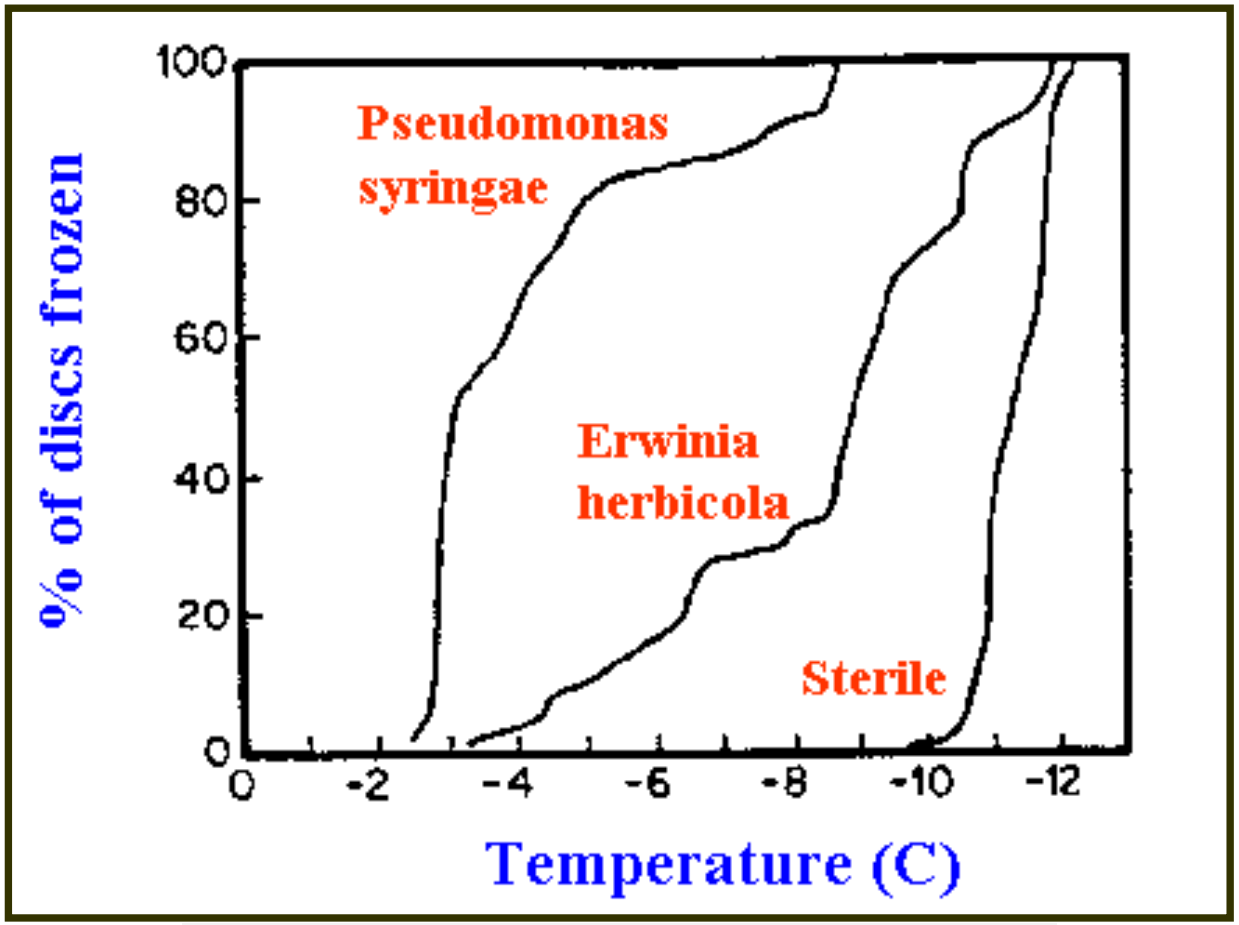
Ice-nucleating bacteria(INBs)

INBs/INAs

- ***Pseudomonas syringae* pv. *syringae***
- ***Pseudomonas syringae* pv. *persicae***
- ***Pseudomonas syringae* pv. *coronafaciens*** (halo blight of cereals)
- ***Pseudomonas syringae* pv. *pisi***
- ***Pseudomonas syringae* pv. *lachrymans***
- ***Pseudomonas savastanoi* pv. *glycinea***
- ***Pseudomonas viridiflava***
- ***Pseudomonas fluorescens*** biotypes A, C, B, G and F
- ***Pantoea ananatis***
- ***Pantoea agglomerans***
- ***Sphingomonas* spp.**
- ***Xanthomonas translucens***

Two efficient ice nucleators

P. syringae and *P. agglomerans* compared with sterile D.H_2O



E. herbicola now *Pantoea agglomerans*

Ice nucleation bacteria or fungi
Subcooling

- Pure water can remain in a liquid phase at temperatures below 0°C in a subcooling state.
- Until now, only a few species of bacteria and a small number of lichen mycobionts were known to produce biological ice nuclei that initiate the crystallization of subcooled water at temperatures above -5.0°C.
- Without bacteria involved, freezing damage on actively growing shoots is usually limited to the tip or outer branches, and the plant will recover.

Pouleur *et al.*,1992; Li *et al.*,2012;..

Ice nucleation

Distilled water vs sterile D.H_2O

Heterogeneous vs homogenous ice nucleation

- To enable ice formation to take place, water molecules must cluster in an ice-like pattern and this cluster must reach a critical size.

1. If the initial aggregation of water molecules takes place on a foreign structure, the process is termed heterogeneous ice nucleation.
2. If the water molecules aggregate without the help of another structure, the nucleation is termed homogenous.

Bisht,2011

Ice nucleation particles

Biological and non-biological ice nucleating particles

- While the nucleation temperature of mineral dusts, carbonaceous combustion products or volcanic ash is mainly limited to temperatures below −12 °C/−15 °C (Murray *et al.*, 2012), some bacteria and fungi nucleate ice at temperatures up to −2 °C (Pouleur *et al.*,1992; Morris *et al.*,2004), and pollen at temperatures up to −10 °C (Pummer *et al.*, 2012).
- Biological ice nuclei (bacteria, pollen, plankton, lichens) are active at relatively warm temperatures, between -2 and -9°C.
- Several bacterial ice nucleation genes have been cloned from all known species with ice nucleation activity.

Georgakopoulos *et al.*,2009; Failor *et al.*,2017

Ice nucleation organisms

Aerial plant surfaces: the most well-known habitat

INA plant tissues

Pollen

Grasses (*Dactylus glomerata*)
Pine (*Pinus sylvestris*)
Oak (*Quercus rubra*)

(-8 to -11℃, contact freezing)

Birch (*Betula alba*) (-5℃, contact freezing)

Other tissues

Lobelia telekii flowers (-4.5℃)

Winter rye mesophyll cells ($1/10^5$ cells active at -7℃)

Woody tissue of *Prunus* spp. (-2℃)

Morris,2006

Ice nucleation organisms

Bacteria, pollen, plankton and lichens

	max T_{ina} ℃
Bacteria	-1°
Fungi (free-living)	-5°
Fungi (lichen symbiont)	-2°
Algae	-5°
Pollen	-5°
Other plant tissues	-2°
Insect haemolymph proteins	-5°

Microbiological Meteorology / ESF workshop / 1-3 March 2006, INRA-Avignon, France

Morris,2006

Ice nucleation organisms

Aerial plant surfaces: the most well-known habitat

Focus on fungi

Free-living

Fusarium acuminatum
F. avenaceum
F. oxysporum
F. tricinctum

soil-borne, vascular plant pathogens
limited amount of air-borne spores

(cell-bound and cell-free ice nuclei)

Lichen-symbionts

Lichens and axenic cultures of fungal symbiont, divers species.

Most active species were collected from rocks (vs epiphytic and soil lichens).

Morris,2006

Ice nucleation organisms

Aerial plant surfaces: the most well-known habitat

Focus on Algae

Algae isolated from Antarctic soils. (Some have also been reported in biotic crusts in deserts.)

Prasiola crispa	-5°	terrestrial
Myrmecia irregularis	-6°	(no info)
Chlorella minutissima	-6°	unicellular; widespread in soils, aquatic (fresh and marine); marine industry and pharmaceutical uses.
Scotiellopsis sp.	-7.5°	unicellular
Bumilleria sp	-8°	filamentous
Elliptochloris subsphaerica	-9°	closely related to *Chlorella*
Pseudophormidium sp.	-9°	filamentous; cyanobacterium?

Microbiological Meteorology / ESF workshop / 1-3 March 2006, INRA-Avignon, France

Morris,2006

Ice nucleation organisms

Aerial plant surfaces: the most well-known habitat

Focus on bacteria

Habitats for proliferation of INA organisms

focus on Bacteria

Aerial plant surfaces: the most well-known habitat of INA bacteria

Microbiological Meteorology / ESF workshop / 1-3 March 2006, INRA-Avignon, France

Morris,2006

Ice nucleation organisms

Formation of raindrops, hailstones and snow

Pseudomonas syringae

- As explained earlier there is enough evidence that *P. syringae* is having more ability to cause rain by precipitation (David sands,1982).
- Studies by meteorologists and plant pathologists are proving that the bacterium plays a crucial role in the formation of all forms of precipitation like:

1. raindrops,
2. hailstones, and
3. snow.

- There are more increasing results to support *P. syringae* in rain drops as there was some researchers detected the presence of *P. syringae* in fresh rain, snow and in ice from locations like Louisiana, the French Alps and even in Antarctica.

Prasanth *et al.*,2015

Ice nucleation organisms

Ice genes plus nutritional and environmental factors

- Even if the gene responsible for this property is present in the genome, bacterial ice nucleation is not always fully expressed.
- Nutritional and environmental factors influence this expression and the subsequent ice nucleation activity of the bacterium.
- E.g.
- The INA characteristic was variable, depending in part upon the host plant from which the strains of *Pseudomonas syringae* were isolated.

Georgakopoulos *et al.*,2009; Morre,1988

Ice nucleation bacteria
Horizontal gene transfer

- A phylogenetic analysis of 16S ribosomal RNA gene sequences from a total of 14 ina^+ and ina^- bacterial strains indicated that the ina^+ bacteria are not monophyletic but instead phylogenetically interspersed among ina^- bacteria.
- Homology of INA genes and 16S ribosomal RNA sequence differences across these species suggest this trait was acquired through horizontal gene transfer from a common ancestor.

Edward *et al.*,1994; Raymond and Kim, 2012 MicrobeWiki,2015

Ice nucleation bacteria or fungi
Subcooling

- INA bacteria have been detected in air above crops under dry conditions and were enhanced ~30-fold during rainfall, and they were relatively abundant in air downwind of harvesting.
- They have also been isolated from cloud water, from ice and rain at up to 2,500 m above a wheat field, and from ~50% of rain and snow samples.
- A typically large reduction in INP (ice-nucleating particles) active at >−12°C after heat treatment or cell wall digestion suggests their ubiquity in precipitation; rainfall itself stimulates the release of biological INP from vegetation and the soil surface.

Hill *et al.*,2013

Ice nucleation organisms

Single ice nucleus is sufficient to initiate ice formation

- According to Lindow *et al.*,1982 and Kaneda,1986, *P. syringae* and other epiphytic bacteria have been found to incite frost damage on numerous plants species and cultivated crops.
- A single ice nucleus is sufficient to initiate ice formation and subsequent frost injury to entire leaves, fruits, flowers, depending on the degree of restriction of ice propagation within the plant (Single & Olien,1967).

They are particularly abundant on crops but are also plentiful on many nonagricultural plants and in habitats such as freshwater and associated biofilms.

Nejad,2005;Hill *et al.*,2013

Ice nucleation bacteria or fungi
Supercooling

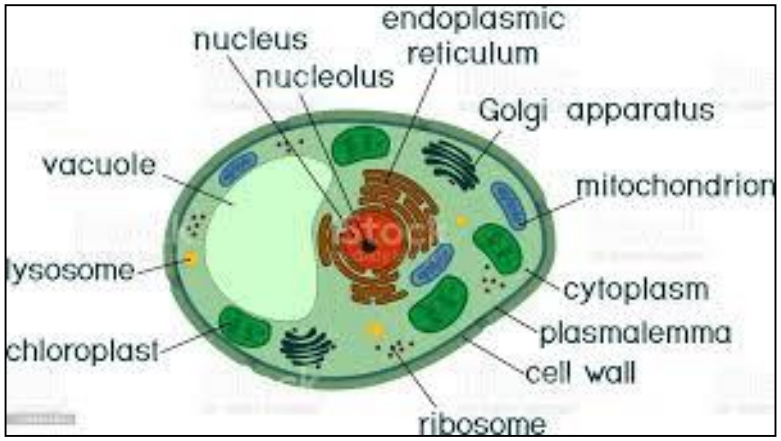
- Frost sensitive plants are injured when ice forms within plant tissues.
- In the absence of heterogeneous ice (ice formation at different temperatures), water associated with leaves will supercool. i.e. water on leaf surfaces typically supercools to temperatures below -5°C before forming ice nuclei and freezing.
- Supercooling in the temperature range of 0 to about - 5°C is primarily limited by the presence of INA bacteria.
- Below -5°C, other heterogeneous ice nuclei, probably also limit supercooling.

Höfte & De Vos,2006

The freezing process in plants
Ice nucleation-active (INA^+) bacteria

- In a tissue, ice nucleation can occur either:

1. in the cell inside the plasmalemma/plasma membrane (intracellular ice crystal formation), or
2. in the intercellular spaces between the cells (extracellular ice crystal formation).



Nejad,2005

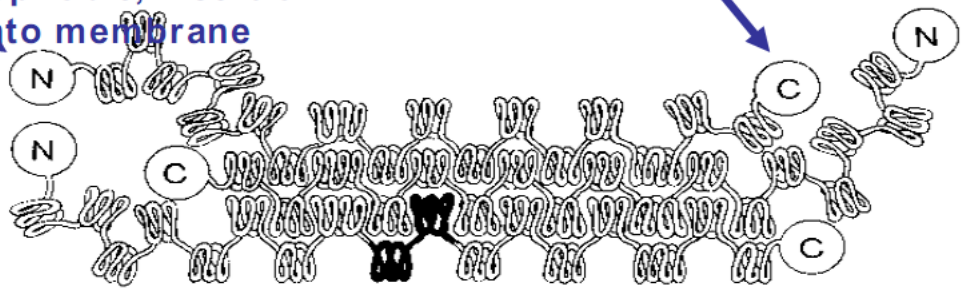
Ice nucleation protein (INP)

The central repeating domain act as a template for ice crystal formation

INA protein

N- terminal: hydrophobic, insertion into membrane

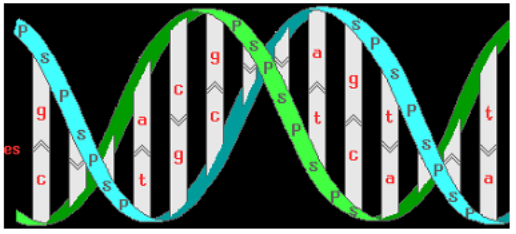
C- terminal: hydrophilic



Core: central domain, repeating units, template for binding water

(Kajava and Lindow 1993)

~4000 pairs of bases (A T G C) constitute the code in the DNA (gene) for this protein.



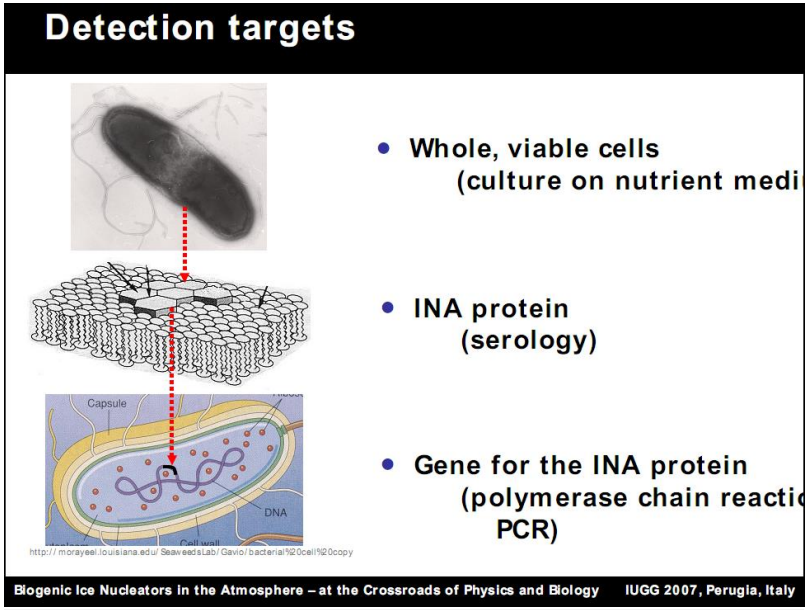
Biogenic Ice Nucleators in the Atmosphere – at the Crossroads of Physics and Biology IUGG 2007, Perugia, Italy

Guilbaud *et al.*,2007

Ice nuclei

Association with the outer bacterial membrane

- Ice nuclei are predominantly located (anchored) on outer membrane bacterial cell (Lindow *et al.*,1989).
- The ice-nucleating secretory protein is extruded out from the cell surface and anchored to the outer cell membrane via phosphatidylinositol (PI).



The 'ice plus' bacteria posses INA protein (Ice nucleation-active protein) found on the outer bacterial wall acts as the nucleating center for ice crystals. This protein located on the outer membrane of these bacteria is responsible for ice nucleation. While 'ice minus' bacteria do not posses Ina proteins and lower the ice nucleation temperature.

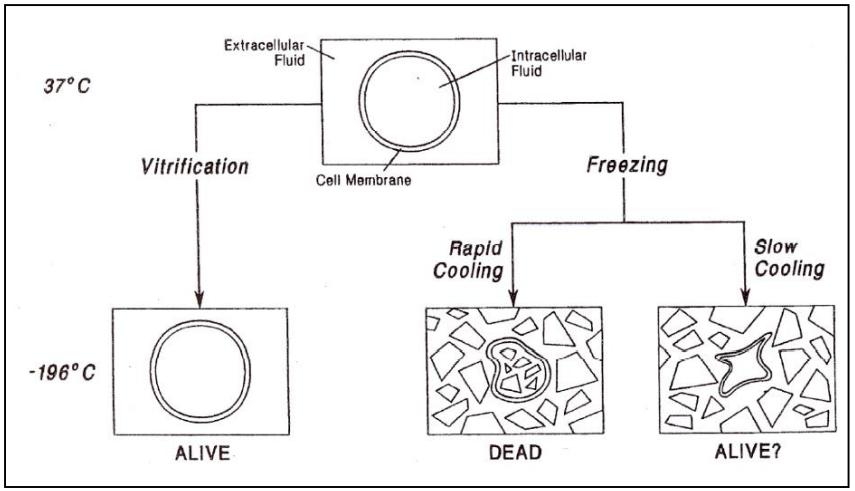
Guilbaud *et al.*,2007; Bisht,2011

The freezing process in plants

Physiochemical process during cryopreservation of the cells

1. Freezing can occur exclusively in the extracellular space (right),
2. it can occur in both the extracellular and the intracellular space (middle), or
3. it may occur not at all (left).

- Adapted from Coger and Toner (1995) in Fahy (1995).



Water that becomes solid without freezing is said to be vitrified. Vitrification(transformation of a substance into a glass) can occur usually through very rapid cooling or the introduction of agents that suppress the formation of ice crystals.

Nejad,2005;..

Ice nucleation bacteria or fungi

Supercooling

Fungal INA vs. Bacterial INA

- Ice-nucleating bacteria (INBs/INA$^+$) are defined as those bacteria that can initiate ice nucleation in water at temperatures above -10°C.
- This ice-nucleating protein INA is responsible for millions of dollars in crop losses each year.
- Some strains of *Fusarium* (Pouleur *et al.*,1992), and related genera of fungi are also active in ice nucleation (Obata *et al.*,1999).
- The maximum temperatures of ice nucleation were -2.5°C for *Fusarium avenaceum* and -1.0°C for the bacteria (Pouleur *et al.*,1992).
- Ice nuclei of *F. avenaceum*, unlike bacterial ice nuclei, pass through a 0.22 μm-pore-size filter.

Ice nucleating (INA^+) bacteria

Heterogeneous ice nuclei

- All Ina^+ bacterial cultures examined so far have a population of cells with varied abilities to nucleate ice formation at different temperatures (heterogeneous ice nuclei).
- Only a small fraction of cells are active at -4.4°C or warmer, whereas almost every cell can nucleate at -8°C or lower.
- e.g. *P. syringae* gene *ina* produces a protein (INA) that acts as a heterogeneous nuclei for ice crystal formation, raising the temperature of ice formation to as high as -1.2°C, thereby causing increased frost damage.

Epiphytic and endophytic bacteria
Ice nucleation bacteria
Quorum Sensing

- Epiphytic populations of INA-bacteria can constitute an inoculum source and be essential for the development of infection when temperatures reach slightly below zero.
- Endophytically appearing bacteria (Nejad & Johnson, 2000) may strategically be at the right place without affecting the plant, building up its forces for a successful attack when the population density becomes high enough, may be by involvement of Quorum Sensing known from several other bacterial species.

Nejad,2005

Ice nucleating (INA$^+$) bacteria
Season/population density

- *P. syringae* pv. *syringae* is characterized by an ice nucleation activity (INA) which may cause severe frost damages especially when sudden decrease of temperature occurs.
- *P. syringae* pvs. are among the most consistently ice nucleation active (85% of strains) and most of them (90%) produce a syringomycin-like toxin.
- A higher percentage of INA strains of Pss on kiwifruit plants was recorded at the beginning of the vegetative kiwifruit seasons (59% of isolates collected in spring) whereas lower percentage of INA strains were observed in the other seasons.

Rossetti and Balestra,2008;Berge *et al.*,2014

Ice nucleation bacteria

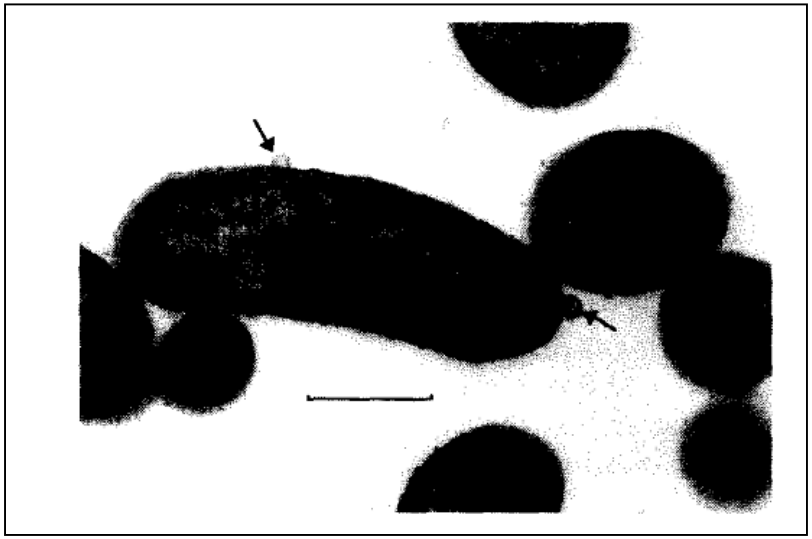
Ice nucleation proteins (INPs)

- Some epiphytic gram-negative eubacteria from genera such as *Pseudomonas*, *Pantoea* and *Xanthomonas* use ice nucleation proteins (INPs) to promote the growth of ice in freezing-sensitive plant tissues at temperatures as high as -2°C.
- These bacterial ice nucleators are composed of 120-kDa lipoglycoproteins that form large membrane-bound aggregates.
- As a result of bacterial ice nucleation, the plants freeze during light frosts and release nutrients that fuel bacterial proliferation.

Muryoi *et al.*,2004

The aggregation of ice nucleation proteins (INP)
Pseudomonas viridiflava

- Transmission electron micrograph of ice-nucleating bacterium, *Pseudomonas viridiflava* KLJIN-2.
- The aggregation of Ice nucleation proteins (INP) as the translation of the ina gene on its plasmid or the vesicle including INP in *P. viridiflava* were transferred to the outer membrane.
- The bar indicates 0.5 μm.
- The arrow shows INP or ice-nucleating material.



Kawahara,2002

Ice-nucleating bacteria(INBs)

INBs/INAs

- ***Pseudomonas syringae* pv. *syringae***
- ***Pseudomonas syringae* pv. *persicae***
- ***Pseudomonas syringae* pv. *coronafaciens*** (halo blight of cereals)
- ***Pseudomonas syringae* pv. *pisi***
- ***Pseudomonas syringae* pv. *lachrymans***
- ***Pseudomonas savastanoi* pv. *glycinea***
- ***Pseudomonas viridiflava***
- ***Pseudomonas fluorescens*** biotypes A, C, B, G and F
- ***Pantoea ananatis***
- ***Pantoea agglomerans***
- ***Sphingomonas* spp.**
- ***Xanthomonas translucens***

Ice-nucleating bacteria(INBs)

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- ***Sphingomonas* spp.**
- ***Xanthomonas translucens***

Ice-nucleating bacteria

Documented ice nucleation active bacteria

Bacterial genus and species	Bacterial genus
Erwinia ananatis (*Pantoea ananas*)	*Bacillus*
Erwinia herbicola	*Coliwellia*
Erwinia uredovora	*Clavibacter*
Erwinia carotovora	*Corynebacterium*
Pseudomonas antarctica	*Curtobacterium*
Pseudomonas aeruginosa	*Exiguobacterium*
Pseudomonas borealis	*Flavobacterium*
Pseudomonas fluorescens	*Frigoribacterium*
Pseudomonas putida	*Kluyvera*
Pseudomonas syringae	*Pedobacter*
Pseudomonas viridiflava	*Pseudoxanthomonas*
Pantoea ananatis	*Sphingobacterium*
Pantoea agglomerans	*Sphingomonas*
Xanthomonas campestris	

Lorv *et al.*,2014

Ice-nucleating bacteria

Ice-nucleating activity of selected bacterial cultures

Bacillus subtilis and *B. cereus*

- Temperatures required to freeze 1% (T_1) and 90% (T_{90}) of the test samples.
- All cultures except *P. syringae* were obtained from the Division's stock collection.

Culture	Ice-nucleating temperatures[a] (C)	
	T_1	T_{90}
Pseudomonas syringae C-9	−2.9	−3.5
Known *P. syringae*[b]	−3.2	−3.9
P. aeruginosa	−7.5	−17.8
Staphylococcus epidermidis	−6.9	−19.5
Escherichia coli	−8.3	−17.1
Enterobacter aerogenes	−9.6	−17.0
Proteus mirabilis	−8.0	−19.4
P. vulgaris	−7.8	−17.0
Bacillus subtilis	−10.6	−18.0
B. cereus	−6.9	−17.0
Uninoculated medium	−9.2	−17.0

Maki *et al.*,1974

Ice-nucleating bacteria
In snow and frost flowers

- Isolated bacteria were identified belonging to different genus, such as: *Afipia genosp*, *Bacillus*, *Paenibacillus*, *Microbacterium*, and *Kocuria*.
- Many of the bacterial isolates in different categories of Arctic samples showed a moderate IN activity e.g. at −15.9 ± 0.4 and −17.2 ± 0.8°C in windpack (WP) snow categories.
- The highest IN activity corresponded to genus: *Bacillus* and *Paenibacillus* with −6.8 ± 0.2 and −15.2 ± 1°C, respectively.

Snow category	Bacterial colony	Ice nucleation temp. (°C)	Accession #	Species	% similarity
WP	A	−18.9 ± 2.5	U87778.1	*Afipia genosp*	86
			JF799916.1	*Bradyrhizobium*	87
			FR691406.1	*Bosea*	87
BS	A	−18.9 ± 1.6	JN082256.1	*Bacillus sp.*	97
			JN092792.1	*Bacillus flexus*	97
			HQ143640.1	*Geobacillus stearothermophilus*	97
	B	−15.2 ± 1	JF343205.1	*Paenibacillus amylolyticus*	95
			NR_044524.1	*Paenibacillus xylanexedens*	95
			HQ202814.1	*Paenibacillus tylopili*	95
FS	A	−6.8 ± 0.2	JN208198.1	*Bacillus sp.*	96
			AB648987.1	*Bacillus megaterium*	96
			JN092792.1	*Bacillus flexus*	96
			HQ857752.1	*Bacillus aryabhattai*	96
	B	−21.6 ± 1	JN085952.1	*Microbacterium sp.*	98
			JF700471.1	*Microbacterium hydrocarbonoxydans*	98
			HQ113206.1	*Microbacterium oxydans*	98
FFs	A	−20.0 ± 1.5	HQ425309.1	*Kocuria sp.*	98
			FR682683.1	*Kocuria rhizophila*	98

Mortazavi *et al.*,2015

Ice-nucleating bacteria
In precipitation

- 23 precipitation events were collected over 15 months in Virginia, USA.
- 33 134 isolates were screened for ice nucleation activity (INA) at -8°C.
- Of 1144 isolates that tested positive during initial screening, 593 had confirmed INA at −8°C in repeated tests.
- Most Ice^+ bacteria were identified as members of known and unknown Ice^+ species in the Pseudomonadaceae, Enterobacteriaceae and Xanthomonadaceae families, which nucleate ice employing the well-characterized membrane-bound INA protein.

Failor *et al.*,2017

Ice-nucleating bacteria
In precipitation

- Two Ice$^+$ strains, however, were identified as *Lysinibacillus*.
- *Lysinibacillus* is a Gram-positive genus not previously known to include Ice$^+$ bacteria.
- Three species of *Lysinibacillus* are:

1. *Lysinibacillus boronitolerans* gen. nov. sp. nov., and
2. transfer of *Bacillus fusiformis* to *Lysinibacillus fusiformis* comb. nov. and
3. *Bacillus sphaericus* to *Lysinibacillus sphaeric*.

- INA of the *Lysinibacillus* strains is due to a nanometer-sized molecule that is heat resistant, lysozyme and proteinase resistant, and secreted.
- Therefore, INA$^+$ bacteria and the INA mechanisms they employ are thus more diverse than expected.

Failor *et al.*,2017

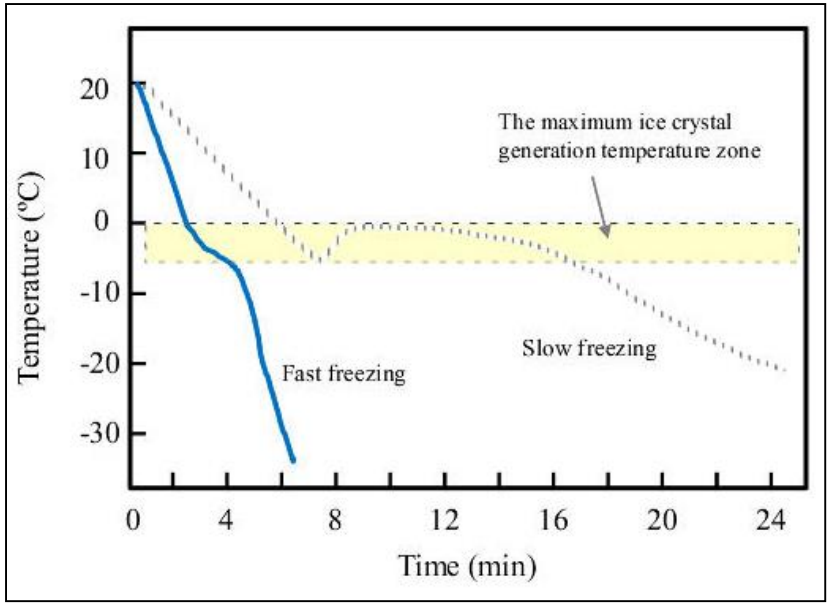
Ice nucleation activities of *P. syringae* A psychrotrophic bacterium

- *P. syringae* is a ubiquist, epiphytic bacterium, naturally present in the environment and generally lives in association with plants.
- It is a psychrotrophic bacterium, having an optimum growth temperature of 25 to 28°C.
- It can be found at densities of up to 10^{14} cells per hectare of agricultural land (Hirano and Upper,1986).
- In water, the bacterium is naturally present at a concentration of 100 to 1000 bacteria per litre and, in snow, this concentration can naturally reach up to 10^5 bacteria per litre (Morris *et al.*,2008).

Lagriffoul *et al.*,2010

The maximum ice crystal generation temperature zone
Ranging from 0°C to -7°C

- The maximum ice crystal generation temperature region is from 0°C to -7°C.
- This temperature region is important for ice crystal structure formation.
- When the time to pass through this temperature region is short, a detailed ice crystal is formed, and when the time is long, a large and rough ice crystal is formed.



Kawarhara *et al.*,2013

Ice nucleation bacteria

Droplet freezing method for effective nucleators

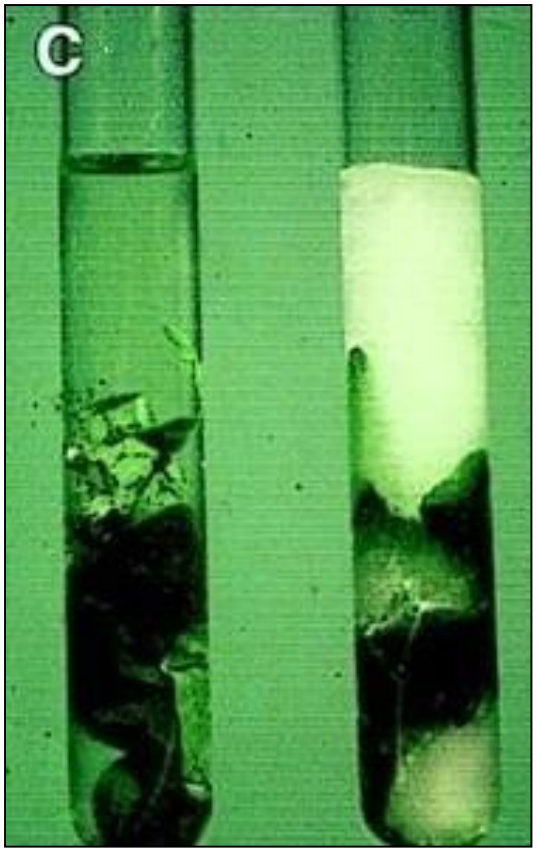
- The presence of INA is not a universal explanation of nucleation of ice in plants.
- Sizes of INA bacterial populations vary greatly between plant species, sites, climates and seasons, and only a small percentage of cells in a population are effective nucleators (Lindow, 1990).
- He argued that a very small population of INA bacteria could nucleate freezing throughout citrus trees since once nucleated freezing would spread rapidly.
- However, this argument would not carry to herbaceous plants, where each leaf (in grasses) or main shoot (in a dicot) would freeze separately because of the higher temperature of the crown compared to the leaves.

Nejad,2005;..

Ice nucleation activity

Tube INA assay

- *P. syringae* strains were grown on KB plates at room temperature for 2 days.
- A single colony of *P. syringae* was suspended in 5 ml of cooled D.H_2O in a test tube held in a bath at -8°C and -9°C.
- Strains were scored positive for ice nucleation activity if there was immediate ice formation in the tube.
- A mix of saturated saline and crushed ice gives a temperature of -9°C (Fahy and Persley,1983).



Ice nucleation test

Tube INA assay

Prechilled in buffer

- Deep frozen cultures of bacteria were revived on TSA for a period of 24-48 hrs, at room temperature (20-25°C) and recultured on TSA after checking purity.
- From a cell concentration of approx. 10^9 colony forming units (cfu) /ml,10 to 50 µl of bacterial suspensions were transferred to test tubes (*n*=5) containing 9 ml sterile ice-nucleus free buffer (0.01 M KH_2PO_4, pH 7.0) vortexed and incubated on a shaker for 10 minutes before placing the tubes in a refrigerated, filled with 96% alcohol.
- The temperature was lowered at a standard rate of 1°C for every half an hour and freezing was recorded from -1°C to -9°C or lower (-16°C).

Nejad,2005;..

Ice nucleation test

Tube INA assay

Prechilled in buffer

- *P. syringae* strains were grown on KB plates at room temperature for 4 to 5 days.
- A single colony of *P. syringae* was suspended in 100 µl of potassium phosphate buffer (10 mM, pH 7) (PPB) by gentle vortexing.
- Then 10 µl of this suspension was added to 2 ml of PPB prechilled in a -10°C ethanol-ice water bath for 5 min.
- Strains were scored positive for ice nucleation activity if there was immediate ice formation in the tube.
- Absence of ice nuclei in tubes had always been observed in assays done at -10°C for 1 h.
- Each strain was need to test in two replicates.

Hawang *et al.*,2005

Ice nucleation test

Droplet freezing method

Prechilled in SDW

- The Pss strains were tested for ice nucleation activity by the method of Lindow *et al.*,1978.
- Briefly, ten 10-µL drops of cell suspension (10^8 cells mL^{-1} in SDW), prechilled at 5°C for 30 min, were applied to the surface of 3- x 3-cm squares of laboratory film within aluminium foil boats floating on a supercooled (-6°C) water/ethanol ice bath (most isolates tested had nucleation activity above -6°C i.e. relatively warm temperature).
- Droplets of SDW and non-ice-nucleating *P. fluorescens* were included as controls.

Droplets were placed on the surface of an aluminium foil sheet (spray coated with a 2% solution of paraffin in xylene and heat-dried to remove the solvent) floating on an anti-freeze bath set at -9°C.

Whitelaw-Weckert *et al.*,2011; Tegos *et al.*,2000

Ice nucleation test
Droplet freezing method

- Bacterial cultures were prepared by inoculating single colonies into 5 ml culture tubes of L broth, growing them overnight with aeration at 37°C, and diluting 1:100 into fresh 5 ml culture tubes and growing with aeration at 37°C to early log phase (A_{600} of 0.15 to 0.25). Cultures were then incubated for 2 h at 24°C with continued aeration, after which A_{600} was measured (to estimate cell titer). The bacterial suspensions were diluted to 10^{-7} in increments of 10^{-1} in sterile distilled water.
- Ten replicate 10 µl drops of each dilution were placed on a par affin-coated tray of aluminum foil which floated on the surface of a refrigerated ethylene glycol(antifreeze) bath.
- Droplet freezing was tallied through stepwise reductions of temperature, and ice nucleation frequencies at each step were calculated according to Vali (1971). Each graph point presented below is a mean of results from two or three replicates of such an assay.

Green *et al.*,1988

Ice nucleation test
Droplet freezing method

- Ice nucleation activity was calculated by the Vali equation (Vali, 1971) using a software programme (Lindow, S.E., University of California, Berkeley, personal communication) and expressed as:
- log ice nuclei per cell, or
- log ice nuclei per millilitre of the

I. whole cultures,

II. cell suspensions, or filtered culture supernatant fluids, respectively.

Tegos *et al.*,2000

Ice nucleation test
Droplet freezing method

- Cells of INA^+ bacteria and fungi e.g. *syringae* were suspended in phosphate buffer (0.1 M, pH 7.0) to ca. 10^8 CFU/ml.
- Tenfold serial dilutions were prepared from this suspension.
- Thirty 10 µl drops of 10-fold dilutions were placed on a aluminum block/thermoelectric cold plate coated with paraffin and cooled from room temperature to -20°C at a rate of 1°C min^{-1} on the surface of ethylene glycol-water (1:1 [vol/vol]) in the refrigerating circulator bath.
- The block was cooled the temperatures at which 10%(T_{10}), 50%(T_{50}) and 90%(T_{90}) of the drops froze were recorded (Muryoi *et al.*,2004).
- i.e. The temperatures required to freeze 10%(T_{10}), 50%(T_{50}) and 90%(T_{90}) of cells.

Pouleur *et al.*,1992;bata *et al.*,1999; Muryoi *et al.*,2004

Ice nucleation test

Droplet freezing method

1. Ice nucleation activity is reported as T_{50} temperatures

- Due to an inability to measure directly ice nuclei formed, ice nucleation activity is reported as the temperature required to freeze 50% of the droplets (T_{50}) added to a thermoelectric cold plate (Kawahara,2008).
- Class C (type III) ice nucleation proteins have the weakest activity, where T_{50} is less than −8.0°C.

Note: The various ice-nucleating bacteria were found to be active in ice nucleation from 22.5°C (T_{50}) to 25.9°C (T_{50}) for 2 days at 18°C.

Lorv *et al.*,2014

Ice nucleation test

Droplet freezing method

2. Ice nucleation activity is measured by alternative model

- Recently, Hartmann and coworkers (2013) have argued that this method inaccurately measures ice nucleation activity since each droplet varies in the number of ice nuclei present and does not account for ice nucleation by substrates in solutions.
- As an alternative, this research group has developed a model that measures ice nucleation rate assuming that ice nucleation complexes in droplets are Poisson distributed.
- However, whether this model becomes the new standard for this measurement remains to be determined.

Lorv *et al.*,2014

Ice nucleation proteins

Three classes of ice-nucleating structures

- The ice nuclei activity has been classified by the range of temperature in which they initiate freezing:
- type 1 ice nuclei are active between -2°C to -5°C,
- type 2 are active between -5°C to -7°C, and
- type 3 are active between -7°C to -10°C.
- Very potent ice nucleators, active at high subfreezing temperature, are produced by bacteria such as *Erwinia herbicola*.
- Other bacterial genera viz., *Pseudomonas*, *Pantoea* (*Erwinia*) and *Xanthomonas* can nucleate the crystallization of ice from super-cooled water.

Bisht,2011

Ice nucleation proteins

Three classes of ice-nucleating structures

- In general and with some differences between strains:

A. Class A structures, which are the most active, nucleate at - 4.5°C or warmer. e.g. *Pseudomonas syringae* and *Pantoea agglomerans*.

B. Class B structures nucleate between - 5°C and - 8°C e.g. *P. s.* pv. *glycinea*.

C. Class C structures are the least active and nucleate at - 8°C or colder. e.g. *P. s.* pv. *glycinea* and *P. s.* pv. *phaseolicola*.

Complex	Stability	Temperature range
Class A	Low	-2°C to -5°C
Class B	Low	-5°C to -7°C
Class C	High	below -7°C

Ice nucleation test

Droplet freezing method

Ice nucleation activity is reported as T_{50} temperatures

- Class C (type III) ice nucleation proteins have the weakest activity, where T_{50} is less than -8.0˚C.
- For example, *Pseudomonas fluorescens* KUAF-68 and *Flavobacterium* sp. GL7 were classified as Class C activity with T_{50} values of -10.6 and -8°C, respectively.
- This class is composed of protein aggregates that can have an overall molecular weight greater than 1000 kDa.

The various ice-nucleating bacteria were found to be active in ice nucleation from 22.5°C (T_{50}) to 25.9°C (T_{50}) for 2 days at 18°C.

Lorv *et al.*,2014

Ice nucleation test

Droplet freezing method

Ice nucleation activity is reported as T_{50} temperatures

- Class B (type II) ice nucleation proteins: Composed of glycoprotein aggregates, and have moderate activity with a T_{50} value around -4.5°C.
- More specifically, these glycoproteins have been found to involve glucosamine and mannose modifications.
- Class A (type I) ice nucleation proteins: The most active ice nucleation proteins composed of lipoglycoprotein aggregates that are anchored to cell membranes via phosphatidylinositol, with T_{50} values up to -2°C. e.g.
  - *Pseudomonas syringae*, was reported with ice nucleation activity of −2°C.
  - *Pseudomonas borealis* DL7 with an activity of −3.7°C.

Lorv *et al.*,2014

Details of droplet freezing method
Calculation of Ice nucleation activity
Ice-nucleation activity of two bacterial isolates

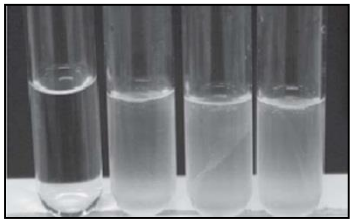
- The ice-nucleation-activity of isolate A4 is higher than isolate A3 with an ice-nucleation frequencies of 59.9/ml and 37.9/ml, respectively.
- Turner *et al.*,1990 classified ice nucleation proteins into three classes:

1. class A which showed the ice nucleation activity between temperature -2°C to -5°C,
2. class B within the temperature range of -5°C to -8°C,
3. class C is below-10°C.

- Based on that system, these two isolates were categorized as class B.

Isolates	f	N(t)(-8°C)
A3	17/20	37.9/ml
A4	19/20	59.9/ml

N(t) = Ice-nucleation activity per ml; f is the proportion of droplets unfrozen.



Waturangi *et al.*2008

Ice nucleation test
Droplet freezing method

- Cultures were grown at 24°C for 24 h.
- Nucleation activity was quantified by droplet-freezing assays.
- Cell suspensions were serially (10-fold) diluted, and 10-µl droplets from each dilution were placed on the surface of an aluminum foil sheet (spray coated with a 2% solution of paraffin in xylene and heat dried to remove the solvent) floating on an ethanol/methanol bath set at -5 or -10°C.
- Ice nucleation activity was calculated by the equation of Vali, using a software program (6a), and was expressed as the logarithm of ice nuclei per CFU of each fraction.

Drainas *et al.*,1995

Ice nucleation test
Droplet freezing method

- A -5°C surface was prepared by spraying aluminum foil with a 1% solution of paraffin in xylene; the xylene was removed at 55°C in a circulating oven, and the foil was folded into a flat-bottomed "boat", which was floated on a methanol-water solution maintained at -5°C in a refrigerated constant temperature bath.
- Discrete 4- to 6-day-old colonies from agar plates were removed with a toothpick and suspended in 0.1 ml of distilled water to yield a turbid suspension (>10^8 cells/ml).
- Five 10 µl droplets of suspension from each colony were placed on the -5°C test surface.
- A colony was considered nuclei active at -5°C if one or more of the five droplets froze within 30 sec.

Lindow *et al.*,1978

Ice nucleation phenomenon

Catalysis of ice crystal formation in supercooled water has been demonstrated for some plant pathogenic bacteria e.g. *Pseudomonas syringae*

These epiphytic bacteria initiate ice formation at temperatures higher than normally required.

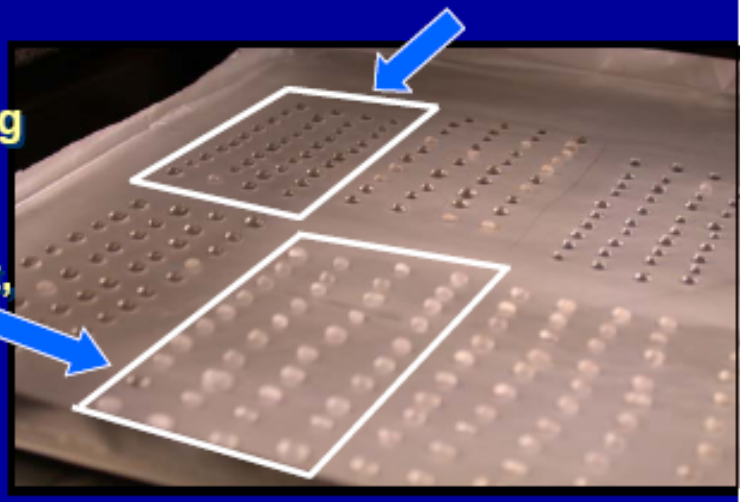
A gene in the bacterium codes for protein in cell wall that confers ice nucleating ability.

Ice damage creates wounds

Droplet freezing assay:

In this sample, ice-nucleating bacteria were not present. The droplets supercool but do not freeze.

In this sample, ice-nucleating bacteria were abundant, and ice was formed



Ice nucleation test

Measurement of frost injury to corn seedlings

Frost sensitivity of intact plants

- Frost injury to three-leaf-stage corn seedlings at -4°C was measured by a method similar to that reported earlier (Arny *et al.*,1976; Lindow *et al.*,1975).
- Plants were sprayed with suspensions of *E. herbicola* growth in 0.1 M phosphate buffer pH 7.0, or buffer alone (about 0.5 ml/plant) at various times before freezing.
- Plants were incubated in a mist chamber (mist treatment) or in ambient air (dry treatment) at about 24°C in the dark until immediately before freezing.
- After incubation, plants were cooled to about -2°C at about 0.2°C/min, then to -4°C at about 0.03°C/min, and finally allowed to warm to 30°C.
- Each of the three leaves of every corn seedling was rated for frost injury. Sixty to 80 plants were included in preliminary experiments, the presence of *E. herbicola* on each treatment.
- A leaf was scored as damaged regardless of the extent of injury. Damage is expressed as the fraction of leaves that showed frost damage in each treatment.

Lindow *et al.*,1978

Ice nucleation test

Measurement of frost injury to corn seedlings

Frost sensitivity of intact plants

- Pots were divided randomly into treatment groups (10 pots/treatment) and either sprayed with bacterial suspensions (approximately $I0^7$ cells/ml) or left untreated. Plants were incubated in a 24°C mist chamber for either 24 h (*P. syringae* treatments and controls) or 48 h (*E. herbicola* treatments and controls).
- After incubation, plants were allowed to dry briefly; treatments were randomized with respect to position on carts and placed in the freezing chamber at about 0°C.
- Plants were then cooled slowly (0.1-0.05°C/min) to the minimum temperature required and were held at that temperature for at least 4 min before rewarming to 30°C.
- Damage was assessed 24 h later and expressed as the fraction of leaves/plant that showed any injury.
- Twelve thermocouples, distributed about the freezing chamber at leaf height, indicated less than 0.2°C spread about the median air temperature at the time of minimum temperature.

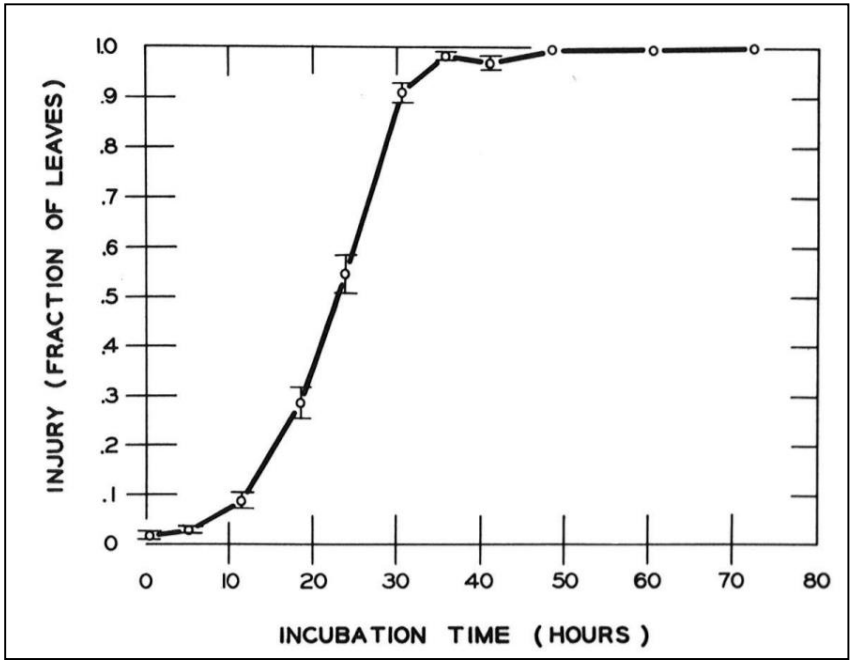
Lindow *et al.*,1978;1982

Ice nucleation test

Measurement of frost injury to corn seedlings

Frost sensitivity of intact plants

- The effect of time of incubation between application of *Erwinia herbicola* and freezing on seedlings.
- Plants were sprayed with a cell suspension of 1.1 X 10^7 cells/ml in 0.1 M phosphate buffer, pH 7.0 (about 0.5 ml/ plant) and incubated in mist chamber for the times given on the abscissa(X-axis) prior to exposure to -4°C.
- The vertical bars represent the standard error of the mean.



Lindow *et al.*,1978

Ice nucleation test

Ice nucleation activity spectra of leaf material

Leaf discs test

- Leaf discs of about 3 mm diameter were cut from random parts of leaves using either a No. 0 cork borer or a paper punch.
- Individual discs were placed on the cooling surface with a dissecting needle and were either submerged in, or floated on, 30 µl droplets of sterile water.
- The surface was cooled, and nucleation events in or on leaf discs were sensed by visually noting when the supporting droplet froze.
- The temperature at which each of 40 to 70 discs per plant sample catalyzed ice formation was recorded manually.
- The cumulative number of nuclei per gram of leaf tissue was determined by substituting the mass of each leaf disc (M) for the volume term (V) in the equation noted below.

$$N(T) = -\ln(f)V^{-1}$$

- This calculation was independent of the volume used since the water drops themselves did not contain ice nuclei active above -15°C. the calculation assumed that the leaf discs were of equal mass (about 1.5 mg each).

Lindow *et al.*,1982

Ice nucleation test

Ice nucleation activity spectra of leaf material

Leaf discs test

- Sterile 9 ml test tubes (18 or 16 x 150 mm) containing sterile K-phosphate(10 mM, pH 7.0, prepared with glass-distilled H_2O) were prepared. (See diagnosis-part1 file)
- The buffer-containing tubes were initially screened for the absence of non-specific ice nuclei by cooling at -10°C for 30 to 60 min
- Tubes do not freeze are then warmed to room temperature, and individual leafs/tubes/.. to be tested are placed in a tube(one leaf in the buffer solution in each test tube), chilled to 0°C, then tested for freezing at -2, -4, -6 and -10°C for periods of 90 minutes.
- Leaves which show ice nucleation activity may then be shaken with the buffer and the frequency of INA^+ bacteria determined by the droplet test.

Hirano *et al.*,1985; Sigee,1993

Ice nucleation test

Ice nucleation activity spectra of leaf material

Leaf discs test

- A total of 180 individual leaflets was harvested
- Each leaflet was submerged in buffer in a test tube [9 ml of sterile Potassium phosphate (10 mM, pH 7.0, prepared with glass-distilled H_20) were placed in test tubes (18 or 16 x 150 mm)].
- All tubes were placed in a refrigerated constant temperature bath maintained at -2.5 ± 0.05°C.
- The number of tubes that contained frozen leaflets was scored at 15-min intervals during the 1st h and then at 30-min intervals for the subsequent 3 h of incubation.

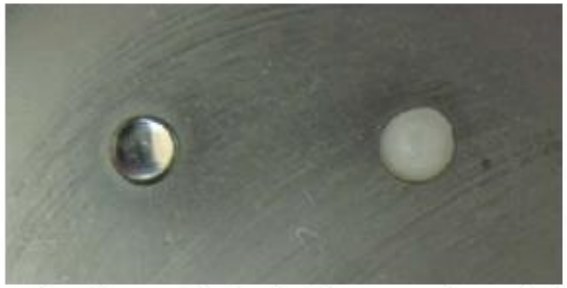
Sampling Time (Duration of Incubation at −2.5°C)	Percentage of Leaflets Frozen	
	Incremental[a]	Cumulative[b]
min	%	
15	56.7	56.7
30	7.8	64.4
45	1.7	66.1
60	0.0	66.1
90	1.7	67.8
120	0.0	67.8
150	1.1	68.9
180	0.0	68.9
210	0.5	69.4
240	0.0	69.4

[a]Incremental percentage of a total of 180 leaflets that froze per 15- or 30-min interval.
[b]Cumulative total frozen since beginning of experiment.

Hirano *et al.*,1985

Ice nucleation test
Droplet freezing method

- Ice Nucleation test produce quick results.
- *P. s.* pv. *morsprunorum* and *P.s.* pv. *actinidiae* did not show ice nucleation activity.
- It is positive for both pvs. *syringae* and *persicae*.



ce nucleation activity. Left – negative; right – positive

Obradovic,2010;..

Ice nucleation test

Droplet freezing method

Vali formulation

- Cell cultures were harvested and resuspended in distilled water to a concentration of 10^8 cells/ml.
- The cell suspension was serially diluted at 1:10, and six 5-µl droplets of the diluted samples were dropped onto an aluminum block.
- The temperature of the block was slowly decreased at a rate of −0.2°C/min, and the number of freezing droplets was counted.
- The number of cumulative ice nuclei was calculated by using Vali's formula as follows:
- $N(t) = [\ln(1/F)]10^D/V$,
- Where
- $N(t)$ is the cumulative number of ice nuclei,
- F is the fraction of droplets unfrozen at temperature t,
- V is the volume of each droplet, and
- D is the number of dilutions.

Kwak *et al.*,1999

Ice nucleation test
Droplet freezing method

- Many strains of *P. syringae*, *P. viridiflava*, and *P. fluorescens*, are ice-nucleation active (INA). These bacteria catalyze ice formation at temperatures above -10°C, some strains even at temperatures as warm as -1.5°C.
- If INA-positive bacteria induce ice formation at temperatures above - 5°C in frost sensitive plants that may lead to freezing injury/frost damage.
- While most strains of pvs. *syringae*, *pisi*, *glycinea*, *lachrymans*, and *coronafaciens* are INA positive, ice-nucleation has never been detected in *P. savastanoi* or *P. syringae* pv. *tomato*.
- A few strains of *P. s.* pv. *phaseolicola* are positive exhibiting ice-nucleation at -10°C and many strains of *P. syringae* pv. *glycinea* express INA only at temperatures approaching -10°C.
- Thus, while a positive INA assay will not unambiguously identify a bacterial strain, it will in many cases eliminate certain species or pathovars from consideration as a causal agent.
- Most bacteria active in ice-nucleation at temperatures greater than -10°C, will express detectable nucleation frequencies at temperatures warmer than - 5°C as well.

Schaad *et al.*,2001

Details of droplet freezing method
Calculation of Ice nucleation activity
Ice nucleation tests

- The single-temperature method of Lindow *et al.*,1978 was used for routine determination of ice nucleation activity at -5°C.
- In this temperature only the most active ice nucleus in each droplet is detectable.
- Thus, a series of dilutions was used to detect less active but more numerous ice nuclei in order to obtain the entire ice nucleus spectrum of bacterial suspensions.
- The ice nucleus concentration in each dilution was normalized to the concentration in the original suspension.

Lindow *et al.*,1982

Details of droplet freezing method
Calculation of Ice nucleation activity
Ice nucleation tests

- Ice nucleation activity was calculated by the equation of Vali, using a software program (6a), and was expressed as the logarithm of ice nuclei per CFU of each fraction.
- Nucleation frequency (NF) is the fraction of cells active at a given temperature (i.e. the number of nuclei/cell).
- Nucleation frequency is determined by the following formula:

$$\text{Nucleation frequency (NF)} = \frac{\text{cells/ml}}{\text{Nuceli/ml}}$$

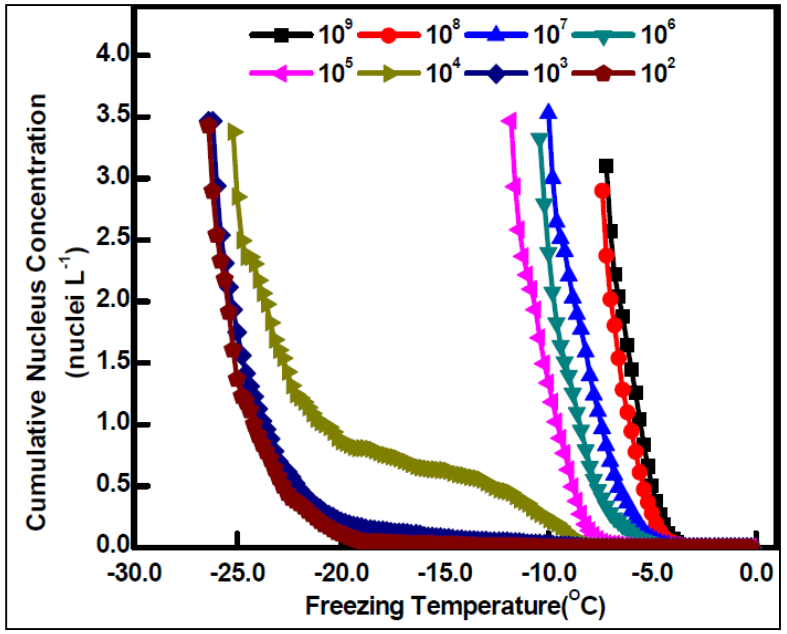
- Nuclei/ml in which the nuclei/ml were calculates by:

$$\text{nuclei/ml (at temperature T)} = \frac{\text{In total number of drops}}{\text{Number of unfrozen drops}} \Big/ \text{Volume of drops}$$

Schaad,1998;..

Details of droplet freezing method
Calculation of Ice nucleation activity
Low cell density, low INA and lower temperature

- With the *Pseudomonas syringae* pv. *lachrymans* suspension concentration decreasing from 10^9 to 10^2 cells/mL, the droplet freezing temperatures changed from –2.8 to –25.8°C.
- Under the low concentration of 10^4, 10^3 and 10^2 cells/mL, the tested droplets' freezing temperatures were changed from –7.2 to –24.6°C, –8.0 to –25.6°C, –9.4 to –25.8°C, respectively, which indicates that the low concentration of INA bacteria cannot catalyze the ice formation at the relatively warm temperatures.



Frequency refers to the ratio between the numbers of active IN at a given temperature to the number of bacterial cells in a culture.

Du *et al.*,2015;..

Example of calculations of differential and cumulative spectra

- There are two methods indicated:

1. one using dN/N,
2. the other ln (1-dN/N) to obtain k (T).

- Also, two ways of obtaining the cumulative spectrum are shown

1. direct summation, and
2. the integral formula.

- The results are nearly indistinguishable for these different approaches, except at the lowest temperature where sample size becomes very small.

Courtesy G. Vali

Example of calculations of differential and cumulative spectra.

3	volume V=0.01cm^3		dT = 0.1		C = 1/(V*dT) = 1/0.01*0.1 = 10^3			
4								
5	temperature	number frozen	number freezing per interval	number remaining unfrozen	differential spectrum	differential spectrum	cumulative spectrum	cumulative spectrum
6	**T**	**N**	**dN**	**Nu**	**k(T) =C* dN/N**	**T) = −C*ln(1−dN/I(T)=sum(k(T)*0.**		**K(T)=ln(Nu/No)/V**
7					$cm^{-3}\,C^{-1}$	$cm^{-3}\,C^{-1}$	cm^{-3}	cm^{-3}
8								
9	-5.0	0		467				
10	-5.1	0	0	467				
11	-5.2	0	2	467	4.28E+00	4.29E+00	0.00E+00	
12	-5.3	2	1	465	2.15E+00	2.15E+00	4.28E-01	4.29E-01
13	-5.4	3	0	464	0.00E+00	0.00E+00	6.43E-01	6.44E-01
14	-5.5	3	2	464	4.31E+00	4.32E+00	6.43E-01	6.44E-01
15	-5.6	5	0	462	0.00E+00	0.00E+00	1.07E+00	1.08E+00
16	-5.7	5	2	462	4.33E+00	4.34E+00	1.07E+00	1.08E+00
17	-5.8	7	5	460	1.09E+01	1.09E+01	1.51E+00	1.51E+00
18	-5.9	12	1	455	2.20E+00	2.20E+00	2.59E+00	2.60E+00
19	-6.0	13	6	454	1.32E+01	1.33E+01	2.81E+00	2.82E+00
20	-6.1	19	4	448	8.93E+00	8.97E+00	4.14E+00	4.15E+00
21	-6.2	23	8	444	1.80E+01	1.82E+01	5.03E+00	5.05E+00
22	-6.3	31	3	436	6.88E+00	6.90E+00	6.83E+00	6.87E+00
23	-6.4	34	3	433	6.93E+00	6.95E+00	7.52E+00	7.56E+00
24	-6.5	37	8	430	1.86E+01	1.88E+01	8.21E+00	8.25E+00
25	-6.6	45	6	422	1.42E+01	1.43E+01	1.01E+01	1.01E+01
26	-6.7	51	10	416	2.40E+01	2.43E+01	1.15E+01	1.16E+01
27	-6.8	61	6	406	1.48E+01	1.49E+01	1.39E+01	1.40E+01
28	-6.9	67	9	400	2.25E+01	2.28E+01	1.54E+01	1.55E+01
29	-7.0	76	10	391	2.56E+01	2.59E+01	1.76E+01	1.78E+01
30	-7.1	86	9	381	2.36E+01	2.39E+01	2.02E+01	2.04E+01
31	-7.2	95	16	372	4.30E+01	4.40E+01	2.25E+01	2.27E+01
32	-7.3	111	21	356	5.90E+01	6.08E+01	2.68E+01	2.71E+01
33	-7.4	132	18	335	5.37E+01	5.52E+01	3.27E+01	3.32E+01
34	-7.5	150	28	317	8.83E+01	9.25E+01	3.81E+01	3.87E+01
35	-7.6	178	30	289	1.04E+02	1.10E+02	4.70E+01	4.80E+01
36	-7.7	208	29	259	1.12E+02	1.19E+02	5.73E+01	5.90E+01
37	-7.8	237	29	230	1.26E+02	1.35E+02	6.85E+01	7.08E+01
38	-7.9	266	21	201	1.04E+02	1.10E+02	8.11E+01	8.43E+01
39	-8.0	287	24	180	1.33E+02	1.43E+02	9.16E+01	9.53E+01
40	-8.1	311	17	156	1.09E+02	1.15E+02	1.05E+02	1.10E+02
41	-8.2	328	29	139	2.09E+02	2.34E+02	1.16E+02	1.21E+02
42	-8.3	357	18	110	1.64E+02	1.79E+02	1.37E+02	1.45E+02
43	-8.4	375	19	92	2.07E+02	2.31E+02	1.53E+02	1.62E+02
44	-8.5	394	25	73	3.42E+02	4.19E+02	1.74E+02	1.86E+02
45	-8.6	419	11	48	2.29E+02	2.60E+02	2.08E+02	2.28E+02
46	-8.7	430	14	37	3.78E+02	4.75E+02	2.31E+02	2.54E+02
47	-8.8	444	14	23	6.09E+02	9.38E+02	2.69E+02	3.01E+02
48	-8.9	458	4	9	4.44E+02	5.88E+02	3.30E+02	3.95E+02
49	-9.0	462	3	5	6.00E+02	9.16E+02	3.74E+02	4.54E+02
50	-9.1	465	1	2	5.00E+02	6.93E+02	4.34E+02	5.45E+02
51	-9.2	466	0	1	0.00E+00	0.00E+00	4.84E+02	6.15E+02
52	-9.3	466	1	1	1.00E+03		4.84E+02	6.15E+02
53	-9.4	467	0	0				
54	-9.5	467	0	0				
55	-9.6	467		0				

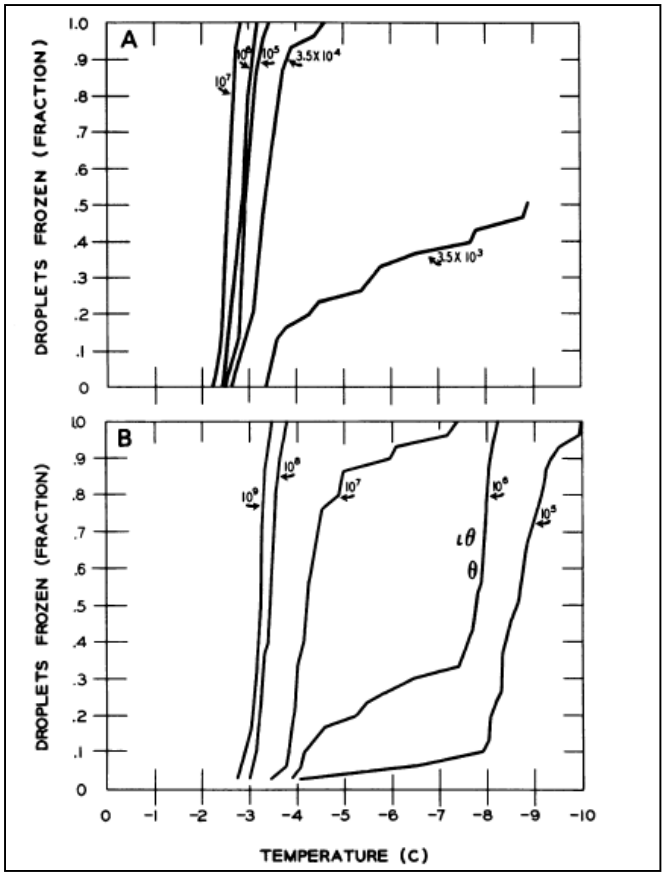
Courtesy G. Vali

Ice nucleation test

Droplet freezing method

Numbers of active ice nucleators (N_T) at the nucleation temperatures were calculated

- Freezing spectra of cell suspensions of:
  - A. *P. syringae* isolate No. 31, and
  - B. *E. herbicola* isolate No. 26.
- Cells were grown on nutrient agar containing 2.5% glycerol for 2 d at 20°C and suspended in sterile water to yield suspensions of 3.5 x 10^7 *P. syringae* cells/ml and 1.6 x 10^9 *E. herbicola* cells/ml.
- The curves represent the freezing of 30 droplets from each of five 10-fold dilutions.



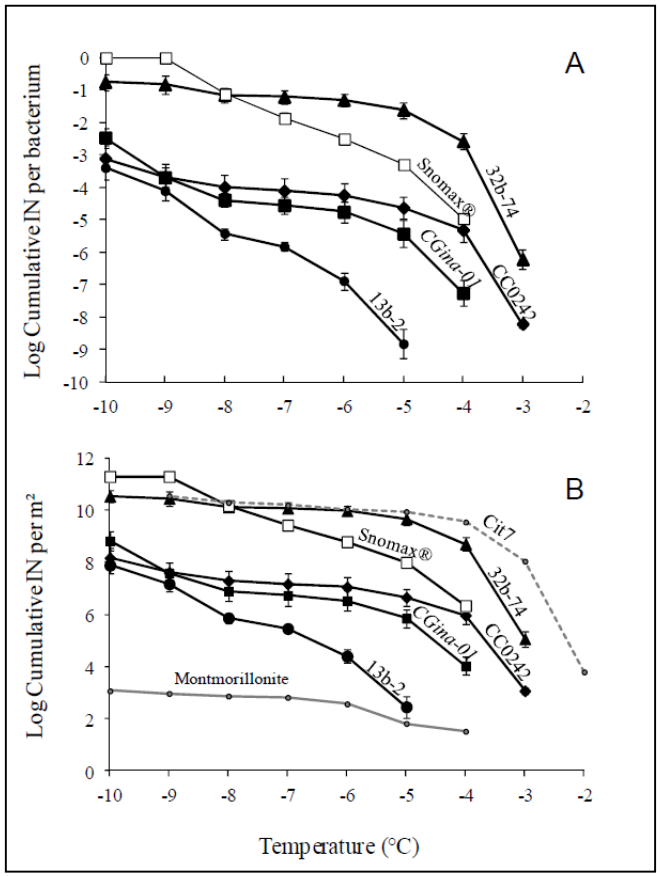
Lindow *et al.*,1982

Ice nucleation test

Droplet freezing method

Numbers of active ice nucleators (N_T) at the nucleation temperatures were calculated

Sample	Temperature of nucleation (°C)	N_T
1	-3.0	1.3×10^3
2	-5.5	9.9×10^4
3	-4.5	4.3×10^3
4	-4.5	1.6×10^4
5	-5.5	6.8×10^3
6	-6.0	7.6×10^3
7	-2.5	9.5×10^3
8	-3.0	3.8×10^3



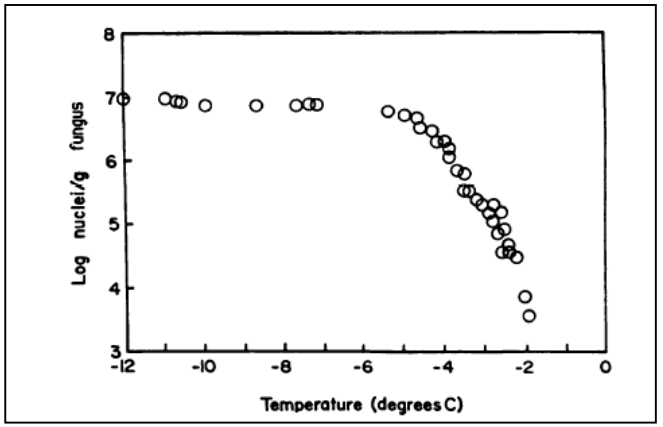
Block,2002, Attard *et al.*,2012

Ice nucleation test
Droplet freezing method

- Isolates were tested for ice nucleation activity by preparing suspension in 3.0 ml of distilled water and chilling the suspension to -1, -2, -3, -4, -5, -6, or -7°C.
- Isolates were considered ice nucleation active at a particular temperature if the water froze within 10 min.

Temp of heat treatment (°C)	T_{50} (°C)[a]
Control (no heat treatment)	−2.3
40	−2.3
50	−2.3
60	−3.3
70	−2.7
80	−14.6
85	−14.6
95	−14.6

[a] T_{50}, Temperature at which 50% of drops froze in drop-freezing assay.



Kieft,1998

Ice nucleation test

Droplet freezing method

LOG(nuclei/cell) of different plant pathogenic bacteria at two nucleation temperatures -5°C and -9°C

Bacterial Strain	Log (nuclei/cell) −5°C	−9°C
Bacillus subtilis	—	—
**Xanthomonas campestris* 1	−4.49	−5.44
X. campestris 2	—	—
X. campestris 3	—	—
X. maltophilia	—	—
**Pseudomonas fluorescens* 1	−1.73	−1.08
**Pseudomonas syringae* 1	−4.43	−2.92
P. fluorescens 2	—	—
P. syringae 2	—	—
Pseudomonas aeruginosa	—	—
Erwinia herbicola 3	—	—
E. herbicola 2	—	—
**E. herbicola* 1	−4.03	−3.86
Escherichia coli	—	—

Note.—Dashes indicate that no significant nucleating activity was detected.

Edwards *et al.*,2015

Ice nucleation test

Droplet freezing method

Vali formulation

- Grow strains on KB for 4-6 days at 18 to 24°C for best expression of INA.
- Single colonies are removed from agar plates with a toothpick and suspended in 0.1 ml of distilled water to yield a turbid suspension (> 10^8 CFU/ml).
- Appropriate dilutions in 10 mM potassium phosphate buffer (pH 7.0) are divided into large numbers (approx. 20 to 40) of small droplets (10 µl) which are then cooled to a given temperature (typically -5 or -100°C) on a test surface. The number of INA events is determined by visual observation.
- It is necessary to test a series of dilutions in order to obtain one or more dilutions for which some but not all of the drops freeze.
- The test surface is prepared by spraying aluminum foil with a 1% solution of paraffin in xylene, removing the xylene at 55°C in a circulating oven, folding the coated foil into a flat bottomed "boat", and floating the "boat" on a methanol water solution maintained at - 5°C or -10°C.
- An ethanol-refrigerated circulating bath is the constant heat sink for such assays.
- A colony is considered to contain nuclei active at 5°C or -10°C if droplets freeze within 30 sec.
- Frozen droplets are generally opaque and non-hemispherical unless a turbid bacterial suspension is being tested.

Schaad *et al.*,2001

Details of droplet freezing method
Calculation of Ice nucleation activity(INA)
Vali,1971

- Bacterial suspensions from each treatment or without treatment were serially diluted in sterile ultrapure water.
- The suspension obtained after the highest treatment was filtered through a 0.22 μm filter (Millipore, France), and the ice nucleating activity of the filtrate was analyzed.
- 40 droplets (10 μl each) from these dilutions or from the filtrate were placed on a paraffin-coated aluminum sheet, which was floated on the surface of a bath that was progressively cooled from 0 to −10°C with a 5 min plateau every 0.5°C.
- The number of frozen drops observed within 5 min was recorded, and the temperature required for freezing 90% of the drops (T_{90}) was determined.
- These two parameters were used to reveal the ice nucleating activity.
- The number of ice nuclei mL^{-1} for a given temperature N(T) was calculated by the Vali equation $N(T)=-\ln(f)\ 10^{D}V^{-1}$, where
- f represents the number of unfrozen droplets at a temperature T,
- D represents the dilution factor,
- V represents the volume of each droplet (0.01 mL) and
- N(T)represents the number of nuclei of mL^{-1}.

Sarron *et al.*,2013

Details of droplet freezing method
Calculation of ice nucleation activity(INA)
Lindow *et al.*,1982 modified method

- The icenucleating temperature was measured with a freezing nucleus spectrometer.
- The concentration of ice nuclei was calculated from the formula of Lindow *et al.*,1982:
- $N(T)=(-\mathrm{IN}\ f)/V$ Where:
- $N(T)$ is the nucleation frequency at temperature T,
- f is the proportion of droplets unfrozen, and
- V is the volume of individual droplets.

1. The INA was calculated by dividing $N(T)$ by the CFU/ml and transforming to $\log_{10}(N/\mathrm{CFU})$.
2. The number of nuclei per cell was calculated by dividing the concentration of nuclei/ml by the density of the cell suspension (cfu/ml).

Obata *et al.*,2014

Details of droplet freezing method
Calculation of ice nucleation activity
Nucleation frequency (NF)

- The lowest effective accumulated ice nuclei concentration is needed to represent the INA of bacterial suspensions at a specific temperature of T°C.
- The cumulative ice nuclei concentration per bacterial suspension at the temperature of T °C was calculated according to the approach given by Vali (1971) as the following equation:
- $K(T) = [\ln(N0) - \ln(NT)]/V$
- $B(T) = [\ln(N0) - \ln(NT)]/A$
- where,
- $K(T)$ represents the accumulated nuclei concentration of the per measured solution droplet.
- $B(T)$ represents the accumulated nuclei concentration of the per bacterial cell.
- NT is the number of the unfreezing droplets at T°C.
- $N0$ is the initial total number of droplets.
- V is the droplet volume.
- A is the number of bacterial cells among each of the test droplets.

Du *et al.*,2015

Details of droplet freezing method
Calculation of ice nucleation activity
Nucleation frequency (NF)

- Before ice nucleation assays (described below), bacterial strains were cultured for 3 days at 17°C on plates of King's medium B (KB).
- Bacterial suspensions were prepared with the newly cultivated bacteria in sterile distilled water to obtain 5×10^8 to 5×10^3 cells mL^{-1}.
- The suspensions were incubated at 4°C for at least 1 hour to allow full expression of the ice nucleation protein.
- Bacterial INA was measured by an immersion freezing test (Vali, 1971).
- Thirty-two droplets of 20 µl of the initial suspension and then 32 droplets of 10 fold serial dilutions of the suspension (from 5×10^8 to 5×10^3 cells mL^{-1}) were distributed on a sterile aluminum plate and floated on a cooling bath.
- This serial dilution was performed to allow the calculations described below such that for at least one dilution series, some drops (at least 1) remained unfrozen.

Attard *et al.*,2012

Details of droplet freezing method
Calculation of ice nucleation activity
Nucleation frequency (NF)

- The initial temperature was -2°C and then reduced at 1°C intervals until -10°C.
- At each temperature step, the droplets were incubated for 8 min and at the end of each incubation step the number of frozen droplets was counted visually.
- The cumulative ice nuclei concentration per bacterial cell at each temperature was calculated according to the method described by Vali (1971) with the following equation:
- $C(T) = [\ln(N^0) - \ln(N^0 - N_x(T)]/A$ where
- $C(T)$ is the number of ice nuclei per bacterial cell active at temperature T, N^0 is the number of droplets tested, N_x is the number of frozen droplets at a given temperature T, and A is the number of bacteria per drop.
- Therefore, for each bacterial strain, the level of the INA was determined by

I. the number of ice nuclei per cell, and

II. the highest temperature at which freezing was first observed.

Attard *et al.*,2012

Details of droplet freezing method
Calculation of ice nucleation activity
Nucleation frequency (NF)

- The lowest effective accumulated ice nuclei concentration is needed to represent the INA of bacterial suspensions at a specific temperature of T°C.
- The cumulative ice nuclei concentration per bacterial suspension at the temperature of T °C was calculated according to the approach given by Vali (1971) as the following equation:
- $K(T) = [\ln(N0) - \ln(NT)]/V$
- $B(T) = [\ln(N0) - \ln(NT)]/A$
- where,
- $K(T)$ represents the accumulated nuclei concentration of the per measured solution droplet.
- $B(T)$ represents the accumulated nuclei concentration of the per bacterial cell.
- NT is the number of the unfreezing droplets at T°C.
- $N0$ is the initial total number of droplets.
- V is the droplet volume.
- A is the number of bacterial cells among each of the test droplets.

Du *et al.*,2015

Details of droplet freezing method
Calculation of Ice nucleation activity
IN assays (ice nucleation (IN) activity)

- The concentration of ice nucleating particles (C_{INP}) in the collection liquid was assayed by the drop-freezing method described previously (Vali, 1971).
- A series of sixteen 0.2ml microtubes containing 20 µl of the impingement liquid, undiluted or diluted 10-fold in distilled water, were placed in a cooling bath and exposed to decreasing temperatures from -2 to -10°C with 1°C steps.
- The number of tubes containing aliquots still in the liquid phase was counted after exposition for 8 min at each temperature step, and C_{INP} was calculated as:

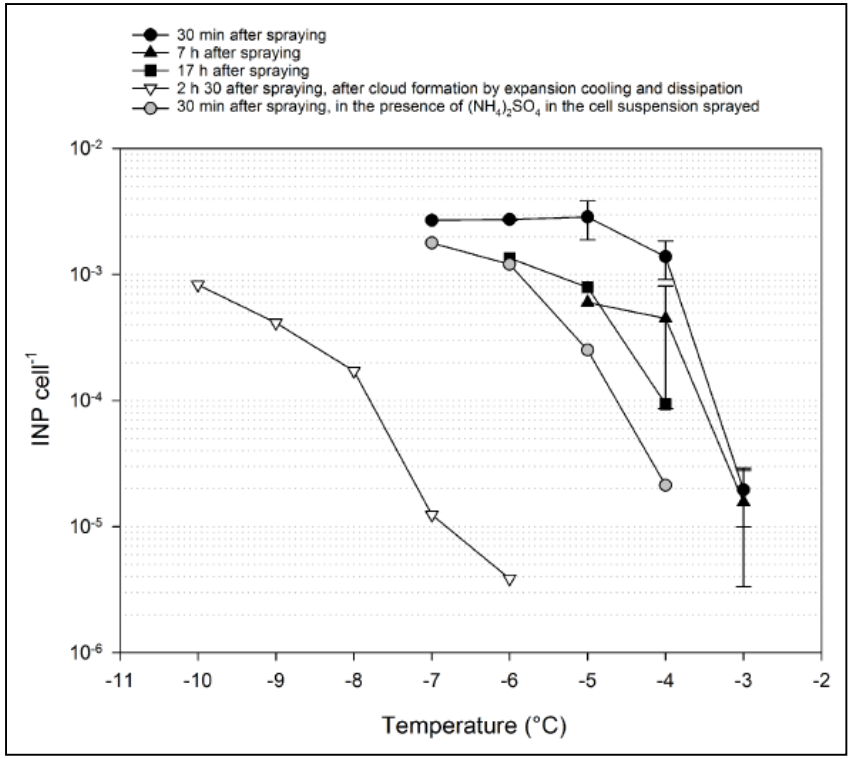
$$C_{\mathrm{INP}} = \frac{[\ln(N_{\mathrm{total}}) - \ln(N_{\mathrm{liquid}})]_T}{V} \times \frac{1}{D_{\mathrm{f}}}, \qquad (1)$$

- Where
- N_{total} is the total number of tubes tested in a given dilution series (16),
- N_{liquid} the corresponding number of tubes still liquid after 8 min at temperature T,
- V the volume of liquid in each tube (0.02 ml or 20 µl), and
- Df the dilution factor (1 or 10).
- CI_{NP} were finally normalized to the corresponding total cell concentrations measured by flow cytometry.

Amato *et al.*,2015

Details of droplet freezing method
Calculation of ice nucleation activity
IN assays (ice nucleation (IN) activity)

- Cumulative frequencies of INP per airborne cell in *P. syringae* 32b-74 within the AIDA chamber 30 min, 7 h and 17 h after aerosolization in the absence of cloud (black symbols), 30 min after aerosolization in the presence of ammonium sulfate (grey symbols), and when the pressure inside the chamber was returned to ambient after cloud formation by expansion cooling (open triangles).
- Error bars are standard deviations from the mean of independent experiments, when available.



Amato *et al.*,2015

Freezing method

Growth conditions

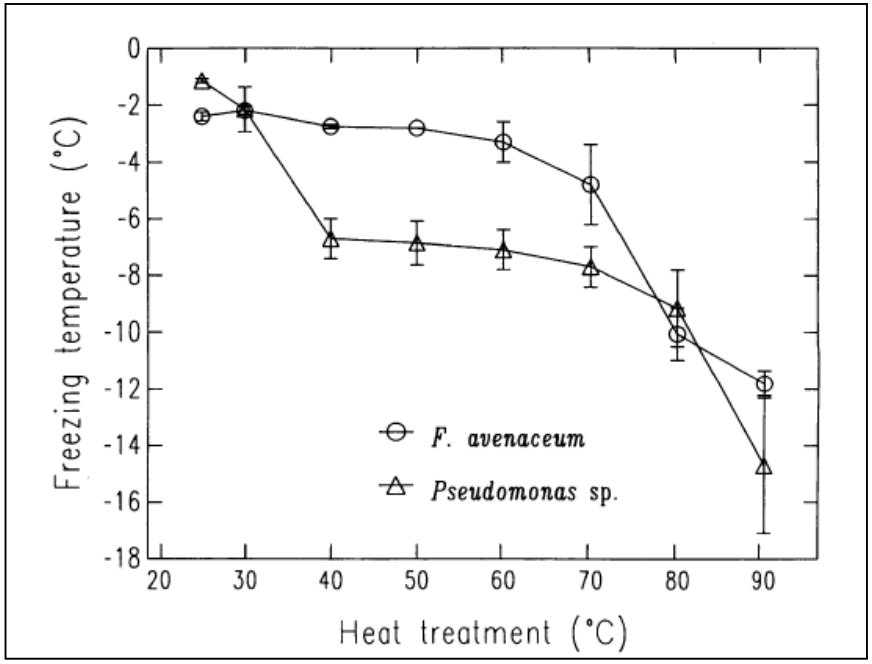
Heat treatment, pH, suspension density….

- The effects of heat, pH, and suspension density of fungal and bacterial INAs were investigated.
- In each experiment, INA was estimated by the temperature at which 50% of 30 10-µl droplets of treated suspensions froze.
- For the heat treatments, fungal and bacterial suspensions were subjected for 10 min to various temperatures (from 24 to 90°C) and immediately cooled to 20°C in melting ice; INA was then estimated.
- To measure the effect of pH, initial suspensions of *F. avenaceum* and *Pseudomonas* sp. were made in sterile deionized distilled water and diluted in different buffers with pHs of 1 to 13 to obtain a final suspension of 1 mg of mycelium ml^{-1} or 10^8 bacterial cells ml^{-1}.
- INA was estimated after 30 min of stabilization.

Pouleur *et al.*,1992

Freezing method
Growth conditions
Heat treatments

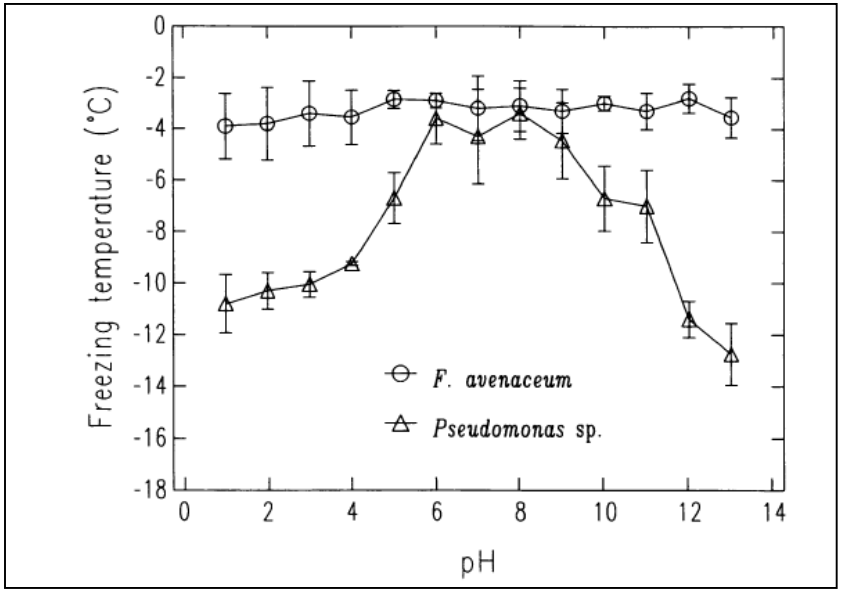
- Effect of 10-min heat treatment on INAs of *F. avenaceum* and *Pseudomonas* sp.
- INA is estimated by the freezing temperature of 50% of droplets containing the tested microorganisms.
- Fungal INA decreased after exposure, for 10 min, to a temperature of 60°C or higher, whereas bacterial INA decreased in two steps, above 30°C and above 80°C.



Pouleur *et al.*,1992

Freezing method
Growth conditions
pH effects

- Effect of pH on INAs of *F. avenaceum* and *Pseudomonas* sp.
- INA is estimated by the freezing temperature of 50% of droplets containing the tested microorganisms.
- Fungal INA was stable at pH levels from 1 to 13, whereas bacterial INA was drastically reduced at pH values below 6 and above 9.



Pouleur *et al.*,1992

Freezing method

Growth conditions

Comparison of Conventional Ice-Nucleating Bacteria and *Pseudomonas* sp. Strain IN-74 of Antarctic Origin

Strain[a]	Incubation		Growth (OD_{660})	pH	Ice-nucleating temperature (°C)[b]		
	Temperature (°C)	Time (day)			T_{10}	T_{50}	T_{90}
P. antarctica IN-74	18	2	18.9	8.3	−15.7	−17.4	−22.7
P. antarctica IN-74	4	6	4.6	8.2	−4.4	−4.4	−4.6
P. antarctica IN-74[c]	0	15	7.5	8.0	−3.7	−3.7	−3.8
P. fluorescens KUIN-1	18	2	17.0	8.3	−2.5	−2.5	−2.6
P. viridiflava KUIN-2	18	2	10.7	8.8	−4.6	−5.9	−8.4
P. syringae IFO-3310[d]	18	2	11.7	8.0	−2.6	−2.7	−2.8
Distilled water	—	—	—	—	−18.7	−21.6	−24.1

[a] Cells were grown for 2 days at 18°C with shaking in the ice nucleation medium.
[b] Temperatures required to freeze 10% (T_{10}), 50% (T_{50}) and 90% (T_{90}) of cells.
[c] Cells were grown for 15 days at 0°C with shaking in the ice nucleation medium.
[d] IFO, Institute for Fermentation, Osaka.

The various ice-nucleating bacteria, other than strain IN-74, were found to be active in ice nucleation from 22.5°C (T_{50}) to 25.9°C (T_{50}) for 2 days at 18°C.

Obata *et al.*,1999

Freezing method

Effect of growth limiting factors on growth and ice-nucleation activity of bacteria after 72 hours incubation in terms of optical density

Bacteria*	Strain	Medium	OD	INA (°C)
Bacillus sp.	(S294)	TSB	1.720	-9.5
		CL	0.830	-9.5
		NL	0.750	-12
		PL	1.270	nf
Erwinia sp.	(S113)	TSB	1.820	-7
		CL	0.412	-8.5
		NL	0.412	-13
		PL	1.060	-5.5
P. fluorescens	(M103)	TSB	1.600	-4.5
		CL	0.820	-7
		NL	0.660	-5
		PL	0.790	-5
P. syringae	(S229)	TSB	1.700	-2.5
		CL	0.500	-3
		NL	0.920	-3.5
		PL	1.255	-4
Sphingomonas yanoikuyae	(E200)	TSB	1.920	-6.5
		CL	0.500	-5.5
		NL	0.520	-13.5
		PL	1.450	-8
Xanthomonas campestris	(S117)	TSB	1.730	-5
		CL	0.480	-9
		NL	1.070	-7
		PL	1.250	-4

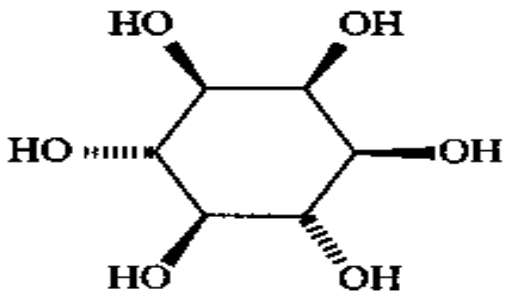
* all strains are isolated from *Salix* stems
TSB as control froze at -16.5°C and phosphate buffer at -16°C
CL= carbon limitation, NL = nitrogen limitation, PL = phosphorus limitation.
nf = not frozen

Nejad *et al.*,2005

Freezing method

Influence of phosphate in Ice-nucleating activity of *Pseudomonas syringae*

- Wheat bran as a natural substrate, contains a relatively high proportion of phytate (*myo*-inositol).
- The double salt of phytic acid is the precursor of a major component of the ice-nucleating site.
- When *Myo*-inositol is metabolized to phosphatidylinositol, it makes up a very significant component of cell membranes.
- The use of the wheat bran medium seems to be specifically efficient on class A bacteria, which is the most active type of *P. syringae*.



Chemical structure and activities of myo-inositol

Ice nuclei

Association with the outer bacterial membrane

Lipoglycoproteins

- Proteins in the outer membrane of the bacteria were thought to cause ice nucleation.
- The highest level of ice nucleation activation is attributed to a protein (lipoglycoprotein) of some bacteria such as *Pseudomonas syringae*.
- Lipoglycoprotein consists of highly repetitive sequences of amino acids in the bacterial outer membrane that serves as a template for ice crystallization.
- This IN protein has a closely related infrastructure with a central region, and can be linked to the membrane by a glycosylphosphatidylinositol (GPI) anchor.
- The sizes of nucleating insoluble IN protein increase logarithmically with increasing nucleation temperature.

Du *et al.*,2015

Freezing method

Filtration on fungal and bacterial INAs

Association with the outer bacterial membrane

- To determine if nuclei were bound to the cell, INAs of fungal and bacterial suspensions were estimated before and after filtration through a 0.22-µm-pore-size filter.
- Part of the *Fusarium* ice nuclei do not seem to be bound to cells because the freezing temperature of 50% of the droplets of the suspension was nearly the same before (-2.3°C) and after (-2.4°C) filtration (data not shown).
- In contrast, the freezing temperature of 50% of the droplets of the bacterial suspensions was -0.9°C before filtration and below -10.0°C after filtration, confirming that active bacterial nuclei are attached to the cell.

Pouleur *et al.*,1992

Freezing method

Effect of cell filtration and centrifugation on ice-nucleating active bacterium (*P. syringae*, strain S229) from *Salix* plants

- Additionally we found that centrifugation of bacterial suspensions for 15 min at 15000×g or filtration of cultures through a 0.20 µm membrane filter separated the activity from the supernatant.
- Thus most of the ice nucleation activity was associated with the cells.

Treatment	INA temperature (°C)
Before centrifuging*	-2.5
Centrifuged (supernatant) [a]	-7.0
Centrifuged (pellet) [a]	-2.5
Before filtering*	-2.6
Filtrate [b]	-8.5
Resuspended cells	-2.8

[a] 15000 x g, 10 min
[b] 0.20µm membrane filter (Non Pyrogenci, Millipore).
* Control

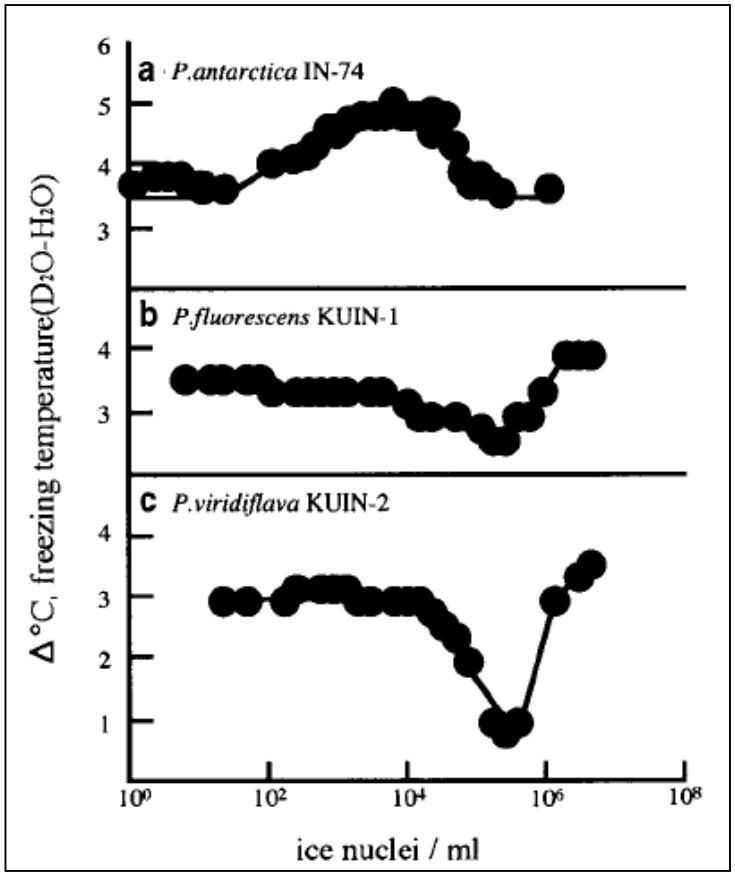
Nejad *et al.*,2005

Droplet freezing method

Growth conditions

Freezing difference spectra in D_2O versus H_2O

- Bacterial suspension can be prepared either in 50 mM phosphate buffer (pH 7.0), sterile H_2O or D. H_2O.
- Freezing difference spectra was found in D_2O and H_2O at ice-nucleating temperature:
- The freezing spectra of *P. fluorescens* KUIN-1 cells and *P. syringae* C-9 cells exhibited similar curves.
- No freezing difference spectra in D_2O and H_2O at ice-nucleating temperature for *Pseudomonas* strain IN-74.
- Since D_2O is known to react more strongly than does H_2O with the hydrophobic domains of proteins, it seemed possible that class A and C structures were much more hydrophobic than were class B structures.



Obata *et al.*,1999

Freezing method

Freezing procedure in *Salix* seedlings/cuttings

1. The plants were placed in large test tubes in an upside-down position during the treatment to cool the plant material in dark, while the roots parts stayed in room temperature (Ramstedt *et al.*,1994).
2. When cuttings were used they were placed in an upright position in the test tubes.

- The plant material was then rewarmed to 4.0+1°C, remained in freezing bath for about 18-24 hrs in dark at 16°C and then acclimated for another few hours in dark before transferring them to greenhouse conditions.

Nejad,2005

Freezing method

Freezing procedure in *Salix* seedlings/cuttings

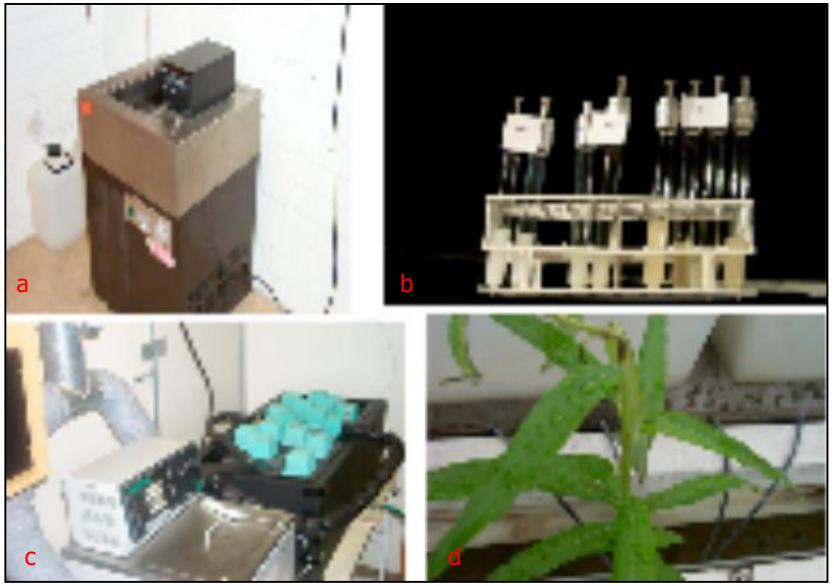
- The procedure for testing of ice-nucleation activity including subsequent pathogenicity tests of willow isolated bacteria:

a) freezing bath for INA;

b) ice nucleation test of isolated bacteria;

c) subjecting inoculated plants to frost treatment using programable refrigerating bath;

d) necrotic infection of Salix plant stem as a result of synergistic effects between bacteria and frost.



Nejad,2005

Ice nucleation protein (INP)

Three domains proteins

Proteins that help ice crystals to form. The crystals pierce holes in plants

- INP is a monomeric protein composed of more than 1,200 amino acid residues with a deduced molecular mass of 118 kDa.
- One monomer is formed by:

1. A N-terminal domain (around 180 amino acids), probably involved in the phenomenon of maturation of the nucleation site,
2. A central repetitive region (around 1000 amino acids), which seems essential in the ice-producing activity, and
3. A C-terminal domain (around 50 amino acids) presumably involved in the aggregation of monomers (Green *et al.*,1988).

Lagriffoul *et al.*,2010;..

Ice nucleation protein (INP)

The central repeating domain act as a template for ice crystal formation

- All INPs (1200 aa to 1500 aa) encoded by these three genes (*inaK*, *inaV* and *inaZ*) comprise of three distinct structural domains:

1. The N-terminal domain
2. C-terminal domain
3. The central repeating domain.

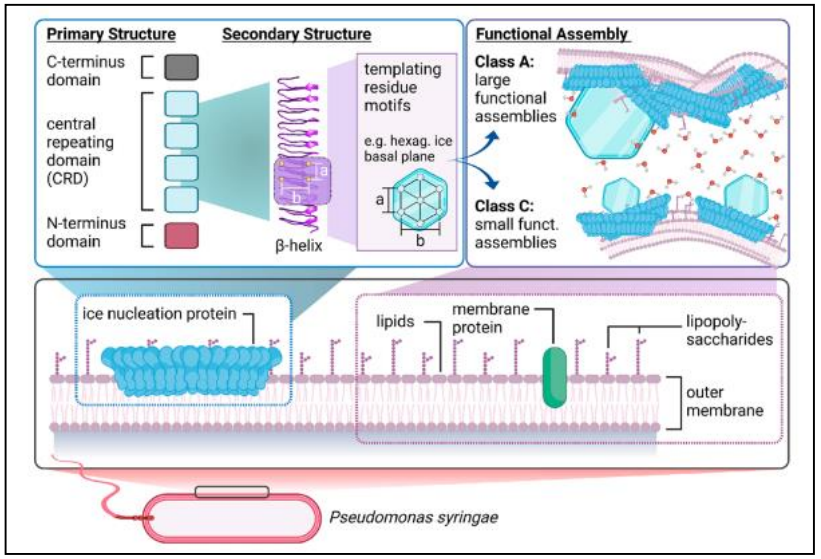
- The central repeating domain (CRD)(approximately 81%), which constitutes contiguous repeats given by 16-residue (or 48-residue) periodicities with a consensus octapeptide (Ala-Gly-Tyr-Gly-Ser-Thr-Leu-Thr).
- This domain presumably acts as a template for ice crystal formation.

Li *et al.*,2012

Ice nuclei

Association with the outer bacterial membrane

- The Ice-nucleating proteins (INpro) consist of:
- an N-terminal, a C-terminal, and a central repeating domain.
- Their general function is to order water molecules into an "ice-like" arrangement to nucleate ice formation.
- This process is facilitated when INPs assemble into larger aggregates.



Overview of the proposed structure and working mechanism of bacterial ice nucleation proteins anchored to the outer cell membrane of *P. syringae*.

Lukas *et al.*,2022

Ice nucleation proteins(INP)

The repeat structure

- A family of large and unusual proteins (ice nucleation proteins, mw 118 kDa, or larger) are a key component of bacterial ice nuclei.
- Predicted amino acid sequences of two such proteins from *P. syringae* and *P. fluorescens*, respectively, have revealed:
- An internal repeating consensus octapeptide Ala-Gly-Tyr-Gly-Ser-Thr-Gly-Thr, which makes up 70% of the protein, contains two threonine residues and one serine residue.
- All three bacterial ice genes have the same type of unique structure.

Ice nucleation proteins

Three domains proteins

The repeat structure

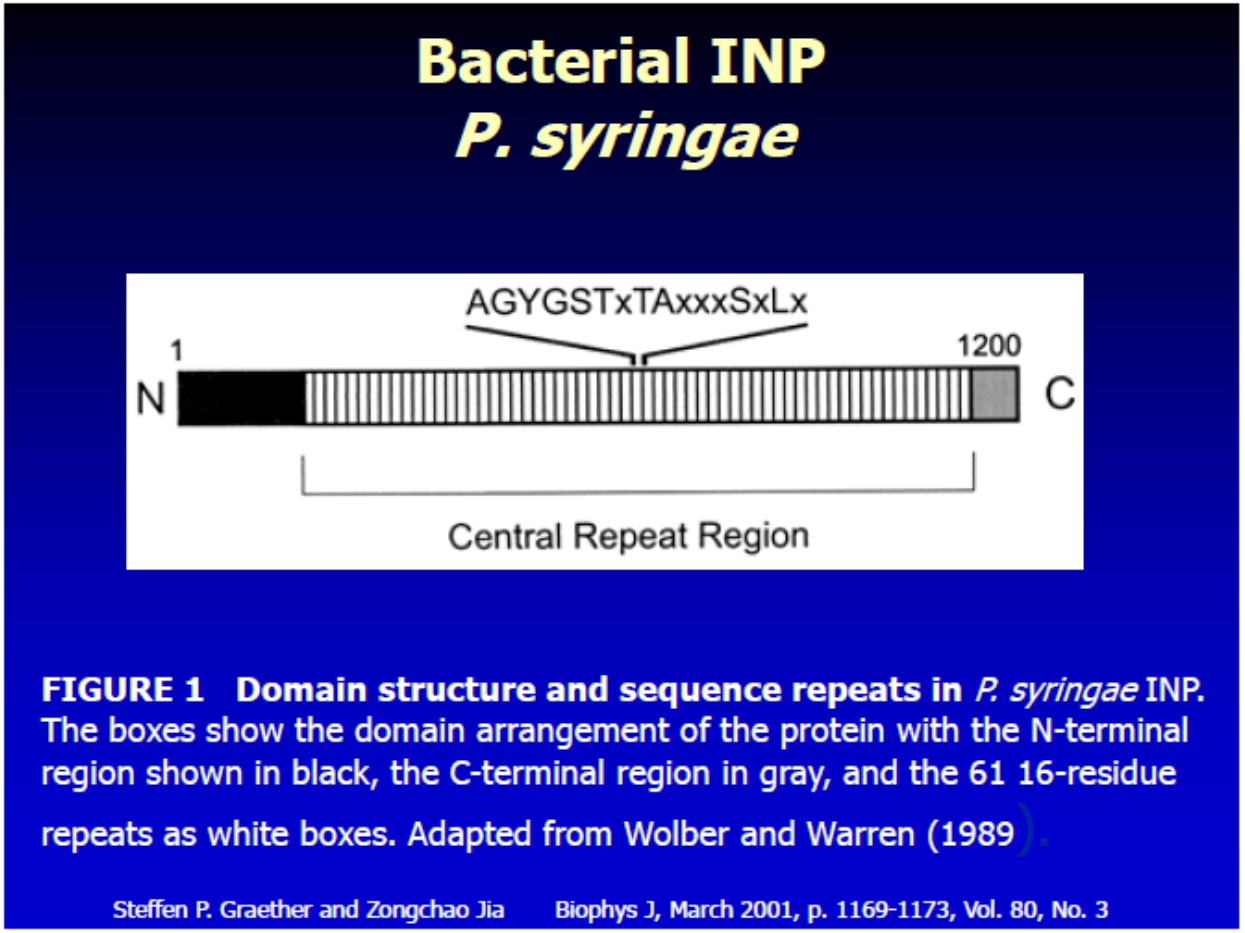


FIGURE 1 Domain structure and sequence repeats in *P. syringae* INP. The boxes show the domain arrangement of the protein with the N-terminal region shown in black, the C-terminal region in gray, and the 61 16-residue repeats as white boxes. Adapted from Wolber and Warren (1989).

Steffen P. Graether and Zongchao Jia Biophys J, March 2001, p. 1169-1173, Vol. 80, No. 3

Ice nucleation protein (INP)

Three domains proteins

- Both the C and N-termini of INP are free and exposed on the cell surface, so foreign proteins fused to the C- or the N-terminus of INP can be localized to the cell surface.
- INP has the ability to maintain its ice nucleation activity after fusion to a foreign protein, which allows the detection of the recombinant proteins on the cell surface by ice nucleation activity assay.

Prasanth *et al.*,2015

Ice nucleation protein (INP)

Three domains proteins

Deletion mutagenesis of the ice nucleation gene from *Pseudomonas syringae*

- Deletions which disrupted the periodicity of 16 codons, in a repetitive region of inaZ, caused the frequencies of ice nuclei in the bacterial population to be significantly depressed.
- Deletions removing part or all of one of the non-repetitive regions (that encoding the amino-terminal domain of the InaZ protein) did not abolish nucleation activity, but caused it to be limited to cooler threshold temperatures.
- In contrast, the non-repetitive carboxy-terminal do main of the InaZ protein was shown to be essential for ice nucleation at all temperatures.

Green *et al.*,1988

Ice nucleation proteins(INPs)

Three classes of ice-nucleating structures

- Properties of ice-nucleating cells indicating that there are three chemically distinct classes:

1. Class A structures,
2. Class B structures, and
3. Class A structures as well as
4. Intermediate or mixed structures on the surfaces of ice nucleation-active cells.

- These suggest that the structures of the classes are chemically heterogeneous.

At colder temperatures, clusters of two to three proteins will trigger freezing, so that by −10 to −12°C the nucleation frequency may be 1 in every 10 cells or higher.

Specific ice genes

The bacterial phenotype is due to a protein product of a single gene

- The genes corresponding to the INA$^+$ phenotype:

1. *inaZ* for *P. syringae*
2. *inaW* for *P. fluorescens*
3. *iceE* for *E. herbicola*
4. *inaU for Pantoea ananatis* pv. *uredovora*
5. *inaK*, *inaV* and *inaZ* which were characterized from *P. syringae* strains, exhibit high similarities in sequences and in primary organization.
6. *inaQ* from a *P. syringae* MB03 strain(a newly identified INP-gene variant) was cloned.

Note: Unlike most bacteria, *Pseudomonas syringae* has been documented to produce several variants of ice nucleation proteins (i.e., InaK, InaQ, InaV, and InaZ) and has been studied extensively.

Li *et al.*,2012: Lorv *et al.*,2014

Specific ice genes

Origin, activity, identity, and closest *ina* allele affiliation of isolates

				16S rRNA		*ina*	
Isolate	Source	Location	Ice nucleation activity[a] (°C)	Closest isolate(s) and GenBank accession no.	Similarity (%)	Closest isolate, *ina* gene allele/gene name, and GenBank accession no.	Similarity (%)
Cit7[b]	Navel orange leaf	Near Exeter, CA[b]	−3.5 (−2.5)	*Ps. syringae* (AY574914)	100	*Ps. syringae inaZ* (X03035)	99.2
BXIN4	Bean pod or leaf lesion	Lingle, WY; 42.1315 N, 104.392 W	−3.0 (−2.5)	*Ps. syringae* (AB680547) *Ps. congelans* (NR_028985)	100 100	*Ps. syringae inaQ* (EU360731)	99.5
PCa2a	Cabernet sauvignon grapevine cane lesion	Coonawarra, South Australia; 37.361 S, 140.838 E	−3.0	*Ps. syringae* (AB680547), *Ps. congelans* (NR_028985)	100 100	*Ps. syringae inaQ* (EU360731)	97.4
HCh1a	Chardonnay grapevine shoot, healthy	Hallston, Victoria, Australia; 38.3465 S, 146.028 E	−3.0	*Ps. syringae* (AJ889841)	100	*Ps. syringae inaQ* (EU360731)	97.2
BXIN3	Bean pod or leaf lesion	Lingle, WY; 42.1315 N, 104.392 W	−3.5 (−2.5)	*Ps. syringae* (CP000075), *Ps. congelans* (NR_028985)	100 100	*Ps. syringae* Psyr1608 (CP000075)	99.2
GCh5Fc	Chardonnay grapevine shoot killed by frost	Glenlofty, Victoria, Australia; 37.120 S, 143.217 E	−4.0 (−3.5)	*Ps. syringae* (AB680547), *Ps. congelans* (NR_028985)	100 100	*Ps. syringae inaV* (AJ001086)	100
Sco1009b	Corn (maize) leaves, senescent	Lingle, WY; 42.1315 N, 104.3965 W	−2.5 (−2.0)	*Ps. syringae* pv. *atropurpurea* (AB001440)	100	*Ps. syringae inaV* (AJ001086)	83
PCa2bi	Cabernet sauvignon grapevine cane lesion	Coonawarra, South Australia; 37.361 S, 140.838 E	−3.0	*Ps. viridiflava* (AY574912)	100	*Ps. syringae inaV* (AJ001086)	85
GrF[c]	Grape	CA[c]	−4.0 (−3.0)	*Pa. ananatis* (CP001875)	99.6	*Pa. ananatis iceA* (AF387802)	99
SBPci	Bean pod lesion	Lingle, WY; 42.1315 N, 104.392 W	−3.5 (−3.0)	*Pa. agglomerans* (FJ756354)	99.9	*Pa. ananatis inaA* (CP001875)	76
Sba1007a	Barley green leaves and heads	Lingle, WY; 42.1315 N, 104.395 W	−3.0	*X. campestris* pv. *campestris* (CP000050)	100	*X. campestris* pv. *raphani* XCR 4000 (CP002789)	92
Sba1007bi	Barley green leaves and heads	Lingle, WY; 42.1315 N, 104.395 W	−3.5	*X. campestris* pv. *campestris* (CP000050)	100	*X. campestris* pv. *raphani* XCR 4000 (CP002789)	92
Sbr1009a	Smooth brome (*B. inermis*) green leaves	Lingle, WY; 42.1320 N, 104.395 W	−2.0	*X. translucens* (NR_036968)	99.9	*X. campestris* pv. *translucens inaX* (X52970)	97
MU26[d]	Wood frog (*Rana sylvatica*) gut	Adams County, OH[d]	−3.0	*Ps. fluorescens* (JF327445)	99.9	*Ps. fluorescens inaW* (X04501)	89
BF81Fb[e]	Air during rain in ponderosa pine forest	Manitou Forest, CO; 39.1028 N, 105.104 W	−4.0 (−2.5)	*Ps. koreensis* (NR_025228) *Ps. fluorescens* (JN679853), *Ps. putida* (HM217118)	99.9 99.9 99.9	*Ps. fluorescens inaW* (X04501)	86
LSb	Peat moss (*S. capillifolium*)	Bog, Lewis, Outer Hebrides, United Kingdom; 58.1 N, 6.7 W	−3.0	*Pseudomonas* sp. strain LD002 (HQ713573), *Pseudomonas* sp. strain NZ099 (AF388207)[f]	100 99.9	*Ps. fluorescens inaW* (X04501)	87
MM3b	Dwarf birch (*B. nana*) and bog rosemary (*A. polifolia*)	Martimoaapa mire, Finland; 65.81 N, 25.22 E	−3.0	*Pseudomonas* sp. strain NZ099 (AF388207)[f]	100	*Ps. fluorescens inaW* (X04501)	87
χ16	Snow	Mt. Parnassos ski center, Greece; 38.5393 N, 22.60645 E	−3.5	*Ps. auricularis* (AB681727)	99.9	*Ps. fluorescens inaW* (X04501)	87
χ17	Snow	Mt. Parnassos ski center, Greece; 38.5393 N, 22.60645 E	−3.0	*Ps. poae* (GU188955)	99.9	*Ps. fluorescens inaW* (X04501)	86
GraPa8	Grass	Mt. Parnitha, Greece; 38.172444 N, 23.728622 E	−3.5	*Pseudomonas* sp. strain JCM5484 (AB685689), *Ps. auricularis* (AB681727)	99.9 99.8	*Ps. fluorescens inaW* (X04501)	86

[a] The warmest temperature of activity from ~10^7 cells (tested in 0.5°C increments). Numbers in parentheses are the warmest temperatures of activity, if higher, in other tests using L_{ND} medium but where numbers of bacteria were not determined.

Hill *et al.*,2013

Ice$^+$genes

Chromosomal or plasmid genes?

HGT

- In spite of many studies being done, the location of ice nucleation gene whether in chromosomal or plasmid DNA has not been identified.
- The ice-nucleation gene for *P. viridiflava* KUIN-2 is in the plasmid DNA.
- The ice nucleation gene are all highly conserved between species and genera, leading to the hypothesis that the Ina$^+$ gene only evolved once and has since been horizontally transmitted among diverse species and genera (Edwards *et al.*,1994).

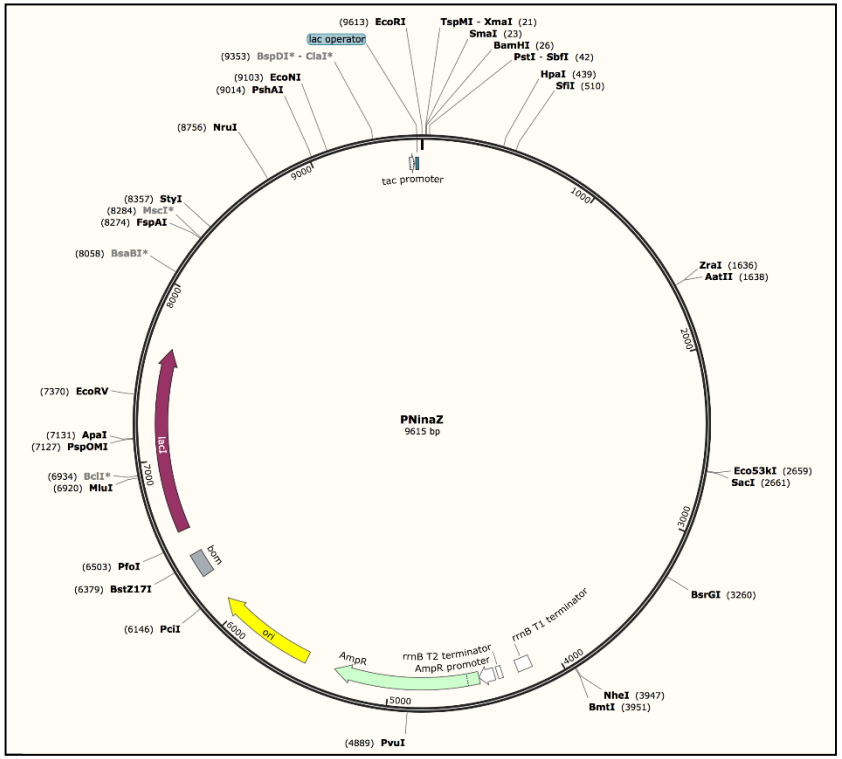
McCorkle,2009

Ice^{+}genes

Chromosomal or plasmid genes?

Plasmid map of the plasmid containing inaZ gene

- The plasmid with the inaZ gene in *P. syringae*.
- After putting the samples in the cooling bath again, one with INP froze after a few minutes while the control remained liquid.



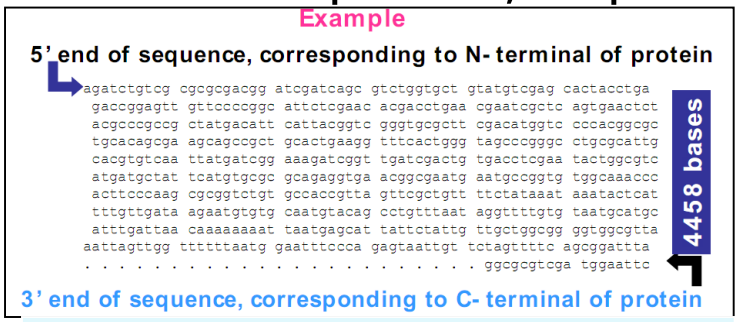
Ksobecka,2015

Specific ice genes

Ice nucleation proteins

The bacterial phenotype is due to a protein product of a single gene

- The sequence of bases in this gene has been determined for several strains of INA bacteria.
- These sequences (for a single strand) are available at an open data base (GenBank).
- All INPs (1200 aa to 1500 aa) encoded by these three genes (*inaK*, *inaV* and *inaZ*).
- The sequence alignments of the *inaQ* and three other genes (*inaK*, *inaV*, and *inaZ*) show similarities of at least 89% and 92% in the gene and aa sequences, respectively.



Guilbaud *et al.*,2007; Li *et al.*,2012

Specific ice genes

Ice nucleation proteins

Complete sequence of InaZ protein

- >sp|P06620|ICEN_PSESY Ice nucleation protein OS=*Pseudomonas syringae* pv. *syringae* GN=inaZ PE=1 SV=1. Length:1,200, Mass (Da):118,588.
- MNLDKALVLRTCANNMADHCGLIWPASGTVESRYWQSTRRHENGLVGLLWGAGTSAFLSVHADAR
  WIVCEVAVADIISLEEPGMVKFPRAEVVHVGDRISASHFISARQADPASTSTSTLTPMPTAIPTPMPAV
  ASVTLPVAEQARHEVFDVASVSAAAAPVNTLPVTTPQNVQTATYGSTLSGDNHSRLIAGYGSNETAGN
  HSDLIAGYGSTGTAGSDSWLVAGYGSTQTAGGDSALTAGYGSTQTAREGSNLTAGYGSTGTAGSDSS
  LIAGYGSTQTSGGDSSLTAGYGSTQTAQEGSNLTAGYGSTGTAGSDSSLIAGYGSTQTSGGDSSLTAG
  YGSTQTAQEGSNLTAGYGSTGTAGVDSSLIAGYGSTQTSGSDSALTAGYGSTQTAQEGSNLTAGYGS
  TGTAGSDSSLIAGYGSTQTSGSDSSLTAGYGSTQTAQEGSILTAGYGSTGTAGVDSSLIAGYGSTQTS
  GSDSALTAGYGSTQTAQEGSNLTAGYGSTGTAGADSSLIAGYGSTQTSGSESSLTAGYGSTQTAREGS
  TLTAGYGSTGTAGADSSLIAGYGSTQTSGSESSLTAGYGSTQTAQQGSVLTSGYGSTQTAGAASNLTT
  GYGSTGTAGHESFIIAGYGSTQTAGHKSILTAGYGSTQTARDGSDLIAGYGSTGTAGSGSSLIAGYGS
  TQTASYRSMLTAGYGSTQTAREHSDLVTGYGSTSTAGSNSSLIAGYGSTQTAGFKSILTAGYGSTQTA
  QERTSLVAGYGSTSTAGYSSSLIAGYGSTQTAGYESTLTAGYGSTQTAQENSSLTTGYGSTSTAGYSS
  SLIAGYGSTQTAGYESTLTAGYGSTQTAQERSDLVTGYGSTSTAGYASSLIAGYGSTQTAGYESTLTA
  GYGSTQTAQENSSLTTGYGSTSTAGFASSLISGYGSTQTAGYKSTLTAGYGSTQTAEYGSSLTAGYGS
  TATAGQDSSLIAGYGSSLTSGIRSFLTAGYGSTLIAGLRSVLIAGYGSSLTSGVRSTLTAGYGSNQIASY
  GSSLIAGHESIQVAGNKSMLIAGKGSSQTAGFRSTLIAGAGSVQLAGDRSRLIAGADSNQTAGDRSKLL
  AGNNSYLTAGDRSKLTGGHDCTLMAGDQSRLTAGKNSVLTAGARSKLIGSEGSTLSAGEDSILIFRLW
  DGKRYRQLVARTGENGVEADIPYYVNEDDDIVDKPDEDDDWIEVK

UniProt Consortium,2012

Specific ice genes

Ice nucleation proteins

Complete sequence of InaZ protein

- A number of investigations of the specific genes or proteins produced by them that are responsible for ice nucleation have been reported.
- For example, Green and Warner in a letter to Nature 317 p. 645 (1985) describe the determination of the sequence of the ice nucleation gene from *Pseudomonas syringae* which they called inaZ.
- They noted that this contains several repeats of a sequence reiteration with the consensus repeat having the sequence GCCGGTTATGGCAGCACGCTGACC, the gene having a total size of 4458.

Green R Land Warren GJ. 1985. Physical and functional repetition in a bacterial ice nucleation gene. Nature 317: 645-648.

US 4978540 A Patent

Specific ice genes

Ice nucleation proteins

Complete sequence of InaZ protein

Green RL, Warren GJ. Physical and functional repetition in a bacterial ice nucleation gene. Nature. 1985; 317: 645-648.

Characterization of ice nucleation proteins

- So far, six different genes that transcribe INPs have been sequenced from six different bacterial strains.
- The genes that have been isolated thus far are known as:

1. *inaW* from *Pseudomonas fluorescens* (Warren *et al.*,1986),
2. *inaZ* from *Pseudomonas syringae* (Green and Warren, 1985),
3. *inaA* and *inaU* from *Pantoea ananatis* (Abe *et al.*,1989, Michigami *et al.*,1994),
4. *iceE* from *Erwinia herbicola* (*Pantoea agglomerans*) (Warren and Corotto, 1989), and
5. *inaX* from *Xanthomonas campestris* (Zhao and Orser,1990).

Orser et al. (1983) demonstrated that the INA phenotype is coded by a single gene localized in a 3.5- to 4.0-kb DNA region.

McCorkle,2009

Characterization of ice nucleation proteins

- Stability characterization
- Structural characterization
- Immunological characterization
- Molecular characterization

Ice nucleation proteins

Protein classification based on freezing temperature

Stability characterization ice proteins

- Positive colonies was suspended in 0.5 ml of phosphate buffer ($6x10^4$ CFU/ml) and 0.1 ml of suspension was added to 0.9 ml of phosphate buffer and tested for ice nucleation activity at -2 to -10^o C in a circulating alcohol bath.

Waturangi and Tjhen,2009

Ice nucleation proteins

Protein classification based on freezing temperature

pH and temperature stability

- The INA bacteria have protein that acts as a core for the nucleation processes.
- This result showed the stability of INP at a wide range of pH, from pH 3 to 10.
- The INP was also tested for the stability of heat endurance.
- Commonly, many proteins will be denaturated or inactive after heat treatment.
- We found that the INP also has the same characteristics as other proteins.

Waturangi and Tjhen,2009

Ice nucleation proteins

Protein classification based on freezing temperature

pH and temperature stability

- pH stability and optimization of heat endurance assays were conducted to understand the ice nucleation protein (INP) characteristics.
- Most of the isolates that contained INP were active and stable in pH and heating treatment.
- INP from Class A bacterium was more stable than other class in reference to pH and temperature stability.

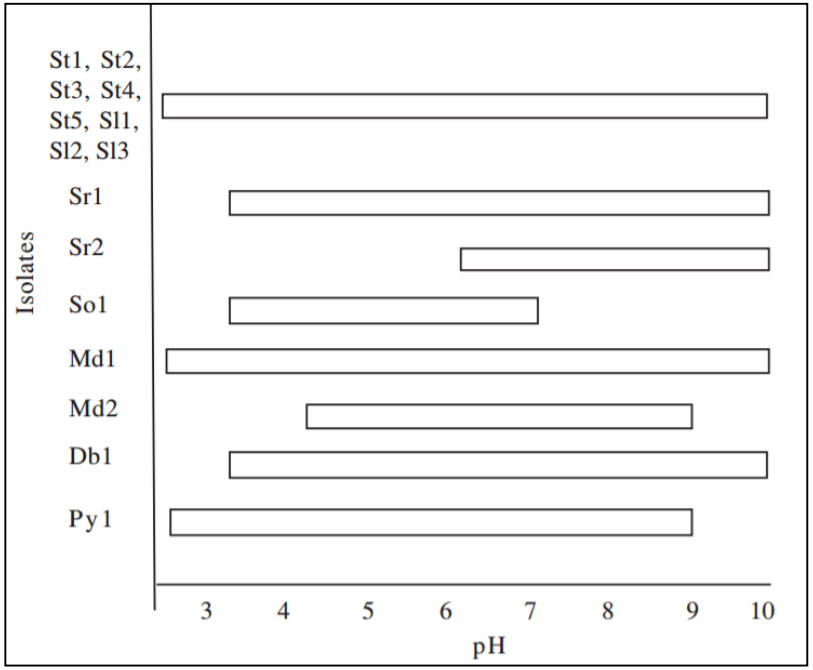
Waturangi and Tjhen,2009

Ice nucleation proteins

Protein classification based on freezing temperature

pH stability

- **pH Stability Assay:**
- All of the positive colonies were suspended in 0.5 ml of phosphate buffer (6 x 10^4 CFU/ml) and 0.1 ml of suspension was added to 0.9 ml of phosphate buffer pH 3-10 in each reaction tube and tested for ice nucleation activity at -10^o C in a circulating alcohol bath.
- Positive ice nucleation activity was indicated by the formation of ice after 5 minutes incubation (Kieft & Ruscetti 1990).



Waturangi and Tjhen,2009

Ice nucleation proteins

Protein classification based on freezing temperature

Heat endurance

- **Optimization of heat endurance:**
- The colonies were suspended in 0.5 ml of phosphate buffer (6 x 10^4 CFU/ml) and 0.1 ml was added to 0.9 ml of phosphate buffer in reaction tube.
- The suspensions were heated at 30 to 90°C and tested for ice nucleation activity in a circulating alcohol bath (Kieft & Ruscetti 1990).

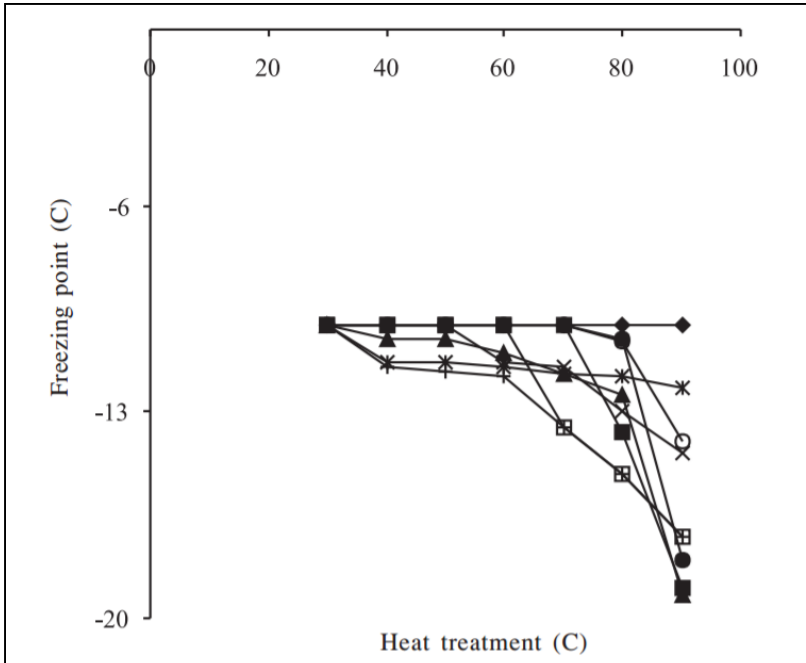


Figure 2. Optimization of heat endurance. ♦: St1, St2, St3, St4, St5, Sl1, Sl2; ■: Sl3; ▲: Sr1; ×: Sr2; ✱: So1; ●: Md1; l: Md2; □: Db1; ○: Py1.

Waturangi and Tjhen,2009

Ice nucleation proteins

Ice-nucleating proteins (INPs)

inaZ gene

- These genes have been extensively studied and various models have been proposed for the tertiary structure of their proteins. E.g.
- The *inaZ* gene specifies a structure containing 1,200 amino acids, and
- A molecular mass of 120 kDa, the isolated protein had an apparent mass on polyacrylamide gel electrophoresis (PAGE) of about 155 kDa.

Pseudomonas fluorescens KUAF-68 and *Flavobacterium* sp. GL7 were classified as Class C activity with T_{50} values of -10.6 and -8°C, respectively. This class is composed of protein aggregates that can have an overall molecular weight greater than 1000 kDa.

Ice nucleation proteins

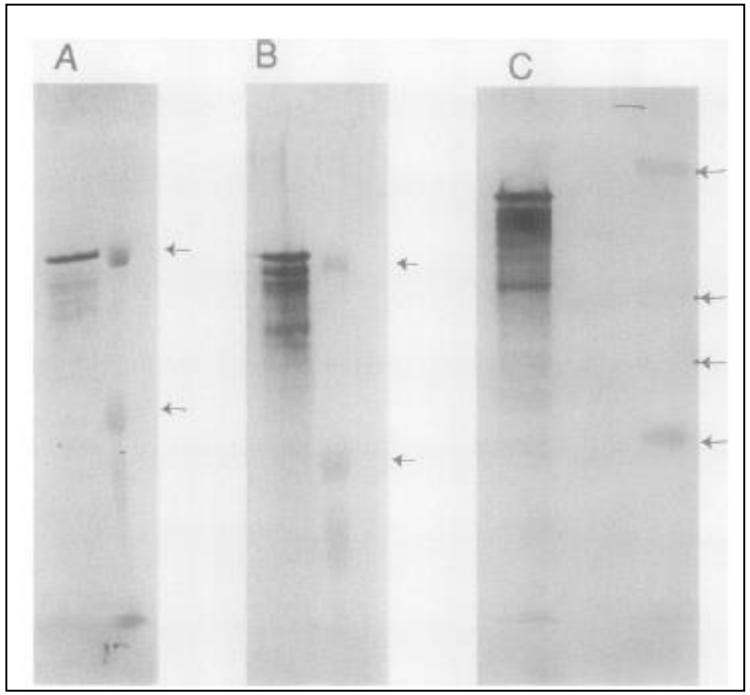
Ice-nucleating proteins (INPs)

inaZ gene

- Genomic DNA from *P. syringae* was amplified.
- The PCR product was cloned in pTrc99A vector and transformed in *Escherichia coli* DH5.
- The resultant recombinant *E. coli* conferred the INA^+ phenotype.
- To obtain the antigen, the vector was constructed to express a fusion protein, the product of GST (glutathion S-transferase) and a DNA fragment of about 1,200 bp was transformed in *E. coli* JM105.
- This recombinant *E. coli* expressed the fusion protein in quantity.
- The fusion protein was cleaved in GST and Ina90' protein, and the latter was collected from the band of gel fractionated by SDS-PAGE.

PAGE analysis on 8.5% polyacrylamide-SDS gels of whole-cell extracts of Ina$^+$ bacteria

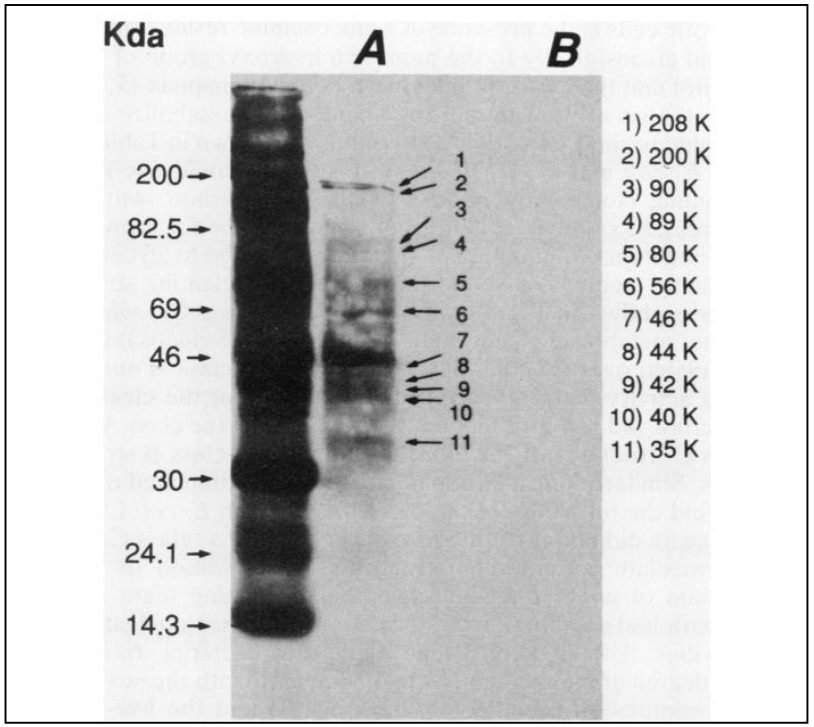
- PAGE analysis on 8.5% polyacrylamide-SDS gels of whole-cell extracts of Ina$^+$ bacteria showing immoblotting against the inaZ protein:
- (A) *P. syringae* C9, (B) *E. coli* C9la, (C) *P. syringae* S203.
- The material applied to each gel came from 5 x 10^9 cells.
- The arrows indicate the following standards run simultaneously (from the top) 200, 92.5, 69, and 43 kDa.
- For gels A and B, only the 200- and 92.5-kDa standards have arrows.



Turner *et al.*,1991

PAGE analysis on 8.5% polyacrylamide-SDS gels of whole-cell extracts of Ina$^+$ bacteria

- Radiogram (62 days) of PAGE analysis on an 8.5% polyacrylamide-SDS gel of extracts of *P. syringae* C9 (lane A) and Ina$^-$ *E. coli* CR63 (lane B) grown in the presence of radioactive compound D-[3H]mannose.



Turner *et al.*,1991

Ice nucleation proteins
Mechanism of ice formation

- *Pseudomonas syringe* has a special protein located in the outer membrane called an Ice Nucleating Protein.
- Under favorable environmental conditions the INP changes configuration to form a "nucleation site".
- It is speculated that when water enters the nucleation site it becomes crystallized.
- The small, newly formed crystal then acts as a seed and locks other water molecules around it into an ice structure.
- However, the exact mechanism used by the bacteria remains unknown.

Shannon,2010

Ice nucleation proteins

Production of artificial snow at the Olympics

- *Pseudomonas syringe*, are responsible for the production of artificial snow.
- *Pseudomonas syringe* can naturally nucleate ice crystals at -2°C on land or in the sky, making snow easier to produce.
- If the Olympic competitors need a little more snow under their skis, it is likely that *Pseudomonas syringe* will come to the rescue.
- Since the 1970s, *P. syringae* has been implicated as an atmospheric "biological ice nucleator", with airborne bacteria serving as cloud condensation nuclei for rain production.

Shannon,2010;BSPP;..

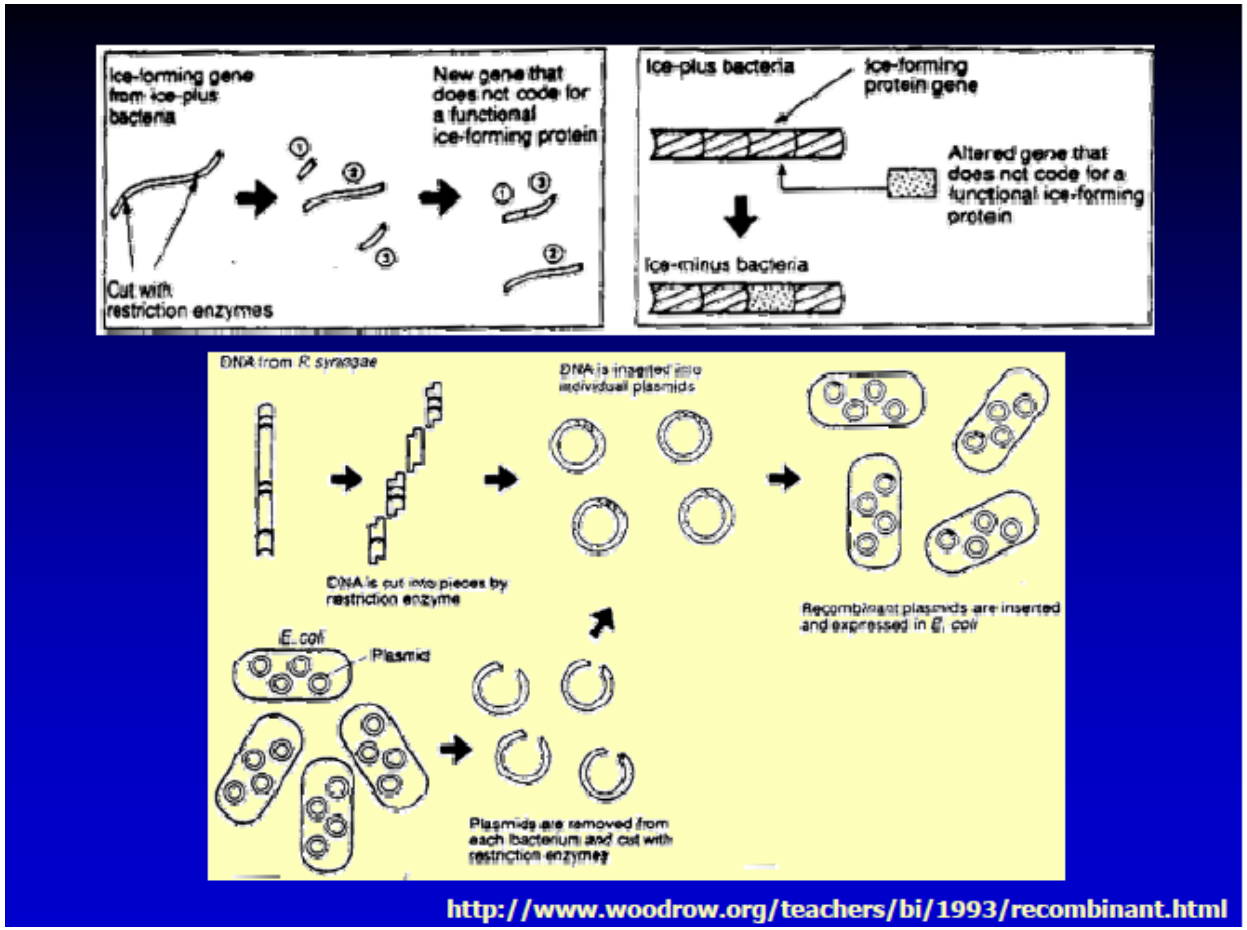
Chemical control of Ice bacteria
Spectinomycin

- Treatment of cells with antibiotics or dyes did not generally seem to cause rapid termination of INA in the most active strains, a finding also supported by several other authors (Lindow *et al.*,1982b; Anderson and Ashworth, 1986).
- Some chemicals are effective in reducing INA as we found in laboratory conditions but how they work in nature is yet to be clarified.
- While spectinomycin has been reported to cause rapid reduction in ice nucleation activity at higher temperatures, its effect may also be dependent on the environmental conditions during or after such treatment (Anderson and Ashworth,1986; Lindow *et al.*,1989).

Nejad,2005

Biocontrol of Ice bacteria

Genetically-engineered Ice$^-$ strains by recombinant DNA



BacteriaFall,2008

PCR Primers for *Pseudomonas* spp.

PCR protocols for detection or identification of several *Pseudomonas* species including *P. syringae* pathovars

Species	Target DNA
Pseudomonas spp.	16SrRNA
P. avellanae	*hrp W*
P. corrugata	Unknown
P. syringae:	IS *50*,Tabtoxin
pv. *actinidiae*	*arg K* (OCTase gene)
pv. *atropurpurea*	Coronatine, *cfl*
pv. *phaseolicola*	Phaseolotocin,Tox-, *arg K*
pv. *papulans*	*hrp L*
pv. *pisi*	RAPD
pv. *syringae*	*syr* B, *syr* D
pv. *tagetis*	Tagetitoxin
pv. *tolaasii*	Tolaasin
pv. *tomato*	*hrp 2*
pv. *cannabina*	*efe* (Ethylene-forming enzyme gene)

López *et al.*,2008

PCR Primers for *Pseudomonas* spp.

PCR Primers for detection or identification of several *Pseudomonas* species including *P. syringae* pathovars

Table 4. PCR Primers for *Pseudomonas* spp.

Specificity	Primer Designation	DNA-Sequence	Size (bp)	Reference
Pseudomonas genus-specific	Ps-for	(5'-GGT CTG AGA GGA TGA TCA GT-3')	1018	71
	Ps-rev	(5'-TTA GCT CCA CCT CGC GGC-3')		
P. putida	XyLR-F1	(5'-TCG CTA AAC CAA CTG TCA-3')	259	35
	XyLR-R1	(5'-GCA CCA TAA GGA ATA CGG-3')		
Phloroglucinol-producing species (*P. fluorescens*, etc.)	Phl2a	(5'-GAG GAC GTC GAA GAC CAC CA-3')	745	54
	Phl2b	(5'-ACC GCA GCA TCG TGT ATG AG-3')		
Phenazine-producing species (*P. fluorescens*, *P. aureofaciens*, *etc.*)	PCA2a	(5'-TTG CCA AGC CTC GCT CCA AC-3')	1150	54
	PCA3b	(5'-CCG CGT TGT TCC TCG TTC AT-3')		
many species and *P. syringae* pathovars	A1	(5'-GAG TTT GAT CAT GGC TCA G-3')	1550	45
	B6	(5'-TTG CGG GAC TTA ACC CAA CAT-3')		
many species and *P. syringae* pathovars	N1	(5'-GGT GGA TGC CTT GGC AGT CA-3')	2900	45
	N2	(5'-AGA TGC TTT CAG CGG TTA TC-3')		
many *P. syringae* pathovars	D21	(5'-AGC CGT AGG GGA ACC TGC GG-3')	558	45
	D22	(5'-TGA CTG CCA AGG CAT CCA CC-3')		
many *P. syringae* pathovars	ERIC1R	(5'-ATG TAA GCT CCT GGG GAT TCA C-3')	DNA-fingerprint 200-6000	44, 70
	ERIC2	(5'-AAG TAA GTG ACT GGG GTG AGC G-3')		

(continued on next page)

Schaad *et al.*,2001

PCR Primers for *Pseudomonas* spp.

PCR Primers for detection or identification of several *Pseudomonas* species including *P. syringae* pathovars

Table 4. (continued)

Specificity	Primer Designation	DNA-Sequence	Size (bp)	Reference
many *P. syringae* pathovars	REP1-I	(5'-III ICG ICG ICA TCI GGC-3')	DNA-fingerprint 200-6000	44, 70
	REP2-I	(5'-ICG ICT TAT CIG GCC TAC-3')		
many *P. syringae* pathovars	BoxA1R	(5'-CTA CGG CAA GGC GAC GCT GAC G-3')	DNA-fingerprint 200-6000	44
many *P. syringae* pathovars	IS50	(5'-CAG GAC GCT ACT TGT GT-3')	DNA-fingerprint 200 - 6000	70
pv. *tomato, atropurpurea, glycinea,* (coronatine)	Primer 1	(5'-GGCGCTCCCTCGCACTT-3')	650	5
	Primer 2	(5'-GGTATTGGCGGGGGTGC-3')		
pv. *syringae, atrofaciens, aptata,* (lipodepsinonopetide)	B1	(5'-CTTTCCGTGGTCTTGATGAGG-3')	752	63
	B2	(5'-TCGATTTTGCCGTGATGAGTC-3')		
pv. *phaseolicola,* (phaseolotoxin)	HM 6	(5'-CGTGTCCGTGGATAAAAGC-3')	1900	53
	HM 13	(5'-GTTGAATTTCACTACCCG-3')		
	P 5.1 P 3.1	(5'-AGCTTCTCCTCAAAACACCTGC-3') (5'-TGTTCGCCAGAGGCAGTCATG-3')	500	60
	followed by: P 5.2 P 3.2	 (5'-TCGAACATCAATCTGCCAGCCA-3') (5'-GGCTTTTATTATTGCCGTGGGC-3')	 450	

Schaad *et al.*,2001

Psy-PCR

P. syringae-specific polymerase chain reaction

Detect all strains of *P. syringae* group

- Primer design for the specific detection of strains of the *P. syringae* group required targeting discriminating regions of the genome.
- Psy_F 5′-ATG ATC GGA GCG GAC AAG-3′ and
- Psy_R 5′-GCTCTTGAGGCAAGCACT-3′
- Allowed the amplification of a 144-bp DNA fragment.
- This PCR was named *Pseudomonas* syringae-specific polymerase chain reaction(Psy-PCR).

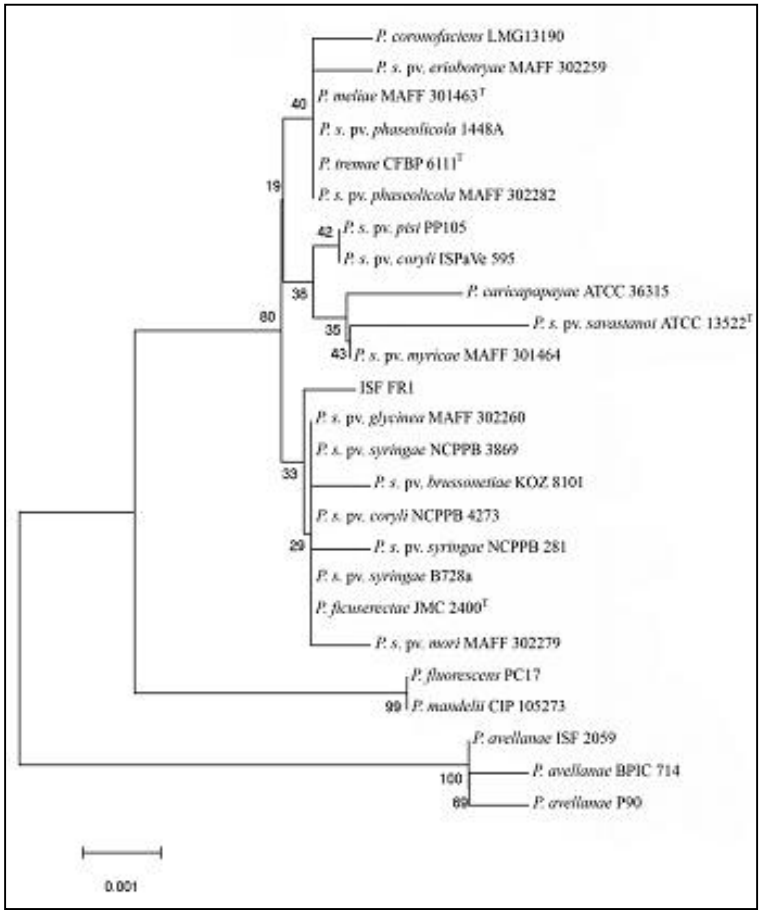
formation): Psy_F 5′-ATG ATC GGA GCG GAC AAG-3′ and Psy_R 5′-GCT CTT GAG GCA AGC ACT-3′ and allowed the amplification of a 144-bp DNA fragment. This PCR was named *Pseudomonas syringae*-specific polymerase chain reaction (Psy-PCR).

Guilbaud *et al.*,2016

PCR detection of Pseudomonads including *P. syringae*

Amplification of the gene 16S rRNA

- Dendrogram based on 16S rDNA gene sequences of endophytic *Pseudomonas syringae* ISF FR1, *P. syringae* pathovars and *Pseudomonas* spp. obtained with neighbor-joining algorithm.
- Multiple alignment of 16S rDNA sequences were performed using the Clustal W algorithm.
- Cluster analysis was conducted using MEGA, version 3.1 (Kumar *et al.*,2004) software.
- The scale bar represents the number of substitutions in each sequence.
- Bootstrap values (1,000 replicates) are also shown.



Scortichini and Loreti,2000

PCR detection of *Pss* and *P. tolaasai* Amplification of both genes of 16S rRNA and *rpoB* (β-subunit of RNA polymerase)

- The two genes (16S rRNA and rpoB) were PCR-amplified using an Applied Biosystems 9700 thermal cycler.
- The 16S rRNA primers were 27f (5'- AGAGTTTGATCMTGGCTCAG) and 765r (5' CTGTTTGCTCCCCACG) (Lane, 1991).
- The cycle conditions were: 94°C for 3 min; 35 cycles of 94°C for 1 min, 47°C for 1 min and 72°C for 2 min; a single cycle of 47°C for 1 min; and a final extension step of 72°C for 10 min.
- Primers LAPS (5'-TGGCCGAG AACCAGTTCCGCGT) and LAPS27 (5'-CGGCTTCGTCCAGCTTGTTCAG) were used for rpoB gene amplification.
- The cycle conditions were: 94°C for 3 min; 40 cycles of 94°C for 45 s, 55°C for 1 min and 72°C for 90 s; and a final extension step 72°C for 10 min.
- The sequence data of approximately 666 bp (16S rRNA) and 728 bp (rpoB) were compared visually.

Whitelaw-Weckert *et al.*,2011;..

PCR detection of *Pss*

Amplification of both genes of 16S rRNA and *rpoB* (β-subunit of RNA polymerase)

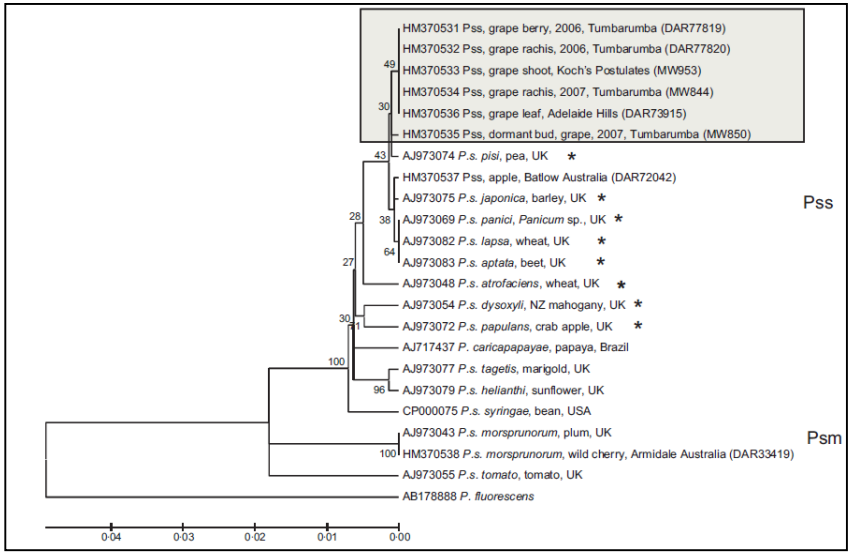
- The 16S rRNA sequences were not able to separate Pss and *Pseudomonas syringae* pv. *morsprunorum* (Psm).
- The rpoB sequences (housekeeping gene) produced a phylogenetic tree which clearly separated the Pss and Psm sequences with strong bootstrap support (100%).
- The Pss cluster contained Pss sequences downloaded from the GenBank data base (pv. *aceris*, pv. *aptata*, pv. *atrofaciens*, pv. *dysoxyli*, pv. *japonica*, pv. *lapsa*, pv. *panici*, pv. *papulans* and pv. *pisi*) that were probably mislabelled as it has been suggested that these are all synonyms of pv. *syringae*.

Whitelaw-Weckert *et al.*,2011

PCR detection of *Pss*

Amplification of both genes of 16S rRNA and *rpoB* (β-subunit of RNA polymerase)

- UPGMA tree derived from *rpoB* gene sequences from representative collected Australian grape strains (boxed); Australian apple and Prunus strains; and published sequences from GenBank.
- *Pseudomonas syringae* pv. *syringae* (Pss) and *P.s.* pv. *morsprunorum* (Psm) groups are clearly separated.
- Sequence for *Pseudomonas fluorescens* (AB178888) was included as an outgroup.



rpoB gene, a reliable biomarker to distinguish some of *P. syringae* pathovars.

Whitelaw-Weckert *et al.*,2011

Rep-PCR genomic fingerprinting

Procedure of BOX-PCR

BOX-PCR-based identification of bacterial species belonging to Pseudomonas syringae: P. viridiflava group

- The 22-mer BOXA1R oligonucleotide/primer was used to generate BOX-PCR profiles (Versalovic *et al.*,1991).
- Amplification reactions were performed in volumes of 25 µL, containing 2 µM of the single BOX primer, 200 µM each of dATP, dCTP, dGTP and dTTP, PCR reaction buffer (10 mM TrisHCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% TritonX100 and 0.2 mg mL^{-1} bovine serum albumin), 1.5 units of *Taq* DNA polymerase and, as template DNA, 5 µL of a bacterial cell suspension at 10^8 cfu mL^{-1}.
- Amplification was performed in an MJ Research, Inc. PTC-100 Thermal Cycler programmed for an initial denaturation step of 7 min at 95°C, followed by 30 cycles of 1 min at 94°C, 1 min at 53°C and 8 min at 65°C with a final elongation step of 15 min at 65°C.

Marques *et al.*,2008

Rep-PCR genomic fingerprinting

Procedure of BOX-PCR

BOX-PCR-based identification of bacterial species belonging to Pseudomonas syringae: P. viridiflava group

- PCR amplification products were detected by electrophoresis of 12 µL aliquots through 1.4% agarose gels in Tris-borate-EDTA (TBE) buffer, which were stained with ethidium bromide (EtBr 1.25 mg/L), visualized under UV light.
- DNA standards (1-kb DNA ladder Gibco BRL) were included in each electrophoresis gel.
- DNA fingerprints of strains were first compared for similarity by visual inspection of band patterns. They were considered identical when all scored bands in each pattern had the same apparent migration distance, even if a slightly different molecular weight was assigned to the same band over two or three different electrophoreses.
- Variations in intensity were not taken as differences.

Marques *et al.*,2008

BOX-PCR analysis

Used to discriminate to identify *Pseudomonas syringae - P. viridiflava* group at species level

- BOX-PCR, independent from the other rep-PCR techniques, has revealed the possibility of delineating *P. syringae* genomospecies.
- BOX-PCR-based identification of bacterial species belonging to *Pseudomonas syringae - P. viridiflava* group.

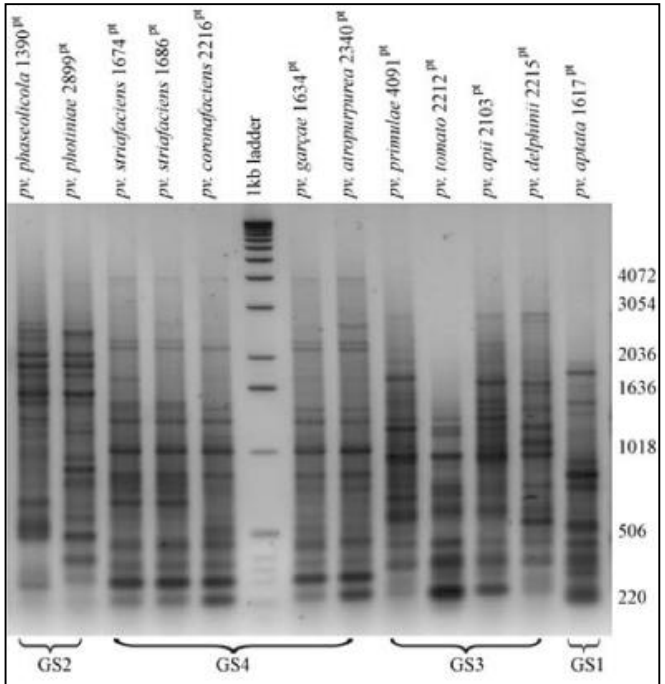


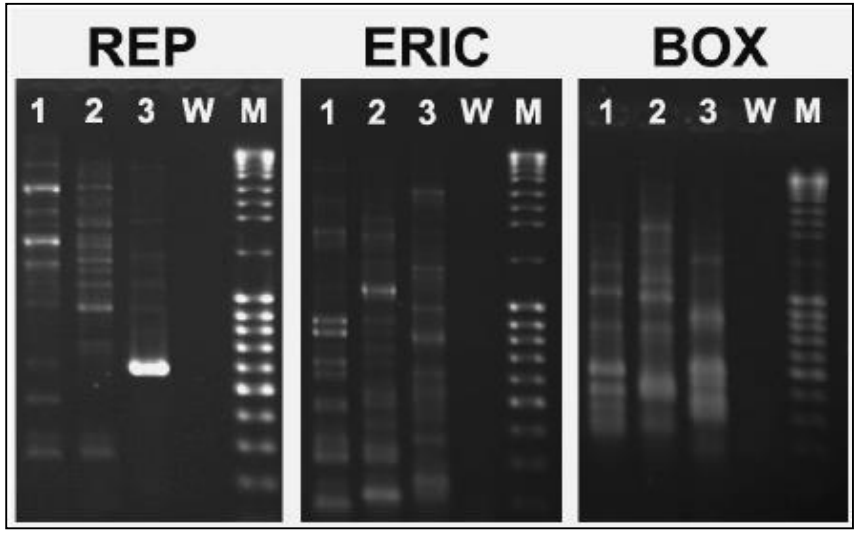
Figure 4 - Agarose gel electrophoresis of BOX-PCR fingerprinting patterns from genomic DNA of different pathovars belonging to *Pseudomonas syringae* (pathovars *photiniae*, *striafaciens*, *coronafaciens*, *garçae*, *atropurpurea*, *primulae*, *tomato*, *apii*, *delphinii* and *aptata*) and *P. savastanoi* pv. *phaseolicola*, which represent some of the nine known genomic species (GS), assigned after Gardan *et al.* (1999). Isolates obtained from the "Collection Française des Bactéries Phytopathogènes" (CFBP, Angers, France). The molecular size marker is a 1-kb ladder (Gibco BRL Life Technologies Inc.) and the sizes are indicated in base pairs. Shown is a negative image of an ethidium bromide gel. pt: pathotype strain.

Marques *et al.*,2008

Rep-PCR genomic fingerprinting

Pseudomonas syringae pv. *persicae* in comparison with pathovars *syringae* and *morsprunorum*

- *P. s. persicae* belongs, like *P. s. syringae* and *P. s. morsprunorum* , to LOPAT Group Ia of the determinative scheme of Lelliott *et al.*,1966.
- It can be distinguished from the other two pathovars attacking stone fruits on the basis of REP profiles (REP-PCR).



Obradovic,2010

ERIC-PCR analyses

P. syringae pv. *syringae*

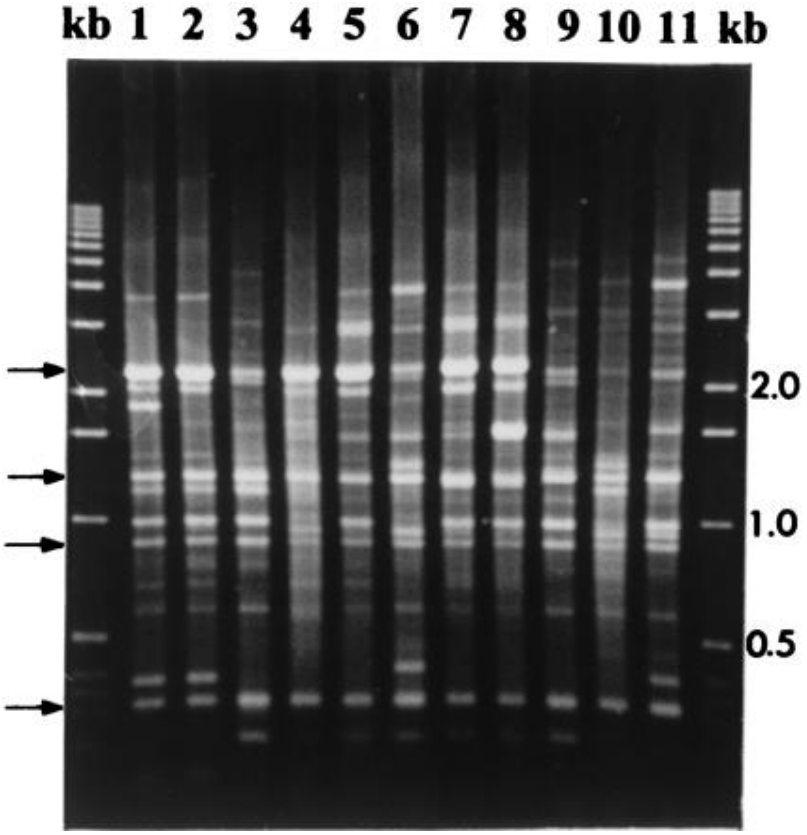
- ERIC sequences are short repetitive DNA sequences with highly conserved central inverted repeats that are dispersed throughout the genomes of diverse bacterial species.

Little *et al.*,1998

ERIC-PCR analyses

P. syringae pv. *syringae*

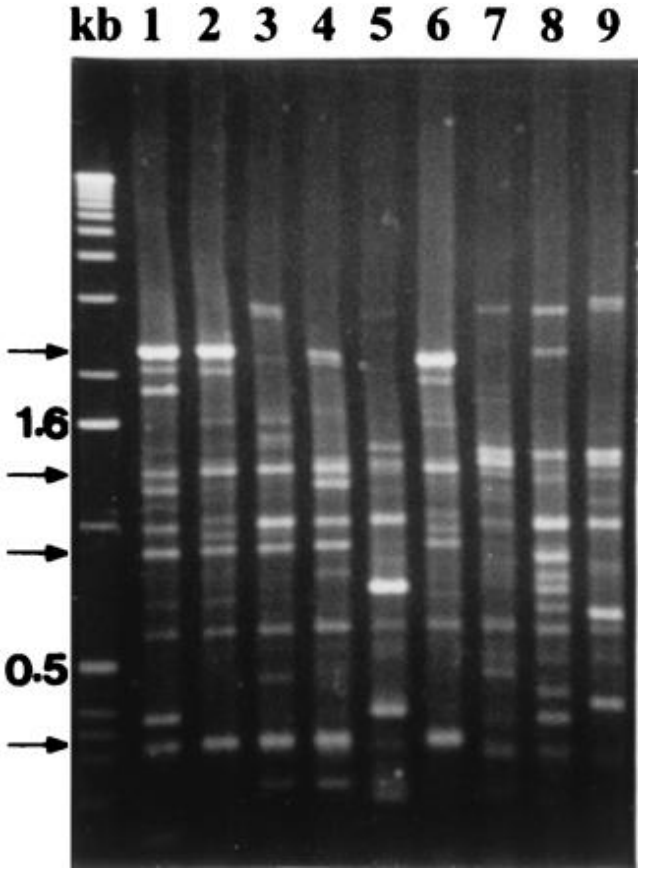
- The 11 ERIC genomic fingerprint patterns which shared four fragments of similar mobilities generated by 95 of the 104 *P. syringae* pv. *syringae* strains tested.
- Lanes:
- kb, the 1-kb molecular marker;
- 1 to 11, ERIC fingerprint patterns 1 to 11, respectively.
- The arrows on the left indicate the four fragments common to the 11 ERIC patterns.



Little *et al.*,1998

ERIC-PCR analyses
P. syringae pv. *syringae*

- ERIC fingerprints of *P. syringae* pv. *syringae* strains isolated from various plant hosts, showing strain variability within the pathovar.
- Lanes:
- kb, the 1-kb molecular marker;
- 1, B3 peach (pattern 1);
- 2, B301 pear (pattern 4);
- 3, B728a bean;
- 4, B37 rose (pattern 3);
- 5, B42 lemon;
- 6, 84-160 kiwi (pattern 4);
- 7, B18 millet;
- 8, B40 foxtail;
- 9, 321 tomato.
- Arrows on the left indicate the four fragments common to 95 of the 104 strains tested.



Little *et al.*,1998

PCR for detection of numerous pathovars of *P. syringae*

Insertion sequence (IS) elements

- Insertion sequence (IS) elements of various families as movable DNA sequences are widespread in genomes of bacteria and other organisms (Berg and Howe, 1989).
- IS50 has been used as primer for the assessment of genetic diversity in several bacteria genera including pseudomonads (Weingart and Volksch, 1997; Noble *et al.*,2006).

Izadiyan and Taghavi, 2017

PCR for detection of numerous pathovars of *P. syringae*

Insertion sequence (IS) elements

- These consist of inverted-repeat sequences at their termini and an open reading frame.
- They typically only encode the transposase protein required for transposition, as well as one or more additional proteins that regulate the rate of transposition (Berg & Berg, 1983).
- Their exact biological meaning is unknown but, as they are able to cause various types of genome rearrangements, they presumably play an important role in genome evolution.

Mazzaglia *et al.*,2010

PCR for detection of numerous pathovars of *P. syringae*

hrcV gene

- The greatest limitation of these genes is inability to detect numerous pathovars of *P. syringae*.
- Herein, by using bioinformatic analysis, we found the hrcV gene located at pathogenicity islands of bacterial genome with the potential of being used as a new marker for phylogenetic detection of numerous pathovars of *P. syringae*.
- Following design of specific primers to hrcV, we amplified a 440 bp fragment.
- Of 13 assayed pathovars, 11 were detected.

Vaseghi *et al.*,2014

PCR for detection of two toxins and siderophore yersiniabactin

Differentiate pvs. of *P. syringae, morsprunorum* and *avii*

- Differential PCR's for races of *P. syringae* pv. *morsprunorum*, *P. syringae* pv. *syringae* and *P. syringae* pv. *avii*.

Bacterium	PCR *cfl* coronatine	PCR *syr B* or *syrD*	PCR irp1 yersiniabac
P.s. pv. *morsprunorum* race 1	+, few -	-	-
P.s. pv. *morsprunorum* race 2	-	-	+
P.s. pv. *syringae*	-	+, few -	-
P.s. pv. *avii*	-	-	+

- = negative; + = positive

Janse,2010

PCR Primers for *Pseudomonas* spp.

PCR for detection of two toxins and siderophore yersiniabactin in Pseudomonds associated stone fruits

- Toxin and siderophore encoding genes in Pseudomonds associated stone fruits are:
- Syringomycin regulation(*syrP*),
- Syringomycin synthesis (*syrB* and *syrC*),
- Syringomycin secretion (*syrD*) genes;
- Coronatine toxin production *cfl* gene; and
- Siderophore gene Yersininabactin(*irp1*).

Obradovic,2010

PCR test

PCR for detection of two toxins and siderophore yersiniabactin in Pseudomonds associated stone fruits

P. syringae, P. morsprunorum (race 1 and 2) and atypical *P. syringae* pv. *syringae*

Group[a]	Strain	Host	Year of isolation	Genes encoding toxin production		
				*syr*B (syringomycin)	*cfl* (coronatine)	*irp*1 (yersiniabactin)
Psm race 1	701A	Sweet cherry	2005	–	+	–
	702	Plum	1994	–	–	–
	704	Sweet cherry	1994	–	+	–
	710	Sweet cherry	1996	–	+	–
	755	Plum	1999	–	–	–
	771	Plum	1999	–	–	–
	782	Sweet cherry	2001	–	–	–
	787	Plum	2001	–	–	–
	788	Plum	2001	–	–	–
	793	Plum	2001	–	–	–
	LMG 2222*	*Prunus avium* cv. 'Napoleon'		–	+	–
Psm race 2	701	Sour cherry	1994	–	–	+
	719	Sour cherry	1997	–	–	+
	732	Sour cherry	1997	–	–	+
	733	Sour cherry	1997	–	–	+
	745	Sour cherry	1999	–	–	+
	764	Sour cherry	1999	–	–	+
	CFBP 3800*	*Prunus cerasus*		–	–	+
Atypical[b]	791	Sour cherry	2001	–	–	–
Pss	702A	Plum	2005	+	–	–
	753	Apricot	1999	+	–	–
	757	Plum	1999	+	–	–
	760	Sour cherry	1999	+	–	–
	762	Apricot	1999	+	–	–
	763	Sour cherry	1999	+	–	–
	2905*	Sour cherry	1978	+	–	–
	LMG 1247*	*Syringa vulgaris*		+	–	–

Kałużna & Sobiczewski,2009

PCR amplification

Molecular detection of syringomycin related genes

Primer B1 and B2 were used to detect *syrD* or *syrB* gene

- Detection of toxic lipodepsipeptides (toxins) by PCR.
- Many strains of *P. syringae* pv. *syringae* produce toxiclipodepsipeptides and possessed the *syrD* and *syrB* genes, which enables their detection.
- Note the gene *syrD* involved in the secretion of the toxins:

1. Syringomycines, and
2. Syringopeptines.

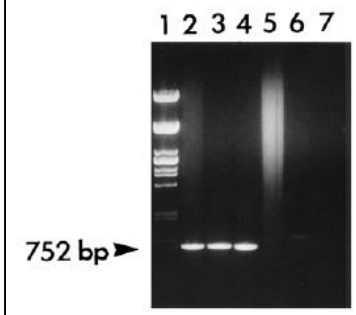


Fig. 1. PCR amplification of the 752-bp fragment of *syrB* (Sorensen et al., 1998)

For *syrB*, the oligonucleotide sequences are:

primer B1: 5'-CTTTCCGTGGTCTTGATGAGG-3'

primer B2: 5'-TCGATTTTGCCGTGATGAGTC-3'

Obradovic and Young,2010; Alain Bultreys

PCR amplification

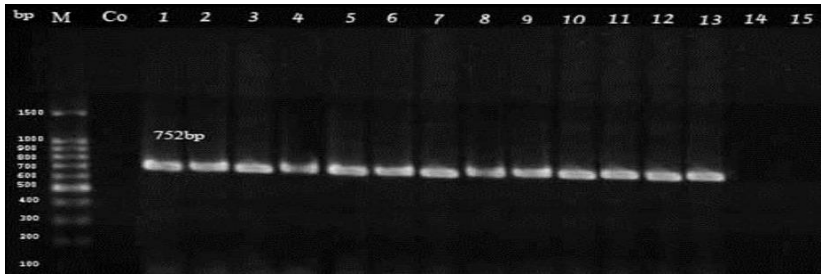
Molecular detection of syringomycin related genes

Primer B1 and B2 were used to detect *syrD* or *syrB* gene

- The primers B1 and B2 locate into the open reading frame of the *syrB* gene and yield a 752-bp product (Sorensen *et al.*,1998).

Components	Volume of one reaction	n x Reaction
Deionise distil water	15.8µl	n x15.8µl
10x buffer with $Mgcl_2$	2.5µl	n x 2.5µl
dNTP mixture	2.5µl	n x 2.5µl
Reverse primer	1µl	n x 1µl
Forward primer	1µl	n x 1µl
Taq polymerase	0.2µl	n x 0.2µl
Made a master mix and then sub-divided as 23µl aliquots to n eppendorf tubes		
Sample DNA	2µl to each respective tube	
Total	25µl final reaction volume each	

The component required for specific primers syrB1 and syrB2 amplification.



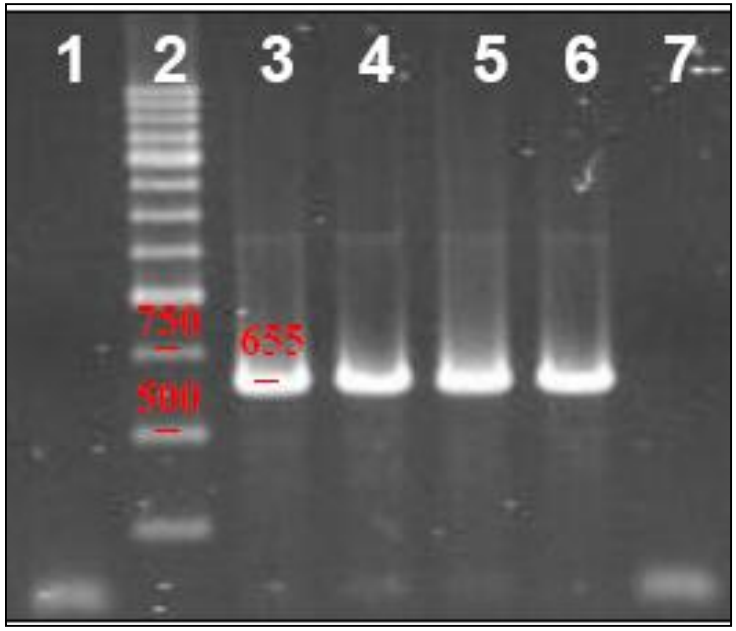
Agarose gel Electrophoresis of PCR with primers B1 and B2 corresponding to gene *syrB*. Co: Negative control,lanes 1-13: *Pseudomonas syringae* pv. *syringae* strains 752bp, 14 &15 strains of *P. syringae* , M: 100-1500bp DNA molecular marker.

Wazeer *et al.*,2014

PCR test

Detection of the *cfl* gene for coronatine (cor A)

- Many strains of *P. syringae* pv. *morsprunorum* race 1 produce coronatine and possessed the *cfl* gene, which enables their detection by PCR.
- Detection of a 655-bp fragment of the *cfl* gene, involved in coronatine synthesis.
- Lane1, water;
- Lane 2 DNA-marker XVI;
- Lanes 3 to 6, *P. syringae* strains from horse-chestnut;
- Lane 7, coronatine-non-producing *Pseudomonas* strain.



The sequence of the primers are as follows:
5`- GGC GCT CCC TCG CAC TT- 3` (**Primer 1**)
and 5`- GGT ATT GGG GGT GC-3` **(Primer 2)**

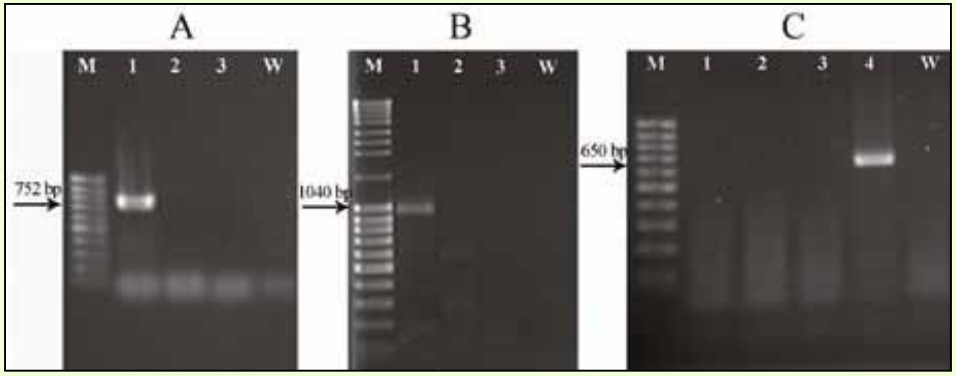
Alain Bultreys

Molecular detection of syringomycin related genes

PCR detection of the *syrD* or *syrB* and *cfl* genes

- PCR detection of genes involved in phytotoxins production: *syrB* (A), *syrD* (B) and *cfl* (C).
- M–marker (MassRuler Low Range (A and C) and Mix Range (B) DNA Ladder, Fermentas, Lithuania), W–negative control.

1. *P. s.* pv. *morsprunorum* (KFB 0101),
2. *P. s.* pv. *persicae* (KFB 0102),
3. *P. s.* pv. *syringae* (KFB 0103),
4. *P. s.* pv. *morsprunorum* (KFB 0120).



Gašić *et al.*,2012

PCR test

Molecular detection of coronatine

For *cfl* gene the oligonucleotide sequences are:

Primer 1: 5`-GGC GCT CCC TCG CAC TT-3`
Primer 2: 5`-GGT ATT GGC GGG GGT GC-3`

PCR Mix	Final conc.	Quantity per reaction (µl)
Water (molecular grade)		16.7
PCR Buffer with KCl and $MgCl_2$ (10X)	1X	2.5
dNTP (10mM)	0.2mM	0.5
Primer 1 (10µM)		2
Primer 2 (10µM)		2
Taq Polymerase (5U/µl)	1.5U	0.3
[a]Sample		1
Total volume		25 µl

[a]Use a standard procedure to isolate genomic DNA or use whole-cells of the strain

Amplification program

Temperature	Time	No. of cycles
93°C denaturation	2 min	1
93°C denaturation	2 min	
67°C annealing	1 min	37
72°C elongation	2 min	
4°C	∞	∞

Obradovic and Young,2010

PCR test
Role of siderophore yersiniabactin

- The siderophores pyoverdin and yersiniabactin play a (possibly indirect) role in pathogenesis and in competition with other microorganisms on plant surfaces(ecological role).
- Yersiniabactin is positive for pv. *morsprunorum* race 2 and pv. *avii*.

Bultreys and Kaluzna,2010;..

PCR test

Molecular detection of Yersiniabactin gene *irp1*

Differentiate pvs. *morsprunorum* race 2 and *avii* from *P. syringae* pv. *syringae*

- The development of a PCR protocol for detecting the gene *irp1* involved in the production of the siderophore yersiniabactin.
- Detection of the yersiniabactin gene *irp1* by PCR using primers PSYE2/PSYE2R helps to differentiate pvs. *morsprunorum* race 2 and *avii* from *P. syringae* pv. *syringae*.
- The strains of pathovar *morsprunorum* race 2 and *avii* both give positive results.

Alain Bultreys

PCR test

Identification and characterization of *Pseudomonas syringae* pv. *mori* affecting white mulberry

Pseudomonas syringae species-specific PCR primer pair (D21/D22)

- The following universal bacterial 16S rDNA primers were used: 16S04 (AACTCAAAGGAATTGACGG) (Gerischer,2008) and pH 2 (AAG GAG GTG ATC CAG CCG CA) (Edwards *et al.*,1989).
- For the gyrB gene partial nucleotide sequence amplification, the 1480F/2242R primer pair (Bonasera *et al.*,2014) was used.
- PCR products of ~1400 bp for 16S rDNA and ~ 900 bp for gyrB were obtained using a touch-down PCR protocol (Korbie and Mattick,2008).
- The expected size amplicons were extracted from the agarose gel using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI), and sequenced (Genomed S.A., Warsaw, Poland).
- Additionally, pathogen identification was verified by using and sequencing the products of the PCR primer pair D21/D22, which was published as *P. syringae*-specific, (Schaad *et al.*,2001).

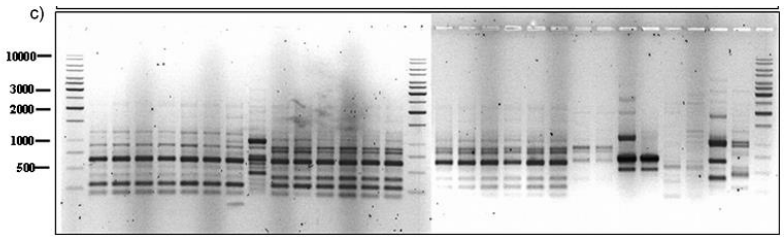
Krawczyk & Łochyńsk,2020

PCR for detection of numerous pathovars of *Pseudomonas syringae* pv. *actinidiae* Insertion sequence (IS) elements

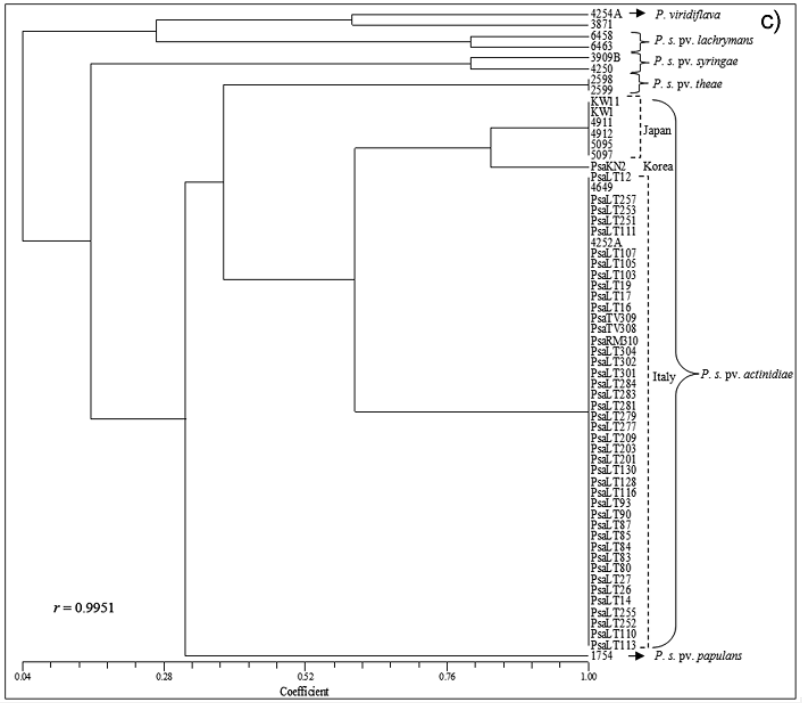
- IS50-PCR reactions were performed witha single 20 bp oligonucleotide primer (IS50 GGT TCC GTT CAG GAC GCT AC) complementary to the IS50 portion of Tn5, as described by Sundin & Murillo (1999).
- Following the amplification step, 10 µL of the products were separated on 1% agarose gel at 4 V/cm for 4-5 h.

Mazzaglia *et al.*,2010

PCR for detection of numerous pathovars of *Pseudomonas syringae* pv. *actinidiae* Insertion sequence (IS) elements



Examples of PCR fingerprints of *Pseudomonas syringae* pv. *actinidiae* and other *P. s.* pathovars obtained with IS50(c) primer.



Computer generated dendrogram of genetic similarity resulting from the data set of IS50-PCR amplifications, using UPGMA analysis and Dice's coefficient. The values of the cophenetic correlation coefficient (r) are reported.

Mazzaglia *et al.*,2010

RAPD

Pseudomonas syringae pv. *actinidiae*

- One hundred and twenty random primers, including 40 commercial 10-mer oligonucleotide primers and 80 commercial 12-mer oligonucleotide primers (Sangon Biotech, Shanghai, China), were used to select primers for screening the specific fragment of *P. syringae* pv. *actinidiae*.
- Amplification was performed in a total volume of a 25 μl reaction mixture with 50 pmol of primer, 100 ng of genomic DNA, 1 Unit of Taq DNA polymerase, 10× reaction buffer (500 mM KCl, 100 mM of Tris-HCl pH 8.3, 25 mM of $MgCl_2$, 0.01% of gelatin), 10mM of dNTP (dCTP, dGTP, dATP, dTTP), and extra sterilized distilled water.

Liu *et al.*,2012

RAPD

P. syringae pv. *actinidiae*

- The conditions of DNA amplification were optimized and followed the procedure of Williams et al. (1990) with few modifications.
- The PCR program consisted of an initial denaturation of 5 min at 94°C, followed by 40 cycles at 94°C for 60 s (denaturation), 36°C for 60 s (annealing) and 72°C for 2 min (extension), with an additional extension period of 10 min at 72°C.
- A negative control, without genomic DNA, was run with every set of samples to confirm that no contaminating DNA was present in the reaction.

Liu *et al.*,2012

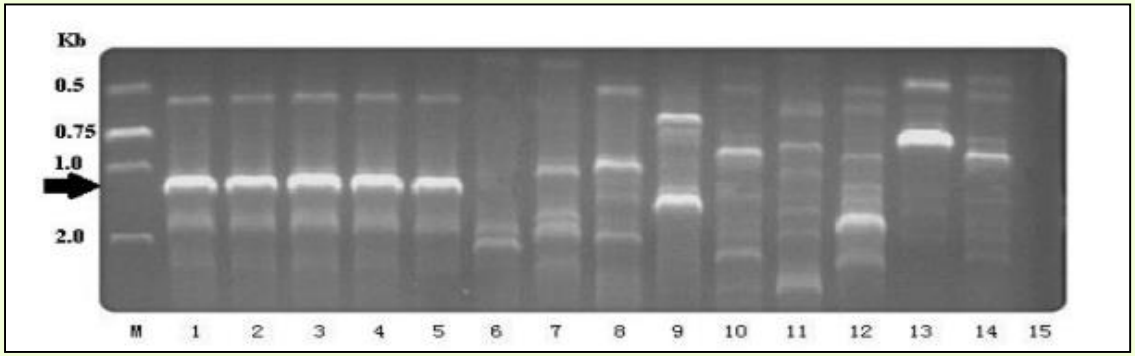
RAPD
P. syringae pv. *actinidiae*

- Amplifications were carried out in a Mastercycler.
- The amplified PCR products were separated by electrophoresis on 1.5% agarose gels in 1×TAE buffer, visualized and imaged by gel documentation system (GelDoc 2000, Bio-Rad, USA) after staining with ethidium bromide.
- According to the RAPD profile (Figure 1), only the primer P7 (5′-CGCAGCCGAGAT-3′) repeatedly produced a specific, stable and distinct DNA fragment, which was produced only from the strains of *P. syringae* pv. *actinidiae*, but not from the other strains.

Liu *et al.*,2012

RAPD
P. syringae pv. *actinidiae*

- The size of specific fragment is about 1300 bp that is suitable for the development of a primer to detect the pathovar-specific DNA fragment of *P. syringae* pv. *actinidiae*.



RAPD profiles are generated with primer P7. Lane M, 2 kb DNA ladder; lanes 1-14, strains of *P. syringae* pathovars or similar strains of *Pseudomonas* or other species listed in Table 1; lane15, CK (ddH_2O).

Liu *et al.*,2012

PCR test

Duplex-PCR assay

P. syringae pv. *actinidiae*

- The duplex-PCR assays were carried out in a 50 µl reaction mixture containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM each KN-F and KN-R primers (Koh and Nou, 2002), 0.4 µM each primer AvrDdpx-F and -R primers (reported in next slide), 0.05 U/µl Platinum *Taq* DNA Polymerase, 1X PCR buffer.
- Bacterial genomic DNA (20 ng), or an aliquot (5 µl) of the bacterial suspension at a concentration of 2×10^7CFU ml^{-1} were used as template.

The PCR thermal profile consisted of an initial denaturation step (95°C for 3 min), followed by 30 cycles at 94°C for 30 sec, 63°C for 45 sec, 72°C for 50 sec and a final elongation step of 5 min at 72°C.

Gallelli *et al.*,2011

PCR test

Duplex-PCR assay

P. syringae pv. *actinidiae*

- The amplification of homologues of *hrpW*, *hrpL*, *hopAB1*, *rpoD* and of the 492 bp KN-amplicon of Koh and Nou (2002) was done by PCR using the primers reported in Table below.

Primer target	Primer name	Primer sequence	Reference
hopAB1	AvrPtoB-LF	5'-GGAGAGGATCAGCATATG-3'	This study
	AvrPtoB-LR	5'-TCAGGGGACTATTCTAAAAG-3'	
hrpL	L1	5'-ACCTGGTTGTGTGGCATTGC-3'	Cournoyer *et al.*, 1996
	L2	5'-CCGTGAGCGGACGGTGCC-3'	
rpoD	PsrpoD FNP1	5'-TGAAGGCGARATCGAAATCGCCAA-3'	Parkinson *et al.*, 2011
	PsrpoDnprpcr1	5'-YGCMGWCAGCTTYTGCTGGCA-3'	
hrpW	WthFor	5' -AAGCGGCAAGAGTCCTCAAC -3'	
	WthRev	5'- GATGCCTGGGTTTTATCGTAG -3'	This study
	WthFint	5'- ACAGCCTGATAGCCAGGCTC -3'	
	Wthfint	5'- GCGTGCACGTTGTCAATGGT -3'	
KN-PCR amplicon	KN-F	5'-CACGATACATGGGCTTATGC-3'	Koh and Nou, 2002
	KN-R	5'-CTTTTCATCCACACACTCCG-3'	
	KN-F/ R and		Koh and Nou, 2002
KN-amplicon and *avrD1*	AvrDdpx-F	5'-TTTCGGTGGTAACGTTGGCA-3'	
	AvrDdpx-R	5'-TTCCGCTAGGTGAAAAATGGG-3'	This study

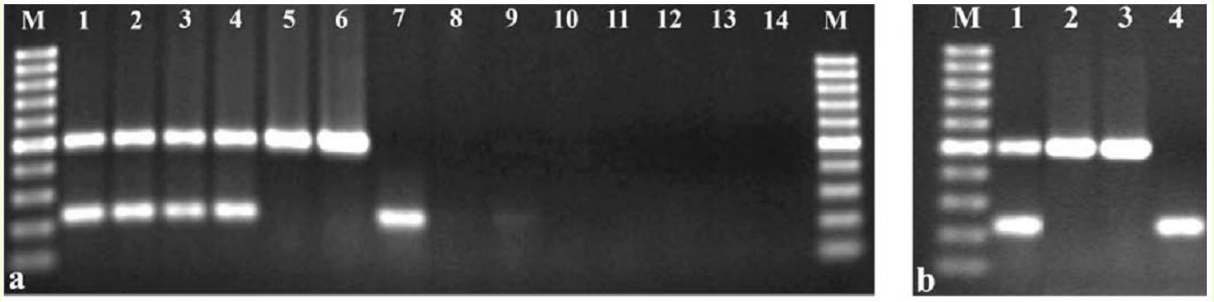
Gallelli *et al.*,2011

PCR test

Duplex-PCR assay

P. syringae pv. *actinidiae*

- Duplex-PCR from bacterial suspension (10^8 CFU/ml). a. *Pseudomonas syringae pv. actinidiae* ISF Act.1, ISPaVe 019, ISPaVe 020, NCPPB 3740 (lanes 1-4), *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *theae* CFBP 4097 (lanes 5-6), *Pseudomonas avellanae* NCPPB 3872 (lane 7), *P. s. phaseolicola* 1448A (lane 8), *P. syringae* pv. *glycinea* ISPaVe 1155 (lane 9), *P. syringae* pv. *syringae* OMP-BO 4250,1 (lane 10), *P. syringae pv. papulans* NCPPB 2848 (lane 11), *P. viridiflava* OMP-BO 4254A,1 (lane 12), *P. syringae pv. syringae* OMP-BO3909B,1 (lane 13), water control (lane 14). b. *Pseudomonas syringae* pv. *actinidiae* ISF 8.57 (lane 1), *P. syringae* pv. *tomato* NCPPB 2563 and *P. syringae* pv. *theae* NCPPB 2598 (laned 2-3), *P. avellanae* ISPaVe 1267 (lane 4). M: molecular markers (Gene Ruler™100 bp DNA ladder, Fermentas, Lithuania).



Gallelli *et al.*,2011

RAPD

Determination of genetic diversity

Pseudomonas syringae pv. *garcae*

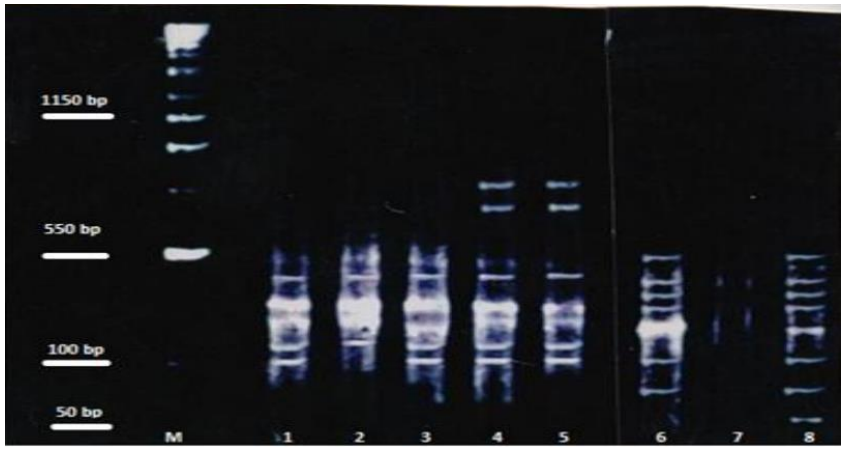
- The following random primers were used for RAPD analysis in eight isolates.
- OPAC 01 5′- TCC CAG CAGT -3′
- OPAC 02 5′- GTC GTC GTCT -3′
- OPAC 04 5′- ACG GGA CCTG-3′
- OPAC 05 5′- GTT AGT GCGG -3′
- OPAC 07 5′- GTG GCC GATG -3′
- The amplified fragments/bands ranged from 50bp to above 1100 bp.
- Out of 8 isolates, the highest number of bands were observed in isolate 8 Gordahama FII T2. T

Hinkosa *et al.*,2017

RAPD

Pseudomonas syringae pv. *garcae*

- Bacterial blight of coffee (BBC) isolates using analysis of RAPD.
- M- Primer OPAC 04 5'- ACG GGA CCTG-3';
- isolate 1 Manche FII T2;
- isolate 2. Boa FII T3,
- isolate 3 Hallo FIII T1,
- isolate 4 Hayilo FI T3;
- isolate 5 Sugale FIII T1,
- isolate 6 Shigado FI T2;
- isolate 7 Kara FII T1; and
- isolate 8 Gordahama FII T2.

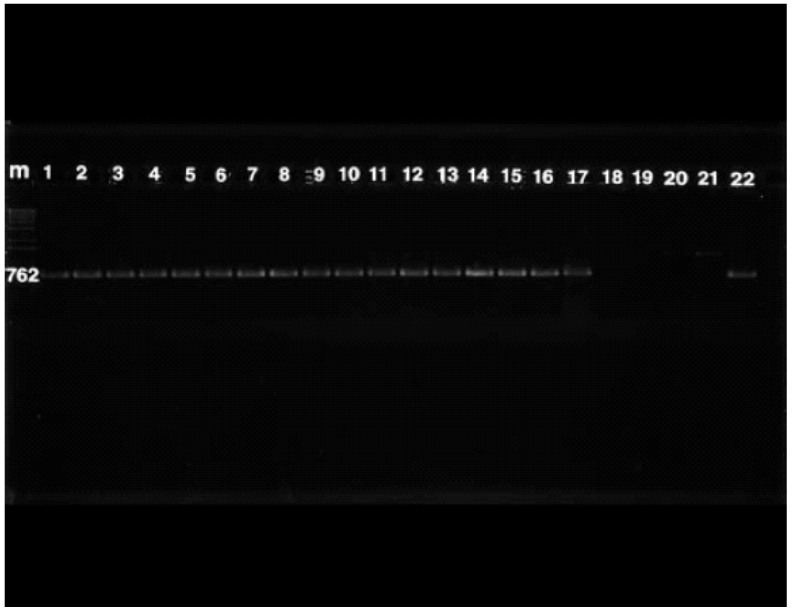


Hinkosa *et al.*,2017

PCR Test

Detection of *Pseudomonas avellanae* using primers based on 16S rRNA gene sequences

- Electrophoretic analysis of PCR-amplified 16S rRNA gene of different strains of *Pseudomonas avellanae* using PAV 1 and PAV 22 primers.
- m, molecular size marker (1 kb ladder).
- A PCR amplification product of 762 bp that was specific for all 40 strains of *P. avellanae*.
- No other bacterial species among those tested showed an amplification product under optimized PCR conditions.



Scor Liu *et al.*,2012 nesi,2001

PCR detection of *P. agarici*

Specific R16-1/R23-1R primer set

Amplify ITS1 regions

- DNA preparation and PCR amplification of the ITS I regions.
- Genomic DNAs were isolated by the method of Ausubel et al. (1987), except that the lysates were extracted twice with chloroform to remove residual phenol.
- ITS I regions were amplified by using the following primers:
- R16-1 (5'-CTTGTA CACACCGCCCGTCA-3') and
- R23-1R (5'-GGTACTTAGATGTTTCAGTTC-3') redesigned from the primer set of Nak agawa et al. (1994).
- Each PCR mixture (50 μl) contained primers (each at a concentration of 20 pmol), a mixture of deoxynucleotide triphosphates (each at a concentration of 200/μM), *Taq* polymerase buffer, chromosomal DNA (ca. 50 ng), and *Taq* polymerase (2.5 Unit). DNA thermal cycler used for PCR amplification was programmed as follows: (i) an initial extensive denaturation step at 94°C for 5 min; (ii) 35 cycles, with each cycle consisting of 94°C for 1 min, 58°C for

Kwon *et al.*,2000

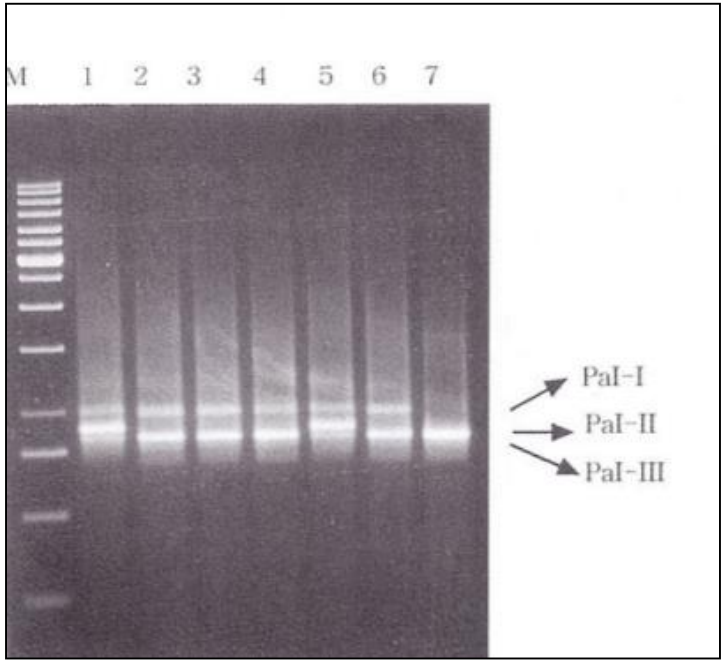
PCR detection of *P. agarici*

Specific R16-1/R23-1R primer set

Amplify ITS1 regions

- ITS I regions of *P. agarici* strains were amplified with primers R16-1 and R23-1R, which divided *P. agarici* strains into three groups:

1. LMG 2112 and LMG 2116 produced three bands, Pal-I, -II and -III, while
2. LMG 2110, LMG 2111, LMG 2113 and LMG 2115 formed two bands of Pal-I and -III, and
3. LMG 5374 produced one band, Pal-III.



P. agarici strains contained from one up to three putative ITS I regions. All strains of *P. agarici* had one common band (Pal-III) of ca. 800 bp in size.

Kwon *et al.*,2000

PCR detection of *P. tolaasii*

Amplify the v4 region of the 16S rRNA gene

- Specifically, we used the universal bacterial primer set 515F (59-GTGYCAGCMGCCGCGGTAA-39) and 806R (59-GGACTACNVGGGTWTCTAAT-39) to amplify the v4 region of the 16S rRNA gene (Apprill *et al.*, 2015; Parada *et al.*,2016).
- PCR conditions were as follows: 8 µl of 5 Prime HotStart MasterMix (Quanta BioSciences Inc., Beverly, MA, U.S.A.), 0.1 to 1 µl of template DNA, 1 µl of each primer from 10 µM stocks, and the appropriate volume of molecular biology grade water to bring the reaction to 20 µl. Thermal cycling was carried out on Mastercycler Nexus Gradient PCR machines (Eppendorf, Hamburg, Germany) under the following conditions: 94°C for 3 min; 25 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s; followed by a final elongation for 10 min at 72°C.

Martins *et al.*,2019

PCR detection of *P. tolaasii*

Amplify genes required for tolaasin production

- Two sets of PCR primers were developed to amplify genes required for tolaasin production.
- **First set of primers:**
- Primers:
- Pt-1A (5'-ATCCCTTCGGCGTTTACCTG-3'), and
- Pt-1D1 (5'-CAAAGTAACCCTGCTTCTGC-3').
- PCR product: 449 bp.
- **Second set of primers:**
- Primers:
- Pt-PM (5' TGCCTTACGCGCTGATTGGC3') ,and
- Pt-QM (5' TGATCA AACTCCAGCAATAG3'), internal primers used for nested PCR.
- PCR product: 249 bp.

Lee *et al.*,2002

PCR detection of *P. tolaasii*

Amplify genes required for tolaasin production

- The PCR reaction with the *Ps. tolaasii* specific primers, designed from the nucleotide sequence of DNA involved in tolaasin production.

Primers	Sequence of the primers
Pt-1A	5'-ATCCCTTCGGCGTTTACCTG-3'
Pt-1D1	5'-CAAAGTAACCCTGCTTCTGC-3'
REP1RI	5'-III ICG ICG ICA TCI GGC-3'
REP2I	5'-ICG ICT TAT CIG GCC TAC-3'
ERIC1R	5'-ATG TAA GCT CCT GGG GAT TCA C-3'
ERIC2	5'-AAG TAA GTG ACT GGG GTG AGC G-3'
BOXA1R	5'-CTA CGG CAA GGC GAC GCT GAC G-3'
pA	5'-AGA GTT TGA TCC TGG CTC AG-3'

Sajben *et al.*,2002

PCR detection of *P. tolaasii*
Specific Pt-1A/Pt-1D1 primer set

- Presence of the tolaasin gene at each strain was revealed by the specific primers:
- Pt-1A (5'-ATCCCTTCGGCGTTTACCTG-3') and
- Pt-1D1 (5'-CAAAGTAACCCTGCTTCTGC-3') (Lee *et al.*,2002).
- The PCR was performed in a total volume of 20µl containing:
- 2.0 µl of 10X Standard *Taq* Reaction Buffer, 200µM of each dNTP, 1.6mM $MgCl_2$, 0.25µM of primers, 1U *Taq* polymerase and 100 ng total DNA template in distilled water.
- PCR amplifications were carried out with an initial incubation at 95°C for 10 min, followed by 35 cycles of 94°C for 1 min, 63°C for 1 min and 72°C for 2 min, and a final elongation step at 72°C for 10 min.

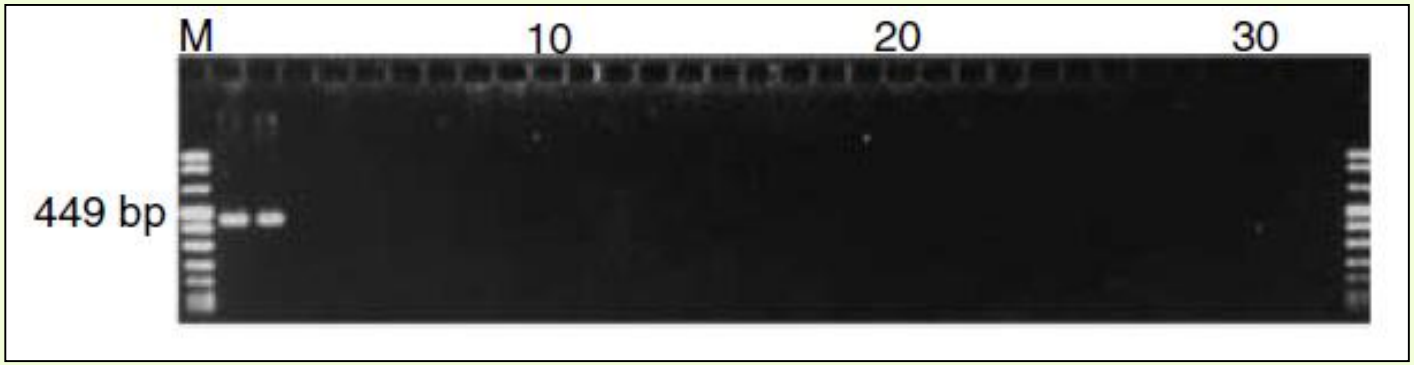
Sajben *et al.*,2011

PCR detection of *P. tolaasi*

First Pt-1A/Pt-1D1 primer set

Amplify genes required for tolaasin production

- Only a PCR product of 449 bp was produced in PCR reactions with the Pt-1A/Pt-1D1 primer set.
- Lanes 1-2 belong to *Pseudomonas tolaasii* amplified by Pt-A/Pt-1D1 primer set.
- No other Pseudomonds was amplified with this primer set (Lanes 3-32).

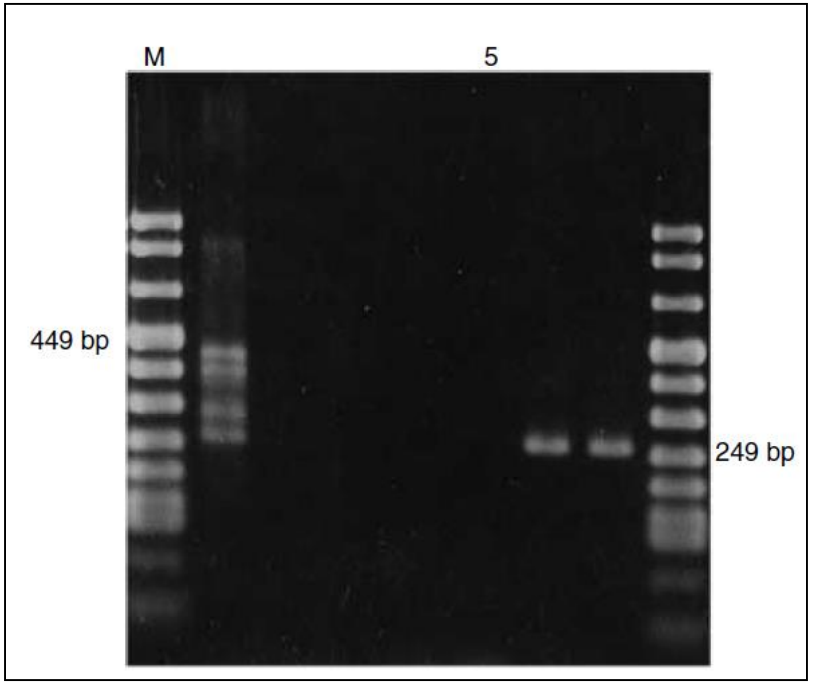


Lee *et al.*,2002

PCR detection of *Ps. tolaasii*

Amplify genes required for tolaasin production

- Primers Pt-PM and Pt-QM, internal primers for nested PCR, amplified 429 bp DNA with DNA from all *Ps. tolaasii*, but they did not amplify any DNA from other bacteria.



Lee *et al.*,2002

Microbial diversity

Genotyping of bacterial isolates by ARDRA

Pseudomonas tolaasii

- Amplified ribosomal DNA restriction analysis (ARDRA) was performed on the PCR-amplified 16S rDNA products from each of the isolates using three specific restriction enzymes *Cfo*I, *Hae*III, *Hinf*I and *Sac*I.
- Five microliters of each PCR product was digested for 2 h at 37°C with 1.5 U of each restriction endonuclease.
- Aliquots (5 µL) of each digested product were analyzed by gel electrophoresis in an 8% nondenaturing acrylamide gel (acrylamide: N,N'-Methylenebisacrylamide, 29:1) and by silver nitrate staining, as described previously.
- Fragment sizes were estimated using a low range, 50 bp DNA ladder and a final grouping of isolates was performed by a visual comparison of the restriction patterns.

ARDRA

Amplified Ribosomal DNA Restriction enzyme Analysis

Pseudomonas tolaasii

- The high degree of polymorphism cause problems in the identification of the *P. tolaasii*.
- Therefore, DNA samples were isolated from *P. tolaasii* strains and PCR reactions were performed with special primers.
- So called ARDRA was performed with *Cfo*I, *Hae*III, *Hinf*I and *Sac*I restriction enzymes, respectively.

1. The *Cfo*I *enzyme* provided 4 groups, but
2. The Hinf*I* and *Sac*I digestion resulted 8 different patterns.

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ARDRA

Amplified ribosomal DNA restriction enzyme analysis

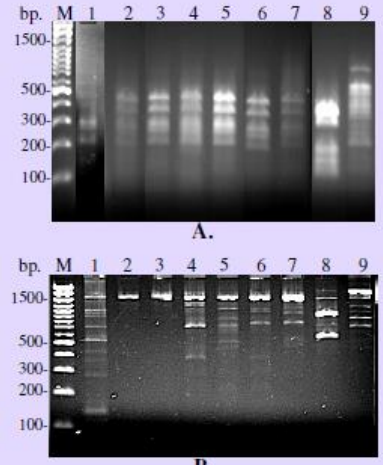
Pseudomonas tolaasii

- The ARDRA pattern of the strains of *P. tolaasii*.

A. Digestion with *Cfo*I enzyme.

B. Digestion with *Sac*I enzyme.

- The strains in the lines as the following: 1:6, 2:7, 3:8, 4:29, 5:17,6:15, 7:37, 8:25, 9:56.
- M: GeneRuler 100bp DNA Ladder Plus.



Pseudomonas strains	*Cfo*I	*Hae*III	*Sac*I	*Hin*fI
6	1	1	1	1
7	2	2	2	2
8	2	2	2	2
29	2	3	3	3
17	2	3	4	4
15	2	4	5	5
37	2	4	6	6
25	3	5	7	7
56	4	6	8	8

Table 2. Classification of the strains on the basis of ARDRA results

Sajben *et al.*,2002

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

Genus *Pseudomonas*

Species/subspecies	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
Pseudomonas (*sensu stricto*)	**Ps-for/Ps-rev** *Pseudomonas* 16S rRNA gene	Conventional and RFLP	Bacteria or soil (DNA extraction)	Widmer *et al.*, 1998	
P. avellanae	**PAV 1/PAV 22** 16S rRNA gene	Conventional	Bacteria (boiled), plant (BLOTTO)	Scortichini and Marchesi, 2001; Scortichini *et al.*, 2002	
P. avellanae	**WA/WC** Harpin-encoding *hrpW* gene	Conventional	Bacteria, plant (DNA extraction)	Loreti and Gallelli, 2002	
P. corrugata	**PC1/1–PC1/2 (group I)** **PC5/1–PC5/2 (group II)** RAPD fragments	Conventional Multiplex	Bacteria, plant (alkaline extraction)	Catara *et al.*, 2000	
P. corrugata *P. mediterranea* (*P. corrugata* Type II)	**PC1/1–PC1/2** ***(P. corrugata)*** **PC5/1–PC5/2** ***(P. mediterranea)*** RAPD fragments	Conventional	Bacteria (DNA extraction)	Catara *et al.*, 2002	Protocol slightly modified from Bereswill *et al.* (1994). Differentiation between Type I (*P. corrugata*) and Type II (proposed new species, *P. mediterranea*).
P. savastanoi pv. *glycinea*	**Tn5-derived**	Random primer-dependent PCR	Bacteria (DNA extraction)	Ullrich *et al.*, 1993	*Pseudomonas syringae* pv. *glycinea*
P. savastanoi pv. *phaseolicola*	**HM6/HM13** Phaseolotoxin gene cluster	Conventional	Bacteria, seed (DNA extraction)	Prosen *et al.*, 1993	*Pseudomonas syringae* pv. *phaseolicola*
P. savastanoi pv. *phaseolicola*	**P 5.1/p 3.1 (external)** **P 5.2/P 3.2 (internal)** Phaseolotoxin gene cluster	Nested	Seed washes (untreated)	Schaad *et al.*, 1995	*P. syringae* pv. *phaseolicola*
P. savastanoi pv. *phaseolicola*	**HB14F/HB14R** Phaseolotoxin gene cluster	Conventional	Bacteria (boiled)	Borowicz *et al.*, 2002	*P. syringae* pv. *phaseolicola* Specificity improved by annealing temperature of 80°C.

Palacio-Bielsa *et al.*,2009

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P. savastanoi pv. *phaseolicola*	**AVR1-F/AVR1-R** Locus *avrPphF* **PHTE-F/PHTE-R** Locus *pthE*	Conventional	Bacteria (DNA extraction)	González *et al.*, 2003	Toxigenic and nontaxigenic strains amplified.
P. savastanoi pv. *phaseolicola*	**PHA19/PHA95** *amtA* gene BIO **P5.1/P3.1+P3004L/ P3004R** Locus *phtE* Multiplex		Seed washes (previously plated on semiselective medium MT)	Schaad *et al.*, 1995; Rico *et al.*, 2006	Toxigenic and nontaxigenic strains differentiated.
P. savastanoi pv. *phaseolicola*	**Real-time PsF-tox/PsR-tox Probe PsF-tox-286P** *tox-argK* chromosomal cluster	Real-time (TaqMan)	Bacteria, seed washes, plant (untreated)	Schaad *et al.*, 2007	*P. syringae* pv. *phaseolicola*
P. savastanoi pv. *savastanoi*	**IAALF/IAALR** *iaal* gene	Conventional	Bacteria, plant (DNA extraction)	Penyalver *et al.*, 2000	
P. savastanoi pv. *savastanoi*	**IAALF/IAALR (external) IAALN1/IAALN2 (internal)** *iaal* gene	Nested	Bacteria, pre-enriched plant (DNA extraction)	Bertolini *et al.*, 2003b	
P. savastanoi pv. *savastanoi*	**iaaMf/iaaMr** *iaaM* gene (IAA biosynthesis) **iaaHf/iaaHr** *iaaH* gene (IAA) (IAA biosynthesis) **ptzf/ptzr** *ptz* gene (cytokinin biosynthesis) **lscCf/lscCr** *lscC* gene (levan biosynthesis)	Conventional	Bacteria (DNA extraction)	Marchi *et al.*, 2005	
P. syringae pv. *actinidae*	Genomic DNA (unknown) RAPD-fragment	Conventional	Bacteria (alkaline lysis)	Koh and Nou, 2002	
P. syringae pv. *alisalensis*	**BOXA 1R** Repetitive DNA sequences	BOX-PCR	Bacteria (DNA extraction)	Cintas *et al.*, 2002, 2006	Bacterial identification.
P. syringae pv. *atropurpurea*	**P1/P2, P3/P4,P1-P4, P5/P8, P7/P8** Plasmid COR1 (coronatine synthesis)	Conventional	Bacteria, plant (untreated)	Takahashi *et al.*, 1996	

Palacio-Bielsa *et al.*,2009

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P. syringae pv. *coryli*	**P0/P6** Entrire 16S rRNA gene **L7/L8** Full-length *hrpL* gene **L1/L2** Internal region hrpL gene **B1/B2** *SyrB* gene	Conventional Rep-PCR (BOX and ERIC)	Bacteria (DNA extraction)	Scortichini *et al.*, 2005	
P. syringae pv. *papulans*	**Pap1/Pap2** *hrpL* gene **Pap1/Pap3** *HrpL* gene	Conventional	Bacteria (boiled), plant (DNA extraction)	Kerkoud *et al.*, 2002	Pap1/Pap2 amplify only *P. syringae* pv. *papulans*, wehereas Pap1/Pap3 also amplify other *P. syringae* of genomospecies 1.
P. syringae pv. *papulans*	**PapHrp1/papHrp2** *Hrp*L gene	Conventional	Bacteria	Kerkoud *et al.*, 2002; Vanneste and Yu, 2006	
P. syringae pv. *pisi*	**AN3/1 Type I** **AN3/2 Type I** RAPD fragment **AN7/1 Type II** **AN7/2 Type II** RAPD fragment	Conventional Multiplex	Bacteria (untreated)	Arnold *et al.*, 1996	
P. syringae pv. *syringae* (strains producers of cyclic lipodepsinonapeptides)	**B1/B2** *syrB* gene **D1/D2** *SyrD* gene	Conventional	Bacteria (DNA extraction)	Sorensen *et al.*, 1998	
P. syringae pv. *tagetis*	**TAGTOX9** **FP1/TAGTOX9 RP1** *exbD* gene **TAGTOX10** **FP10/TAGTOX10 RP1** *Asnb* gene	Conventional	Bacteria (DNA extraction)	Kong *et al.*, 2004	*Pseudomonas syringae* pv. *helianthi* also amplified and considered as nontoxigenic form of *P. syringae* pv. *tagetis*.
P. syringae pv. *tomato*	**MM5F/MM5R** $hrpZ_{Pst}$ gene	Conventional	Bacteria (boiled), plant (DNA extraction)	Zaccardelli *et al.*, 2005	
P. syringae pv. *tomato*	**RealFor1/RTRev** RAPD fragment **27F/1492R+HSP1/HSP2** 16S rDNA+specific to *P. syringae* pv. *tomato* **Rtimefor/RTRev** **Probe** (molecular beacon)	Conventional Multiplex Real-time (molecular beacon)	Bacteria, plant (DNA extraction)	Fanelli *et al.*, 2007	

Palacio-Bielsa *et al.*,2009

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P. tolaasii	**Pt-1A/Pt-1D1** **Pt-1A/Pt-1D1+Pt-PM/PtQM** Tolaasin biosynthesis genes	Conventional Nested and immunocapture-nested	Bacteria (untreated)	Lee *et al.*, 2002	
P. syringae (pathovars producers of toxic lipodepsipeptide)	***SyrD1ISyrD2*** *SyrD* gene	Conventional	Bacteria (DNA extraction)	Bultreys and Gheysen, 1999	
P. syringae (pathovars producers of tabtoxin)	**tblA1/tblA2** *tblA* (tabtoxin gene) **tabA1/tabA2** *tabA* (tabtoxin gene)	Conventional	Bacteria (untreated)	Lydon and Patterson, 2001	
P. savastanoi pv. *phaseolicola* *P. syringae* pv. *actinidae*	**OCTF/OCTR** *argK* gene (phaseolotoxin resistance)	Conventional	Bacteria (DNA extraction)	Sawada *et al.*, 1997	*P. syringae* pv. *phaseolicola*
P. savastanoi pv. *savastanoi* Four viruses: *Cucumber mosaic virus* (CMV) *Cherry leaf roll virus* (CLRV) *Strawberry latent ringspot virus* (SLRSV) *Arabis mosaic virus* (ArMV)	***P. savastanoi* pv. *savastanoi*** **IAALF/IAALR (external)** **IAALN1/IAALN2 (internal)** *iaal* gene **CMV1/CMV2+CMVi1/CMVi2** **CLRV1/CLRV2+CLRVi1/CLRVi2** **SLRV1/SLRV2+SLRVi1/SLRVi2** **ArMV1/ArMV2+ArMVi1/ArMVi2**	Nested Multiplex nested RT-PCR	Pre-enriched, plant (DNA extraction)	Bertolini *et al.*, 2003a, b; Penyalver *et al.*, 2000	Colorimetric detection of amplicons using digoxigenin marked internal probes.
P. syringae pv. *morsprunorum* *P. syringae* pv. *syringae*	**REP1R/REP2I** **ERIC1R/ERIC2** **BOXA1R**	Rep-PCR	Bacteria (DNA extraction)	Vicente and Roberts, 2007	Bacterial identification.
P. fuscovaginae *P. syringae* pv. *syringae*	**R16-1/R23-2R** 16S-23S rDNA spacer region	Conventional	Bacteria (DNA extraction)	Kim and Song, 1996	*Acidovorax avenae* (*Pseudomonas avenae*), *Burkholderia glumae* (*Pseudomonas glumae*), *Pantoea agglomerans* (*Erwinia herbicola*), *X. oryzae* (pathovars *oryzae* and *oryzicola*) also amplified and differentiated by primary and secondary fragments.
P. avellanae *P. syringae* pv. *theae* *P. syringae* pv. *actinidae*	**PAV 1/PAV 22** 16S rRNA gene	Conventional	Bacteria (DNA extraction)	Scortichini and Marchesi, 2001; Scortichini *et al.*, 2002	

Palacio-Bielsa *et al.*,2009

PCR detection and identification of plant pathogenic bacteria

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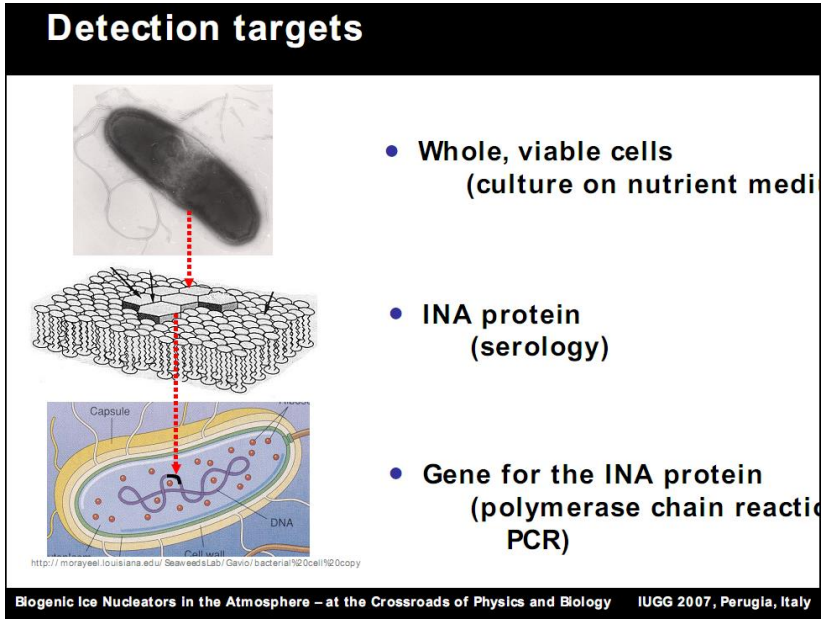
P. cannabina *P. savastanoi* pv. *glycinea* *P. savastanoi* pv. *phaseolicola* *P. syringae* pv. *sesami*	**ETH-1/ETH-2** Entire *efe* gene (ethylene-forming enzime) **ETH-1/ETH-3** Partial *efe* gene	Conventional	Bacteria (boiled)	Sato *et al.*, 1997	*P. syringae* pv. *cannabina* *P. syringae* pv. *glycinea* *P. syringae* pv. *phaseolicola*
P. syringae pv. *atropurpurea* *P. syringae* pv. *glycinea*, *P. syringae* pv. *maculicola* *P. syringae* pv. *morsprunorum* *P. syringae* pv. *tomato*	**cff primer 1/ cff primer 2** Coronatine biosynthesis gene cluster	Conventional and RFLP	Bacteria (DNA extraction)	Bereswill *et al.*, 1994	
P. savastanoi pv. *phaseolicola*	**HB 14F/HB 14R** **(*Pseudomonas*)** Phaseolotoxin gene cluster **X4c/X4e** **(*Xanthomonas*)** Plasmid DNA **HB 14F+HB 14R+ X4c+X4e (simultaneous detection)**	Conventional	Seeds (alkaline lysis)	Audy *et al.* 1996	*Pseudomonas syringae* pv. *phaseolicola* *Xanthomonas axonopodis* pv. *phaseoli* (*X. campestris* pv. *phaseoli*) also amplified.
P. syringae pv. *tomato*	**COR1/COR2** **(*Pseudomonas*)** Coronafacic acid *cfa7* gene **BSX1/BSX2** **(*Xanthomonas*)** Genomic DNA (unknown)	Conventional	Pure cultures or plant tissue (frozen-boiled method DNA extraction)	Cuppels *et al.*, 2006	Other coronatine-producing *P. sryringae* pathovars also amplified with COR primers. *X. axonopodis* pv. *vesicatoria* and *X. gardneri* are not valid names according to ISPP list. BSX primers also amplify *X. vesicatoria*.

Palacio-Bielsa *et al.*,2009

PCR Primers for ice$^+$ *Pseudomonas* spp.

Detecting ice-nucleating bacteria in environmental samples using PCR of the gene conferring ice nucleation activity

- Primers available for core region of gene for diverse INA bacteria including *P. syringae*.
- Additional primers needed for *P. fluorescens*, etc.
- To determine the sequence of base it is a need to use primers to amplify large regions of the genes.



Guilbaud *et al.*,2007

PCR Primers for INA+ bacteria

Primers used successfully to amplify ice nucleation genes from environmental isolates *ina*A, *ina*W, *ina*Z and *ina*Z/K/V/W/Y

Allele	Primers developed (forward & reverse positions from the start codon of the IN gene)	Annealing temp (°C)	No. of isolates used	Reference
*ina*A	5-ATGAGTGTATCGCTATTGAAACTCATGG-3 (221-248) 5-ACGATATTATTGTCCTCATCCATCTGG-3 (3924–3950)	58	1	Dimos et al. (2006)
*ina*W	5-GCGGTCTGGTATGGCCTATTT-3 (59-79) 5-CCGGCGTATCGCTATTGTCC-3 (3588–3607)	47	2*	Castrillo et al. (2000)
	5-AACCAGATTGCGAGTCATAAG-3 (3052–3072) 5-CATGGCTGAATCTGAGACTGG-3 (3612–3632)	59	1**	Ahern (2007)
*ina*Z	5-GAGAATGGTCTGGTCGGTTTACTGTGG-3 (124–150) 5-TCAACACCGTTCTCACCCGTTCTGG-3 (3494–3518)	58	5	Dimos et al. (2006)
	5′-CAAGTGTCACGTTACCGGTG-3′ (404–423) 5′-ATCCAGTCATCGTCCTCGTC-3′ (3571–3590)	55	3*	Castrillo et al. (2000)
*ina*Z/K/V/W/Y	Primers targeting core and C-terminal under development	–	~100	Guilbaud et al. (2007)

*Product shown in paper 1 kb larger than expected.

Georgakopoulos *et al.*,2009

1. *inaZ* for *P. syringae*; *inaW* for *P. fluorescens*; *P. putida* carrying *InaZ* gene was cloned.
2. *inaK*, *inaV* and *inaZ* were characterized from *P. syringae* strains.
3. In Real-time qPCR reactions, primer pair 3308f/3463r amplified most *ina* genes strongly and with good specificity. The pair successfully amplified all alleles from *P. syringae*, *P. viridiflava* and all Pseudomonads from non-agricultural sources.

PCR primers for INA$^+$ bacteria

Primers used to amplify ina genes from environmental isolates

Organism	ina gene	NCBI ID	Forward/ Reverse	Primer sequence	Primer ID	Primer Length (bp)	Tm (°C)	GC%	Amplicon Length (bp)
P. syringae	inaZ	X03035.1 GI:45828	F	ACGGAAGCACGTCGACTGCG	PS-F	20	60.05	65	718
	inaK	AF013159.1 GI:2331278							
	inaV	AJ001086.1 GI:2467259	R	CCGGCGATCAAAACGCTGCG	PS-R	20	59.92	65	
	ice4	FN650702.1 GI:283837102							
P. fluorescens	inaW	X04501.1 GI:45507	F	ACGGCCGGTTATGGCAGCAC	PF-F	20	60.04	65	767
			R	AGTTTGCTCCGGTCGCCAGC	PF-R	20	59.97	65	
X. campestris	inaX	X52970.1 GI:48531	F	CAGTTCGCTGACCGCCGGTT	XC-F	20	59.98	65	904
			R	GGCGGTCGAGTTGCTGCCAT	XC-R	20	60.04	65	
Erwinia spp.	iceE	M26382.1 GI:148419	F	ACGGCAGTTCGCTGACCAGC	EA-F	20	59.98	65	550
	inaU	D14992.1 GI:493641	R	CCGGCGGTCAGGGTTGAACC	EA-R	20	59.97	70	
	inaA	X17316.1 GI:296095							

D'souza *et al.*,2013

PCR primers for INA$^+$ bacteria

Primers used to amplify ina genes from environmental isolates

- PCR amplification of *ina* genes in environmental isolates and control strains. Only the PF-F/R primer set was successful in amplifying DNA sequences in the environmental isolates. None of these products, however, were of the predicted size and when referenced against the NCBI database, did not identify as known genes involved in ice nucleation. The PF-F/R primers did amplify *ina* genes in the positive controls. The PS-F/R primer set was successful in amplifying *ina* gene sequences in the *P. syringae* control strain, but did not amplify *ina* genes in any of the other samples tested. None of the primers used amplified *ina*-like genes in *P. fragi* (negative control). Primers designed for ice nucleation genes from *Xanthomonas campestris* and *Erwinia* spp. did not amplify appropriate gene sequences in any of the strains tested.

Isolate/Strain	Primer	Blast results		
		Organism	Gene	% identity
Environmental isolates				
E11_ICE2	PF-F/R	**NR**	none	-
E11_ICE7	PF-F/R	***P. fluorescens***	putative ABC transport system, ATP-binding protein	82%
E11_ICE11	PF-F/R	***P. fluorescens***	putative ABC transport system, ATP-binding protein	84%
E11_ICE12	PF-F/R	***P. fluorescens***	putative ABC transport system, ATP-binding protein	82%
E11_ICE16	PF-F/R	**NR**	none	-
E11_ICE17	PF-F/R	***P. fluorescens***	putative ABC transport system, ATP-binding protein	80%
E11_ICE25	PF-F/R	***P. fluorescens***	putative ABC transport system, ATP-binding protein	83%
E11_ICE26	PF-F/R	***P. fluorescens***	putative ABC transporter, ATP binding/permease protein	85%
E11_ICE27	PF-F/R	**NR**	none	-
E11_ICE28	PF-F/R	***P. fluorescens***	PTS system, fructose-specific IIBC component	87%

D'souza *et al.*,2013

PCR primers for INA$^+$ bacteria

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Isolate/Strain	Primer	Blast results		
		Organism	Gene	% identity
***P. fluorescens* - CPBG5**	PF-F/R	***P. fluorescens***	putative iron transport receptor protein	78%
***P. fluorescens* - RSG**	PF-F/R	***P. fluorescens***	inaW	86%
***P. putida* - CPBG8**	PF-F/R	***P. fluorescens***	inaW	86%
***P. putida* - CPBG10**	PF-F/R	***P. fluorescens***	inaW	86%
***P. putida* - RSG**	PF-F/R	***P. fluorescens***	inaW	86%
P. syringae	PF-F/R	***P. syringae***	inaK	91%
P. syringae	PF-F/R	***P. syringae***	ice-nucleation proteins octamer repeat protein	99%
P. syringae	PS-F/R	***P. syringae* pv. *syringae* B728a**	ice-nucleation proteins octamer repeat protein	99%
P. fragi	PF-F/R	***P. fluorescens***	carbon starvation protein CstA	88%
P. fragi	PF-F/R	***P. brassicacearum***	carbon starvation protein A	90%

D'souza *et al.*,2013

PCR primers for INA+ bacteria
Isolation of ice genes

- DNA was extracted from bacteria *Shewanella* (marine bacterium) and *Psychrobacter* (cold tolerant bacterium). PCR was run with primers previously designed from *inaA* and *inaE* genes, isolated from *Pantoea ananatis* and *P. fluorescens*, respectively (Watanabe and Sato, 1998).
- These sequences were:
- IceE1-R (5´-GGT-TTAGACATGAAAGAAGACAAGG-3´),
- IceE2-L (5´-TTATTCTTCGGGTTTATTCACGA-TA-3´),
- IceE1-1R (5´-GATTTCAATCGTTTGTGTCGGCTGC-3´),
- IceE1-IL (5´-GCAGGCT-ACGGCAGTTCGCTGACCA-3´) and
- IceE1-L (5´-CCTCTGTTATGGCGATTATTCTTCG-3´).
- In addition, two primers designed based on *inaZ* cloned from *Pseudomonas putida* were used with *Psychrobacter* and *Shewanella* DNA.
- These primers were 5´-ATCCAGTCATCGTC-CTCGTC-3´ and 5´-CAAGTGTCACGTTACCGGTG-3´.

McCorkle,2009

Real-time qPCR for bacterial *ina* gene

ina gene

- The two best performing pairs were:
- 3308f (5' GGCGATMGVAGCAAACTSAC 3'), with
- 3463r (5' STGTAVCKTTTNCCGTCCCA 3'), and

- 3341fb (5' AHTGTRYBYTSATGGCBGGVGA 3'), with
- 3462r1 (5' TGTAVCKTTTSCCGTCCCAG 3').
- Product sizes were 194 bp and 162 bp, respectively.

Primer pair 3341fb/3462r1 was often complementary to 3308f/3463r. Primer pair 3308f/3463r amplified most *ina* genes strongly and with good specificity such as *P. syringae*, *P. viridiflava* and all Pseudomonads from non-agricultural sources. But primer pair 3341fb/3462r1 failed to amplify *ina* gene from *P. viridiflava*.

Hill *et al.*,2013

Real-time qPCR for bacterial *ina* gene

Origin, activity, identity and closest *ina* allele affiliation of isolates

ina gene(*inaZ*,*inaQ*,*inaV*,*IceA*)

Isolate	Source	Location	Ice nucleation activity (°C)[1]	16S rRNA		*ina*	
				Closest isolate(s) and GenBank accession number	Similarity (%)	Closest isolate, *ina* gene allele/gene name and GenBank accession number	Similarity (%)
Cit7[2]	Navel orange leaf	Near Exeter, CA[2]	-3.5 (-2.5)	*P. syringae* (AY574914)	100	*P. syringae ina*Z (X03035)	99.2
BXIN4	Bean pod or leaf lesion	Lingle, WY 42.1315 N 104.392 W	-3.0 (-2.5)	*P. syringae* (AB680547) *P. congelans* (NR 028985)	100 100	*P. syringae ina*Q (EU360731)	99.5
PCa2a	Cabernet sauvignon grapevine cane lesion	Coonawarra, S. Australia 37.361 S 140.838 E	-3.0	*P. syringae* (AB680547) *P. congelans* (NR 028985)	100 100	*P. syringae ina*Q (EU360731)	97.4
HCh1a	Chardonnay grapevine shoot, healthy	Hallston, Victoria, Australia 38.3465 S 146.028 E	-3.0	*P. syringae* (AJ889841)	100	*P. syringae ina*Q (EU360731)	97.2
BXIN3	Bean pod or leaf lesion	Lingle, WY 42.1315 N 104.392 W	-3.5 (-2.5)	*P. syringae* (CP000075) *P. congelans* (NR 028985)	100 100	*P. syringae* Psyr1608 (CP000075)	99.2
GCh5Fc	Chardonnay grapevine shoot killed by frost	Glenlofty, Victoria, Australia 37.120 S 143.217 E	-4.0 (-3.5)	*P. syringae* (AB680547) *P. congelans* (NR 028985)	100 100	*P. syringae ina*V (AJ001086)	100
Sco1009b	Corn (maize) leaves, senescent	Lingle, WY 42.1315 N 104.3965 W	-2.5 (-2.0)	*P. syringae* pv. *atropurpurea* (AB001440)	100	*P. syringae ina*V (AJ001086)	83
PCa2bi	Cabernet sauvignon grapevine cane lesion	Coonawarra, S. Australia 37.361 S 140.838 E	-3.0	*P. viridiflava* (AY574912)	100	*P. syringae ina*V (AJ001086)	85
GrF[3]	Grape	CA[3]	-4.0 (-3.0)	*Pa. ananatis* (CP001875)	99.6	*Pa. ananatis Ice*A (AF387802)	99
SBPci	Bean pod lesion	Lingle, WY	-3.5 (-3.0)	*Pa. agglomerans* (FJ756354)	99.9	*Pa. ananatis ina*A (CP001875)	76

Hill *et al.*,2013

Real-time qPCR for bacterial *ina* gene

Origin, activity, identity and closest *ina* allele affiliation of isolates

ina gene(*ina*W)

		42.1315 N 104.392 W					
Sba1007a	Barley green leaves and heads	Lingle, WY 42.1315 N 104.395 W	-3.0	*X. campestris* pv. *campestris* (CP000050)	100	*X. campestris* pv. *raphani* XCR 4000 (CP002789)	92
Sba1007bi	Barley green leaves and heads	Lingle, WY 42.1315 N 104.395 W	-3.5	*X. campestris* pv. *campestris* (CP000050)	100	*X. campestris* pv. *raphani* XCR 4000 (CP002789)	92
Sbr1009a	Smooth brome (*B. inermis*) green leaves	Lingle, WY 42.1320 N 104.395 W	-2.0	*X. translucens* (NR 036968)	99.9	*X. campestris* pv. *translucens ina*X (X52970)	97
MU26[4]	Wood frog (*R. sylvatica*) gut	Adams County, OH[4]	-3.0	*P. fluorescens* (JF327445)	99.9	*P. fluorescens ina*W (X04501)	89
BF81Fb[5]	Air during rain in ponderosa pine forest	Manitou Forest, CO 39.1028 N 105.104 W	-4.0 (-2.5)	*P. koreensis* (NR 025228) *P. fluorescens* (JN679853) *P. putida* (HM217118)	99.9 99.9 99.9	*P. fluorescens ina*W (X04501)	86
LSb	Peat moss (*S. capillifolium*)	Bog, Lewis, Outer Hebrides, UK 58.1 N 6.7 W	-3.0	*P.* sp. LD002 (HQ713573) *P.* sp. NZ099 (AF388207)[6]	100 99.9	*P. fluorescens ina*W (X04501)	87
MM3b	Dwarf birch (*B. nana*) and bog rosemary (*A. polifolia*)	Martimoaapa mire, Finland 65.81 N 25.22 E	-3.0	*P.* sp. NZ099 (AF388207)[6]	100	*P. fluorescens ina*W (X04501)	87
χ16	Snow	Mt. Parnassos ski center, Greece 38.5393 N 22.60645 E	-3.5	*P. auricularis* (AB681727)	99.9	*P. fluorescens ina*W (X04501)	87
χ17	Snow	Mt. Parnassos ski center, Greece 38.5393 N 22.60645 E	-3.0	*P. poae* (GU188955)	99.9	*P. fluorescens ina*W (X04501)	86
GraPa8	Grass	Mt. Parnitha, Greece 38.172444 N 23.728622 E	-3.5	*P.* sp. JCM5484 (AB685689) *P. auricularis* (AB681727)	99.9 99.8	*P. fluorescens ina*W (X04501)	86

Hill *et al.*,2013

PCR primers for INA$^+$ bacteria

inaZ

P. syringae

- Specific designed INA primers:
- *Pseudomonas syringae* upper primer (5´GCA GAC TGC GGG TTA TGA GAG C 3´, ina Z);
- *Pseudomonas syringae* lower primer (5´CGC CGG TCA GTT TGC TTC TAT C 3´).

Nejad *et al.*,2006

PCR primers for INA+ bacteria *ina*A, *ina*W and *ina*X

- *Pantoea* (ex *Erwinia*) *ananatis* upper primer (5´AGG CTT TGA GAA CGG ACT AAC G 3´, inaA);
- *Pantoea* (ex *Erwinia*) *ananatis* lower primer (5´ TTT CTG TCG GCT GCG TAC TG 3´);
- *P. fluorescens* upper primer (5´GCA GTA CGC AGA CGG CAC AG 3´, inaW);
- *P. fluorescens* lower primer (5´ TTT CGT AGC CAG CAG TTG ATG TG 3´);
- *Xanthomonas campestris* upper primer (5´GCA AGG GCA GCG ATG TCA C 3´, inaX); and
- *Xanthomonas campestris* lower primer (5´ TCT GCG TGC TGC CGT AAC C 3´).

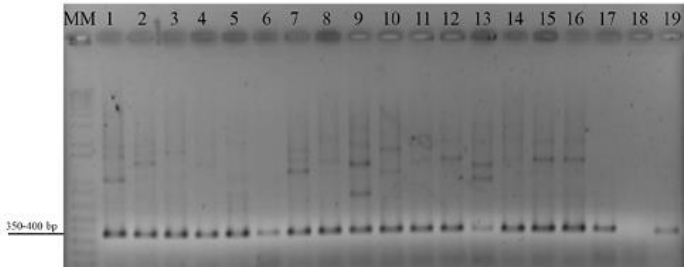
Nejad *et al.*,2006

PCR primers for INA$^+$ bacteria

*ina*A

Pantoea ananatis

- *inaA* gene expression was determined by PCR using the method described by Nejad et al. (2006b) utilizing species-specific primers (INA A upper primer 5'-AGG CTT TGA GAA CGG ACT AAC G-3' / INA A lower primer 5'-TTT CTG TCG GCT GCG TAC TG-3') for the inaA gene of *P. ananatis* (syn. *Erwinia ananas*), designed to amplify a DNA sequence of approximately 350-400 bp.

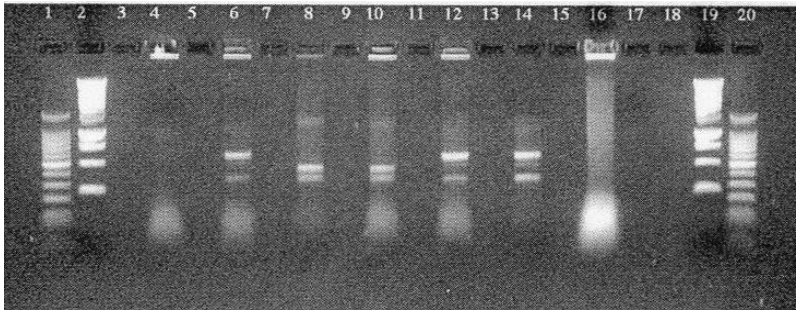


Miller *et al.*,2015

PCR Primers for INA$^+$ bacteria

Agarose gels showing amplification products from polymerase chain reaction (PCR) of genomic DNA by specifically designed *Psedomonas syringae* INA primers

- Lane 1 and 20: Ladders, 1kb.
- Lane 2 and 19: Ladders, 100 bp.
- Lane 18 negative control (water),
- Lane 6: *P. syringae* (V1D1) as positive control,
- Lane 8, 10, 12 and 14 are different *P. syringae* strains.
- Lanes 3, 5, 7, 9, 11, 13, 15 and 17 are different *Erwinia* spp, *Xanthomonas* spp. or *Pseudomonas* spp.



Nejad *et al.*,2006

PCR Primers for *Pseudomonas* spp.

Detecting ice-nucleating bacteria in environmental samples using PCR of the gene conferring ice nucleation activity

Sequences of bases of inaZ gene at NCBI

INA gene

The sequence of bases in this gene has been determined for several strains of INA bacteria. These sequences (for a single strand) are available at an open data base (GenBank).

Example

5' end of sequence, corresponding to N- terminal of protein

```
agatctgtcg cgcgcgacgg atcgatcagc gtctggtgct gtatgtcgag cactacctga
 gaccggagtt gttccccggc attctcgaac acgacctgaa cgaatcgctc agtgaactct
 acgcccgccg ctatgacatt cattacggtc gggtgcgctt cgacatggtc cccacggcgc
 tgcacagcga agcagccgct gcactgaagg tttcactggg tagcccgggc ctgcgcattg
 cacgtgtcaa ttatgatcgg aaagatcggt tgatcgactg tgacctcgaa tactggcgtc
 atgatgctat tcatgtgcgc gcagaggtga acggcgaatg aatgccggtg tggcaaaccc
 acttcccaag cgcggtctgt gccaccgtta gttcgctgtt ttctataaat aaatactcat
 tttgttgata agaatgtgtg caatgtacag cctgtttaat aggttttgtg taatgcatgc
 atttgattaa caaaaaaaat taatgagcat tattctattg ttgctggcgg ggtggcgtta
aattagttgg ttttttaatg gaatttccca gagtaattgt tctagttttc agcggattta
. . . . . . . . . . . . . . . . . . . . . . . . ggcgcgtcga tggaattc
```

4458 bases

3' end of sequence, corresponding to C- terminal of protein

Guilbaud *et al.*,2007

PCR Primers for *Pseudomonas* spp.

Detecting ice-nucleating bacteria in environmental samples using PCR of the gene conferring ice nucleation activity

INA bacteria

Species reported to have active strains

Pseudomonas syringae ♣
P. fluorescens
P. viridiflava ♣
Pantoea agglomerans (Erwinia herbicola) ♣
Xanthomonas campestris pv. *translucens* ♣

Gram – negative
non spore- forming
♣ plant pathogens

Test strains

> 50 from the above species
plants, water, snow, rain
US, Europe, Asia, Antarctica
10 'negative' strains: no INA gene or other species

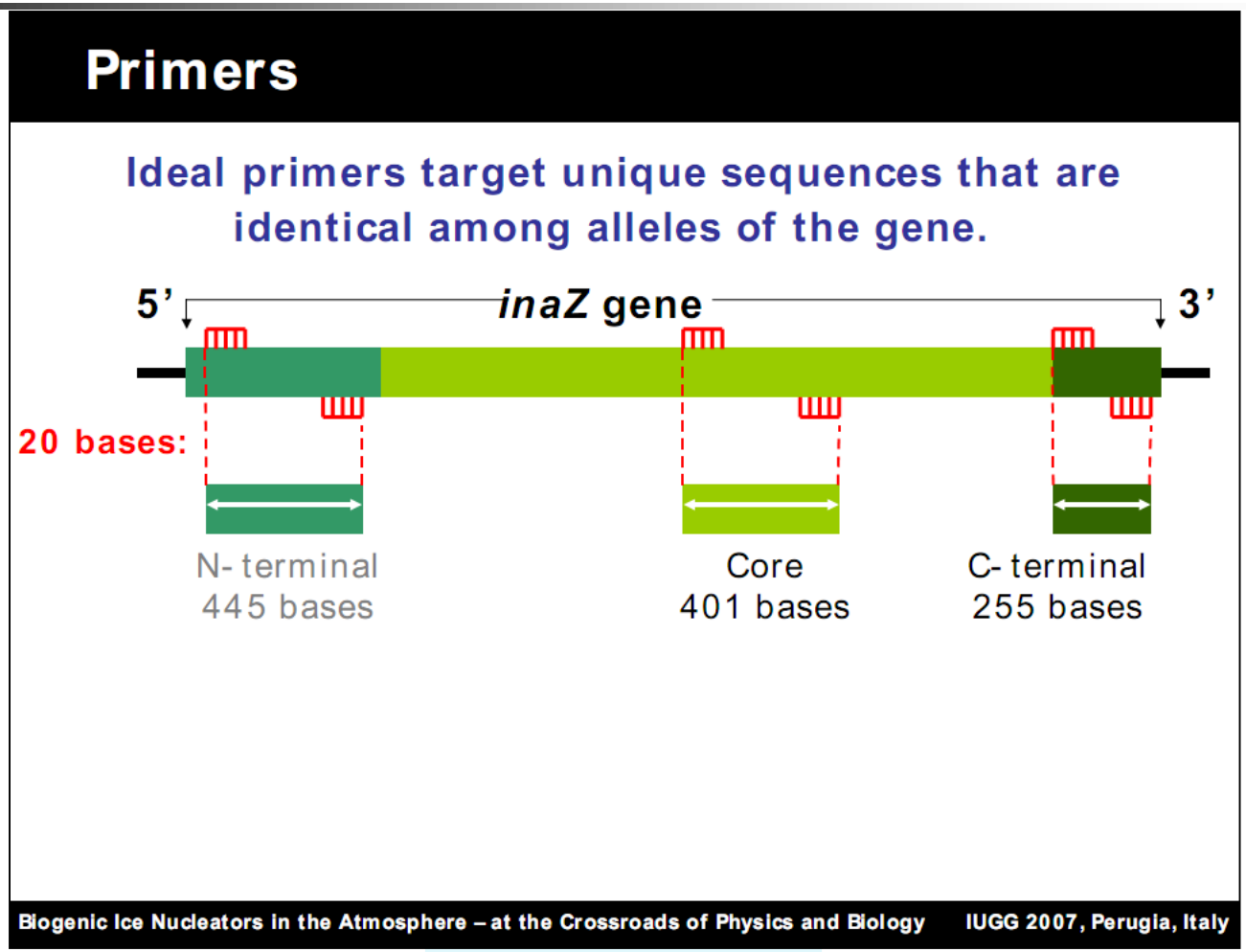
Biogenic Ice Nucleators in the Atmosphere – at the Crossroads of Physics and Biology IUGG 2007, Perugia, Italy

Guilbaud *et al.*,2007

PCR Primers for *Pseudomonas* spp.

Detecting ice-nucleating bacteria in environmental samples using PCR of the gene conferring ice nucleation activity

InaZ gene

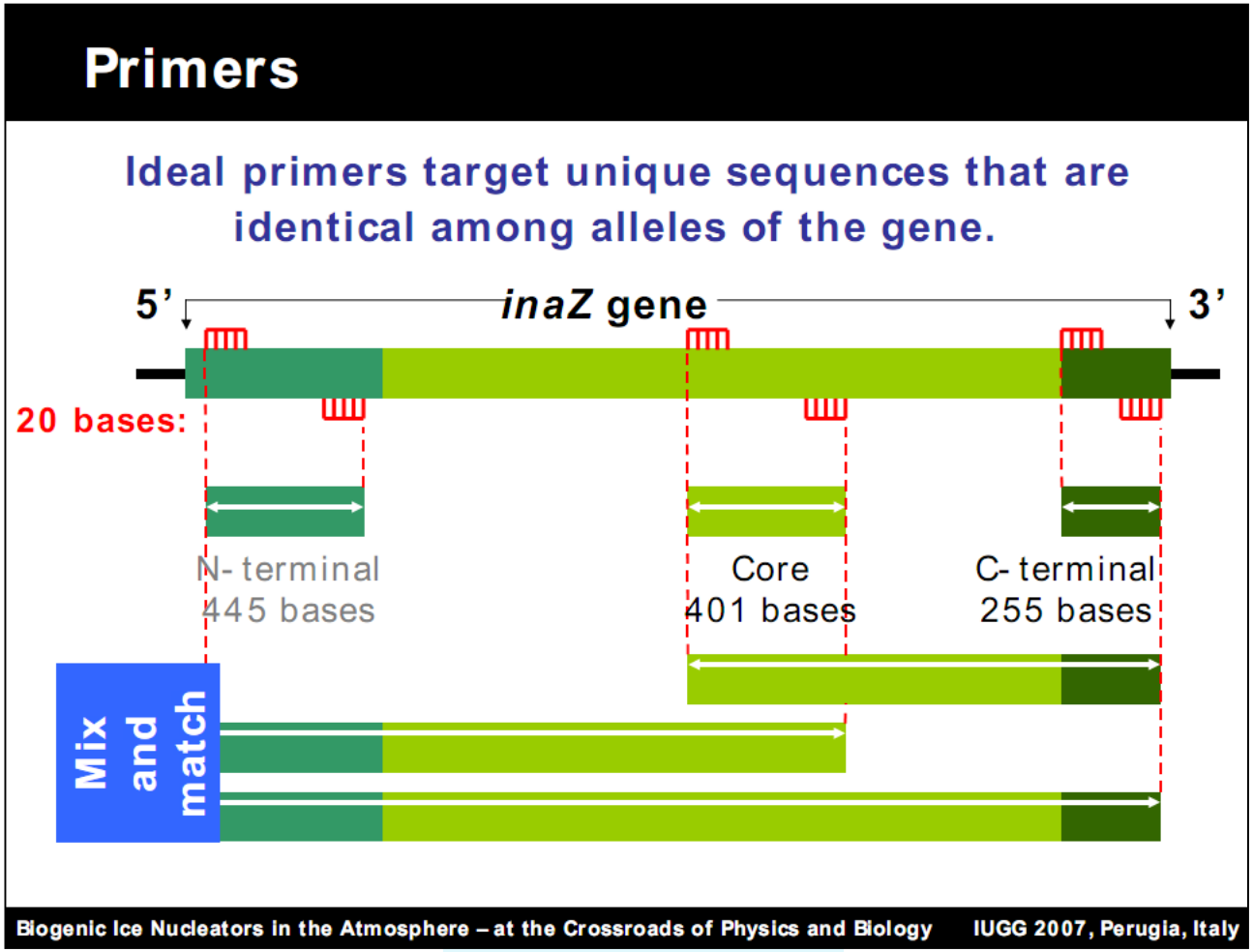


Guilbaud *et al.*,2007

PCR Primers for *Pseudomonas* spp.

Detecting ice-nucleating bacteria in environmental samples using PCR of the gene conferring ice nucleation activity

InaZ gene



Guilbaud *et al.*,2007

PCR Primers for *Pseudomonas* spp.

Detecting ice-nucleating bacteria in environmental samples using PCR of the gene conferring ice nucleation activity *InaZ* gene

Primers : based on the *inaZ* sequence

C- terminal

GGG AGA CCA AAG CAG ATT GA
5'→

→3'
CTG CTC CTG CTA CTG ACC TA

Core

ACC GCG AGT TAC AGA AGC AT
5'→

→3'
CTG GAA TAG CCC GCA GTA GA

CTG GAA TAG CCC GCA GTC GA

CTG GAA TAG CCC GCA GTG GA

CTG GAA TAG CCC GCA GTT GA

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Guilbaud *et al.*,2007

PCR Primers for *Pseudomonas* spp.

Detecting ice-nucleating bacteria in environmental samples using PCR of the gene conferring ice nucleation activity

Primer specificity

Strains without an ice nucleation gene

# strains		C- terminal	Core A	Core C	Core G	Core T
1	*Agrobacterium radiobacter*	-	-	-	-	-
1	*Escherichia coli*	-	-	-	-	-
2	*Erwinia carotovora*	+ (2 bands)	-	-	-	-
1	*Erwina chrysanthemi*	+	-	-	-	-
1	*Flavobacterium* sp.	+	-	-	-	-
1	*Aureobacterium* sp.	-	-	-	-	-
1	*Micrococcus* sp.	+	-	-	-	-
1	*P. syringae* 1448A	-	-	-	-	-
1	*P. syringae* DC3000	-	-	-	-	-

Guilbaud *et al.*,2007

PCR Primers for *Pseudomonas* spp.

Detecting ice-nucleating bacteria in environmental samples using PCR of the gene conferring ice nucleation activity

Primer specificity

Ice nucleation active strains (- 2° to - 8°C)

# strains		C- terminal	Core A	Core C	Core G	Core T
1	*P. viridiflava* (China)	-	-	-	-	-
2	*P. viridiflava* (Montana)	+	-	+	-	-
2	*P. fluorescens* (Antarctica)	-	-	-	-	-
2	*Pantoea agglomerans*	+	-	-	-	-
1	*Pantoea agglomerans*	+	+	+	+	+
1	*Pantoea agglomerans*	+	+	+	-	-
1	*Pantoea agglomerans*	+	+	+	+	+
33	*P. syringae* (diverse)	+	-	+	-	-
1	*P. syringae* (irrigation lake)	-	+	+	+	+
1	*P. syringae* (pv. *pisi*)	-	-	+	+	+
1	*P. syringae* (pv. *melea*)	-	-	-	+	-
2	*X. campestris* pv. *transluscens*	+	+	+	-	-
1	*X. campestris* pv. *transluscens*	+	+	-	-	-
1	*X. campestris* pv. *transluscens*	+	-	+	-	+

Biogenic Ice Nucleators in the Atmosphere – at the Crossroads of Physics and Biology IUGG 2007, Perugia, Italy

Guilbaud *et al.*,2007

PCR Primers for *Pseudomonas* spp.

Detecting ice-nucleating bacteria in environmental samples using PCR of the gene conferring ice nucleation activity

Primer specificity

Inactive strains of ice nucleation species (< - 8°C)

# strains		C- terminal	Core A	C	G	T
1	*P. fluorescens*	-	-	-	-	-
3	*P. fluorescens*	+	-	-	-	-
2	*P. syringae*	-	-	-	-	-
6	*P. syringae*	+	-	-	-	-
1	*P. syringae*	+	-	-	+	-
1	*P. syringae*	+	-	+	+	-
1	*P. syringae*	+	+	+	+	-
7	*P. syringae*	+	+	+	+	+

Guilbaud *et al.*,2007

PCR Primers for *Pseudomonas* spp.

Detecting ice-nucleating bacteria in environmental samples using PCR of the gene conferring ice nucleation activity

Applications

Limits of detections, and direct detection of INA bacteria in environmental samples

Validation

1 L of 'test water' filtered on 0.22 µm membrane
DNA extracted in 50 µl solvents
PCR (core) with 2 µl of extracted total DNA

'test water' =

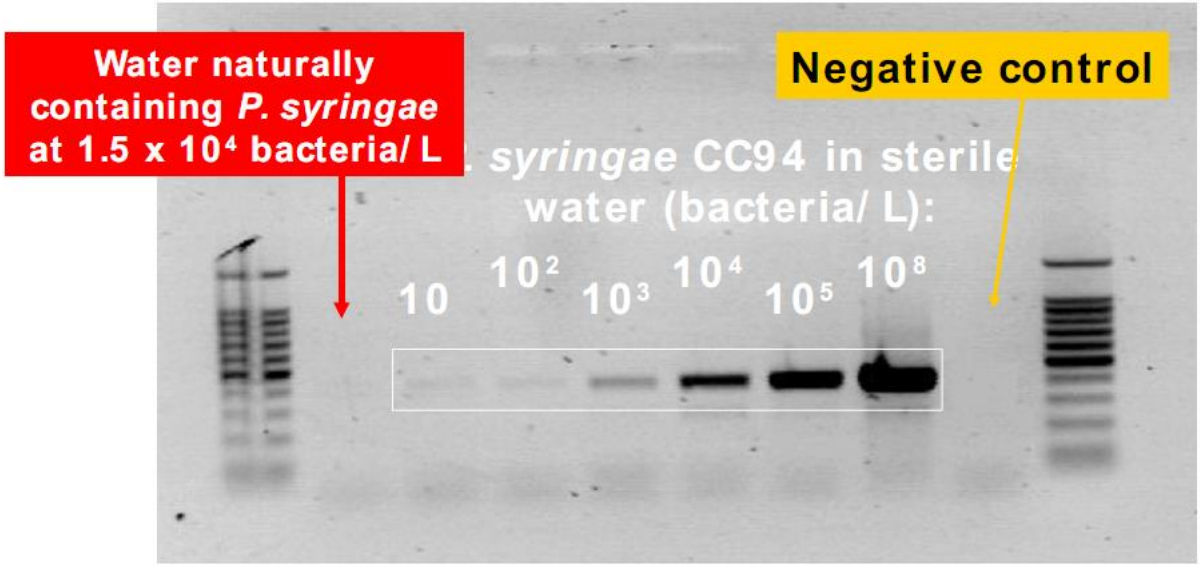
- *P. syringae* (CC94) at 10^6 and 10^7 bacteria/ L in river water
- *P. syringae* (CC94) in sterile distilled water at 10 - 10^8 bacteria/ L
- River sample from which *P. syringae* was isolated at 1.5 x 10^4 bacteria/ L

Guilbaud *et al.*,2007

PCR Primers for *Pseudomonas* spp.

Detecting ice-nucleating bacteria in environmental samples using PCR of the gene conferring ice nucleation activity

Applications



P. syringae seeded into river water at 10^7 and 10^6 bacteria/ L was detected (lower concentrations not yet tested).

Biogenic Ice Nucleators in the Atmosphere – at the Crossroads of Physics and Biology IUGG 2007, Perugia, Italy

Guilbaud *et al.*,2007

Taxonomic characterization of Ice bacteria

PCR, DNA sequencing, sequence alignment

- Sequence alignment of the 16S rRNA gene region of each isolate was performed using CLUSTALW option of the software Molecular Evolutionary Genetics Analysis (MEGA) software v4.0. (Tamura *et al.*, 2007).
- The sequences were deposited at GenBank and were subjected to a NCBI BLAST search.
- In a second alignment, the 16S rRNA gene region sequences were aligned using CLUSTALW together with the homologous sequences (retrieved from GenBank) of closely related species of bacteria.

Singh *et al.*,2013

Pathogenicity test

Pathovar system of taxonomy

Pseudomonas syringae* pv. *syringae – bacterial canker fruit trees

P. s.* pv. *tomato – tomato bacterial speck

P. s.* pv. *tabaci – tobacco wildfire

P. s.* pv. *phaseolicola – bean halo blight

host→ / pathovar↓	*Prunus*	tomato	bean
syringae	disease	HR	HR
tomato	HR	disease	HR
phaseolicola	HR	HR	disease

Pathogenicity tests

P. syringae pv. *syringae* isolated from stone fruits

- All 89 strains of *P. syringae* pv. *syringae* isolated from healthy and diseased stone fruit tissues(peach, European plum and French prune, Japanese plum, sweet cherry, apricot and almond) were moderately to highly pathogenic on Lovell peach seedlings regardless of the host of origin.
- Bacterial cells grown for 24 h on solid KB at 24°C were suspended in PB to a concentration of ~5×10^7 CFU/ml.
- Bacterial suspensions (~0.1 ml) were injected into the stems of 10- to 12-week-old Lovell peach seedlings by using a 22-gauge needle inserted tangentially under the cambium.
- PB was injected as a control.
- Each seedling was inoculated in three places with a strain, and an average pathogenicity rating for each strain was used to determine the mean and standard deviation of the pathogenicity for all strains isolated from a particular host.

Little *et al.*,1998

Pathogenicity tests

P. syringae pv. *syringae* isolated from stone fruits

- The plants were maintained in a greenhouse at 28°C and rated after 10 days for disease development on a scale of 0 to 3 as follows:
- 0, light necrosis associated with wounding at the area of inoculation;
- 1, dark, water soaked necrosis confined to the immediate area of inoculation, with some streaking in the cambium;
- 2, streaking in the cambium extending away from the site of inoculation, necrosis around the wound up to 2 mm above and below the wound with gumming; and
- 3, necrotic lesion and streaking involving the entire stem, often with girdling and death of distal portions and extensive gumming.

Little *et al.*,1998

Pathogenicity tests

For different pathovars of *Pseudomonas syringae*

- Pathogenicity assays in plants are essential in addition to the well known procedures for HR in tobacco leaves or bioassays on attached and detached organs (small pear and cherry fruits, yellow lemon fruits, lilac leaves or shoots).
- Since *P. syringae* pv. *syringae* is a genetically heterogeneous pathovar and having a very broad range of plant hosts, the strains of this pathovar (pv. *syringae*) can show different virulence on lilac, wild cherry and pear.

López *et al.*,2010; Gilbert *et al.*,2010

Pathogenicity tests

Leaf pathogenicity tests

Pseudomonas syringae pv. *syringae*

- Five fully expanded detached leaves of lilac, wild cherry and pear were wounded (a 5-mm wound made with a scalpel) on the petiole at approximately 5 mm from the lamina.
- A 10 µl drop of bacterial suspension (10^8 cfu ml^{-1}) or water was deposited on the wound.
- The treated leaves were incubated in Petri dishes at 20°C for 7 days under daylight conditions.
- The lengths of the necroses, including the 5-mm wound, were measured.
- Five repetitions per strain and test were used.

Gilbert *et al.*,2010

Pathogenicity tests

Leaf and hypocotyl pathogenicity tests

For different pathovars of *Pseudomonas syringae*

- These tests were carried out to evaluated the virulence of *P. syringae* pathovars, *Pss* strains and the strains of the pvs. *atrofaciens* and *aptata*.
- Two 8- to 10-cm long young wheat leaves were wounded three times each with a sterile needle, 5 µl of bacterial suspension(10^8 cfu ml^{-1}) were deposited on the wounds and the seedlings were incubated at 20°C for 7 days under daylight.
- The necroses were evaluated and repartited in 5 classes.
- For sugar beet, 3-5 cm long hypocotyls were inoculated with a needle preliminary dipped in a 10^8 cfu ml^{-1} bacterial suspension.
- The seedlings were incubated at 20°C for 7 days under daylight.
- Four symptom severity classes were described.

Gilbert *et al.*,2010

Pathogenicity tests

Twig pathogenicity tests

Pseudomonas syringae pv. *syringae*

- Two kinds of tests were carried out in cherry.

2.1. Inoculation of cut ends of twigs (twig E test):

- It is well adapted for *P. syringae* pv. s*yringae* (*Pss*) when a frost period is followed by an incubation at 15°C.
- This test was carried out on one year-old dormant twigs of sweet cherry and pear collected during the November-March period.
- The collected detached twigs were 20 cm long and the inoculated twigs were incubated in the dark at 15°C for 7 days, −10°C for 3 days and 15°C for 10 days.
- The entire twigs were transferred to glass tubes.
- The lengths of the necrozed zones starting from the cut end were measured.

Gilbert *et al.*,2010

Pathogenicity tests

Twig pathogenicity tests

P. syringae pvs.*syringae* and *morsprunorum*

2.2. Lateral inoculation of cortical tissues of detached twigs (twig W test)

- The lateral inoculation of an exposed 'window' of cortical tissues (twig W test) followed by incubation at 15°C is more adapted for *Pseudomonas syringae* pv. *morsprunorum* (Psm) race 1.
- This test was also performed on one year-old dormant twigs of sweet and sour cherry with the pathovars of *P. syringae* collected during the November-March period.
- 17 cm-long twigs were prepared as described above.
- A drop of 20 µl of bacterial suspension containing 10^8 cfu ml^{-1} was deposited on an exposed window of cortical tissue (5 mm wide and 20 mm long) resulting from the removing of the superficial bark.
- The drop was spread in the zone by using the lateral part of the inoculation tip, without direct contact between the tip and the exposed tissue.
- The entire twigs were transferred to glass tubes as described above and incubated at 15°C for 30 days in the dark.
- Test reading consisted in visual estimation of the necrosed zone in the inoculated window.

Gilbert *et al.*,2010

Pathogenicity tests

Flower pathogenicity test

Pseudomonas syringae pv. *syringae*

- The *P. syringae* strains from pear were tested for their virulence on pear flowers.
- The flowers were collected closed in early spring and suspended in plastic boxes (see fruit tests).
- 10 µl of bacterial suspension (1.5×10^{6} cfu ml^{-1}) were deposited on the flower calyx.
- The flowers were incubated at 15°C for 7 days under daylight, conditions encountered in spring in Belgium.
- Test reading consisted of visual estimation of necrosis progressions.

Gilbert *et al.*,2010

Pathogenicity tests

Apple trees

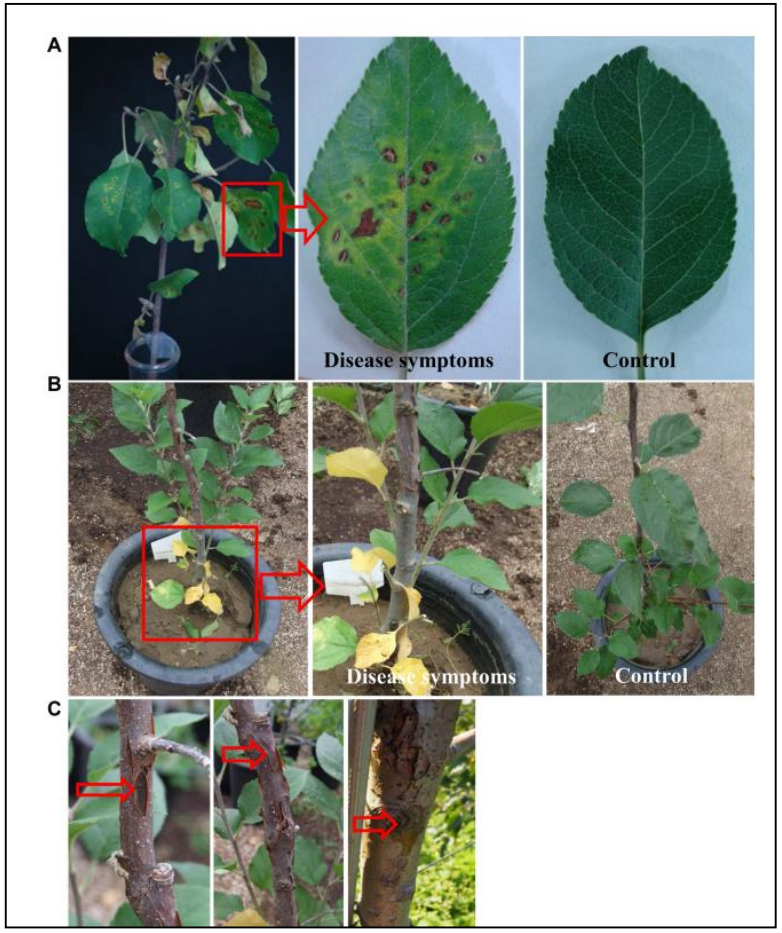
Pseudomonas syringae pv. *syringae*

- Pathogenicity test of *Pseudomonas syringae* pv. *syringae* (Pss) WSPS007 cell suspensions by foliar spray and soil drench methods on detached leafy branches of an apple tree.

A. Development of disease symptoms on leaves of detached leaves 7 days after inoculation (DAI) by foliar spray method. Inoculated leaves showed yellow halos around the black spots, whereas no symptoms appeared on non-inoculated (control) leaves and stems.

B. Symptoms on bottom leaves (shown in red square) appear as yellowing, 14 days after inoculation by using the soil drench method. In enlarged images, the bottom (indicated by an arrow) and upper leaves of the infected plant appeared healthy. No symptoms were observed in non-treated plants (control).

C. This disease symptoms (indicated by arrows) appeared on the stem two months after inoculation. The experiment was performed two times with six replicates per treatment.



Lee *et al.*,2023

Pathogenicity tests

Fruit pathogenicity test

Pseudomonas syringae pv. *syringae*

- The pear, sour cherry and sweet cherry fruit fruits were collected 6 weeks after flowering, disinfected with 1% sodium hypochlorite and rinsed twice with sterile osmosed water.
- The pear and sweet cherry fruits were wounded with the ellipsoidal edge of a sterilized needle, inoculated on the wounds with 20 µl of bacterial suspension (10^8 cfu ml^{-1}) and incubated for 7 days at 24°C.
- In each test, two pears were wounded four times or five sweet cherries were wounded once.
- The sour cherry fruit test included no wounding: five fruits were soaked for 1 min in a bacterial suspension (10^8 cfu ml^{-1}) and incubated for 5 days at 25°C.
- The fruits were suspended under daylight conditions on sterile trays in closed sterile plastic boxes containing 20 ml of water agar (10 mg of agar l^{-1}).
- The necrotic lesions were measured.

Gilbert *et al.*,2010

Pathogenicity tests

Fruit pathogenicity test

Pseudomonas syringae pv. *syringae*

- In the case of wounded fruits, the narrowest sides of the necroses resulting from the needle wounds or the diameter of the necroses resulting from strain pathogenicity were measured.
- For unwounded fruits, as multiple and variable necroses were observed, the diameters of the necroses were measured individually, summed and allocated into six classes.

Gilbert *et al.*,2010

Pathogenicity tests

Bud pathogenicity test

Pseudomonas syringae pv. *syringae*

- The *P. syringae* strains from pear were tested for their virulence on pear buds.
- 20 cm-long detached twigs were collected during the November-March period and washed (see twig tests).
- Five buds per shoot were cut at 1 mm from the apical part and 5 µl of bacterial suspension (1.5×10^6 cfu ml^{-1}) were deposited on the cut.
- The twigs were transferred to glass tubes (see twig tests) and incubated in the dark at 10°C for 7 days, -10°C for 1 day, and 10°C for 6 days.
- These conditions reproduce temperatures encountered in winter in Belgium during bud dormancy.
- Disease progression from the cut was measured.

Gilbert *et al.*,2010

Description of classes in the leaf, twig E, fruit and bud pathogenicity tests *P. syringae* pvs.*syringae* and *morsprunorum*

- The percentages of pathogenic strains among Belgian strains were:
- 100% within *Psm* race,
- 88.9% within *Psm* race 2,
- 00% within TLP (Toxic lipodesipeptide-producing strains of *Pss*,
- 72.7% within TLP$^-$ *Pss* and
- 73.3% within unclassified *P. syringae*.
- [a] The strains inducing no (class 0) or very limited symptoms (class 1) were not considered pathogenic in this study
- [b] Under brackets are the maximal lengths observed in the respective tests.

Organ tested	Pathogenicity[a]	Class	Necrose length intervals (mm) corresponding to classes[b]			
			Pear	Sweet cherry	Sour cherry	Plum
Leaf	Non-pathogenic	0	5–6	5.6	5–6	5–6
		1	6.1–10.9	6.1–10.9	6.1–10.9	6.1–10.9
	Pathogenic	2	11–20.9	11–20.9	11–20.9	11–15.9
		3	21–30.9	21–30.9	21–30.9	16–21
		4	31–41	31–41	31–41	>21 (23)
		5	>41 (50)	>41 (63)	>41 (47)	
Twig E	Non-pathogenic	0	0–5.9	0–5.9	0–5.9	
		1	6–9.9	6–10.9	6–10.9	
	Pathogenic	2	10–14.9	11–20.9	11–15.9	
		3	15–20	21–30.9	16–20.9	
		4	>20 (26)	31–41	21–30	
		5		>41 (67)	>30 (37)	
Fruit	Non-pathogenic	0	0.4–0.6	0.4–0.6		
		1	0.61–1.0	0.61–2.0		
	Pathogenic	2	1.1–2.0	2.1–5.0		
		3	2.1–3.0	5.1–7.5		
		4	3.1–4.0	7.6–10		
		5	>4 (12.8)	>10 (12)		
Bud	Non-pathogenic	0	0.0–1.0			
		1	1.1–2.0			
	Pathogenic	2	2.1–3.0			
		3	3.1–4.0			
		4	>4 (4.3)			

Gilbert *et al.*,2010

Pathogenicity tests

Twig pathogenicity tests

P. syringae pvs.*syringae* and *morsprunorum*

2.2. Lateral inoculation of cortical tissues of detached twigs (twig W test).

- Classes in the pathogenicity test on cortical windows of cherry twigs.

Gilbert *et al.*,2010

Pathogenicity tests

Flower pathogenicity test

Pseudomonas syringae pv. *syringae*

- Symptoms on detached pear flowers after inoculation with *P. syringae* strains.

a. Class 0;
b. Class 2;
c. Class 3.

Gilbert *et al.*,2010

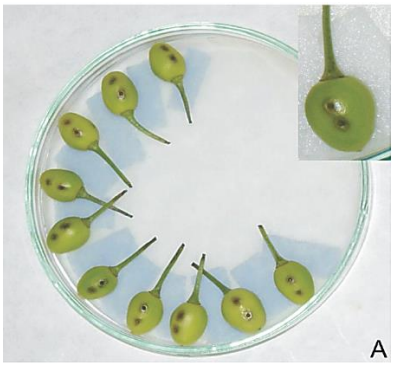
Pathogenicity test on sweet cherry fruitlets

P.s. pv. *syringae* and *P.s.* pv. *morsprunorum*

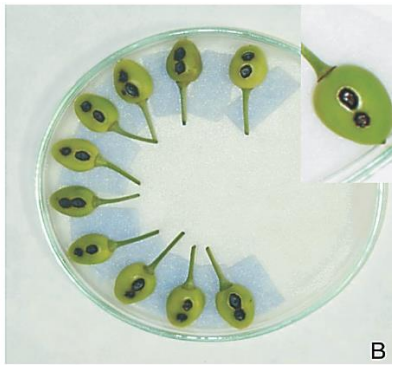
- Symptoms occurring on immature sweet cherry fruits cv. 'Napoleon' after inoculation with
- *Pseudomonas syringae* strains:

A. Fruits inoculated with strains of *P. syringae pv. morsprunorum* (races 1 and 2). *Psm* strains caused brownish water soaked superficial lesions Brownish water soaked superficial lesions.

B. Fruits inoculated with strains of *P. syringae* pv.*syringae*. All *Pss* strains caused deep black brown necroses.



Psm



Pss

The following Scale was used:
0 – no symptoms,
1 – necroses up to 1 mm in diameter,
2 – necroses up to 2 mm,
3 – up to 3 mm, 4 – up to 4 mm,
5 – up to 5 mm,
6 – necroses over 5 mm.

Kałużna and Sobiczewski,2009

Host tests for canker pathogens

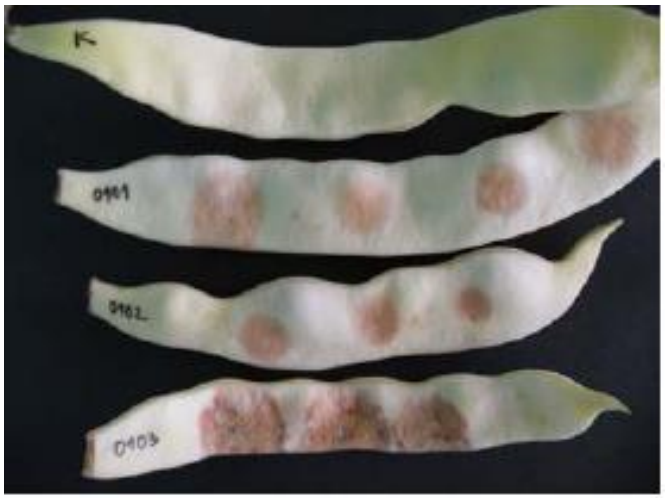
P.s. pvs. *syringae, morsprunorum* and *persicae*

- The canker phase of some diseases cannot be produced by inoculation at all time of the year.
- For example, inoculations of cherry with should be carried out in autumn and winter.
- Stab soft stems of plants with needle charged with bacterial cluture(10^6-10^7 cfu/ml).
- Symptoms appear more reliably and quickly if the site of inoculation is protected from desiccation with polythene for 48 hours.
- Many pathogens will produce spreading lesions and dieback on young, detached shoots standing in water.

Lelliott and Stead,1987

Pathogenicity test on string bean pods
P.s. pvs. *syringae*, *morsprunorum* and *persicae*

- Pathogenicity test can be performed in dormant 1 year-old shoot of young trees from mid September to the end of January.
- It took more than a month to reproduce typical symptoms on woody tissue of the host plants.
- Whereas, HR, reaction of unripe nectarine fruits as well as lemon fruits and string bean pods will be observed within few days.
- Usually *P. s.* pv. *syringae* strains can be distinguished from the other two pvs by severity of symptoms they induce in these plant organs.



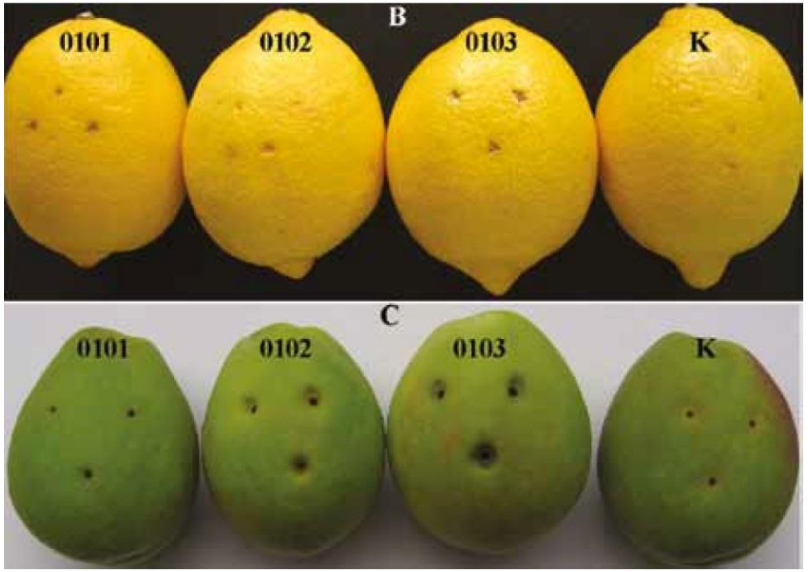
K: sterile water
0101: pv. *morsprunorum*
0102: pv. *persicae*
0103: pv. *syringae*

Obradovic,2010

Pathogenicity test on lemon fruits (B) and nectarine fruits(C)

P.s. pvs. *syringae*, *morsprunorum* and *persicae*

- Pathogenicity test on:
- lemon fruits (B) and
- nectarine fruits (C).
- 0101– *P. s.* pv. *morsprunorum*,
- 0102 – *P. s.* pv. *persicae*,
- 0103 – *P. s.* pv. *syringae*,
- K – negative control.



Gašić *et al.*,2012

Pathogenicity test

Interactions of bacteria with bacteria

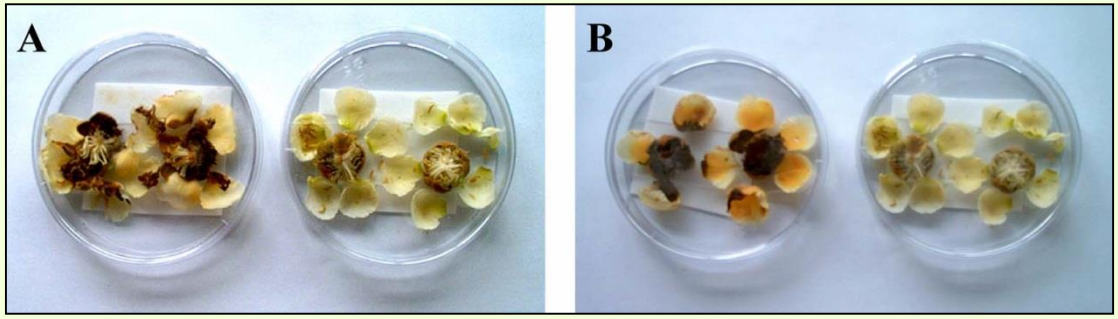
Partnerships (synergists) of *Pseudomonas syringae* pv. *syringae* and *P. fluorescens*

- Pathogenicity tests demonstrated that the blossom blight of kiwifruit in Korea is caused by two pathogens, *Pseudomonas syringae* pv. *syringae* and *P. fluorescens*.
- *Pss* primarily affected the stamen, while *P. fluorescens* caused rotting of all internal tissues of buds or flowers.
- Fig. Symptoms on buds inoculated with:

A. Group I (*Pseudomonas syringae* pv. *syringae*)

B. Group VII (*P. fluorescens*) isolates.

- Left plate was infected flowers and right plate was uninfected flower.



Lee *et al.*,2009

Pathogenicity test

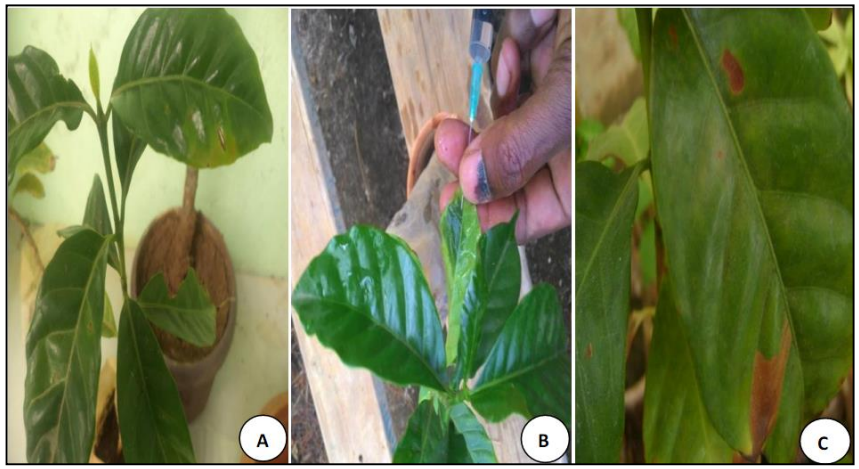
Pseudomonas syringae pv. *actinidiae*

- Ten 2-year-old healthy Kiwi plants (*Actinidia*) cvs. Jin Tao and Hayward, were inoculated with bacterial suspensions of about 1×10^{7} CFU ml^{-1} (per ml)as described by Takikawa *et al.*,1989.
- Symptoms on the leaves, twigs and branches were observed within 3-4 weeks after artificial inoculation with the pathogen.
- No symptoms were observed on the control plants inoculated with SDW.
- The original bacterial strains were reisolated from all the developed symptoms.

Balestra *et al.*,2009

Pathogenicity test
Bacterial blight of coffee(BBC)
Pseudomonas syringae pv. *garcae*

- Coffee seedlings inoculated with the isolates of *Pseudomonas syringae* at bacterial concentrations of $1x10^8$ cfu/ml induced colour changed from green to yellow and collapse of host tissue in the inoculated region within 16 hrs of inoculation.
- After 24 to 48 hrs of injection, the injected leaf area became necrotic and in 3-4 days, the treated tissue was entirely dry and yellow.



A, B & C) Coffee seedling inoculation with *Pseudomonas syringae* and B) Chlorosis and induced necrotic blight symptoms on leaves.

Hinkosa *et al.*,2017

Seedling test
P. syringae pv. *atrofaciens*

- **Seedling test:**
- Only *P.s.* pv. *atrofaciens* causes typical dark margins around the inoculation points on barley and wheat.

Figure 3.7. Seedling test: only *Pseudomonas syringae* pv. *atrofaciens* causes typical dark margins around the inoculation points on barley and wheat seedlings. (Photo: A. Fessehaie.)

Photo: A. Fessechaie

Pathogenicity test

Pseudomonas syringae pv. *lachrymans*

- The bacterial suspensions were adjusted to about 10^8 CFU/ml using a spectrophometer (optical density of 0.1 at 640 nm).
- Each isolate was inoculated to five potted seedlings at the second true leaf stage of cucumber (cultivar Admirable F1), of melon (cultivar *Lavi*) and zucchini (cultivar Blitz F1).
- The inoculation was performed by dusting the cotyledons and the leaves with rubbing with a piece of cheesecloth previously dipped in the inoculum.
- Control seedlings were inoculated with sterile distilled water.
- After inoculation the plants were covered separately by plastic bags for 24 h.
- The plants were kept in a greenhouse under favorable temperature (25°C) and relatively high humidity conditions.
- The plants were watered and fertilized as required.
- The plants were observed regularly for disease symptoms development.
- Plants that showed dark green water soaked spots were recorded positive.

Fatmi *et al.*,2008

Pathogenicity test

Bacterial inflorescence rot of grape

Pseudomonas syringae pv. *syringae*

- **Grapevine leaf assay**
- Attached young leaves on potted Chardonnay plants were inoculated by puncturing with a 26-gauge needle and applying aliquots (100 µL) of cell suspension (cells from 4-day-old nutrient agar (NA; Oxoid) culture suspended in SDW to a concentration of 10^5 cells mL^{-1}).
- Control leaves were treated identically with SDW.
- The potted vines were maintained in a growth cabinet at 24°C day/18°C night with a 12-h fluorescent light/dark cycle.
- Each treatment was replicated three times.
- After 1 week, the leaves were surface-sterilized and plated onto PS agar for reisolation of *P. syringae*.
- The isolates were tested for their ability to cause necrotic leaf lesions on grapevine leaves.

Whitelaw-Weckert *et al.*,2011

Pathogenicity test

On alfalfa(*Medicago sativa* also called lucerne)

Pseudomonas syringae pv. *syringae*

- Pathogenicity of four selected bacterial strains was determined by inoculating 6-week-old alfalfa (*M. sativa* var. *Hamedani*) leaves on potted plants.
- Strains of the bacteria were grown in nutrient broth (NB) at 28°C for 48 h. Cells were centrifuged (5 min, 3615 × *g*) and the pellet resuspended in sterile-distilled water to a concentration of approximately 1×10^7 CFU/ml as determined by OD reading (A_{600}= 0.05) with a spectrophotometer (Jenway (Jenway Ltd., Essex, UK) 6505 uv/vis).
- Bacterial strains were applied to the alfalfa leaves either by spraying with an airbrush or injecting the suspension into leaf tissue from the underside of leaves using a sterile syringe.
- Sterile distilled water was used as a control. Plants were maintained in the greenhouse at 22-25°C until symptoms were assessed 14-21 days after inoculation.
- Bacteria from the lesions produced were isolated and characterized as previously described.

Harighi,2007

Pathogenicity test

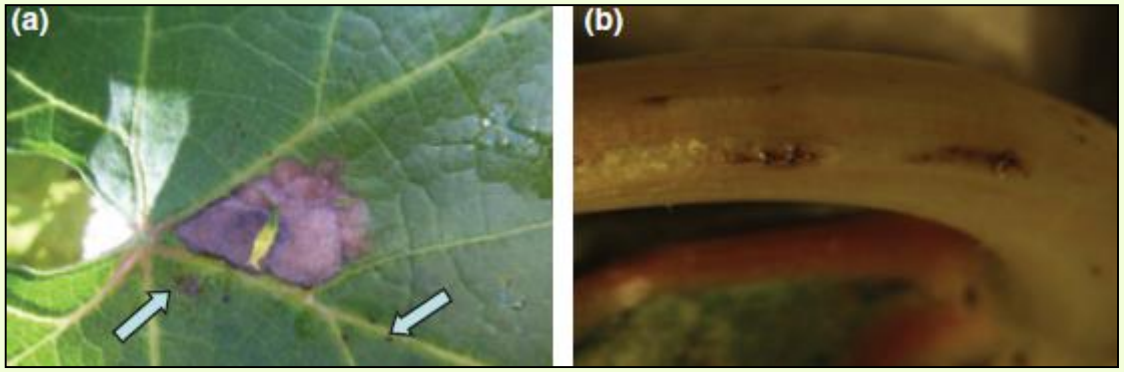
Bacterial inflorescence rot of grape

Pseudomonas syringae pv. *syringae*

- **Pot experiments**
- Visible symptoms 2 weeks after Semillon grapevines were inoculated with *Pseudomonas syringae* pv. *syringae* in pot experiment 2 (high humidity);

a) necrotic split HR lesion within inoculation site and necrotic spots along vein (arrows);

b) brown longitudinal striations on leaf petioles.



Whitelaw-Weckert *et al.*,2011

Pathogenicity test

Bacterial blight of white mulberry

Pseudomonas syringae pv. *mori*

- All bacterial isolates obtained were tested in a pathogenicity test in greenhouse conditions (temperature 20-25°C, humidity 50-80%) to confirm Koch's postulates.
- Each isolate was tested on five healthy mulberry seedlings of the Zolwinska wielkolistna cultivar.
- The plants were inoculated with an aqueous bacterial suspension (10^6-10^7 cfu/ml) applied under high pressure on both sides of the leaves with a compressor sprayer, after wounding the leaves by abrasion with carborundum (EPPO Bulletin,2006).
- After inoculation, each plant was kept in a humid chamber for 72 h, and then in the greenhouse until symptom development. After incubation, symptomatic plants were subjected to reisolation to confirm Koch's postulates.
- All reisolated strains were investigated as described above. Sterile distilled water sprayed instead of the bacterial suspension was used as a negative control, and an aqueous suspension (10^6-10^7 cfu/ml) of *P. syringae* pv. *syringae* 2238, from the Institute of Plant Protection Bank of Pathogens collection was used as a positive control.

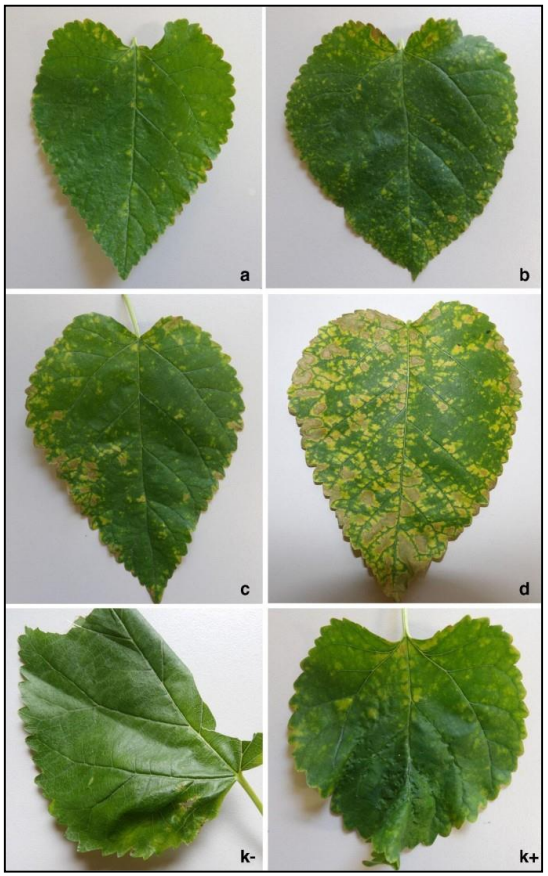
Krawczyk & Łochyńsk,2020

Pathogenicity test

Bacterial blight of white mulberry

Pseudomonas syringae pv. *mori*

- Symptoms observed after spraying bacterial suspensions of *Pseudomonas syringae* on mulberry leaves.
- A-D – the pictures were aligned according to increasing symptoms development.
- Negative control: leaves treated with sterile distilled water (K-);
- Positive control: leaves treated with the *Pseudomonas syringae* pv. *syringae* 2238 strain (K+)



Krawczyk & Łochyńsk,2020

Pathogenicity test
P. syringae pv. *theae*

- *P.s.* pv. *theae* causes bacterial shoot blight of tea (*Thea sinensis*).
- Bacterial suspensions were photometrically adjusted to an optical density corresponding to 1-2 × 10^6 cfu ml^{-1}.
- Each strain was inoculated on to *C. sinensis* cultivar 'Perfection'.
- *C. sinensis* was inoculated in the spring by puncturing the new leaves and by wounding the twig and placing 10 µl of the suspension into the wounds.
- For each strain, ten leaves and ten twigs were inoculated.
- The appearance of symptoms were checked over the spring that followed the inoculation.
- *P.s.* pv. *theae* strains induced wilting of the new leaves and necrosis around the wounds along the twigs of *C. sinensis*.

Scortichini and Prospero,2002

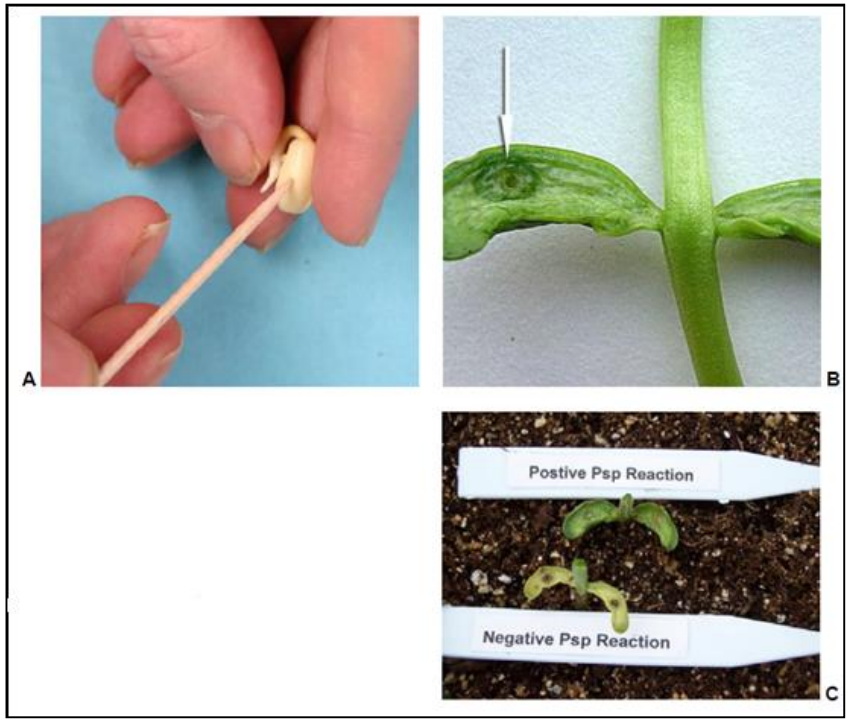
Pathogenicity test

Pseudomonas savastanoi pv. *phaseolicola*

- Germinated bean seedlings.

A. The method of inoculation.

B. Typical *Pseudomonas savastanoi* pv. *phaseolicola* symptoms in a pathogenicity test, indicated as a typical 'greasy' spot (C) at the point of inoculation.



Kurowski and Remeeus,2008

Race differentiation

Pseudomonas savastanoi pv. *phaseolicola*

- Race differentiation of *P. svastoni* pv. *phaseolicola* on 8 differential cultivars and lines (Taylor *et al.*,1996).

		Races								
Differential	R-genes	1	2	3	4	5	6	7	8	9
Canadian Wonder	-	+	+	+	+	+	+	+	+	+
A52 (ZAA 54)	4	+	+	+	+	-	+	+	+	+
Tendergreen	3	+	+	-	-	+	+	+	+	+
Red Mexican UI 3	1,4	-	+	+	+	-	+	-	+	-
1072	2	+	-	+	-	-	+	-	+	+
A53 (ZAA 55)	3,4	+	+	-	-	-	+	+	+	+
A43 (ZAA 12)	2,3,4,5	+	-	-	-	-	+	-	-	-
Guatemala 196-B	3,4	-	+	-	-	-	+	-	+	-

+, compatible (susceptible); -, incompatible (resistant)

Pathogenicity test

Pseudomonas savastanoi pv. *glycinae*

- Soybean seeds (cv. Balkan) were sown in plastic boxes with wet and sterile sand covered with plastic lids.
- After 5 days, soybean seedlings (cotyledon stage) were used for pathogenicity test.
- Pathogenicity test was done on fully expanded soybean cotyledons by dipping and spraying with aqueous suspensions of the investigated isolates.

Ignjatov *et al.*,2008

Pathogenicity test

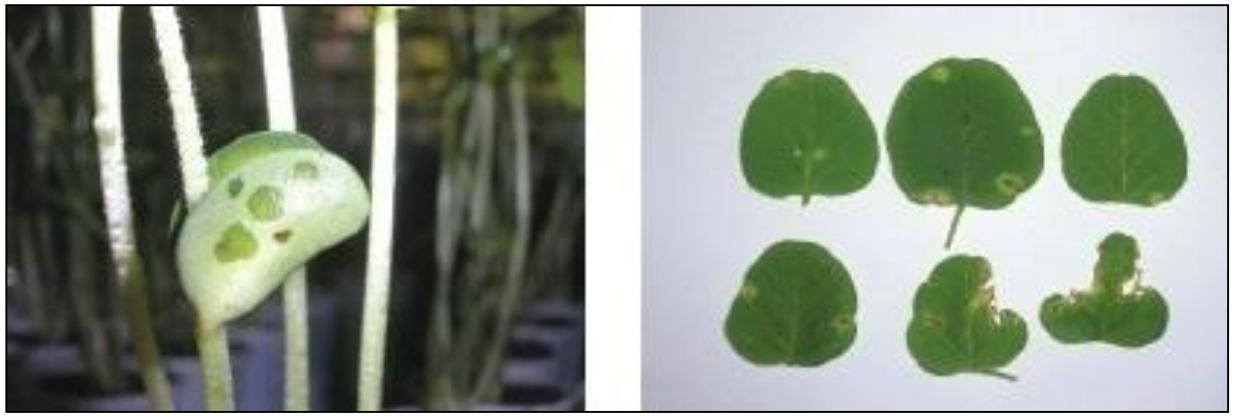
Pseudomonas savastanoi pv. *glycinae*

- **Inoculation by dipping:** the method applied by Šutić (1951) for testing susceptibility of cotton varieties to *Xanthomonas campestris* pv. *malvacearum*, was used as a basis in this investigation.
- Wounded (punctured with sterile needle) and intact cotyledons of soybean were dipped into a bacterial suspension (conc. 10^8 CFU/ml) for 2 h.
- Plant roots were covered with wet paper in order to prevent drying.
- After 2 h, plants were transplanted to pots containing a 3:1 mixture of sterile substrate (Klasmann 2) and sand.
- After inoculation, the seedlings were kept at room temperature (≈25°C).
- **Inoculation by spraying:** Wounded cotyledons were inoculated by spraying with an aqueous bacterial suspension (conc. 10^8 CFU/ml).
- The plants were incubated for 48 h in a mist chamber at 25°C, then transferred to room temperature and observed daily during 15 days.
- During the experiments plants were watered regularly and humidified with a hand sprayer.
- Control plants were inoculated with distilled water.

Ignjatov *et al.*,2008

Pathogenicity test

Pseudomonas savastanoi pv. *glycinae*



Symptoms on soybean cotyledons and leaves

Ignjatov *et al.*,2008

Pathogenicity test
Pseudomonas viridiflava

- Chicory (*Cicorium intybus*) inoculated with *Pseudomonas viridiflava* causing soft rot, 5 days after inoculation of a 10^6 cells ml^{-1}(per ml) bacterial suspension into the parenchymal leaf tissue.

Janse,2006

Pathogenicity test

Pseudomonas syringae pv. *apii*

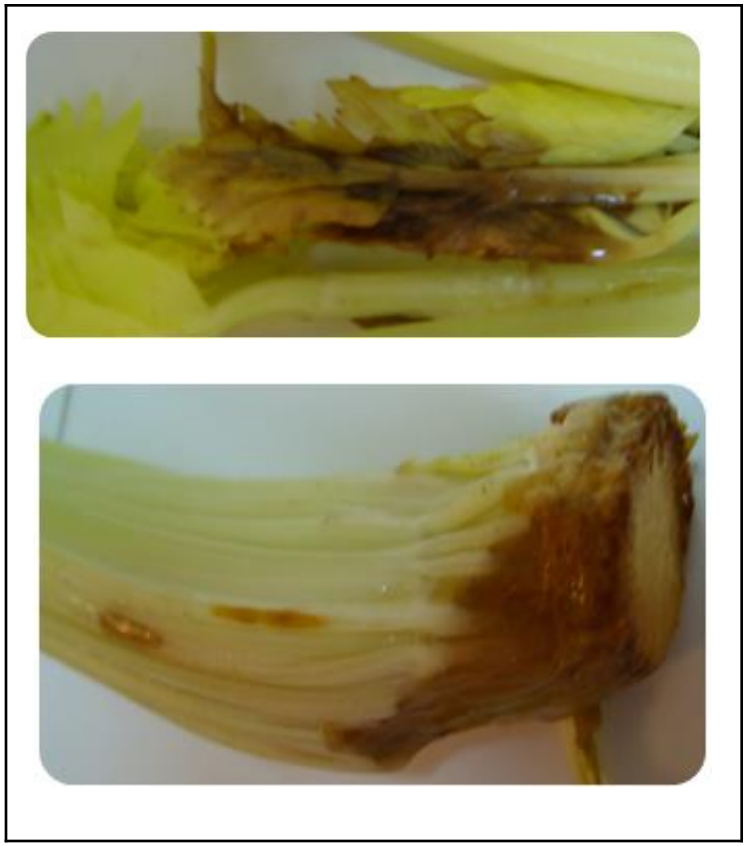
- Carborundum, an abrasive powder used to make minute wounds in leaf tissue, was added to the culture.
- Ten- to 12-wk-old celery plants (*Apium* sp.) were inoculated by dipping sterile cotton swabs into the culture and rubbing the swabs onto leaves that had expanded one-half to three-quarters.
- For negative controls, plants were inoculated with nutrient broth plus Carborundum; as a positive control, the *P. syringae* pv. *apii* strain was used.
- Plants were maintained in a greenhouse, and 7 to 10 days after inoculation, we rated disease severity on inoculated leaves on the following scale:
- 0 = no disease reaction.
- + = localized necrosis or chlorosis around area of inoculation.
- ++ = water-soaked brown lesions developing at and around the point of inoculation.
- +++ = large expanding brown water-soaked lesions with entire area becoming necrotic.

Koike *et al.*,1994; Venkatesh,2002

Pathogenicity test

Pseudomonas syringae

- The healthy excised stalks of celery were disinfected with 1% NaOCl solution and rinsed with water.
- The suspension of *P. syringae* at a conc. of 10^8 cfu/ml was prepared and injected into inner leaves and stalk bases.
- For negative controls, tissues were treated with water.
- Stalks were incubated in warm and humid conditions
- Large brown lesions will be evident 3 to 4 days after inoculation.



Taheri *et al.*,2012

Pathogenicity test

Mushroom brown blotch pathogens

Pseudomonas agarici and *P. tolaasii*

- **Dipping method:**
- Bacterial inoculum (e.g. *P. tolaasii*) was prepared from 24 h old cultures by suspending bacterial growth in sterile tap water and adjusting the concentration to approximately 10^8 CFU/ml.
- Caps of excised *A. bisporus* sporocarps (fruiting body, a multicellular structure on which spore-producing structures are borne) were either cut longitudinally in 5 mm thick slices or sectioned in cube-shaped pieces.
- Pathogenicity was tested by placing a 10 µl drop of the inoculum on the central part of the slice or top of the cube, followed by incubation in a humid chamber at room temperature.
- Occurrence of symptoms was recorded daily.
- Brown discoloration around the inoculation spot/pitting within 72 h was considered a positive reaction.

Obradovi'c *et al.*,2008

Pathogenicity test

Mushroom brown blotch pathogens

Pseudomonas tolaasii

- The bacterial suspension of *P. tolaasii* (20ml) was centrifuged (6,500 x g) and rinsed twice with sterile distilled water.
- From this stock concentration, several bacterial suspensions, 0.1-2.0 OD_{450} were prepared.
- A minimal concentration of $5x10^6$ cfu bacterial cells (0.3 OD_{450}) per inoculation site was necessary for symptom expression on surfaces of cut pileus tissue.
- Bacterial number was determined by dilution and plating.
- Concentrations of 0.3 OD_{450} approximately yield 10^8 cfu/ml.

Estrada and Royse,2007

Pathogenicity test

Mushroom brown blotch pathogens

Pseudomonas agarici and *P. tolaasii*

- **Dipping and spraying methods:**
- Bacterial suspension was prepared by adjusted to yield an optical density of 0.1.
- Using a sterile Pasteur pipette, we placed 1 drop of the adjusted inoculum onto each of several primordia (initial mushroom growth stage) of oyster mushroom (*Pleurotus ostreatus*).
- In another set of experiments, the adjusted inoculum was applied as an atomized spray.
- The oyster mushrooms were incubated at 60 to 65°F (15 to 18°C) at a relative humidity of 85 to 95% and were continuously illuminated by fluorescent light.
- Developing sporocarps were monitored daily for up to 10 days for the appearance of symptoms.
- Negative controls consisting of sterile distilled water were used whenever isolates were tested.

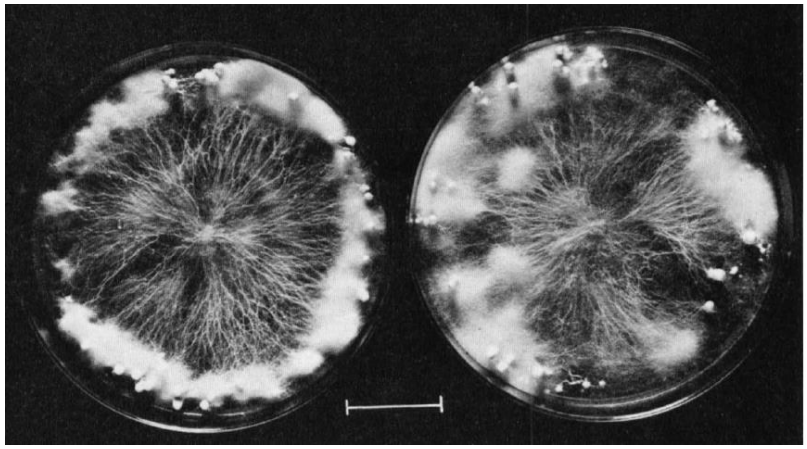
Besstete,1985

Pathogenicity test

Mushroom growth stages

Primordium

- A primordium was defined as a structure of greater than 1 mm diam., composed of:
- A dense hyphal mesh, with a smooth surface, and
- Visibly distinct from knots and strands of hyphae.
- At later stages, sporophores (fruiting bodies) are developed from the primordia.



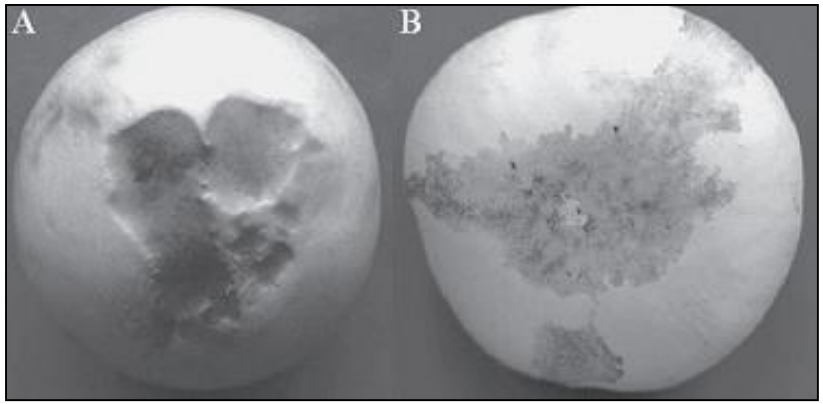
Mycelium and primordia of *A. bisporus* on malt agar plates.

Wood,1976

Pathogenicity tests

Pseudomonas agarici and *P. tolaasii*

- Prepare a 10^7-10^8 cfu/ml suspension.
- Add drops of suspension to some *Agaricus bisporus* caps.
- Use sterile water controls and, known culture controls.
- For bacterial pit tests prick some of the caps through droplets.
- Incubate in a humid chamber for 2 days.
- Where drippy gill caused by *P. agarici* is suspected inject the undersurface of unopened caps or very young gills and incubate as above.



Pseudomonas tolaasii ***P. agarici***

A) Browning of the caps in the inoculated area with *P. tolaasii*, and
B) Droplets of ooze and watersoaking of the gills (*P. agarici*).

Lelliott and Stead,1987; Cantore and Iacobellis,2004

Pathogenicity tests

Pseudomonas agarici vs. *P. tolaasii*

- *P. agarici* strains on whole sporophores (fruiting bodies) cause only superficial brown discoloration of the inoculated caps were caused.
- Whereas *P. tolaasii* strains caused high degree of browning, sunken brown lesions and rotting of the mushroom tissues.

Cantore and Iacobellis,2004

Pathogenicity tests

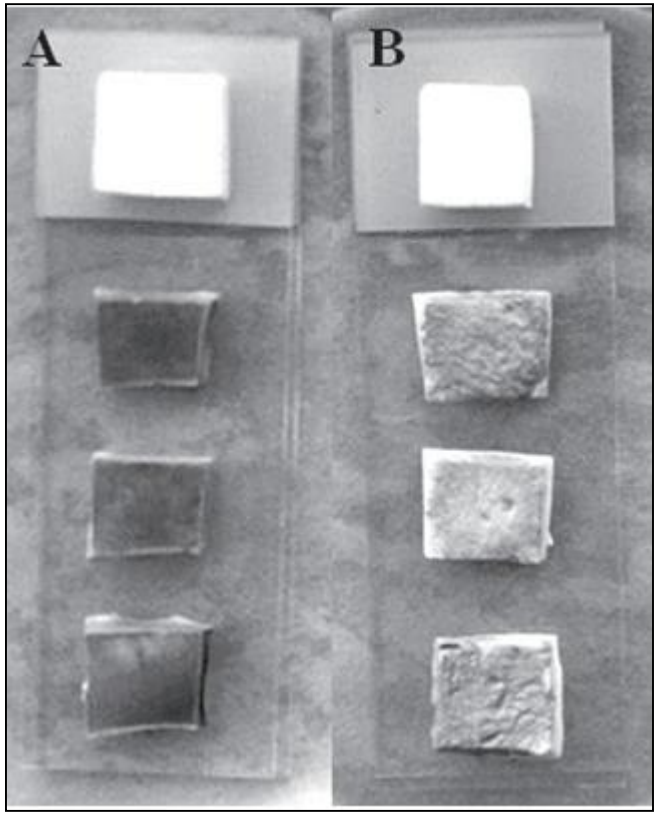
Pseudomonas agarici and *P. tolaasii*

- Deterioration of *Agaricus bisporus* tissue blocks inoculated with 10µl drops of:

A. *Pseudomonas tolaasii* NCPPB2192 or with

B. *P. agarici* USB78 10^8 cfu/ml suspensions.

- The tissue blocks in upper rows were treated with sterile distilled water.



Pt: Browning, sunken brown lesions and rotting of the mushroom tissues.

Pa: Superficial brown discoloration of the inoculated caps.

Cantore and Iacobellis,2004

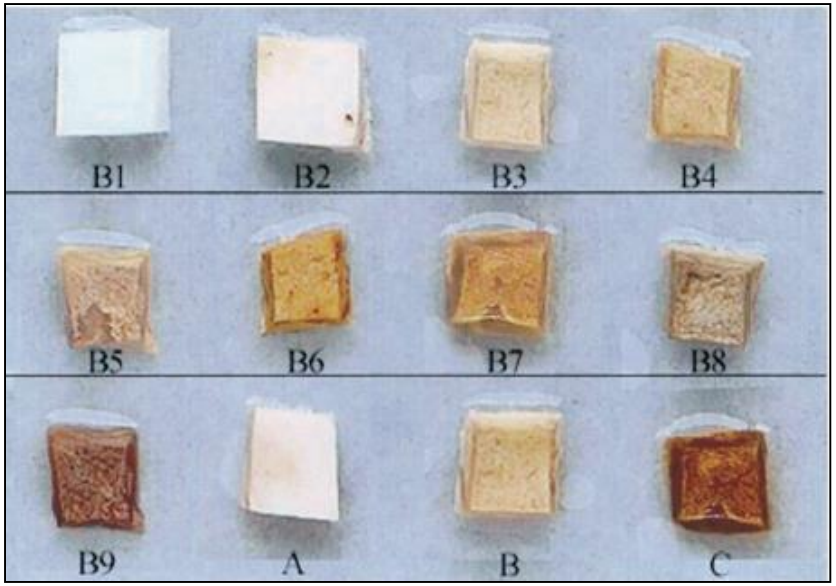
Qualitative measurement of symptoms
Cube pathogenicity bioassay
Pseudomonas tolaasii

- 1-day-old *A. bisporus* cubes (1 cm^3) of cap tissue were excised with sterile scalpel blades and placed into a sterile petri dish containing a 50-mm-pore-size paper filter dampened with 800 ml of sterile double-distilled water.
- Four cubes were placed 2 cm apart to eliminate cross-contamination by motile pseudomonads.
- Bacterial strains were cultured in KB medium to a density of 10^9 CFU/ml^{-1}, and a 50-µl aliquot of cells was placed onto three cubes.
- The fourth cube was inoculated with a 50-µl control of uninoculated KB.
- Petri dishes were sealed with parafilm and incubated under ambient conditions for 24 h.
- Mushroom caps incubated with bacterial isolates were scored for the degree of blotch discoloration on a scale of B1 to B9 (where B 5 blotch).

Godfrey *et al.*,2001

Qualitative measurement of symptoms
Cube pathogenicity bioassay
Pseudomonas tolaasii

- Bioassays to determine the capability of bacterial isolates in inducing discoloration of *A. bisporus* tissue.
- Pictured are cubes within the assigned color scale, B1 through B9.
- B1, cube inoculated with KB alone(control).
- B2, 3.1%, B3, 36.8%, B4, 10.5%, B5, 11.6%, B6, 11.6%, B8, 2.1% and B9, 4.2%.



Godfrey *et al.*,2001

Pathogenicity on shoots

Pseudomonas avellanae

- *Pseudomonas avellanae* is the causal agent of bacterial canker of hazelnut (*Corylus avellana* L.).
- Adult *C. avellana* trees cv. 'Tonda Gentile Romana' and 'Nocchione' were chosen for the pathogenicity tests.
- At the beginning of October 1998, one-year-old twigs, 30-40 cm long, were chosen on different branches of the trees.
- For each strain evaluation 15 twigs per cultivar were inoculated.
- For each twig,10 µl of the suspension in sterile saline (1-2x10^5 cfu/ml, i.e. approximately 1000 bacterial cells) were placed by means of a micropipette, on a leaf scar located midway from the tip of the twig immediately after the removal of the leaf at the base of the petiole.
- The inoculated leaf scars were marked by placing a plastic ring below the corresponding node.
- A control treatment using only sterile saline was also evaluated.
- The pathogenicity of strains were assessed in early May 1999 by recording:

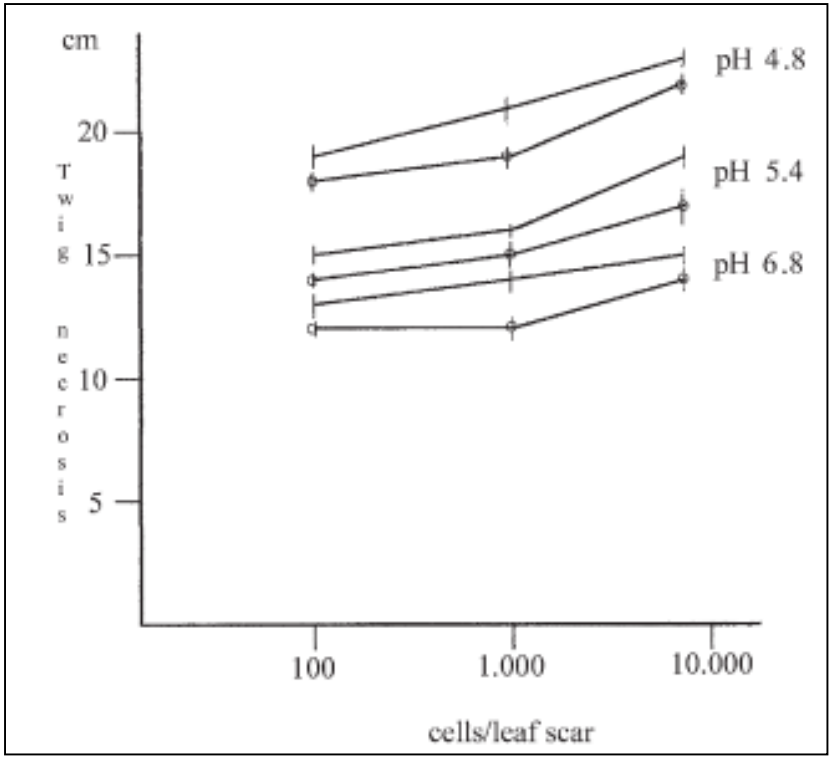
i. The number of wilted twigs; and

ii. The length of the internal necrosis after the removal of the epidermis in the un-wilted twigs.

Scortichini *et al.*,2000

Virulence Tests
Pseudomonas avellanae

- Virulence of *Pseudomonas avellanae* strains expressed as mean length necrosis of twigs.
- The plants used for the artificial inoculations were grown for three years in pots containing soils of different pH.
- To perform the tests,10 µl each suspension was placed with a micropipette on the surface of a leaf scare immediately after removal of the leaf at the base of the petiole.
- I: *P. avellanae* ISPaVe 011, isolated in Italy;
- °: *P. avellanae* BPIC 631, isolated in Greece.
- At the highest dose (i.e., 10,000 cells per leaf scar), with both strains, a twig necrosis greater than 20 cm was observed in the twigs of plant grown in soil with pH of 4.8.



Scortichini,2008

Pathogenicity test
Pseudomonas marginalis strains 7M& 8M

- **Pathogenicity on calla lily leaf petioles:**
- Observations performed within 3 days after dipping the bases of petiole segments into bacterial suspension of isolates.
- Total rotting of all petiole segments (from upper, middle and lower part of leaf petioles) was caused by *Pseudomonas marginalis* strains 7M and 8M.

Fig. 3 Pathogenicity test of two bacterial isolates on calla lily cv. Treasure leaf petioles (7M, 8M *Pseudomonas marginalis*)

Mikiciński *et al.*,2009

Preservation of Pseudomonad Cultures

- Lyophilization using skim milk works well.
- Today, most investigators also freeze cultures at −70°C or in liquid nitrogen (-196°C) for convenience.
- Loss of viability is much less at the low temperatures.
- Some investigators store bacteria in broth and water cultures and maintain them at room temperatures.
- In general this is done by placing about 5 loopfuls of bacteria in 10 ml of a rich broth or water.
- We have maintained many cultures up to seven or more years using these techniques.
- However, the occurrence of mutations is a problem.
- Mutations are also inevitable when cultures are maintained by mass transfers.
- Our recommendation is to immediately freeze bacteria of interest to reduce the occurrence of mutations.

Identification of the bacterial pathogens *Acidovorax*

Disease diagnosis and pathogen diagnostics

Domain: Bacteria
Phylum: Proteobacteria

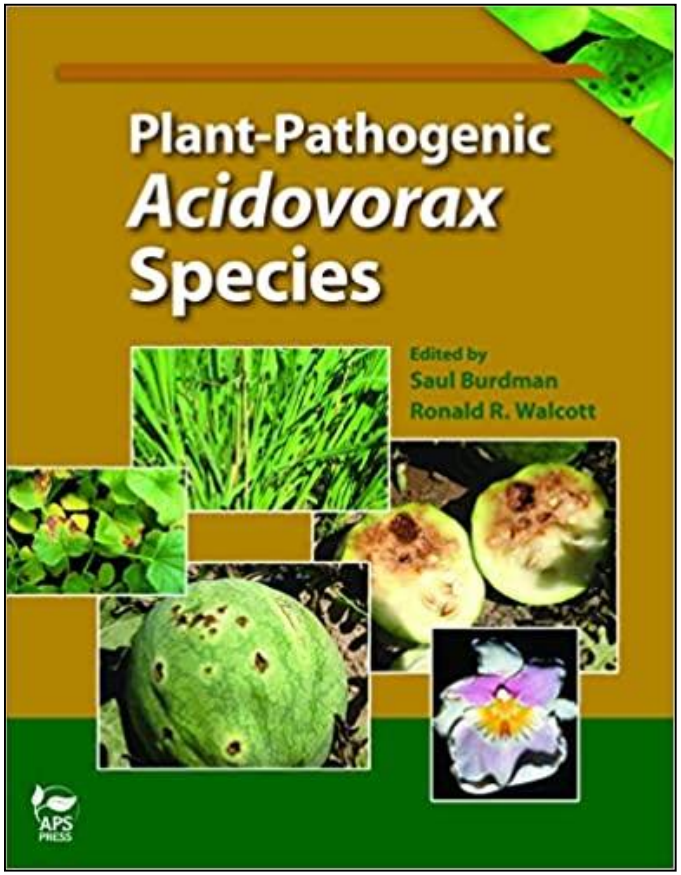
- Class: Betaproteobacteria
  - Order: Burkholderiales
    - Family: Comamonadaceae
      - Genus: *Acidovorax*
    - Family: Burkholderiaceae
      - Genus: *Burkholderia*
    - Family: *Ralstoniaceae*
      - Genus: *Ralstonia*
    - Family: ----------
      - Genus: *Xylophilus*

Etymology: L. n. *coma*, the hair of the head, hair; L. fem. n. *monas*, a unit, monad; N.L. fem. n. *Comamonas*, cell with a polar tuft of flagella.

J.P. Euzéby,2020

Plant-Pathogenic *Acidovorax* Species

- **Plant-Pathogenic *Acidovorax* Species.**
- Burdman, S. and Ronald R. Walcott.
- Publisher: Amer Phytopathological Society
- 2018
- 200 pages.



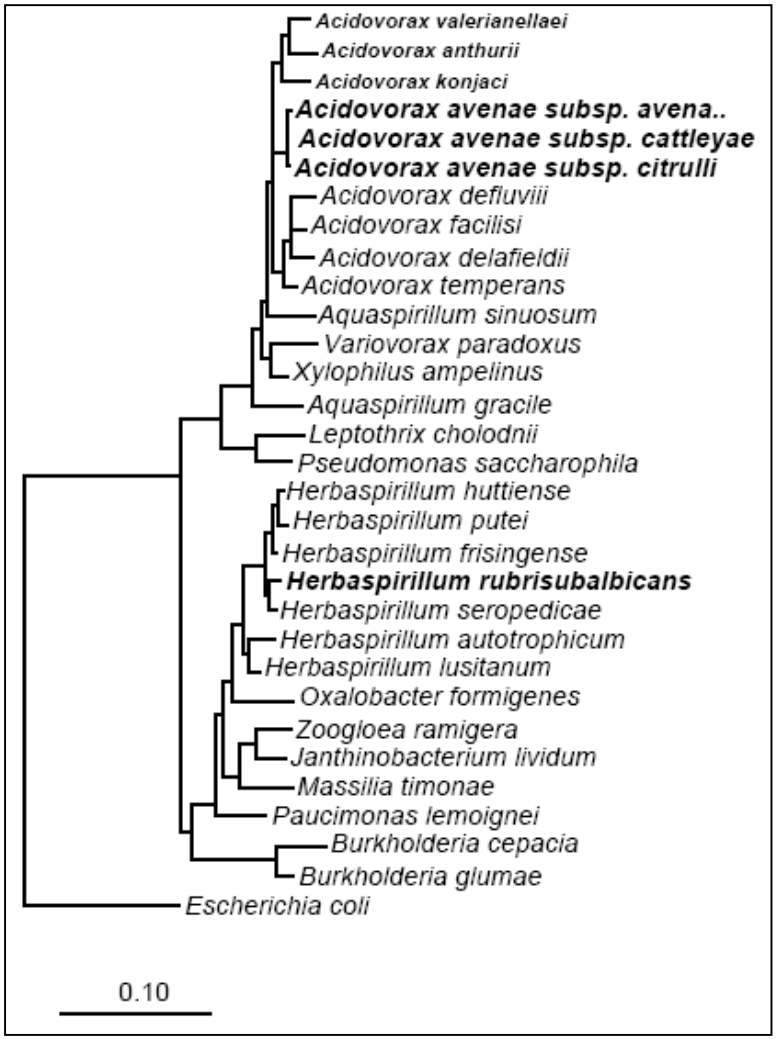
The genus *Acidovorax*

- All *Acidovorax* spp. form a separate rRNA sub-branch within the acidovorans rRNA complex.
- DNA-rRNA hybridization, 16S rRNA sequence and fatty acid analysis confirmed that this new species belongs to the *β*-subclass of *Proteobacteria* and to rRNA superfamily III, to the family of *Comamonadaceae* encompassing the acidovorans rRNA complex and to the genus *Acidovorax*.
- The name *Acidovorax anthurii* is proposed for this new phytopathogenic bacterium.

Phylogenetic analysis

Based on 16S rRNA sequences

- Phylogenetic analysis of the 16S rRNA sequences of the members of the genus *Acidovorax* has shown that the plant pathogenic members of the genus cluster together on one branch within the genus with the other, non-plant pathogenic, organisms clustering together on a separate branch.

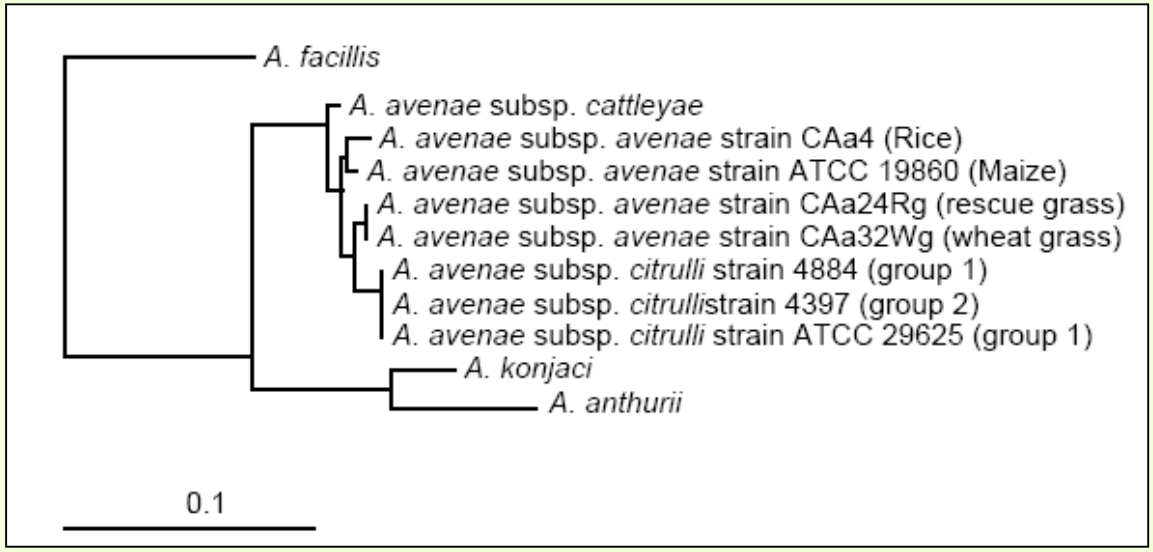


Fegan,2006

Phylogenetic analysis

Based on analysis of sequences of the 16S-23S rRNA gene spacer region

- Phylogenetic tree inferred from the analysis of sequences of the 16S-23S rRNA gene spacer region of *Acidovorax* sp. strains.
- The tree was generated using the ARB software program (Ludwig *et al.*,2004) from sequences available in the GenBank database.



Fegan,2006

Acidovorax
Plant pathogenic species

- The genus *Acidovorax* contains four described plant pathogenic species:
- *A. anthurii*, *A. avenae*, *A. konjaci*, and *A. valerianellae*.
- *A. avenae* includes three subspecies:

1. *A. avenae* subsp. *avenae*
2. *A. avenae* subsp. *citrulli*
3. *A. avenae* subsp. *cattleyae*.

- The species *A. facilis*, *A. defluvii*, *A. temperans* and *A. delafeldii* commonly found in soil, water, activated sludge or clinical environments.

Fegan,2006

Diseases caused by *Acidovorax* spp.

- Plant pathogenic strains within this genus cause seedling blights, leaf spots, leaf blights, and fruit blotches.

Acidovorax anthurii	**Bacterial leaf-spot of *Anthurium***
Acidovorax avenae* subsp. *avenae	**Wide host range:** **Pathogenic on members of the *Gramineae* including bacterial leaf blight of maize and sorghum, red stripe of sugarcane, etc.**
Acidovorax avenea* subsp. *cattlyae	**Bacterial leaf spot of orchid**
Acidovorax avenea* subsp. *citrulli	**Bacterial fruit blotch of watermelon & melons**
Acidovorax valerianellae	**Bacterial spot of lamb's lettuce (*Valerianella locusta*)**
Acidovorax konjaci	**Bacterial leaf blight of Konjac**
Acidovorax oryzae	**Bacterial stripe of rice**

Bacterial fruit blotch of cucurbits

Acidovorax avenae subsp.*citrulli*

	
Watermelon fruit naturally infected by *Acidovorax avenae* subsp. *citrulli*, showing irregular, water-soaked lesions and brown cracks on the surface. Infected fruits are usually small.	Section of watermelon fruit naturally infected by *Acidovorax avenae* subsp. *citrulli*, showing water-soaked areas in the rind underneath the surface lesions and watery rot of the flesh.

Holeva *et al.*,2009

Bacterial leaf blight of fishtail palm
Acidovorax avenae pv. *avenae*

Initial symptoms of bacterial leaf blight of fishtail palm (*Caryota mitis*) are small, water-soaked, translucent to light yellow banded areas running along leaf veins.

Nelson,2008

Older lesions may coalesce and develop tan to light-colored central regions that become colonized by fungi that are not necessarily pathogenic to *C. mitis*, especially under wet conditions for leaves.

Under dry conditions, the bacterial lesions may stop expanding altogether.

The main Characteristics
Acidovorax

- All members of the genus have the following characteristics:
- Gram-negative,
- Strictly-aerobic,
- Motile by a single polar flagellum,
- Oxidase positive,
- No pigment is produced on nutrient agar.
- The strains of *A. konjaci* turn certain media (i.e. King medium B, YP agar & PDA) deep brown.
- A clear brown pigment also diffuse around the colonies *Acidovorax valerianellae*.
- Accumulate PHB but not 3-hydroxyoctanoic acid [P(3HO)] as storage lipid granules,
- Utilize D-arabinose.

Isolation

1. Agar media

Acidovorax aveane subsp. *citrulli*

- Phosphate buffer is routinely used for bacterial extraction from seeds.
- It was confirmed MOPS buffer is superior to phosphate buffer for extraction.
- Extraction can be done for 4 hours at RT (room temperature) or 24 hours at 4 °C.
- Agar based enrichment is superior to liquid enrichment.
- Small sample is the key to detect the pathogen.
- Strains can be stored in 40% sterilized glycerol at -80°C until use (Jeng *et al.*,2011).

Colony morphology

Colony morphology on three agar media

Acidovorax aveane subsp *citrulli*

- **Modified Tween:**
- *Aac* colonies appear grayish, flat, round to oval, with a crystallized zone surrounding the colonies.
- **YDC:**
- *Aac* colonies appear tanish, domed and round.
- Colonies range in size from 1 mm to 3 mm after 2-3 days incubation at 28ºC.
- **Kings B medium**
- *Aac* colonies are non-fluorescent, cream colored and slightly domed. Colonies remain constricted up to 5 days.

Vegetable crops, Pdf

Colony morphology

Colony morphology on three agar media

Acidovorax aveane subsp *citrulli*

- **Semi selective agar medium:**
- AacSM (Shirakawa et al.,2000a) is common use for Aac isolation in Japan.
- The medium contains are the following per liter: 2.5 g of $Na_2HPO_4.12H_2O$, 0.5 g of KH_2PO_4, 2.0 g of $(NH4)_2SO_4$, 10.0g of diammonium adipate, 10 mg of yeast extract, 29 mg of $MgSO_47H_2O$, 67 mg of $CaCl_2$, 25 mg of $Na_2MoO_4.2H_2O$, 12.5 mg of bromothymol blue, 15 g of agar, and antibiotics such as 10 mg of ampicillin, 100 mg of phenethicillin potassium, 2 mg of novobiocin and 25 mg of cycloheximide.
- Incomplete AacSM, free of both antibiotics of ampicillin and phenethicillin allows the size of *A. avenae* subsp. *citrulli* colony to be bigger than original AacSM.

Sato,2009

Colony morphology

On nutrient agar

Acidovorax aveane subsp *citrulli*

- *Acidovorax aveane* subsp *citrulli* (aac), is seed borne bacterial pathogen.
- To screen for bacterium use small sub samples (approximately 200 seeds) in MOPS buffer for 4 hours at room temperature.
- 24 hours in refrigerator also gave consistently positive recoveries.
- On nutrient agar, white colonies are formed.
- All isolates could grow at 41°C.

Visual reading of liquid plating on P-278 media. Small colonies (arrow) are of the pathogen.

Randhawa *et al.*,2001;..

Isolation

2. Amended EBBA medium

Acidovorax aveane subsp *citrulli*

- The protocol can be used with 1,000 to 10,000 seeds depending upon the assay sensitivity desired (Geng et al.,1983).
- Because of relatively large number of saprophytes verses the small numbers of Ac per seed, the likelihood of detecting a single infested seed in 10,000 is greater with five to ten reps of 1,000 seeds each than with one to three reps of 10,000 seeds.

Zaho *et al.*,2009

Isolation

Amended EBBA medium

Acidovorax aveane subsp *citrulli*

- To improve the selectivity of EBB agar (ethanol bromcresol purple/brilliant blue R), several antibiotics were screened.
- Results of preliminary tests showed ampicillin to be the most effective at inhibiting saprophytes, and the amended medium is referred to as EBBA (Schaad and Sechler,1999).
- After 5 days incubation on EBB and EBBA agar at 37°C, colonies of Ac were 1.5 to 2.0 mm diameter, convex, slightly spreading with clear margins, and were green to bluish-green in color, compared to colonies of saprophytic bacteria which were convex without clear margins and blue.

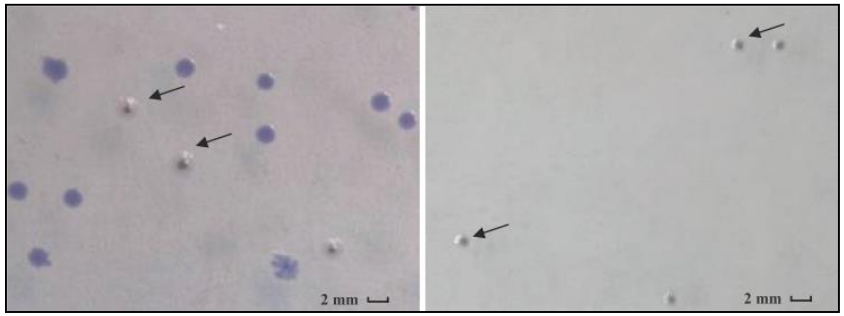
Zaho *et al.*,2009

Isolation

Amended EBBA medium

Acidovorax aveane subsp *citrulli*

- Appearance of colonies from seed extracts on ethanol bromothymol blue (EBB) agar (left) and EBB plus ampicillin (EBBA) agar (right), after 5 days.
- Note the wide, clear margin of colonies of Ac and dark blue colonies of saprophytic bacteria with little or no margin (left).
- The arrows indicate colonies *Acidovorax citrulli* (Ac).



Normally no colony of saprophytic bacteria was larger than 2 mm in diameter and most were easily differentiated from Ac.

Zaho *et al.*,2009

Detection/Diagnosis
Acidovorax spp.

- Characterization and identification of *Acidovorax* spp. is commonly based on phenotypic properties such as colony morphology, physiological and biochemical properties, disease symptoms, host range and hypersensitivity reaction in tobacco.
- The Biolog GN Microplate automated identification system and fatty acid analysis has been used to identify these species.
- Molecular tests such as amplification products of 16S and the spacer regions between the 16S and 23S rRNA gene were also used for diagnosis purposes.

Gnanamanickam,2007

Colony morphology
Acidovorax valerianellae

- On YBGA (0.7% yeast extract, 0.7% bactopeptone, 0.7% glucose and 1.5% agar; pH 7.3), colonies are white/cream, circular with a clearer margin and reach diameters of 1-2 mm after 4 days incubation at 25°C.
- A clear brown pigment can diffuse around the colonies after 10 days.

Gardan *et al.*,2003

Differential biochemical characteristics of some non-fluorescent plant pathogenic bacteria *Acidovorax*, *Ralstonia solanacearum*, *Burkholderia* and *Pseudomonas*

Test	*Ralstonia solanacearum*	*Burkholderia cepacia*	*Burkholderia gladioli*	*Burkholderia caryophylli*	*Pseudomonas corrugata*	*Acidovorax avenae*
Diffusible pigment	+	+	+	+	–	–
Oxidase	–	+	V	+	+	+
Arginine dihydrolase	–	–	–	+	–	–
Nitrate reduction	–	–	–	+	+	+
Growth at 41 °C	–	V	V	+	–	+
Oxidation of:						
Galactose	+/V	+	+	–		
Glycerol	+ W	–	–	+ W		
Mannose	+/V	–	+	–		
Utilization of:						
Cellobiose	V	+	+	V	–	
Trehalose	V	V	+	+		
D-Arabinose	–	+	+	+	–	–
D-Tartrate	–/V	–/W	+	–	V	
Mannitol	V	+	+	+		+
Sorbitol	V	+	+	+		+
L-Rhamnose	–	–	–	–	–	
Levulinate	V	+	–/W	–		
Sucrose	+	+	+	+		–
Glucose	+	+	+	+	+	+
Benzoate	V	–/V	+	–		
n-Propanol	+	+	–	+ W	–	–
β-Alanine	V	–	–	–	–	+
Betaine	–	+	+	+		
L-Arginine	–	+	+	+		
L-Lysine	–	+	+	V		
Heptanoate	–	+	+	–		
D-Fucose	–	+	+	+		
D-Raffinose	–	V	–	+		

+ = positive reaction; – = negative reaction; V = variable; W = weak.

OEPP/EPPO Bulletin,2004

Differentiation of plant pathogenic *Acidovorax* and *Xylophilus* from other genera of *Comomonadaceae*

Characteristics of the genus *Acidovorax*

Characteristic	*Acidovorax*	*Xylophilus*	*Comomonas*	*Hydrogenophaga*	*Variovorax*
Cell morphology	Rods	Rods	Rods to spirilla	Rods	Rods
Flagella	Polar, 1 to 2	Polar, 1	Bipolar,1 to 5	Polar, 1	peritrichous
Yellow insoluble pigment on nutrient agar	-	+	-	+	+
Growth at 41°C	+	-	+	+	+
Carbon sources utilized for growth:					
Adonitol or L-arabitol	-	-	-	-	+
D-glucose	+	-	-	+	+
Glycolate	-	ND	$+^{D}$	+	+
L-mandelate	-	ND	$+^{D}$	+	$+^{D}$
L-tyrosine	+	ND	$+^{D}$	$+^{D}$	-
Isolated from infected plant	+	+	-	-	-
Hypersensitive reaction	+	ND	-	-	-

+, 80% or more strains positive; $+^{D}$, 80% or more strains positive but delayed; -, 80% or more strains negative; ND, not determined.

Schaad *et al.*,2001

Comparison of phytopathogenic species of *Acidovorax* with other phenotypically similar plant pathogens

Characteristics of the genus *Acidovorax*

Characteristic	*Acidovorax*	*Pseudomonas*	*Ralstonia*	*Burkholderia*
Flagella	Polar, 1 to 2	Bipolar, 1 to 5	Polar, 1 to 4	Polar, 1 to 4
Growth at 41°C	+	-	-	-
Accumulates poly-β-hydroxybutyrate	+	-	+	+
Fluorescent pigments produced by most strains	-	+	-	-
Carbon sources utilized for growth:				
Arginine	-	+	-	+
Betaine	-	+	-	+
Citraconate	+	ND	-	V
L-arabinose	+[a]	-	-	+
Sucrose	-[b]	+	+	V
Ethanol	+	-	-	-
n-Proponal	+	-	-	-

+, 80% or more strains positive; V, between 21 - 79 % of strains positive; -, 80% or more strains negative.

[a] All positive except *A. konjaci*.

[b] All negative except *A. avenae* subsp. *cattleyae*.

Schaad *et al.*,2001

Determinative tests for *Acidovorax* spp., *Burkholderia* spp., and *Herbaspirillum rubrisubalbicans* (*H.*)

	Acidovorax				*Burkholderia*					*H.*
	avenae	*citrulli*	*konjaci*	*andropogonis*	*caryophylli*	*cepacia*	*gladioli*	*glumae*	*plantarii*	*rubrisubalbicans*
Number of strains tested	29	8	3	23	5	5	7	5	6	9
Growth at 41°C	*	+	-	-	+	+	-	+	-	-
Levan	-	-	-	-	+	*	-	-	-	*
Oxidase	+	+	+	-	-	-	*	-	-	-
Nitrate reductase	-	-	-	-	+	*	-	*	+	-
Urease (Dye's method)	+	+	+	*	-	-	*	-	-	+
Gelatin hydrolysis	-	*	-	-	-	*	+	+	*	-
Soluble starch hydrolysis	*	*	-	+	-	-	-	-	-	-
Tween 80	+	+	+	-	+	+	*	*	+	-
Cellobiose	-	-	-	*	*	+	+	+	+	-
Rhamnose	-	-	-	*	*	-	-	-	+	-
Xylose	+	+	-	-	*	+	+	+	+	+
L-Tartrate	-	-	+	-	-	+	+	-	+	*
Adonitol	-	-	-	*	*	+	+	+	-	+
Dulcitol	-	-	-	-	-	+	+	+	+	-
Erythritol	-	-	-	-	-	-	-	-	-	+
m-Inositol	-	-	-	*	+	+	+	+	+	-
Sorbitol	+	-	-	*	*	+	+	+	+	+
Butane-2,3 diol	+	-	-	-	+	+	-	*	-	*
Benzoate	-	-	-	-	+	+	+	-	-	*
Lactate	+	+	+	-	*	+	+	+	-	+
Laevulinate	+	+	+	-	-	+	+	-	-	*
Propionate	+	*	+	-	+	+	+	+	+	+
Salicin	*	-	-	-	*	+	+	+	+	-
β-alanine	+	+	+	-	*	+	+	*	-	+
Ethanolomine	*	-	-	-	-	+	+	+	+	+
Tryptamine	-	-	-	-	-	+	-	+	-	*

+ = >90% of strains gave positive reactions.
- = <10% of strains gave negative reactions.
* = 11–89% of strains gave positive or negative reactions.

Hu *et al.*,2001

Characteristics of species in the genus *Acidovorax*

Properties	Present strains (n=10)	Reference strains of *Acidovorax avenae* subsp.						*Acidovorax konjaci*[c) d)]
		avenae	*cattleyae*	*citrulli*[a) b) c)]	unidentified			
		ATCC 19860[T]	MAFF 301576	ATCC 29625	MAFF 301027	MAFF 301141	MAFF 301504	
Gram reaction	–	–	–	–	–	–	–	–
Fluorescence on KBA	–	–	–	–	–	–	–	–
Growth at 40 °C	+	+	+	+	+	+	+	+
Levan production	–	–	–	–	–	–	–	–
Oxidase activity	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Arginine dihydrolase	–	–	–	–	–	–	–	–
Nitrate reduction	+	+	+	+	+	+	+	+
Denitrification	–	–	–		–	–	–	
Urease	+	+	+	+	+	+	+	+
Hydrolysis of starch	+	+	+	–	+	+	+	–
Hydrolysis of esculin	–	–	–	–	–	–	–	
Liquefaction of gelatin	–	–	–	+	–	–	–	–
Tobacco HR	+	+	+	+	+	+	+	+
Potato soft rot	–	–	–	–	–	–	–	–
Utilization of :								
Sucrose	–	–	–	–	–	–	–	–
Inositol	–	–	–	–	–	–	–	
Xylose	+	+	+	+	+	+	+	–
Dextrose	+	+	+		+	+	+	
Lactose	–	–	–	–	–	–	–	
Maltose	–	–	–	–	–	–	–	
Trehalose	–	–	–	+	–	–	–	
Sorbitol	+	+	+	–	+	+	+	
Galactose	+	+	+	+	+	+	+	
Mannitol	+	+	+	–	+	+	+	
Mannose	–	–	–		–	–	–	
L–Arabinose	+	+	–	+	–	+	+	–
D–Arabinose	–	–	–		–	–	–	
Adonitol	–	–	–	–	–	–	–	–
Raffinose	–	–	–		–	–	–	
D–Cellobiose	–	–	–		–	–	–	
Threonine	–	–	–		–	–	–	
L–leucine	+	+	+	+	+	+	+	+
Adipic acid	+	+	+		+	+	+	
Sodium citrate	–	–	–		–	–	–	
Phenyl acetate	–	–	–		–	–	–	
Potassium gluconate	+	+	+		+	+	+	
N–Acetyl–D–glucosamine	–	–	–		–	–	–	
L–Cystein	–	–	–		–	–	–	
Salicin	–	–	–	–	–	–	–	–
DL–Alanine	+	+	+		–	+	+	
Propionic acid	–	–	+		–	–	–	+
Benzoic acid	–	–	–	–	–	–	–	–
L–Tryptophan	+	+	–		–	–	–	
Pectic acid	–	–	–		–	–	–	
Phthalic acid	–	–	–		–	+	+	
n–Decanoic acid	–	+	–		–	–	–	+

Plus sign, positive; minus sign, negative

a) Cited from Shirakawa *et al.*, 2000. b) Cited from Rane *et al.*, 1992. c) Cited from Hu *et al.*, 2001. d) Cited from Goto *et al.*, 1983.

Furuya *et al.*,2009

Characteristics of species in the genus *Acidovorax*

All strains react with PCR primers Aaaf5 or Aaaf3/Aaar2, whereas *A. avenae*, *A. citrulli*, and *A. cattleyae* do not.

Character	Species			
	Aa (6)[a]	Ao (6)	Ac (11)	Aca (4)
DNA/DNA relatedness to[b]				
A. avenae	81	53	39	27
A. oryzae	46	97	43	25
A. citrulli	47	43	88	25
A. cattleyae	35	45	39	98
ITS similarity to[c]				
A. avenae	100	99.0	98.0	98.3
A. oryzae	99.0	100	97.8	98.0
A. citrulli	98.0	97.8	100	97.3
A. cattleyae	98.3	98.0	97.3	100
Utilization of				
D-arabitol	–[d]	–	V+	+
Sodium citrate	+	–	+	+
Maltose	+	–	–	–
D-fucose	V+	+	(V+)[3]	–[2]
D-mannitol	+	+	–	+
Ethanol	–	+	+	+
Lipase production	+	+	–	+
Gelatin liquefaction	(+)	(+)	(+)	–
Nitrate reduction	+	+	–	+
Litmus milk	Alk	Alk	Alk (P)	Alk
Reaction to PCR[e]				
Aaaf5, Aaaf3/Aaar2	–	+	–	–
Aacf2/Aacr2	–	–	+	–
Hosts	Corn	Rice	Cucurbits	Orchid

Schaad *et al.*,2008

Differentiation between *A. avenae* subspecies and *A. konjaci*

Characteristic	*avenae*	*cattleyae*	*citrulli*	*A. konjaci*
Growth on:				
D-xylose	+	-	$+^{D}$	-
D-fucose	+	+	$+^{D}$	+
D- mannitol or D-arabitol	+	+	-	+
Sorbitol	+	+	-	ND
Sucrose	-	+	-	-
L-threonine or L-histidine	+	+	-	ND
Nitrate reduction	+	+	-	+
Lactalysate or beef extract ppt.	+	-	-	-
Starch hydrolysis	$+^{D}$	$+^{D}$	-	-
Pathogenicity on:				
Watermelon	-	ND	+	-
Canteloupe	-	ND	+	-
Squash	-	ND	+	-
Pumpkin	-	ND	+	-
Corn	+	ND	-	-
Rice	+	ND	-	-
Phalenopsis spp.	-	+	-	-
Cattleya spp.	-	+	-	-
Amorphophallus konjac	-	-	-	+

+, 80% or more strains positive; $+^{D}$, 80% or more strains positive but delayed; -, 80% or more strains negative; ND, not determined.

Schaad *et al.*,2001

Characteristics of *A. citrulli* with two type strains

- *Acidovorax avenae* subsp. *citrulli* (*Aac*) is pathogenic to inoculated watermelon fruits and to seedlings of watermelon, melon, cucumber and pumpkin; isolates induced tobacco hypersensitivity and failed to hydrolyzes starch.

Characteristic[a]	Strains tested (no.)	Watermelon strains	Type strain[b]	
			PPC	PP
Fluorescence on KMB	15	–[c]	–	–
Oxidase	15	+	+	+
CVP pitting	15	–	–	–
Gelatin liquefaction	4	S	S	NT
Nitrate reduction	5	–	–	–
Gram reaction	5	–	–	–
Oxygen relationship (aerobic)	4	+	NT	NT
Lipolytic activity	15	+	+	–
Starch hydrolysis	15	–	–	–
Maximum growth temperature	15	8 at 39 C 7 at 42 C	39 C	39 C
Hypersensitivity, tomato	5	+	–	NT
Hypersensitivity, tobacco	5	+	–	NT
Arginine dihydrolase	4	–	–	NT

[a] Where appropriate, positive or negative controls were tested to ensure accuracy of the tests.
[b] PPC = *Pseudomonas pseudoalcaligenes* subsp. *citrulli*, ATCC 29625. PP = *P. pseudoalcaligenes*, ATCC 17440.
[c] – = Negative, + = positive, S = slight, and NT = not tested.

Somodi *et al.*,1991; Holeva *et al.*,2009

Distinguishing phenotypic characteristics of *A. avenae* subsp. *citrulli* group I and Group II strains

- Phenotypically, gas chromatography fatty acid methyl ester (GC-FAME) profile analysis, carbon substrate utilization profiles and pathogenicity on different host plants have defined two groups of strains.
- The same two groups of strains have also been identified by using the:

1. Genomic fingerprinting techniques,
2. rep-PCR, and
3. Pulsed field gel electrophoresis.

Distinguishing phenotypic characteristics of *A. avenae* subsp. *citrulli* group I and Group II strains

Phenotypic characteristic	*Group I*	*Group II*
GC-FAME profile		
11:0, 3:OH	-	+
12:0, 3:OH	-	+
17:0 *cyclo*	-	+
Carbon Substrate Utilisation		
L-Leucine	- (96%)	+ (96%)
Disease severity		
Watermelon	Moderate	High
cantaloupe	Moderate	Low

Fegan,2006

Fatty acid analysis

For all plant/non plant-pathogenic *Acidovorax* species

Species: 1, *A. valerianellae* sp. nov. (10 strains analysed/10 profiles obtained); 2, *A. anthurii* (2/9); 3, *A. avenae* subsp. *avenae* (28/35); 4, *A. avenae* subsp. *cattleyae* (1/4); 5, *A. avenae* subsp. *citrulli* (5/11); 6, *A. delafieldii* (1/10); 7, *A. facilis* (1/5); 8, *A. konjaci* (4/7); 9, *D. acidovorans* (2/2). Values are mean percentages ± SD of the total named peak area.

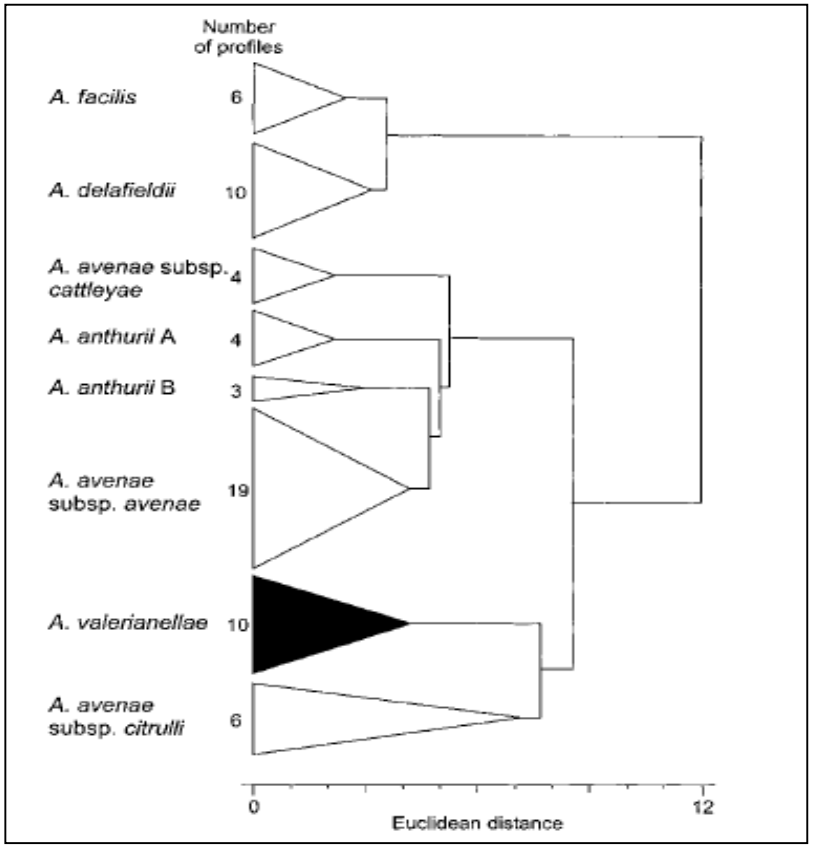
Fatty acid	1	2	3	4	5	6	7	8	9
9:0 3-OH		0·2±0·1							
10:0			0·2±0·1	0·2±0·2				0·2±0·2	
10:0 3-OH	2·1±0·2	1·9±0·3	3·1±0·5	3·7±0·2	3·2±0·4	2·6±0·6	2·3±0·1	3·0±0·1	2·7±0·1
11:0		0·3±0·1			0·1±0·1				
12:0	2·9±0·1	2·3±0·2	2·3±0·1	2·3±0·1	2·4±0·2	3·1±0·5	3·1±0·2	2·5±0	2·6±0·2
12:1 3-OH				0·6±0·1	0·2±0·3				
13:0		0·4±0·1			0·1±0·1				
14:0	3·6±0·1	1·6±0·2	2·2±0·2	1·7±0·1	1·6±0·2	3·5±0·4	3·0±0·1	3·0±0	0·7±0·1
15:1ω6*c*	0·4±0·3	2·8±1·0			1·6±0·9				0·2±0·3
15:0	3·8±0·9	12·5±2·6	0·2±0·1	0·5±0·1	5·5±2·2	0·1±0·2	0·1±0·1	0·4±0·1	1·8±1·6
16:1ω7*c*	46·3±0·5	37·9±1·6	42·9±1·0	40·6±0·3	41·9±0·3	40·9±0·3	43·3±0·6	43·1±0·4	36·3±3·9
16:0	29·4±1·0	20·4±1·7	32·2±1·2	34·9±0·4	32·9±2·7	25·9±0·7	26·0±0·1	29·3±0·5	31·9±1·2
17:1ω8*c*	0·1±0·2	1·0±0·2							0·1±0·1
17:1ω6		1·4±0·3			0·1±0·1				
17:0 cyclo	0·2±0·3		0·3±0·3	0·5±0·2		0·2±0·3	0·1±0·2		6·2±3·2
17:0	0·9±0·2	5·0±0·8	0·1±0·1	0·3±0·2	1·4±0·5			0·3±0	0·7±0·8
18:1ω7*c*	10·3±0·5	11·8±1·9	15·9±0·8	14·4±0·6	9·1±1·6	23·8±0·8	21·9±0·5	17·8±0·6	15·9±1·8
18:0		0·1±0·1	0·2±0·1	0·2±0·2			0·2±0·2	0·2±0	0·2±0·1

Gardan *et al.*,2000

Fatty acid analysis

Clustering of *Acidovorax* spp. by fatty acid profiling

- Fatty acid analysis provided phenotypic support for differentiation of all taxa included in the study.
- The two strains of *A. anthurii* clustered separately in both analyses.



Gardan *et al.*,2000

Serological methods

- ELISA kit and immunostrip kit are released by Agdia and/or Adgen for the plant materials.
- Latex agglutination test is very simple and useful to determine and identify the Aac colonies on agar medium.

Sato,2009

PCR primers for *Acidovorax avenae*

Organism	Primer Designation	Sequence	Size (bp)	Reference
Acidovorax avenae	RST49 RST51	(5'GATGGCCGTGCCCTTC TTCATCCTCG3') (5'CATGGCCACGATGAGG ATCG3')	390	(11), G. V. Minsavage (personal communication)
Acidovorax species and *A. avenae* subsp.	RST63 RST64	(5'TCCGGCGGCGCGCTCA CCGTGGTGCTG3') (5'AGCGCGGCGGCGTAG GCGCGCGAG3')	210	G. V. Minsavage (personal communication)
Acidovorax avenae subsp. *citrulli*	WFB1 WFB2	(5'GACCAGCCACACTGGG AC3') (5'CTGCCGTACTCCAGCG AT3')		(24)

Schaad *et al.*,2001

Specific primers for *Acidovorax avenae* subsp. *citrulli*

BX-L1F/BX-R5F, BX-L1F/BX-S-R2R, 63f/1389r

- Two sets of citrulli specific primers, *viz*.

1. BX-L1F/BX-R5F and
2. BX-L1F/BX-S-R2R (Bahar *et al.* 2008), as well as

- The set 63f/1389r (Osborn *et al.*,2000) amplifying part of the 16S rDNA region, verified the isolates as *Aac*.
- In Rep-, Eric- or Box-PCR, the isolates from plants and *Aac* reference strains produced similar banding patterns.

Holeva *et al.*,2009; Cucuzza and Braun,2007

Specific primers for *Acidovorax avenae* subsp. *citrulli*

SEQ. ID primers amplify the inner spacer regions of 16S-23S rDNA

- A set of *Acidovorax*-specific primers were designed to amplify the inner spacer regions of 16S-23S rDNA of strains *Acidovorax avenae* subspecies.

1. The first primer comprising 5′-CGCGCCGACCGAGACCTG-3′ (SEQ. ID NO:1) and a second primer comprising 5′-GGGGCACGCCAACATCCT-3′ (SEQ. ID NO:2) was used to amplify the target sequence of all strains of *Acidovorax avenae* subsp. *avenae* including subsp. *citrulli*.
2. But the primers identified as SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6 react with all strains of *Acidovorax avenae* subsp. *citrulli*, but not with any other strains of subsp.*avenae*.

Schaad *et al.*,2000; Cucuzza and Braun,2007

Specific primers for *Acidovorax avenae* subsp. *citrulli*

SEQ. ID primers

Forward Primers:

5'-GTCGGTGCTAACGACATGG-3' (SEQ ID NO:1)

5'-GGAAGAATTCGGTGCTACCC-3' (SEQ ID NO:3)

5'-CCTCCACCAACCAATACGCT-3' (SEQ ID NO:5)

Reverse Primers:

5'-AGACATCTCCGCTTTCTTTCAA-3' (SEQ ID NO:2)

5'-TCGTCATTACTGAATTTCAACA-3' (SEQ ID NO:4)

5'-CATGCTCTTAGTCACTTGACCCTA-3' (SEQ ID NO:6)

Probe:

5'-CGGTAGGGCGAAGAAACCAACACC-3' (SEQ ID NO:7)

Schaad *et al.*,2000

Specific primers for *Acidovorax avenae* subsp. *citrulli*

The primers AAC 94-85

- For direct PCR:
- Another specific primers for 16s ribosomal RNA (rRNA) from *A. avenae* subsp. *citrulli* strain (AAC 94-85) were used (Walcott *et al.*, 2000b):
- Forward primer: (5'-CAG CCA CAC TGG GAC-3';
- Reverse primer: 5'-CTG CCG TAC TCC AGC GAT-3').
- The single colony was picked with sterilized toothpick and transferred into King's medium B (KB) liquid medium.
- After 48 h incubation at 28°C in a water-bathing shaker, the concentration of the cell reached 4×10^8 CFU/ml (OD_{600}= 0.627).
- The culture of *A. avenae* subsp. *citrulli* was then diluted with PBS into the final concentration at 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 CFU/ml, respectively.

Jeng *et al.*,2011

Specific primers for *Acidovorax avenae* subsp. *citrulli*

AAC 94-85

- For direct PCR:
- The bacterial suspension and seeds suspension were directly used as PCR template without any pathogen enrichment operation.
- Five microliters of suspension and 31.6 µl sterilized distilled-water added in the tubes, boiled at 99°C for 15 min, and then chilled on ice for 5 min, and then this tube was used for the PCR reaction.

See the original paper for some more PCR detection methods such as IC-PCR, IMS-PCR.

Jeng *et al.*,2011

Specific primers for *Acidovorax avenae* subsp. *citrulli* AAC 94-85

- For direct PCR:

A. A series dilution of bacterial suspension, or

B. Seeds suspension were performed by direct-PCR, and visualized on 1.5% agarose gel.

- P: represents positive control (10^8 CFU/ml Aac cell suspension),
- N: represents negative controls including bacterial suspension, PBS buffer; seeds suspension, seeds extract), and
- M: represents 100 bp Ladder.

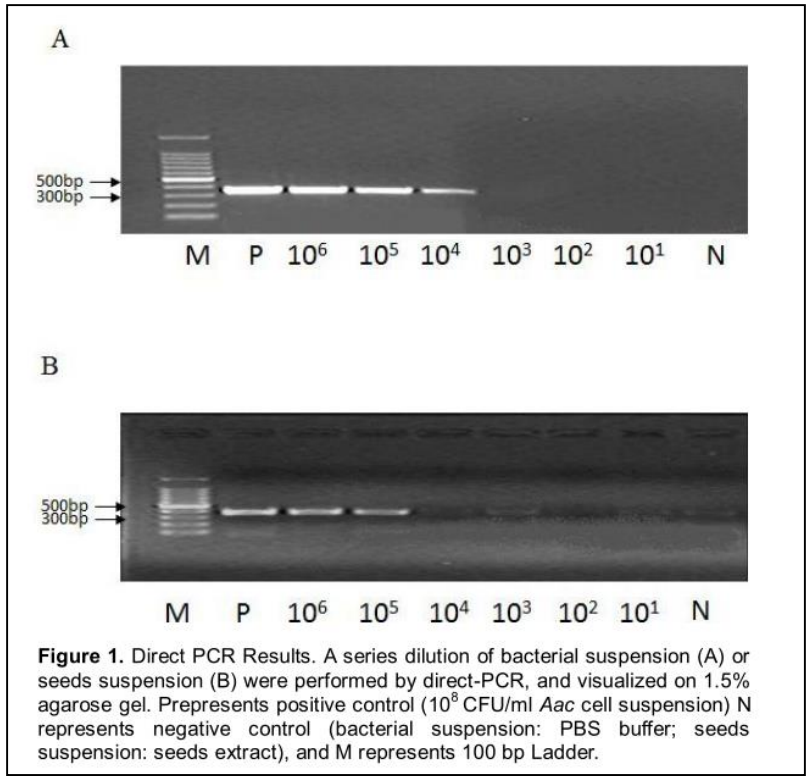


Figure 1. Direct PCR Results. A series dilution of bacterial suspension (A) or seeds suspension (B) were performed by direct-PCR, and visualized on 1.5% agarose gel. Prepresents positive control (10^8 CFU/ml *Aac* cell suspension) N represents negative control (bacterial suspension: PBS buffer; seeds suspension: seeds extract), and M represents 100 bp Ladder.

Jeng *et al.*,2011

Oligonucleotide primer pairs for the detection of *Acidovorax avenae* strains

Organism detected (specificity)	*Primer designation*	*Primer sequence (5'-3')*	*Reference*
Acidovorax sp. and *Delftia acidovorans*	WFB1	GACCAGCCACACTGGGAC	(Walcott & Gitaitis, 2000)
	WFB2	CTGCCGTACTCCAGCGAT	
Acidovorax sp.	RST63	TCCGGCGGCGCGCTCACCGTGGTGCTG	(Jones, Gitaitis, & Schaad, 2001)
	RST64	AGCGCGGCGGCGTAGGCGCGCGAG	
A. avenae	Oaf1	GTCGGTGCTAACGACATGG	(Song et al., 2003)
	Oar1	AGACATCTCCGCTTTCTTTCAA	
A. avenae	RST49	GATGGCCGTGCCCTTCTTCATCCTCG	(Jones et al., 2001; Minsavage, Hoover, Kucharek, & Stall, 1995)
	RST51	CATGGCCACGATGAGGATCG	
A. avenae	Oaf1	GTCGGTGCTAACGACATGG	(Song et al., 2003)
	Oar2	TCCTCGCATCTTATGTTCGGAA	
	AaP1(Probe)	6FAM-TCAGCTGGTTAGAGCACCGTCTAGA-TAMARA	
A. avenae subsp. *citrulli*	Aacf2	GGAAGAATTCGGTGCTACCC	(Song et al., 2003)
	Aacr2	TCGTCATTACTGAATTTCAACA	
A. avenae subsp. *citrulli*	Aacf3	CCTCCACCAACCAATACGCT	(Song et al., 2003)
	Aacr2	TCGTCATTACTGAATTTCAACA	
	Aap2 (Probe)	6FAM-CGGTAGGGCGAAGAAACCAACACC-TAMARA	
A. avenae subsp. *avenae* strains from rice	Aaaf3 (SEQ10)	GTCATCCTCCACCAACCAAG	(Song et al., 2004)
	Aaar2 (SEQ 12)	AGAACAATTCGTCATTACTGAAC	
	Aaaf5 (SEQ 11)[a]	TGC CCT GCG GTA GGG CG	

[a] to be used in conjunction with the primer Aaar2 in a hemi-nested PCR reaction using amplicon from application of primer pair Aaaf3/Aaar2 as template

Fegan,2006

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

Genus *Acidovorax*

Genus *Acidovorax*					
Species/subspecies	Primer name Target DNA	Variant of PCR Protocol	Sample (treatment)	Reference	Synonyms/observations
A. avenae subsp. *avenae*	**Aaaf3/Aaar2 (external)** ITS region + **Aaaf5/Aaar2 (internal)** ITS region	Nested BIO	Seed (washes enrichment)	Song *et al.*, 2004	DNA extraction recommended if high level of other microflora is found after enrichment.
A. avenae subsp. *citrulli*	**WFB1/WFB2** 16S rRNA gene	Conventional	Bacteria (lysed) or crude extract and immunocapture	Walcott and Gitaitis, 2000	
A. avenae	**R16-1/R23-2R** ITS region	Conventional	Bacteria (DNA extraction)	Kim and Song, 1996	*Pseudomonas avenae* *Burkholderia glumae* (*Pseudomonas glumae*), *Pantoea agglomerans* (*Erwinia herbicola*), *Pseudomonas fuscovaginae*, *Pseudomonas syringae* pv. *syringae* and *Xanthomonas oryzae* (pathovars *oryzae* and *oryzicola*) also amplified and differentiated by primary and secondary fragments.

Palacio-Bielsa *et al.*,2009

Loop-mediated isothermal amplification (LAMP)

For detection of *Acidovorax avenae* strains

- LAMP is a new DNA amplification assay developed by Eiken Chemical (http://loopamp.eiken.co.jp/lamp/index.html).
- This assay has unique characters including the use of 4 different primers and reaction process proceeds at constant temperature (about 65°C) using strand displacement reaction.
- The positive reaction of LAMP can be determined visually by checking the cloud of solution in test tubes.

Sato,2009

Detection and diagnosis methods for Aac and their characters

Methods	Specificity	Threshold (CFU/ml)	Time	Cost	Applicable situation
Selective agar media					
AacSM	○	2	3 days	△	in Lab
Serological assay					
ELISA	○	10^5	3-8 hr	△	in Lab
Immunostrip	○	10^5	5 min	○	in Lab. & at Field
Latex assay	△	10^7	5 min	○	in Lab. & at Field
Molecular assay					
PCR	◎	10^3	4 hr <	△	in Lab
LAMP	◎	10^3	1-2 hr	△	in Lab

△ : less good, ○ : good, ◎ : excellent (Shirakawa)

Sato,2009

Pathogenicity test

Bacterial spot of lamb's lettuce

Acidovorax valerianellae

- The pathogenicity test was confirmed on young plants of lamb's lettuce (also known as corn salad (*Valerianella locusta*), based on the presence of symptoms recorded for 2 weeks after inoculation.
- The typical water-soaked spots appeared after 3 days incubation, becoming grey to black after 6 days.
- The leaf spots are circular with a regular margin and can reach 3 mm in diameter.
- They can also be surrounded by a bright-yellow halo.
- Pure cultures were easily reisolated, especially from the watersoaked lesions.
- We demonstrated that the lamb's lettuce strains were not pathogenic to anthurium.

Gardan *et al.*,2003

Pathogenicity test
Acidovorax avenae subsp.*citrulli*

- *Acidovorax avenae* subsp. *citrulli*
- This bacteria can be spread via contaminated seeds.
- By multiplying (watermelon) seeds in relatively warm and moist greenhouses, one can determine whether seed lots are contaminated.
- An alternative method is to use the "sweatbox".
- **Sweatbox test:**
- In the 'sweatbox' test, seeds are incubated under warm and moist conditions in a climate-controlled cell, where favourable conditions for development of the disease are created.
- Whenever the bacteria are present, the seedlings should show symptoms within 14 days.
- However, other species of bacteria can cause similar symptoms.

Koenraadt *et al.*,2005

Pathogenicity test

Acidovorax avenae subsp.*citrulli*

- Plant watermelon seed into any common, sanitized greenhouse potting mixture and maintain under conditions optimal for seed germination until seedling emergence.
- Plant a known *A. avenae* ssp. *citrulli*-infested seed sample as a check on the test conditions and subsequent disease development.
- Maintain the positive check in an isolated area of the greenhouse.
- Maintain relative humidity at 70% or higher, from the time seedlings emerge to final reading. Relative humidity should not be lower than 50% for more than 12 h.

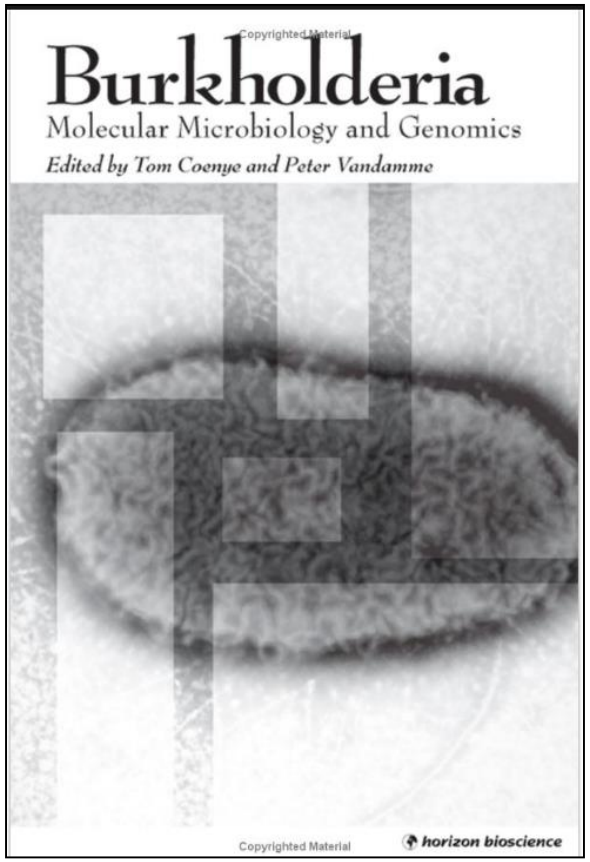
Koenraadt *et al.*,2005

Identification of the bacterial pathogens *Burkholderia*

Disease diagnosis and pathogen diagnostics

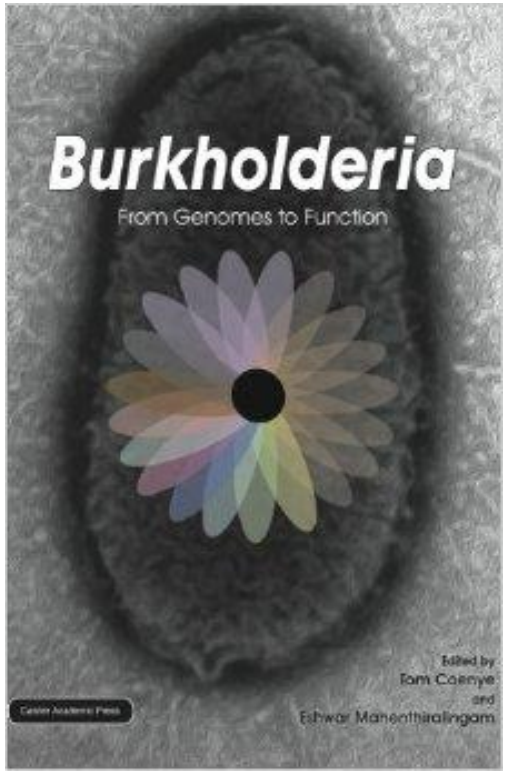
Burkholderia: Molecular Microbiology and Genomics

- by Tom Coenye and Peter Vandamme
- Taylor & Francis
- 2007
- 350 pages.



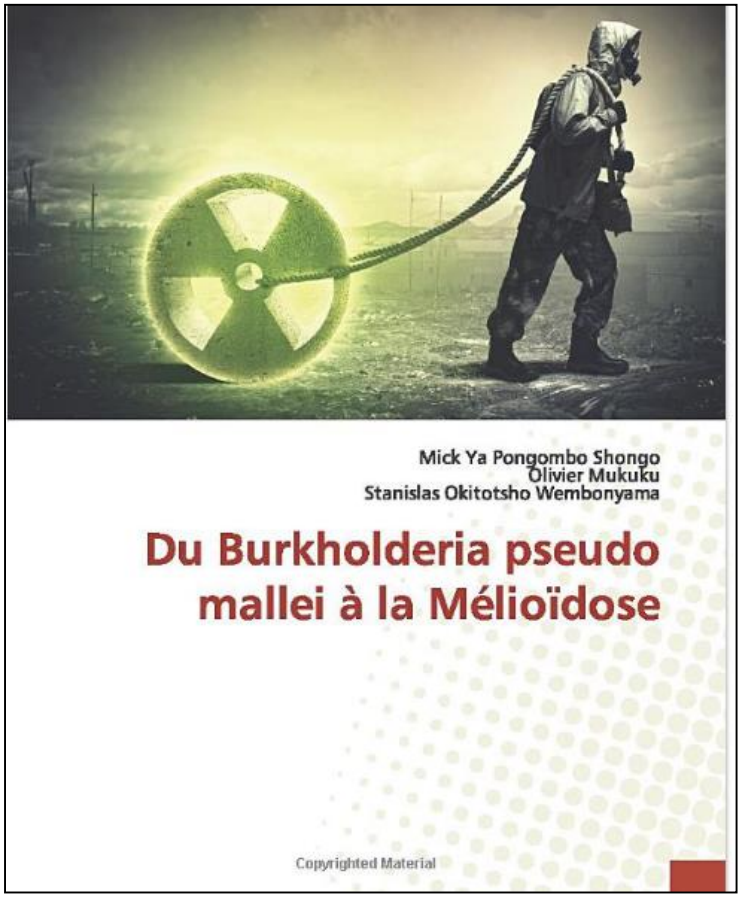
Burkholderia:
From Genomes to Function

- Tom Coenye and Eshwar Mahenthiralingam
- Caister Academic Press
- 2014
- Medical
- 253 pages.



Du Burkholderia pseudo mallei à la Mélioïdose

- by Mick Ya Pongombo Shongo, Olivier Mukuku, Stanislas Okitotsho Wembonyama
- Publisher: Éditions universitaires européennes
- French Edition
- 2020
- 84 pages.



Domain: Bacteria
Phylum: Proteobacteria

- Class: Betaproteobacteria
  - Order: Burkholderiales
    - Family: Comamonadaceae
      - Genus: *Acidovorax*
    - Family: Burkholderiaceae
      - Genus: *Burkholderia*
    - Family: *Ralstoniaceae*
      - Genus: *Ralstonia*
    - Family: ----------
      - Genus: *Xylophilus*

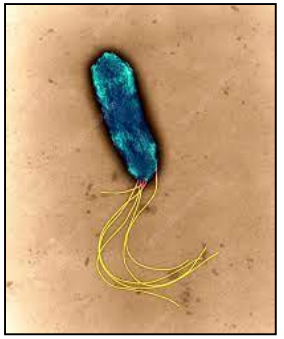
J.P. Euzéby,2010

Domain: Bacteria
Phylum: Proteobacteria

- Class: Betaproteobacteria
  Order: Burkholderiales
  Family: *Burkholderiaceae*
  Genus: *Burkholderia*

J.P. Euzéby,2010

Characteristics of
The genus *Burkholderia*



- The burkholderiads all have the following characteristics:
- Gram-negative,
- Polar or multitrichous flagella. *B. cepacia* is a motile organism, and motility is mediated by polar flagella.
- PHB positive,
- Most are strict aerobe and do not produce fluorescent pigment under iron limiting conditions.
- *B. glumae* is a nonfluorescent bacterium producing a yellow-green, water-soluble pigment on various media.
- *B. cepacia* as the type species.

The genus *Burkholderia*
Taxonomy

- The *Burkholderia* (previously part of *Pseudomonas*) genus name refers to a group of virtually ubiquitous Gram-negative, obligately aerobic, rod-shaped bacteria that are motile by means of single or multiple polar flagella, with the exception of *Burkholderia mallei* (animal pathogen), which is nonmotile.

1. *Burkholderia* harbored about 100 species, and
2. the *Burkholderia cepacia* complex (BCC) consisting of 22 species.

Espinosa-Victoria *et al.*,2020;..

The genus *Burkholderia*

New genera within *Burkholderia*

Updated taxonomy

- In recent years, *Burkholderia* s.l. was divided into several genera and it is currently classified into:

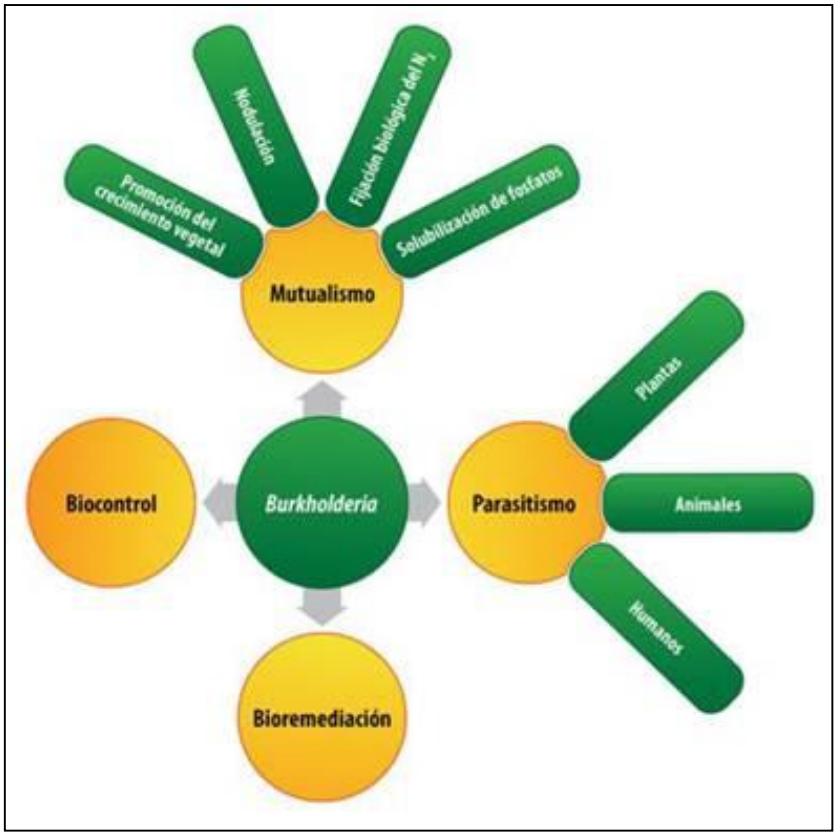
1. *Burkholderia* sensu stricto (s.s.),
2. *Paraburkholderia*
3. *Robbsia*
4. *Mycetohabitans*, and
5. *Trinickia*.

sensu lato- In the broad sense- abbreviation: s.l.
sensu stricto- "in the strict sense", abbreviation: s.s. or s.str.

The functional versatility the *Burkholderia* genus

- *Burkholderia* is a bifunctional genus because:

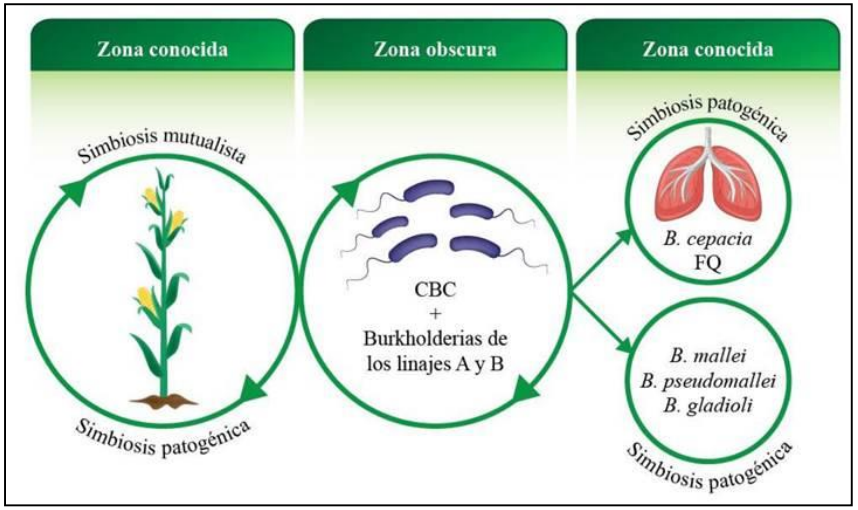
1. some of its species establish symbiotic-mutualistic relationships with plants,
2. while others establish symbiotic-pathogenic associations with plants, animals, and humans.



Espinosa-Victoria *et al.*,2020;..

The functional versatility the *Burkholderia* genus

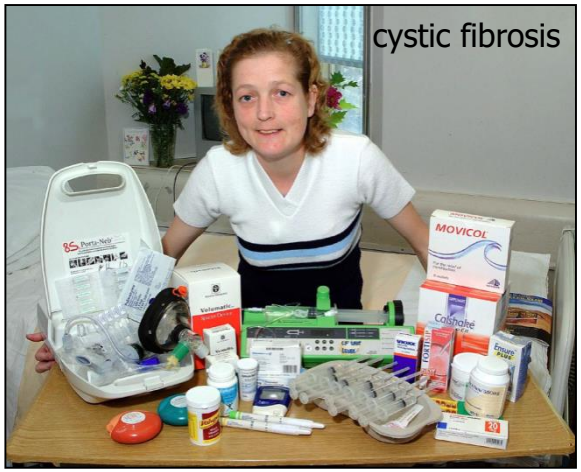
- Model of the mutualistic and pathogenic symbiosis of the *Burkholderia sensu lato* genus.
- The circle in the center shows the *Burkholderia* species pool (BCC and burkholderias of lineages A and B).
- The arrows indicate a dynamic taxonomy with a continuous species reclassification.



complexo *Burkholderia cepacia* (CBC)

Espinosa-Victoria *et al.*,2020

The genus *Burkholderia*
Ubiquitous in nature



- Abundant in:
1. Soil
2. Plants: rhizosphere, roots & shoots
3. Water (including sea water)
4. Humans & animals (infections)
5. Hospital environment.

- Environmental' isolates (safe) and 'clinical' isolates (dangerous).
- Human isolates are not necessarily distinct from environmental isolates.
- Clinical strains from the *B. cepacia* complex responsible for death in patients by **decline and failure of lung function (CF or cystic fibrosis).**

David Stead;Coenye,2003

The genus *Burkholderia*

Diversity in the genus *Burkholderia*

- Strongly underestimated!
- Extremely versatile genus comprising over 29 species including:

1. Primary human pathogen (e.g. *B. pseudomallei*)
2. Primary animal pathogen (e.g. *B. mallei*)
3. Primary plant pathogens (e.g. *B. glumae)*
4. Soil and plant associated organisms (e.g. *B. graminis* and *B. phytofirmans*).

Coenye,2003; Eberla and Vandamme,2016

Bacterial species described in the genus *Burkholderia*

- **Human/animal pathogens:**
- *B. mallei*, *B. pseudomallei.* Recently *B. gladioli* (see the next slides).
- **Plant pathogens:**
- *Robbsia* (ex. *Burkholderia*) *andropogonis*, *Trinickia caryophylli* (ex. *Burkholderia*), *B. glumae*, *B. cepacia*, *B. cenocepacia*, *B. gladioli*, *B. plantarii*
- **Opportunistic pathogens:**
- *B. ambifaria*, *B. cepacia*, *B. cenocepacia*, *B. gladioli*, *B. multivorans*, *B. pyrrocinia*, *B. stabilis*, *B. ubonensis*, *B. vietnamiensis*, *B. cepacia* genomovar VI

Coenye,2003; Eberla and Vandamme,2016

Bacterial species described in the genus *Burkholderia*

- **Nodulating species:**
- *B. phymatum*, *B. tuberum*, *B. cepacia* genomovar VI
- **Soil organisms:**
- *B. caledonica*, *B. caribensis*, *B. fungorum*, *B. graminis*, *B. hospita*, *B. terricola*, *B. thailandensis*, *B. glathei*, *B. kururiensis*, *B. phenazinium*, *B. sacchari*.

Coenye,2003; Eberla and Vandamme,2016

Bacterial species described in the genus *Burkholderia*

- **Plant endosymbiont:**
- *Candidatus B. kirkii*
- **Insect symbionts:**
- A large body of evidence demonstrates that many insect species harbor symbiotic bacteria of the genus *Burkholderia*.
- The association of *Burkholderia* species with the bean bug *Riptortus pedestris* has emerged as a promising experimental model to study the molecular mechanisms involved in insect-bacterium symbiosis.

Eberla and Vandamme,2016

The genus *Burkholderia*

Plant, animal and human causal agent *Burkholderia gladioli*

- *Burkholderia gladioli* are widely distributed and commonly found in different environments.
- The origin of these strains included:

1. plants, such as corn, rice, gladiolus and onion;
2. human clinical samples, including cystic fibrosis, sputum, nose-ethmoid sinus and ethmoid sinus,
3. animal samples including beetle eggs; and
4. environment samples including water and soil.

- Some *B. gladioli* strains live endophytically within various wild and ancient *Zea* plants without causing any disease symptoms.

Peng *et al.*,2021

The genus *Burkholderia*

Plant pathogen and endophyte

Burkholderia cenocepacia

- *Burkholderia cenocepacia* is both a plant pathogen and the cause of serious opportunistic infections, particularly in cystic fibrosis patients.
- *B. cenocepacia* strain with both biocontrol and plant-growth-promoting characteristics was also reported.

Eberla and Vandamme,2016

The genus *Burkholderia*

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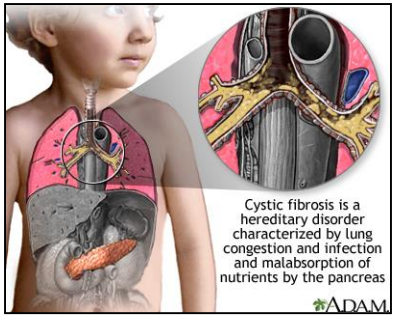

Eberla and Vandamme,2016

The genus *Burkholderia*

Endophyte with plant growth promotion

Burkholderia phytofirmans

- *Burkholderia phytofirmans* PsJN, which was originally isolated from onion roots and was subsequently demonstrated to establish endophytic populations in various plants.
- It is a novel plant-associated bacterium with plant-beneficial properties.
- Nitrogen fixation, plant growth promotion and increasing the yield in halotolerant crops such as quinoa under salinity stress was reported.

Eberla and Vandamme,2016; Yang *et al.*,2020

The genus *Burkholderia*

Endophyte with plant growth promotion

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Eberla and Vandamme,2016; Yang *et al.*,2020

The genus *Burkholderia*
B. cepacia complex(Bcc)

- *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) was once thought to be a single bacterial species but has expanded to the *Burkholderia cepacia* complex (Bcc), comprising 24 closely related opportunistic pathogenic species.
- These bacteria have:

1. a widespread environmental distribution,
2. an extraordinary metabolic versatility,
3. a complex genome with three chromosomes, and
4. a high capacity for rapid mutation and adaptation.

Tavares *et al.*,2020

The genus *Burkholderia*
Burkholderia and *B. cepacia* complex(Bcc)

- Several of the *Burkholderia* spp. including *B. cepacia* complex strains can be used as:

1. Biopesticides,
2. Nitrogen fixation
3. Biological control
4. Plant growth promoters
5. Bioremediation(use of microbes to clean up contaminated soil and groundwater by degrading environmental pollutants)

- **Are the 'good' and the 'bad' strains the same?**
- Because of the link of *Burkholderia* spp. to clinical problems, only a few beneficial *Burkholderia* spp. are used commercially.

Pelt *et al.*,1999; David Stead,Coenye,2003

The genus *Burkholderia*

Beneficial properties of *Burkholderia* spp. for agricultural improvement

Beneficial properties of nonhuman pathogenic *Burkholderia* spp.

Biocontrol

- Siderophore secretion
- Antibiotic secretion
- Elicitor of plant defenses (lippolysaccharide, siderophore,...)

Plant resistance against abiotic stresses

- ISR and others mechanisms

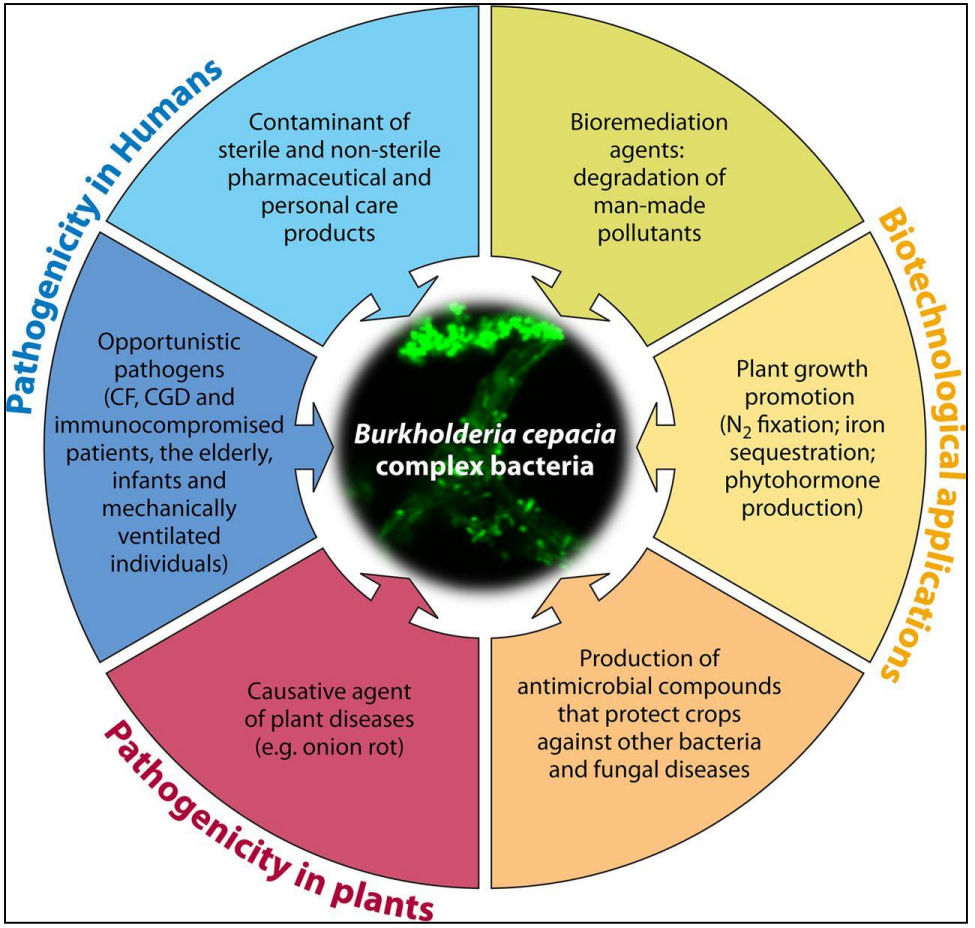
Plant growth-promotion

- N_2-fixation
- Nodulation
- Sequestration of iron
- Phosphate solubilization
- ACC deaminase activities
- Quinolinate phosphoribosyl transferase activity
- Phytohormones production

Compant *et al.*,2008

The genus *Burkholderia*
B. cepacia complex(Bcc)

- Strains of bacteria have potentially beneficial ecological and biotechnological applications, including their role in biocontrol, bioremediation, and plant growth promotion.
- Some members of the Bcc are human opportunistic pathogens.
- Some were associated with infection outbreaks due to contaminated pharmaceutical products.
- Some cause disease in plants.



Tavares *et al.*,2020

The genus *Burkholderia*

B. cepacia complex(Bcc)

CF or cystic fibrosis: decline and failure of lung function.

Species	Natural Environment	Clinical Environment
B. cepacia	Rhizosphere, soil, water	Cystic fibrosis (CF), medical solution
B. multivorans	Rhizosphere, soil, water	CF, CGD, non-CF
B. cenocepacia	Rhizosphere, plant, soil, water, animal	CF, non-CF
B. stabilis	Rhizosphere	CF, (rare) hospital equipment
B. vietnamiensis	Rhizosphere, plant, soil, water, animal	CF
B. dolosa	Maize rhizosphere, plant	CF
B. ambifaria	Rhizosphere, soil	CF (rare)
B. anthina	Rhizosphere, soil	CF (rare)
B. pyrrocinia	Rhizosphere, soil, water, plant	CF, non-CF (rare)
B. ubonensis	Soil	Nosocornial infection
B. letens	No environmental strain reported	CF
B. diffusa	Soil, water	CF, hospital equipment, non-CF
B. abroris	Rhizosphere, soil, water	CF, non-CF
B. seminalis	Rice rhizosphere	CF, nosocornial infection
B. metallica	No environmental strain reported	CF
B. contaminans	Soil, water, animal	CF, hospital equipment, non-CF
B. lata	Soil, water, flower	CF, non-CF

*Based on Ref. 11.

Barlasov *et al.*,2014

The genus *Burkholderia*
Foe: Fatal food poisoning

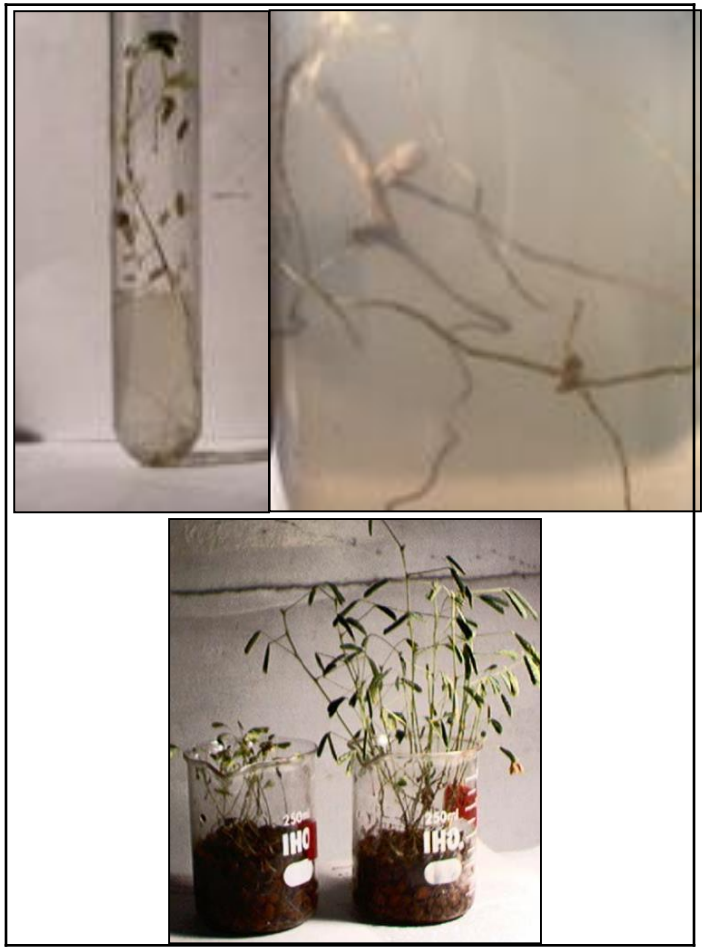
- The environmental bacterium *Burkholderia gladioli* pv. *cocovenenans* (*B. cocovenenans*) has been linked to fatal food poisoning cases in Asia and Africa.
- Bongkrekic acid (BA), a mitochondrial toxin produced by *B. cocovenenans*, is thought to be responsible for these outbreaks.
- *B. cocovenenans* is the only pathovar capable of producing BA and causing human death.

Peng *et al.*,2021

The genus *Burkholderia*

Friend: Nitrogen fixing, nodulating symbionts (*B. phymatum* & *B. tuberum*)

Species	Nodulated plant
Root endosymbionts	*Fabaceae*
B. cepacia complex	*Dalbergia* spp.
B. caribensis	*Mimosa diplotricha*
	Mimosa. pudica L.
B. mimosarum	*Mimosa* spp.
B. nodosa	*Mimosa* spp.
B. phymatum	*Machaerium lunatum* (L. f.) Ducke
	Mimosa invisa Mart.
	Mimosa pudica L.
	Mimosa pigra
	Mimosa casta
B. tuberum	*Aspalathus carnosa* Bergius
	Cyclopia spp.
Leaf endosymbionts	*Rubiaceae*
'*Candidatus* Burkholderia calva'	*Psychotria calva* Hiern
'*Candidatus* Burkholderia kirkii'	*Psychotria kirkii* Hiern
'*Candidatus* Burkholderia nigropunctata'	*Psychotria nigropunctata* Hiern



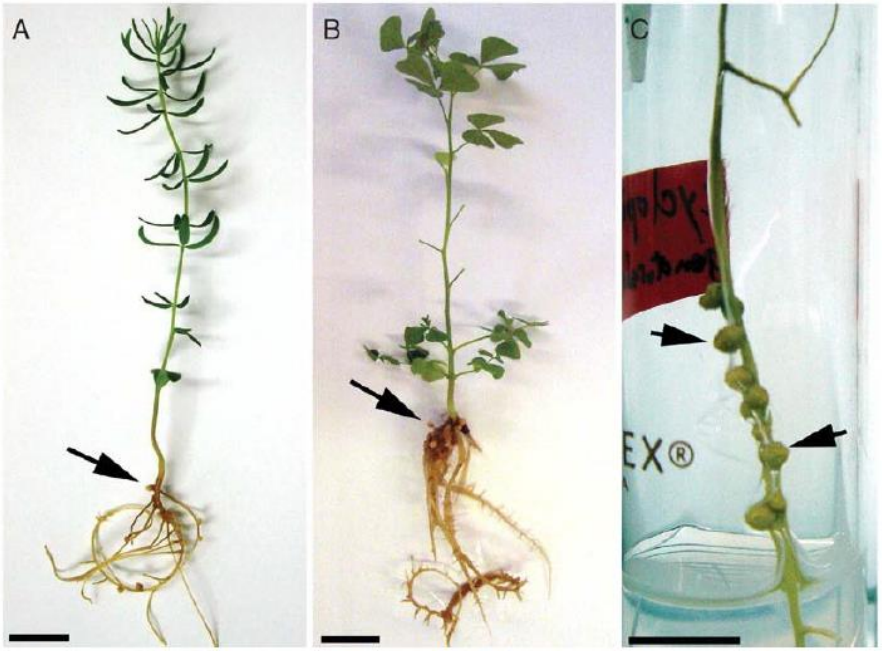
Coenye,2003;Compant *et al.*,2008

The genus *Burkholderia*

Friend: Nitrogen fixing, nodulating symbionts

Ecological diversity within the genus *Burkholderia*

- N_2 fixing and nodulation of *Cyclopia* spp. by *B. tuberum*



C. genistoides *C. galioides* *C. genistoides*

Elliott et al., 2007

T. Coenye

The genus *Burkholderia*

B. cepacia complex(Bcc)

Friend: Plant growth promotion

- Ecological diversity within the *B. cepacia* complex
- Plant growth promotion

Coenye,2003;..

The genus *Burkholderia*

B. cepacia complex(Bcc)

Friend: As biocontrol agents

Parke,2011

The genus *Burkholderia*

B. cepacia complex(Bcc)

Friend: As biocontrol agents

Ecological diversity within the *B. cepacia* complex

- Biocontrol of bacteria, fungi, yeasts, nematodes, protozoa

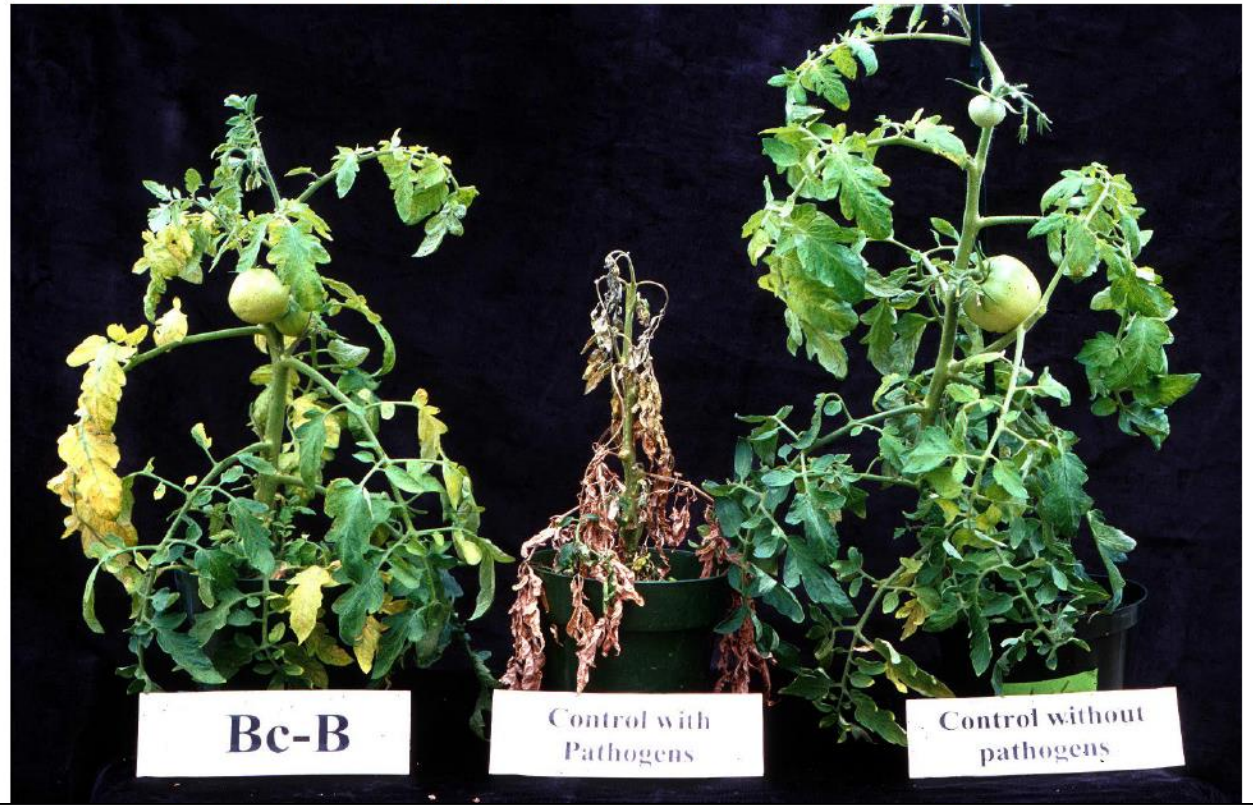
T. Coenye

The genus *Burkholderia*

B. cepacia complex(Bcc)

Friend: As biocontrol agents

- Ecological diversity within the *B. cepacia* complex
  - Biocontrol of bacteria, fungi, yeasts, nematodes, protozoa



T. Coenye

The genus *Burkholderia*

Friend: Biopesticidal protection of peas by *B. ambifaria* in soil infested with *Pythium*

- Pea seeds were planted in soil infested with the damping-off pathogen *Pythium* and as a result failed to germinate (left), coated with *Burkholderia* and germinated successfully when planted in the same infested soil due to protection from antimicrobial cepacin production (centre), and planted in un-infested soil where they germinated successfully (right).

Mullins and Mahenthiralingam,2021

The genus *Burkholderia*

Genome sequences

Burkholderia genome sequences

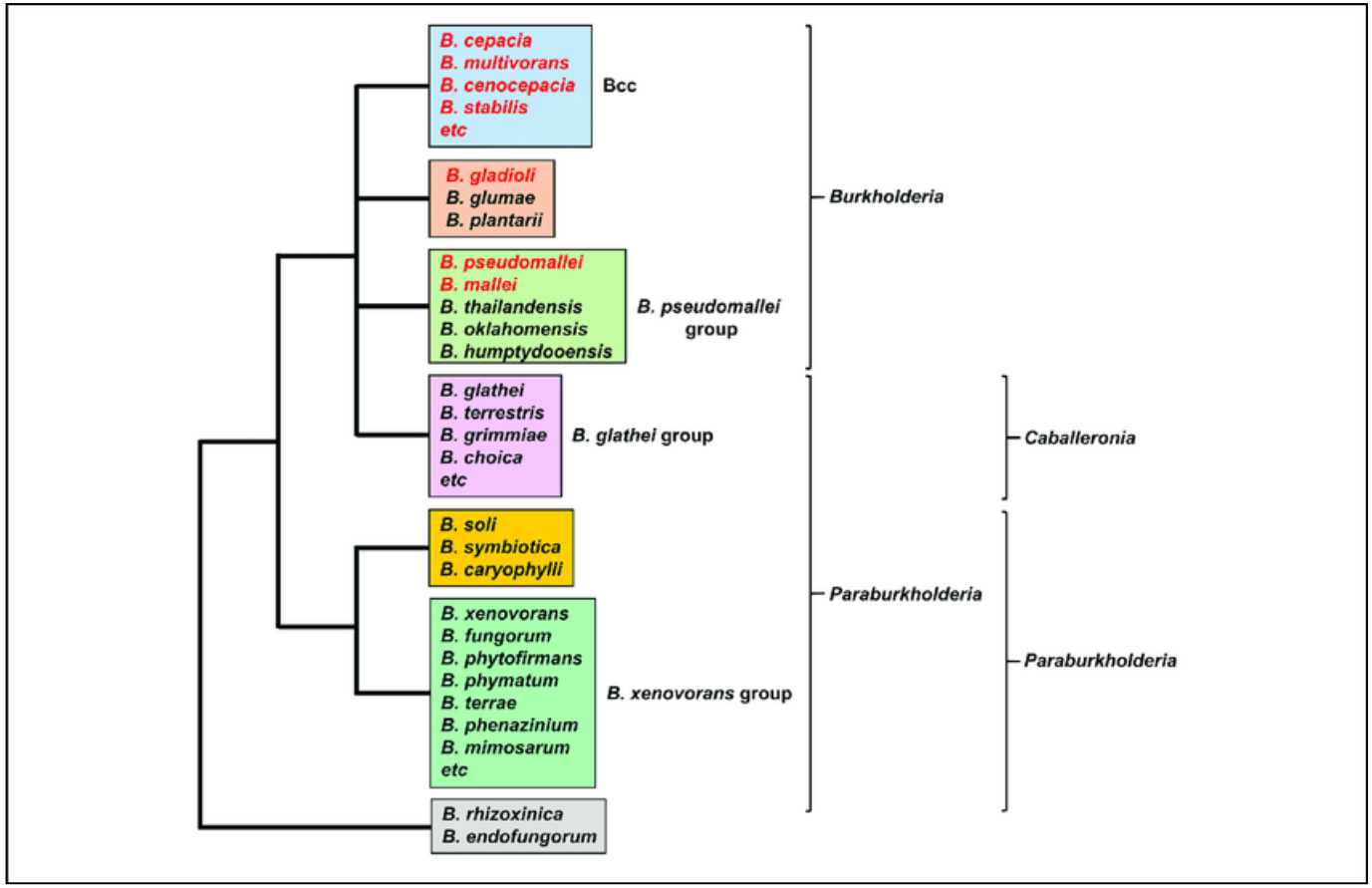
Species	Strain	No. of chr.	Size	No. of CDS
B. ambifaria	AMMD		7.5 Mb	6617
B. cepacia complex K	ATCC 17760	3	8.7 Mb	7836
	SAR-1 (meta)		8.5 Mb	6907
B. cenocepacia	J2315	3+p	8.1 Mb	7240
	AU1054		7.3 Mb	6555
	HI2424		8.1 Mb	7146
	PC184	3	7.0 Mb	
B. dolosa	AU0158	3	6.4 Mb	
B. vietnamiensis	G4		8.4 Mb	7992
B. mallei	>8 strains	2	5.8-6.1 Mb	4831-5201
B. pseudomallei	>7 strains	2	7.0-7.4 Mb	5465-5934
B. thailandensis	E264	2	6.7 Mb	5714
B. xenovorans	LB400	2+p	9.7 Mb	9012

The coding region of a gene, also known as the coding sequence or CDS.

T. Coenye

Taxonomy of the genus *Burkholderia*

Classification of Burkholderias/*B. cepacia* complex based on 16SrDNA analysis



Butt and Thomas,2017

The genus *Burkholderia*
Plant pathogenic spp.

- *Burkholderia cepacia*, appear to infect both plants and animals.
- Current taxonomy recognizes over 29 species, of which 7 are known plant pathogens:
- *Robbsia* (ex. *Burkholderia*) *andropogonis*
- *Trinickia caryophylli* (ex. *Burkholderia caryophylli*)
- *B. cenocepacia* (recently has been renamed from *B. cepacia*)
- *B. gladioli*
- *B. glumae*
- *B. plantarii*

B. graminis is a rhizospheric *Burkholderia* species associated with wheat root (soil and plant associated sp.). *B. gladioli* was also reported as opportunist human pathogen and one strain of *B. cenocepacia* as endophyte.

Coenye,2003; Jacobs *et al.*,2008; Eberla and Vandamme,2016

Diseases caused by *Burkholderia* spp.

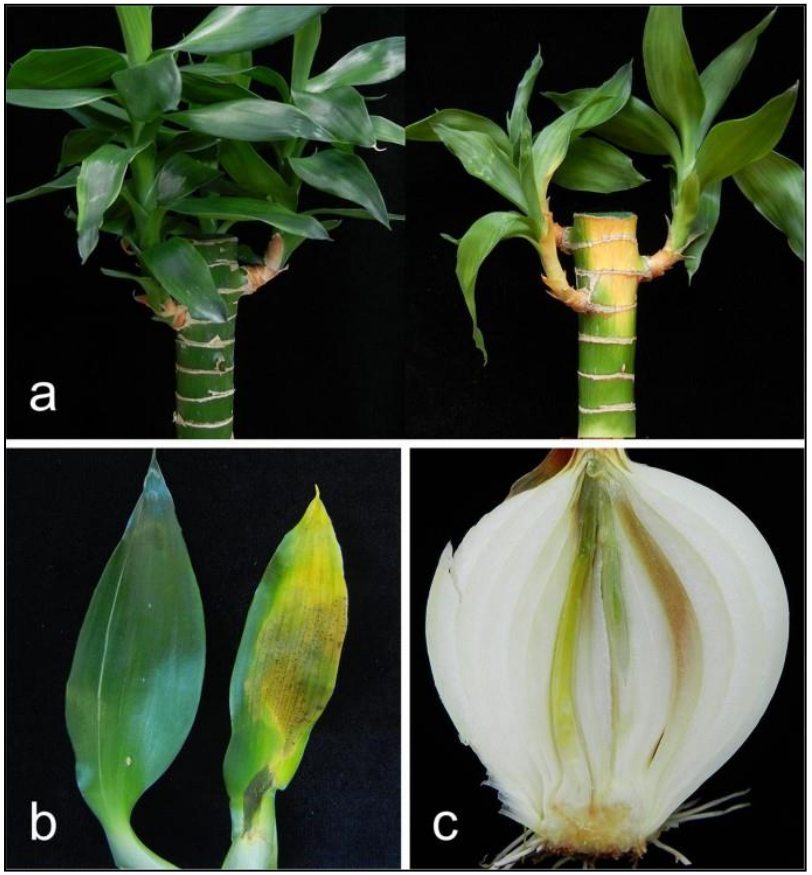
Robbsia* (ex. *Burkholderia*) *andropogonis	**Widest host range:** **Leaf blights on different hosts (Corn, Sorghum, Vicia, Phaseolus, Medicago,..)**
***Trinickia caryophylli* (ex. *Burkholderia caryophylli*)**	**Bacterial wilt or stem crack of carnation (*Dianthus caryophyllus*) and crown rot of statice (*Limonium* sp.)**
B. cepacia	**Disease on several plant hosts e.g. Chinese cabbage, leek (*Allium porrum*), onion (*A. cepa*) and shallot. The main plant disease associated with *B. cepacia* is sour skin of onion. Some are human pathogenic strains**
B. gladioli	**Corm and pseudobulb rot of Gladiolus**
B. gladioli* pv. *agaricicola	**Soft rot of mushrooms (*Agaricus bisporis*)**
B. gladioli* pv. *alliicola	**Slippery skin of onion (*Allium cepa*)**
B. gladioli* pv. *gladioli	**Disease on several plant hosts e.g. *Gladiolus* spp., *Crocus* spp., *Freesia* spp., and *Iris* spp., as well as several ferns (leaf spots and blight) and orchids.**
B. glumae	**Seedling blight and grain/sheath rot in rice (*Oryza sativa*)**
B. plantarii	**Bacterial seedling blight of rice (*Oryza sativa*)**

B. cenocepacia has been isolated as the causal agent of fingertip rot disease of banana and often associated with *B. cepacia* in onion rot diseases.

The genus *Burkholderia*

Burkholderia cepacia

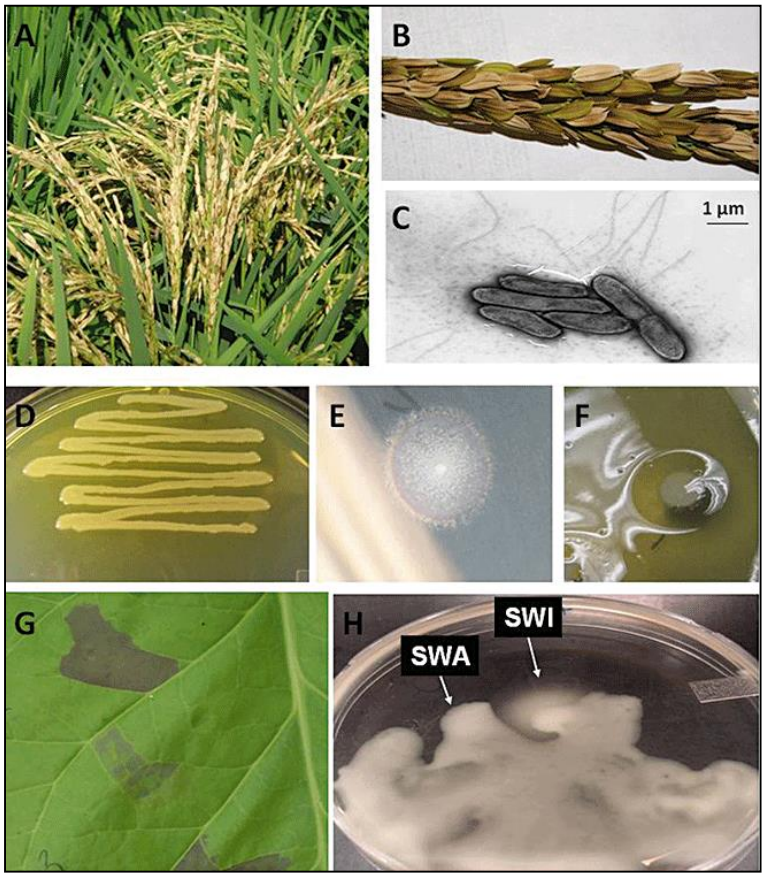
- Bacterial blight on Dracaena sanderiana also called 'Lucky Bamboo' caused by *Burkholderia cepacia*.



Choi *et al.*,2020

The genus *Burkholderia*
B. plantarii

- Symptoms and virulence factors produced by *Burkholderia glumae*:
- (A, B) typical symptoms of bacterial panicle blight on rice panicles;
- (C) *B. glumae* cells and flagella;
- (D) toxin (toxoflavin) production by *B. glumae* indicated by the yellow pigment on a King's B agar plate;
- (E) lipase activity;
- (F) pectinase activity
- (G) hypersensitive reaction (HR) on a tobacco leaf
- (H) swimming (SWI) and swarming (SWA) motilities on an LB with 0.3% agar plate.



Ham *et al.*,2010

Bacterial diseases

Robbsia (ex. *Burkholderia*) *andropogonis*

		
Bacterial leaf stripe of sorghum	**Blight of golden cane palms**	**Leaf spot of white clover**

Scortichini,2006;…

Bacterial diseases of onions, gladiolus, iris

Burkholderia gladioli

		
Gladiolus plant inoculated with *B. gladioli*	slippery skin (*Burkholderia gladioli* pv. *alliicola*)	bacterial panicle blight of rice (*Burkholderia glumae*)

Isolation

Trypan Blue Tetracycline (TBT) Medium

Burkholderia cepacia

- Trypan Blue Tetracycline (TBT) Medium (Hagedorn *et al.*,1987) is one of the recommended media for *B. cepacia* isolation.

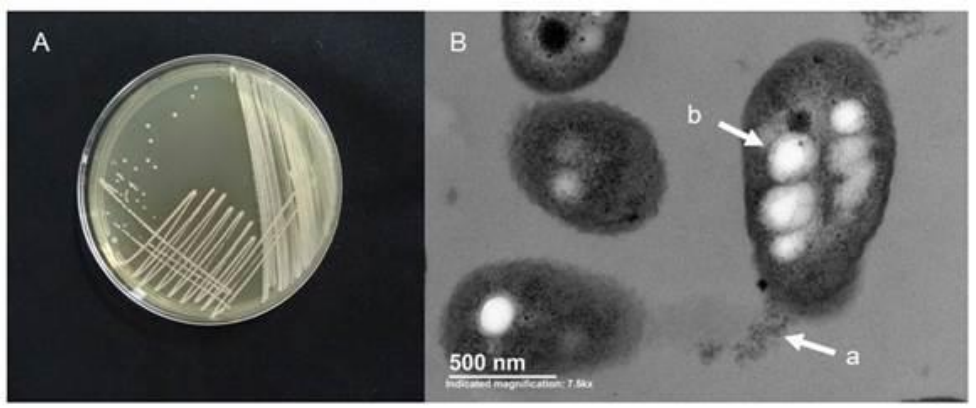
Glucose	20.0 g
L-Asparagine	1.0 g
$NaHCO_3$	1.0 g
KH_2PO_4	500.0 mg
$MgSO_4 \cdot 7H_2O$	100.0 mg
Trypan Blue	50.0 mg
Tetracycline	20.0 mg
Agar	20.0 g
Distilled water	1.0 liter

pH was adjusted to 5.5 with 10% phosphoric acid (4 ml/liter). The tetracycline was filter-sterilized and added after autoclaving. For low soil dilutions it was recommended that crystal violet, 5 mg/l, and filter sterilized nystatin, 50 mg/1, be added.

Colony morphology
Burkholderia cepacia

A. *Burkholderia cepacia* BUAP-AM51 culture growing in trypticasein-soybean agar.

B. Microphotography of the same strain, using transmission electron microscopy, where the multitrichous flagella (a) and polyhydroxybutyrate granules, PHB (b) are observed.



Espinosa-Victoria *et al.*,2020

Selective medium for *P. glumae* S-PG Tsushima *et al.*,1986

- Cetrimide 0.01 g
- EDTA-Fe 0.01 g
- Phenol red 0.02 g
- Na_2 MoO. $2H_2O$ 0.024 g
- $MgSO_4.7H_2O$ 0. 25 g
- Na_2 HPO_4 1.2 g
- KH_2PO_4 1.3 g
- $(NH4)_2SO_4$ 5.0 g
- D-sorbitol 10.0 g
- Agar 20.0 g
- L-cystine 10.0 g
- Methyl violet 1.0 mg
- Distilled H_2O 1.0 L

Mew and Misra,1994

Characteristics of species of *Burkholderia*

Characteristics	*andropogonis*	*caryophylli*	*cepacia*	*gladioli*	*gladioli* pv. *agaricico*	*glumae*	*plantarii*
Oxidase	-	+	+	v	+	ND	ND
Growth at pH 4	ND	-	+	+	ND	-	+
Growth at pH 8	ND	-	+	+	ND	V	-
Growth at pH 9	ND	-	-	-	ND	-	-
Growth at 40°C	-	+	+	+	-	+	+
Growth in 3% NaCl	ND	-	V	-	ND	+	-
Arginine dihydrolase	-	+	-	-	-	+	V
Gel hydrolysis	+	-	-	V	+	+	+
Starch hydrolysis	ND	-	-	-	+	-	-
Pectate hydrolysis	-	-	+	+	ND	-	-
Colonies wrinkled, yellowish	-	-	-	-	-	-	-
Utilization of:							
β-alanine	-	V	V	V	ND	+	+
Arginine	-	+	+	+	+	+	+
Betaine	-	+	+	+	+	+	+
Glycine	-	-	V	+	ND	ND	ND
Isoleucine	-	V	+	+	ND	V	-
L-valine	-	+	+	+	ND	+	+
Adonitol	+	+	+	+	+	+	-
Benzoate	V	-	V	+	ND	-	-
Cellobiose	ND	+	+	+	ND	ND	ND
Lactose	+	-	V	V	+	-	-
Levulinate	-	-	+	-	ND	+	-
N-propanol	ND	V	+	V	ND	+	V
L-rhamnose	ND	V	-	-	+	-	V
D-sorbitol	+	+	+	+	+	+	+
Sucrose	-	+	+	V	+	-	V
D-tartrate	-	-	-	+	ND	-	V
Trehalose	+	V	V	+	+	+	-
D-xylose	-	+	+	+	+	+	0

-, 80% or more strains positive; V, between 21-79% of strains positive; -, 80% or more strains negative; ND, not determined.

Schaad *et al.*,2001

Characteristics of new mushroom soft-rot isolate (OM1) compared with type culture strain of *B. gladioli* pv. *agaricicola*

1. On nutrient agar the colonies were dirty white, flat semi-translucent and smooth with a greenish yellow zone, non-fluorescent, yellow diffusible pigment.
2. Colonies on nutrient agar+1% dextrose were flat convex, cream-coloured, characteristically wrinkled with a diffusible, non-fluorescent yellow pigment.
3. On King's B agar, the colonies were yellow/green, circular, pulvinate, slightly viscid, and produced a diffusible non-fluorescent yellow pigment.

Lee *et al.*,2010

Characteristics of new mushroom soft-rot isolate (OM1) compared with type culture strain of *B. gladioli* pv. *agaricicola*

- Characteristics of *B. gladioli* pv.*agaricicola*, the new soft rot disease of mushrooms.
- Isolate OM1 had an oxidative metabolism of glucose.
- Produces pectinase on potato and carrot slices.

Characteristic	OM1	*B. gladioli* pv. *agaricicola* NCPPB 3580
No. of flagella	1-2	1-2
Gram reaction	-	-
Levan production	-	-
Diffusible pigments	+	+
Poly-β-hydroxybutyrate	+	+
Slime production from sucrose	+	+
Gelatine liquefaction	+	+
Lecithinase (egg yolk)	+	+
Lipase (Tween 80 hydrolysis)	+	+
Oxidase reaction	-	-
Arginine dihydrolase	-	-
Tobacco hydrolysis	+	+
Potato rot	+	+
Nitrate reduction	-	-
H2S production	-	-
Blotch test	-	-
White line	-	-
Growth at 4°C	±[a]	±
Growth at 41°C	-	-

OM1, mushroom soft-rot bacteria; [a]very slight growth at the bottom of the tube.

Lee *et al.*,2010

Differentiation of *B. planterii* from *B. glumae* and other *Burkholderia* species

Tropolone production

- In contrast to other *Burkholderia* and *Pseudomonas* spp. tested, none of the *B. planterii* strains grew at 40ºC and produced the toxin tropolone in a shake culture in Ayers agar supplemented with 1% glucose and 100ppm iron.
- Also, in contrast to *B. glumae*, *B. planterii* was oxidase positive, and grew in Cohn's solution.

Gitaitis and Nischwitz,2006

Identification based on:

Toxin production

Toxoflavin and Tropolone

- Pathogenic *Burkholderia* strains produced a yellow pigment in King's B medium.
- Avirulent strains did not produce this pigment.

1. Toxoflavin

- *Burkholderia glumae* causes bacterial seedling and grain rot in rice (*Oryza sativa*) and other grasses produces the phytotoxin toxoflavin responsible for characteristic symptoms of bacterial grain rot on rice.
- *B. glumae* isolates that did not produce the toxin did not cause bacterial seedling or grain rot.
- In addition to the toxin, it is assumed that *B. glumae* produces enzymes that result in the rotting of invaded plant tissue.

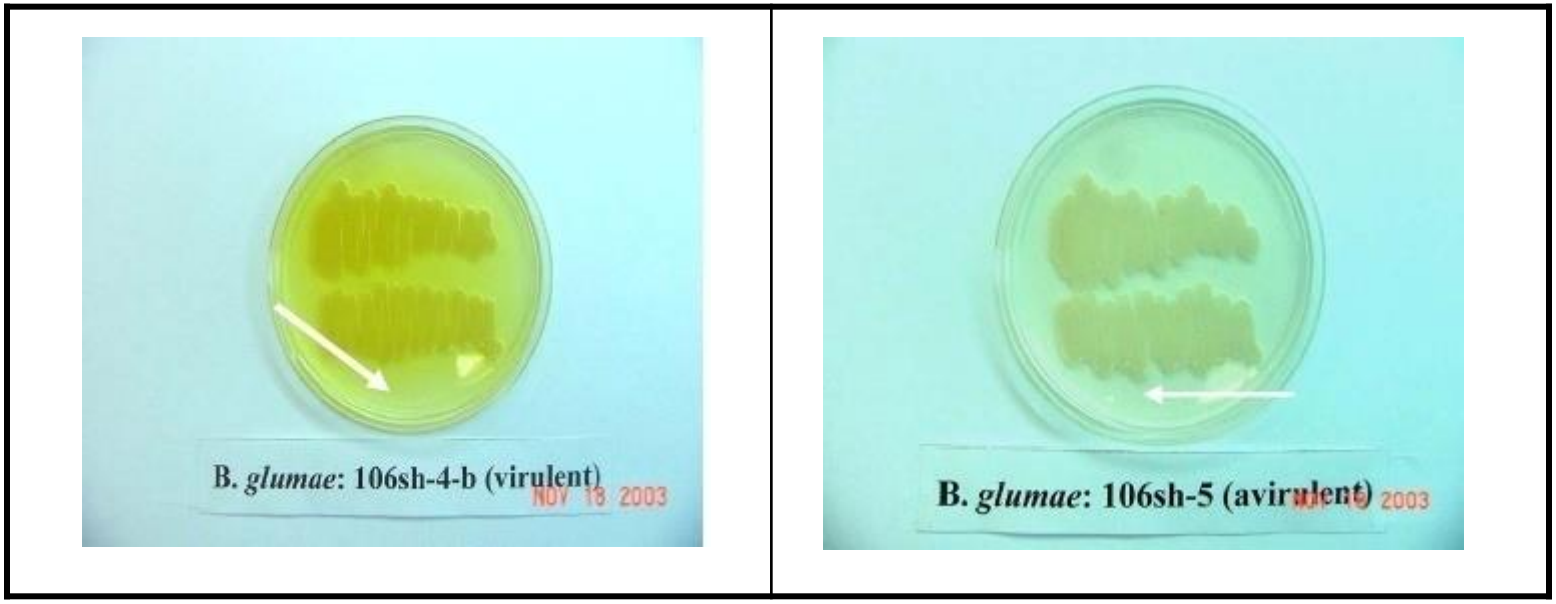
2. Tropolone

- *Burkholderia planterii*, the causal of bacterial seedling blight of rice produces the toxin tropolone which is responsible for the symptoms observed on rice seedlings.

Gitaitis and Nischwitz,2006

Pigment (toxin) production

B. glumae cultures that produced the yellow or green pigments (Toxoflavin) were pathogenic and virulent

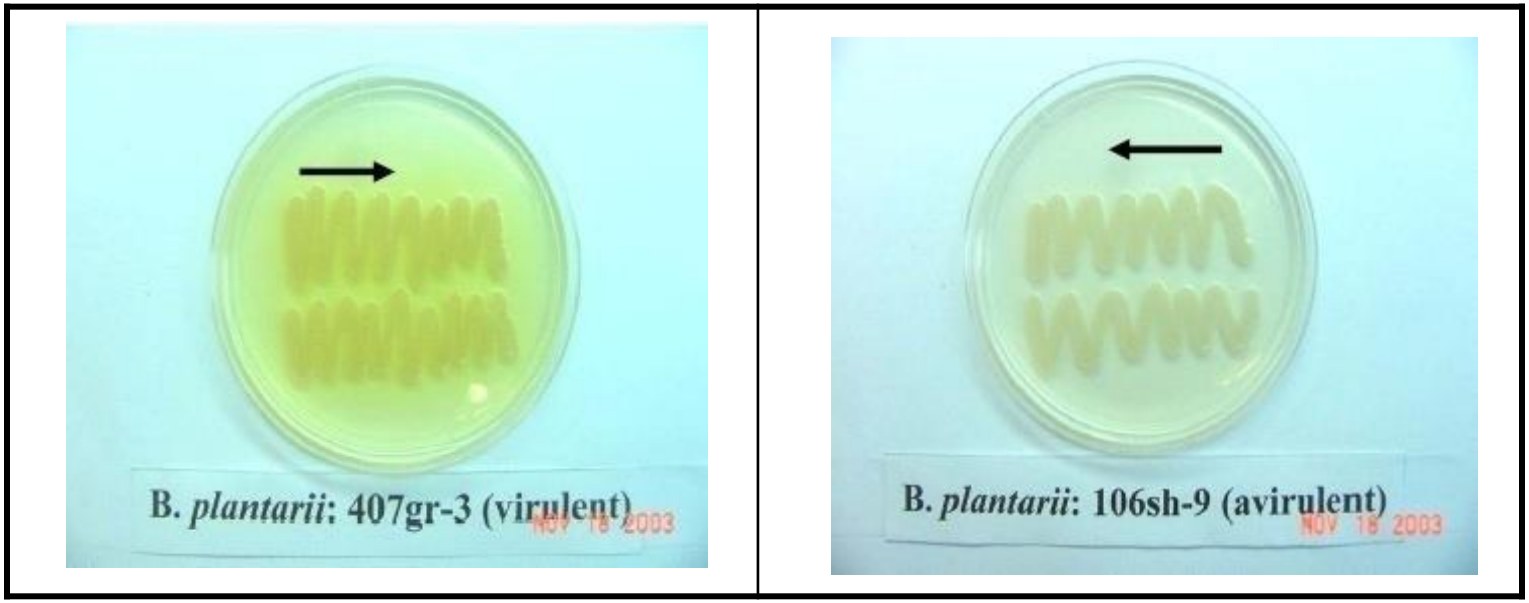


- Cultures of *Burkholderia glumae* with a virulent strain on the left and an avirulent strain on the right.
  - Note the lack of toxin in the medium on the right.

Yuan,2004

Pigment (toxin) production

B. plantarii cultures that produced the yellow or green pigments (Tropolone) were pathogenic and virulent



- Cultures of *B. plantarii*, virulent strain (left) and avirulent strain (right).
- Note the yellow toxin in the virulent culture and the lack of yellow toxin in the medium of the avirulent strain.

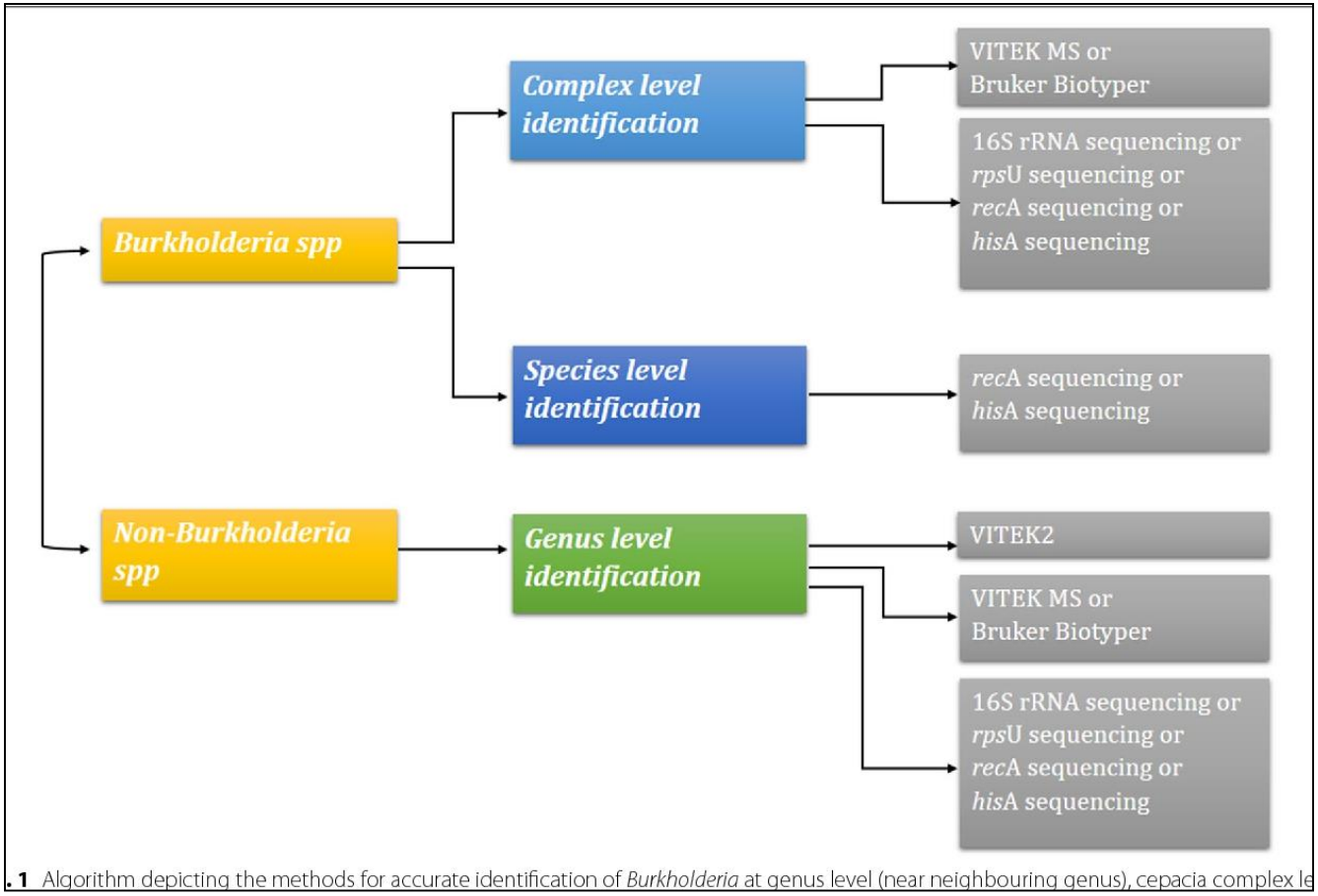
Yuan,2004

Identification based on:
Chitinase production

- Chitin is an essential component of fungal cell walls.
- *Burkholderia gladioli* is the only known bacterial species other than *Streptomyces* where many strains produce family 19 chitinase (chiB gene).
- Chitin hydrolysis was also reported from *Serratia* spp.
- Using specific primers for the chiB gene, PCR could distinguish *B. gladioli* pathovars from other bacterial species and genera.

Gitaitis and Nischwitz,2006

Accurate identification and epidemiological characterization of *Burkholderia cepacia* complex



. 1 Algorithm depicting the methods for accurate identification of *Burkholderia* at genus level (near neighbouring genus), cepacia complex le

Ragupathi *et al.*,2019

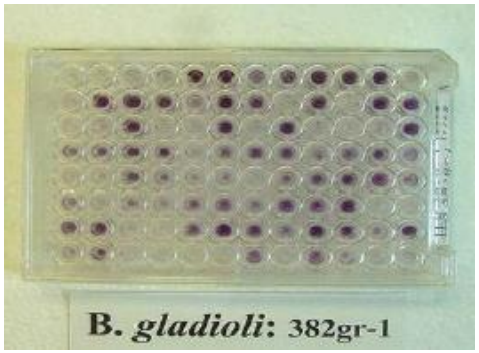
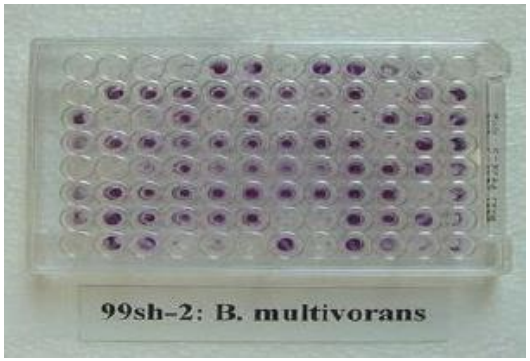
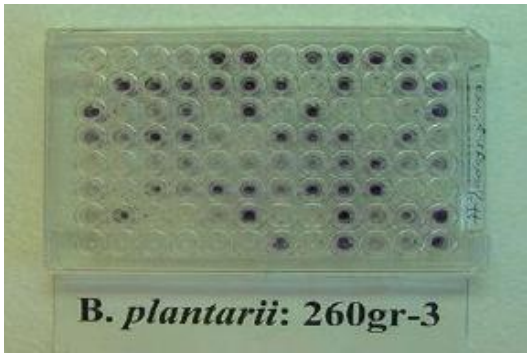
Identification based on:
Diagnostic kits

1. Biolog™ GN2 Microplate System.
2. Vitek GNI reliably identify most of the *B. cepacia* strains, but it encountered major problems with *B. gladioli* strains.
3. The API 20NE gave a doubtful but essentially correct identification.

- The best system for the biochemical identification of *B. cepacia* appeared to be the API 20NE test.
- All of the *B. cepacia* and *B. gladioli* strains were positive in the oxidase assay.

Pelt *et al.*,1999

Identification of strains
Diagnostic kits

		
Biolog GN2 Profile of *B. gladioli* isolate 382gr-1.	BiologGN2 profile of *B. multivorans* isolate 99sh-2.	Biolog GN2 Profile of *B. plantarii* isolate 260gr-3.

Yuan,2004

Identification based on:
Molecular methods

- 16S and *recA* RFLP,
- AFLP,
- Ribotyping
- 16S rDNA and *recA* sequence analysis
- PCR targeting the 16S-23S rDNA spacer region also distinguish *Burkholderia* spp. from other genera.
- Multilocus sequence typing (MLST) analysis is a new technique for strain discrimination.

Pelt *et al.*,1999;Coenye,2003

PCR primers for *Burkholderia* spp.

Specificity	Primer Designiation	Sequence	Size (bp)
B. andropogonis	Pf Pr	(5'AAGTCGAACGGTAACAGGGA3') (5'AAAGGATATTAGCCCTCGCC3')	410
B. gladioli	CMG-23-1 G-23-2	(5'ATAGCTGGTTCTCTCCGAA3') (5'CCTACCATGCAYATAAAT3')	388
	CMG-16-1 G-16-2	(5'AGAGTTTGATCMTGGCTC3') (5'CGAAGGATATTAGCCCTC3')	468
B. cepacia	CMG-23-1 CM-23-2	(5'ATAGCTGGTTCTCTCCGAA3') (5'CTCTCCTACCATGCGYGC3')	388
	CMG-16-1 CM-16-2	(5'AGAGTTTGATCMTGGCTC3') (5'CGAAGGATATTAGCCCTC3')	468
B. glumae	1416S: 1414A: 1417S: 1417A: 1418S: 1418A: 1419S: 1419A:	(5' GAGAGAATCGAGCCATGAAC) (5' GAGCGCATCCAGAACGAAGT) (5' GACTCACACCAGGCAGGAAGT) (5' ATTTCGGACTCGGTATGCAGC) (5' GCGATATGGCAAGACGCAAA) (5' AGTCATACCCTTTGTCAGCGT) (5' ACCCGTTTTGATAGAGGTGCG) (5' ACGAAGGTCTGCTGGTAAATCC)	873 925 571 628

Schaad *et al.*,2001

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

Genus *Burkholderia*

Genus *Burkholderia*					
Species	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
B. andropogonis	**Pf/Pr** 16S rRNA gene	Conventional	Bacteria (DNA extraction)	Bagsic *et al.*, 1995	*Pseudomonas andropogonis*
B. caryophylli	**P1240-5´/P480-5** 16S rRNA gene	Conventional and BOX-PCR	Bacteria (boiled)	Anon., 2006a	Amplify also other species (but shows a distinct profile for *B. caryophilli*). Adviced in the EPPO protocol.
B. cepacia	**PSL1/PSR1** 16S rRNA gene **PSL/PSR** 16S rRNA gene **G1/G2** ITS region	Conventional and RFLP	Bacteria (DNA extraction)	Whitby *et al.*, 1998; McDowell *et al.*, 2001	
B. gladioli	**CMG16-1/G-16-2** 16S rRNA gene **CMG-23-1/G-23-2** 23S rRNA gene	Conventional	Bacteria (DNA extraction)	Bauernfeind *et al.*, 1998	
B. gladioli	**Eub-16-1** Eubacteria 16S rDNAs **Gl-16-2** *B. gladioli* 16S rRNA gene	Conventional	Bacteria (DNA extraction)	Bauernfeind *et al.*, 1999	
B. glumae	**R16-1/R23-2R** ITS region	Conventional	Bacteria (DNA extraction)	Kim and Song, 1996	*Pseudomonas glumae* *Pantoea agglomerans* (*Erwinia herbicola*), *Pseudomonas fuscovaginae*, *Pseudomonas syringae* pv. *syringae* and *Xanthomonas oryzae* (pathovars *oryzae* and *oryzicola*) also amplified and differentiated by primary and secondary fragments.
B. glumae	**GL-13f/GL-14r** ITS region	Conventional	Bacteria or plant tissue (boiled)	Takeuchi *et al.*, 1997	
B. glumae	**Forward/Reverse** ITS region	Real-time (SBYR® Green Master Mix)	Seed washes and plants (without extraction step)	Sayler *et al.*, 2006	
B. plantarii	**PL-12f/PL-11r** ITS region	Conventional	Bacteria or plant tissue (boiled)	Takeuchi *et al.*, 1997	

Palacio-Bielsa *et al.*,2009

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

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B. caryophylli	**P1240-5´/P480-5** 16S rRNA gene	Conventional and BOX-PCR	Bacteria (boiled)	Anon., 2006a	Amplify also other species (but shows a distinct profile for *B. caryophilli*). Adviced in the EPPO protocol.
B. cepacia	**PSL1/PSR1** 16S rRNA gene **PSL/PSR** 16S rRNA gene **G1/G2** ITS region	Conventional and RFLP	Bacteria (DNA extraction)	Whitby *et al.*, 1998; McDowell *et al.*, 2001	
B. gladioli	**CMG16-1/G-16-2** 16S rRNA gene **CMG-23-1/G-23-2** 23S rRNA gene	Conventional	Bacteria (DNA extraction)	Bauernfeind *et al.*, 1998	
B. gladioli	**Eub-16-1** Eubacteria 16S rDNAs **Gl-16-2** *B. gladioli* 16S rRNA gene	Conventional	Bacteria (DNA extraction)	Bauernfeind *et al.*, 1999	
B. glumae	**R16-1/R23-2R** ITS region	Conventional	Bacteria (DNA extraction)	Kim and Song, 1996	*Pseudomonas glumae* *Pantoea agglomerans* (*Erwinia herbicola*), *Pseudomonas fuscovaginae*, *Pseudomonas syringae* pv. *syringae* and *Xanthomonas oryzae* (pathovars *oryzae* and *oryzicola*) also amplified and differentiated by primary and secondary fragments.
B. glumae	**GL-13f/GL-14r** ITS region	Conventional	Bacteria or plant tissue (boiled)	Takeuchi *et al.*, 1997	
B. glumae	**Forward/Reverse** ITS region	Real-time (SBYR® Green Master Mix)	Seed washes and plants (without extraction step)	Sayler *et al.*, 2006	
B. plantarii	**PL-12f/PL-11r** ITS region	Conventional	Bacteria or plant tissue (boiled)	Takeuchi *et al.*, 1997	

Palacio-Bielsa *et al.*,2009

PCR amplification and sequencing of gyrB and rpoD *Burkholderia* spp.

- PCR of gyrB and rpoD was performed using primers UP-1E and AprU, and 70F2 and 70R2, respectively, as shown in next Table.
- PCR was performed with a thermal cycler (TaKaRa) using PCR buffer (TaKaRa) containing 200 μM of each of the dNTPs, 0.5 μM of each primer, 0.2 μg template DNA and 2.5 U Ex-Taq polymerase (TaKaRa), in a total volume of 40 ml.
- A total of 35 amplification cycles were performed with template DNA denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min and primer extension at 72°C for 1 min.
- PCR products were electrophoresed on 1.0% agarose gels and purified using Quantum Prep Freeze 'N Squeeze DNA Gel Extraction spin columns (Bio-Rad), following the manufacturer's instructions.

Maeda *et al.*,2006

PCR and sequencing primers used in this study

Burkholderia spp.

- UP-1E/Apru for amplification of gyrB gene.
- 70F2/70R2 for amplification of rpoD gene.

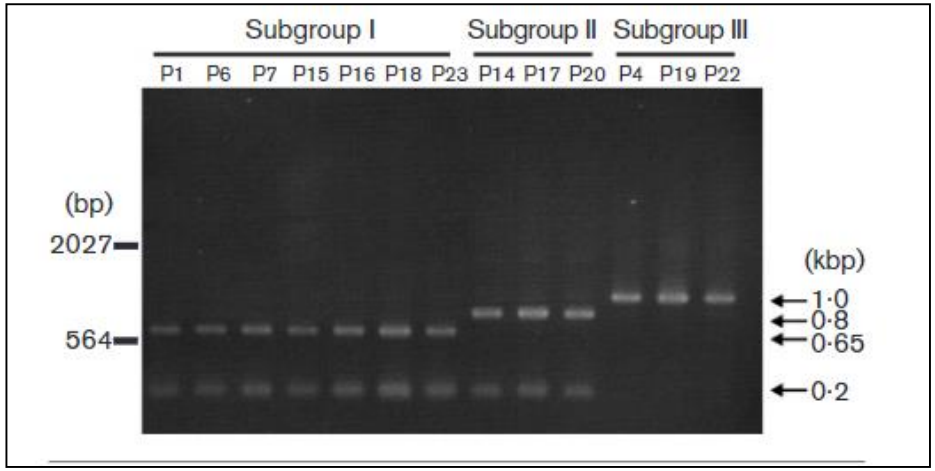
Primer	Target gene	Sequence (5′–3′)	Length (nt)	Reference
UP-1E	*gyrB*	CAGGAAACAGCTATGACCAYGSNGGNGGNAARTTYRA	37	Yamamoto *et al.* (2000)
AprU		TGTAAAACGACGGCCAGTGCNGGRTCYTTYTCYTGRCA	38	Yamamoto *et al.* (2000)
70F2	*rpoD*	ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGG	38	This study
70R2		ATAGAAATAACCAGACGTAAGTTNGTRTAYTTYTTNGCDAT	41	This study
glu-FW	*gyrB* of *B. glumae*	GAAGTGTCGCCGATGGAG	18	This study
glu-RV		CCTTCACCGACAGCACGCAT	20	This study
pla-FW	*gyrB* of *B. plantarii*	TCGAGCTGGCTGCGCCTC	18	This study
pla-RV		GTCGTCGCCCGAGGTCTCG	19	This study
gla-FW	*gyrB* of *B. gladioli*	CTGCGCCTGGTGGTGAAG	18	This study
gla-RV		CCGTCCCGCTGCGGAATA	18	This study

N, any; R, A or G; S, C or G; Y, C or T; M, A or C.

Maeda *et al.*,2006

PCR and sequencing primers used in this study *Burkholderia* spp.

- 23 rice strains were divided into three subgroups.
- Nucleotide sequences of the gyrB PCR products from strains in subgroup I showed one restriction site for both *Hae*II and *Sac*I (data not shown).
- One restriction site for *Hae*II was located in the gyrB PCR products from strains in subgroup II.
- No *Hae*II and *Sac*I restriction sites were present in the gyrB PCR products from strains in subgroup III.
- *Sac*I and *Hae*II digestion of the gyrB PCR products allowed discrimination of strains among subgroups I, II and III.

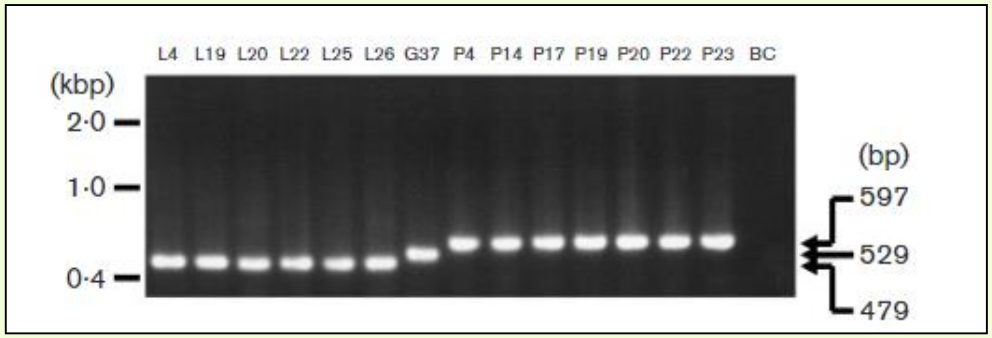


Ethidium bromide-stained gel of *Hae*II- and *Sac*I-digested partial fragments of gyrB amplified by PCR from *B. plantarii* strains using primers UP-1E and AprU.

Maeda *et al.*,2006

Phylogenetic relationships among *B. plantarii* strains *Burkholderia* spp.

- Sensitive detection of *B. plantarii* , *B. glumae* and *B. gladioli* by multiplex PCR.
- The expected sizes of partial fragments of gyrB amplified by PCR from genomic DNA of *B. plantarii*, *B. glumae* and *B. gladioli* were 597, 529 and 479 bp, respectively.



Maeda *et al.*,2006

Pathogenicity test

Rice seedling blight *Burkholderia* spp.

B. plantarii and *B. glumae*

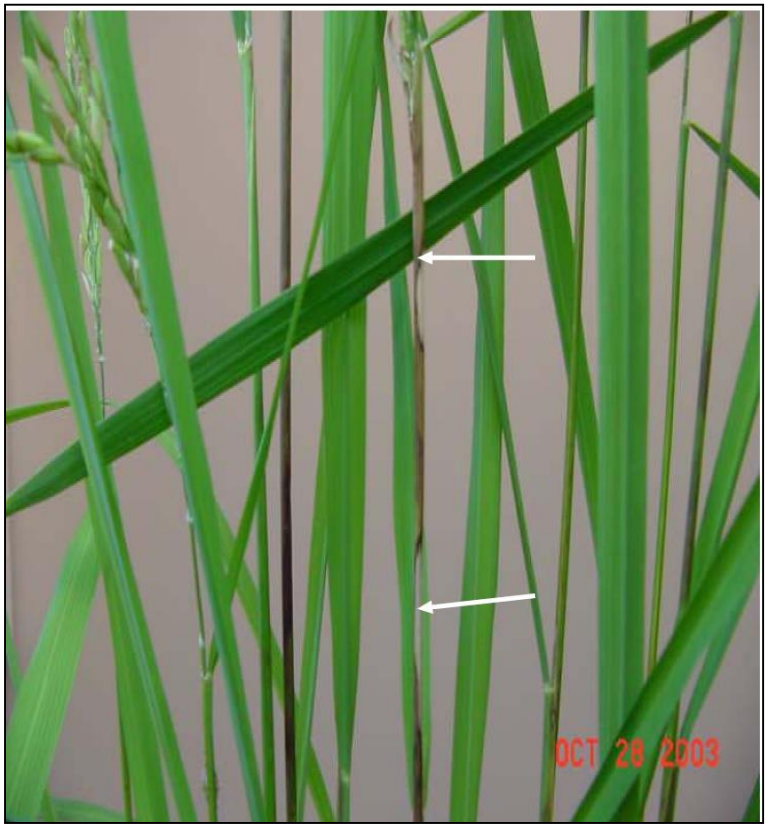
- Inoculated seedlings were rated with a four category scale.
- In this scale:
- 0 = No symptoms produced after inoculation,
- + = Slight browning around the injection site,
- ++ = A brown lesion 1-2 cm in diameter or spreading up and down the stem from the injection site with a distinct darker brown lesion,
- +++ = Brown lesion spreading up the stem from the injection site and with the leaf blade on the inner leaf yellowing or blighted and turning necrotic.
- Plants with a "0" rating were listed as NP or non-pathogenic in the data tables.

Yuan,2004

Pathogenicity test

On Cypress rice

- Flag leaf sheath rot, caused by *Burkholderia glumae* on inoculated Cypress rice.



Yuan,2004

Pathogenicity test
On Cypress rice

- Panicle blighting and grain rot, caused by *Burkholderia glumae*, on inoculated Cypress rice.

Yuan,2004

Pathogenicity test

On detached onion bulb scales

B. cepacia complex

- The thin, papery covering, along with the outer bulb scale, of each onion was removed prior to quartering the bulbs with a sterile knife.
- The quartered individual onion scales were placed in a sterile aluminum pan (22.9 cm by 33.0 cm) containing two sheets of sterile Whatman no.1 filter paper premoistened with 90 ml of sterile distilled water.
- The bacterial isolates used in the assays were grown overnight in KB broth.
- Individual onion scales were wounded on the inner surface with a sterile pipette tip (1- to 200 µl volume), and 5 µl of bacterial culture (10^7 CFU/ml) was inoculated into the wound.
- The onion scales were incubated at 30°C for 48 h.
- The degree of maceration was estimated by probing with a toothpick (toothpick method).
- A rating scale of 0 to 3 was used to indicate the degree of tissue maceration.
- A rating of 0 indicated no maceration, 1 indicated 1 to 33% macerated tissue area, 2 indicated 34% to 66% macerated tissue area, and 3 indicated 67% to 100% macerated tissue area.

Jacobs *et al.*,2008

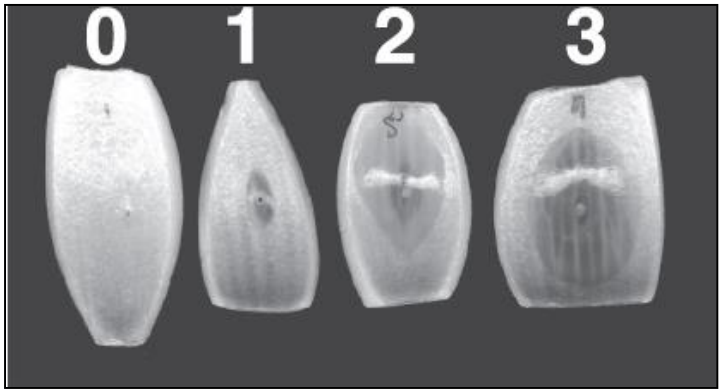
Pathogenicity test

On detached onion bulb scales

B. cepacia complex

- Pathogenicity test was assayed on detached onion inoculated with isolates of each of the species examined in *B. cepacia* complex:
- *B. cepacia*
- *B. cenocepacia*
- *B. ambifaria*, and
- *B. pyrrocinia*.

Species	No. of isolates	No. of isolates with an onion pathogenicity rating of[a]:			
		0	1	2	3
B. cepacia	160	0	23	52	85
B. cenocepacia	480	0	46	174	260
B. ambifaria	623	4	143	223	253
B. pyrrocinia	27	10	13	3	1

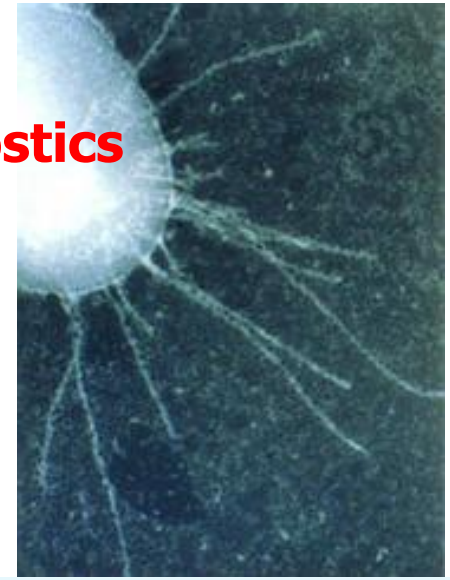


A rating of 0 indicated no maceration, 1 indicated 1 to 33% macerated tissue area, 2 indicated 34% to 66% macerated tissue area, and 3 indicated 67% to 100% macerated tissue area.

Jacobs *et al.*,2008

Identification of the bacterial pathogens
Ralstonia

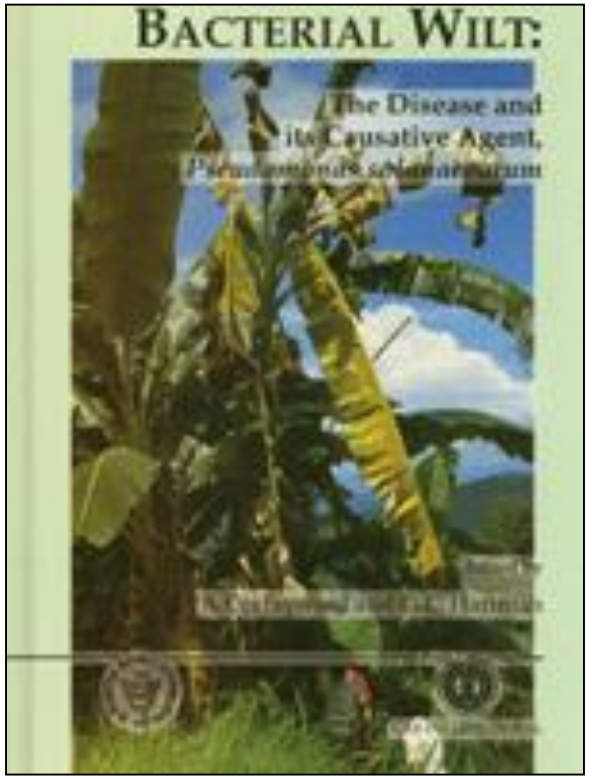
Disease diagnosis and pathogen diagnostics



Micrography of *R. solanacearum*

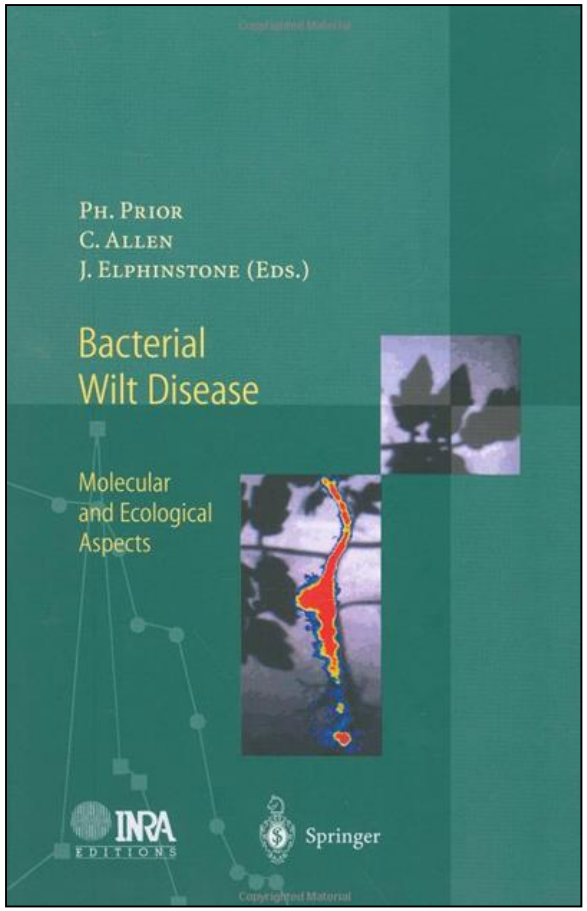
Bacterial Wilt: The Disease and its Causative Agent, *Pseudomonas solanacearum*

- Edited by A. C. Hayward and G. L. Hartman.
- Published by the Center for Agriculture and Bioscience International in association with the Asian vegetable Research and Development Center.
- 1994.



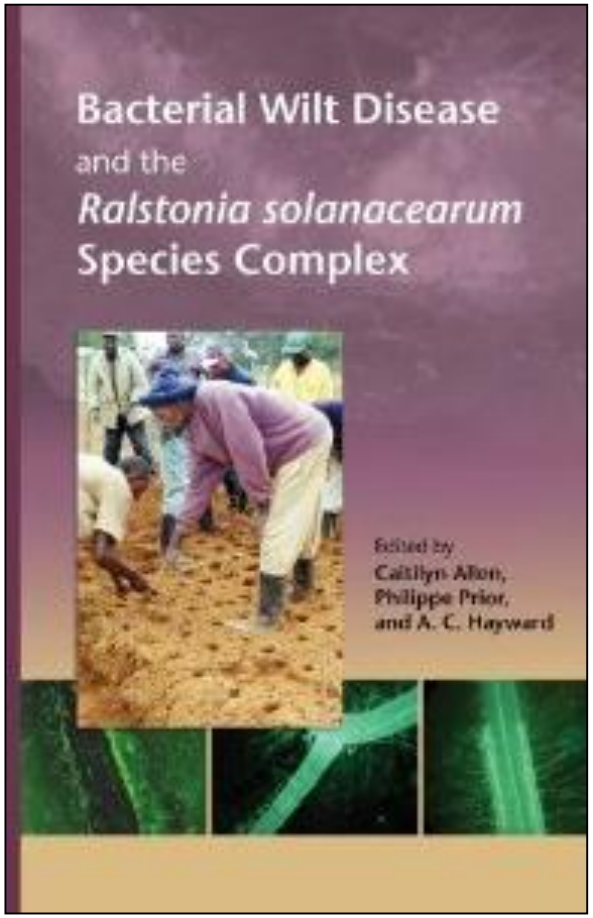
Bacterial Wilt Disease:
Molecular and Ecological Aspects

- Edited by Philippe Prior, Caitilyn Allen and G. Elphinstone.
- Springer. Jointly published with INRA, Paris.
- This book summarizes the current information on bacterial wilt for both the basic research community and for concerned professionals who are faced with the disease in the field, offering the latest approaches to diagnosis and control of the…
- 1998, 447 pages.



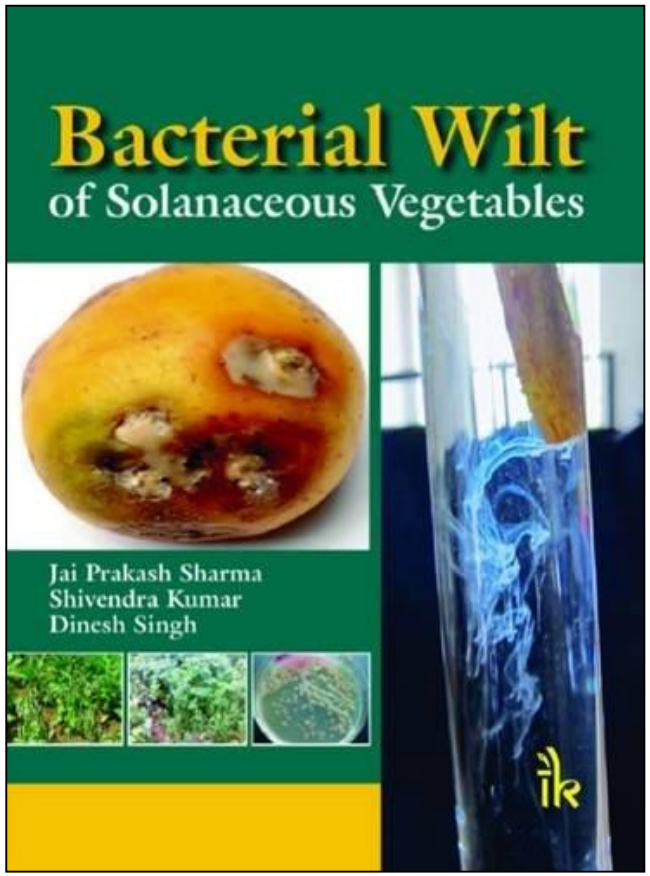
Bacterial wilt disease and the *Ralstonia solanacearum* species complex

- Edited by Caitilyn Allen, Philippe Prior, and A.C. Hayward.
- Amer Phytopathological Society(APS),
- The bacterial wilt diseases caused by members of the *Ralstonia solanacearum* species complex have never been more important.
- This book, based on the 3rd International Bacterial Wilt Symposium, covers topics ranging from the basic biology of the host-pathogen interaction.
- 2005, 528 pages.

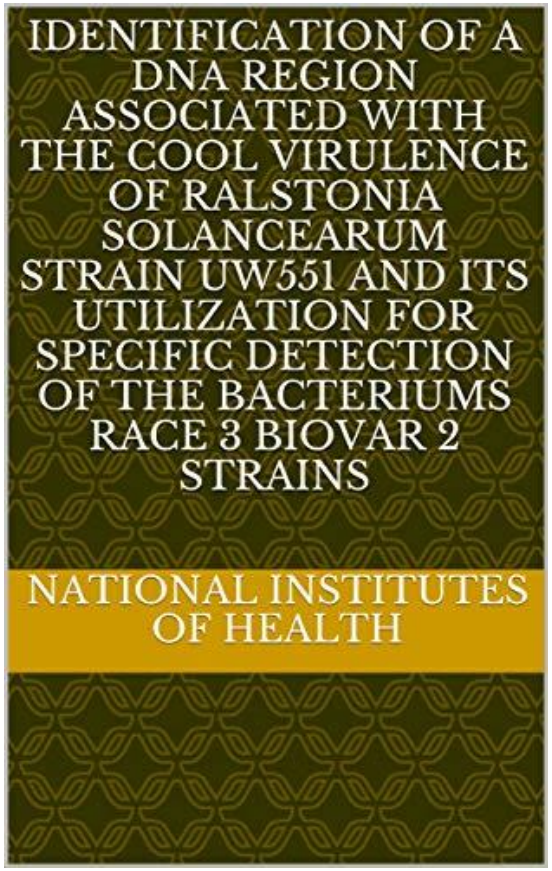


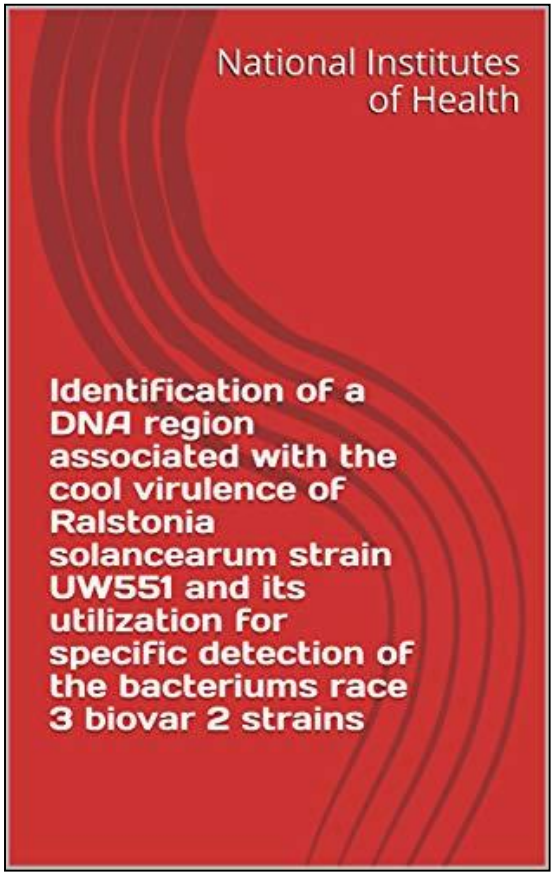
Bacterial Wilt of Solanaceous Vegetables

- Jai Prakash Sharma, Shivendra Kumar, Dinesh Singh (Authors).
- Publisher: I K International Publishing House Pvt. Ltd.
- 30 December 2015
- 280 pages



Identification of a DNA region associated with the cool virulence of *Ralstonia solancearum* strain UW551 and its utilization for specific detection of the bacterium's race 3 biovar 2 strains



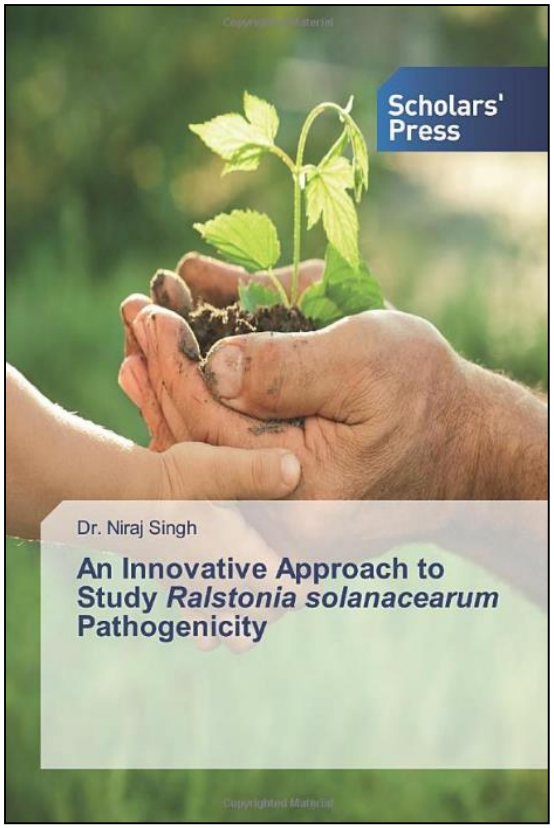


National Institutes of Health (Author) July 12, 2020 and March 23, 2020

Book cited

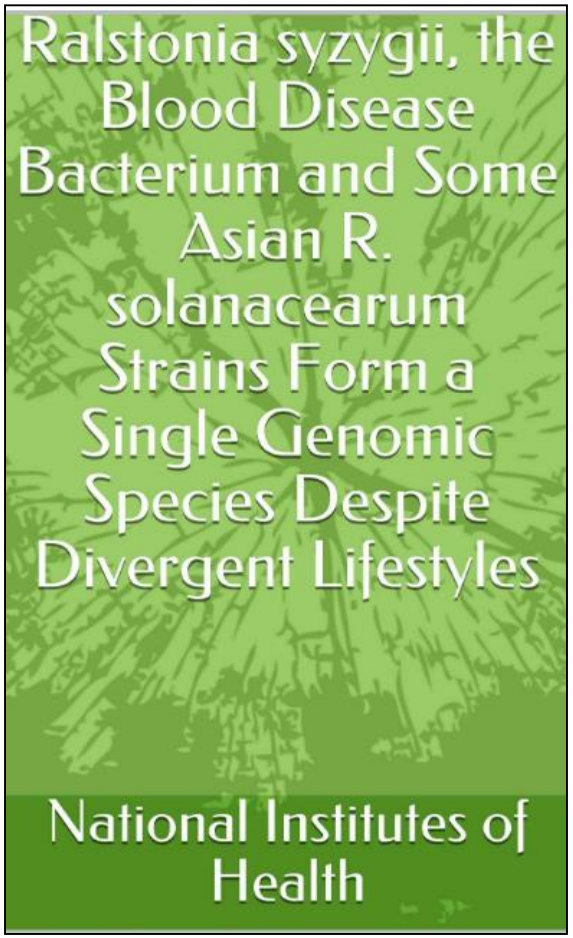
An Innovative Approach to Study *Ralstonia solanacearum* Pathogenicity

- **An Innovative Approach to Study *Ralstonia solanacearum* Pathogenicity**
- Niraj Singh
- Publisher: Scholars' Press
- 2020
- 68 pages.



Ralstonia syzygii, the Blood Disease Bacterium and Some Asian *R. solanacearum* Strains Form a Single Genomic Species Despite Divergent Lifestyles

- by National Institutes of Health (Author)
- Format: Kindle Edition.
- 13 March 2020;
- 34 pages.



Some more Books/Manuals on Bacterial wilt disease *Ralstonia solanacearum*

- Kelman, A. 1953. The bacterial wilt caused by *Pseudomonas solanacearum*: A literature review and bibliography. North Carolina Agricultural Experiment Station, Science,194 pages.
- Hayward, A.C. and Glen L. Hartman. 1994. Bacterial Wilt: The Disease and its Causative Agent, *Pseudomonas solanacearum*. CABI. 272 pages.
- Middleton, K.J. and Hayward, A.C. 1990. Bacterial Wilt of Groundnut (Aciar Proceedings). 58 pages.
- Surhone, Lambert. M., Mariam T. Tennoe and Susan F. Henssonow.2010. *R. solanacearum*. Betascript Publishing. 116 pages.
- Sujan Paudel, S., S. Dobhal, A. M. Alvarez and M. Arif. 2020. Taxonomy and Phylogenetic Research on *Ralstonia solanacearum* Species Complex: A Complex Pathogen with Extraordinary Economic Consequences. Pathogens 9 (11), 886.

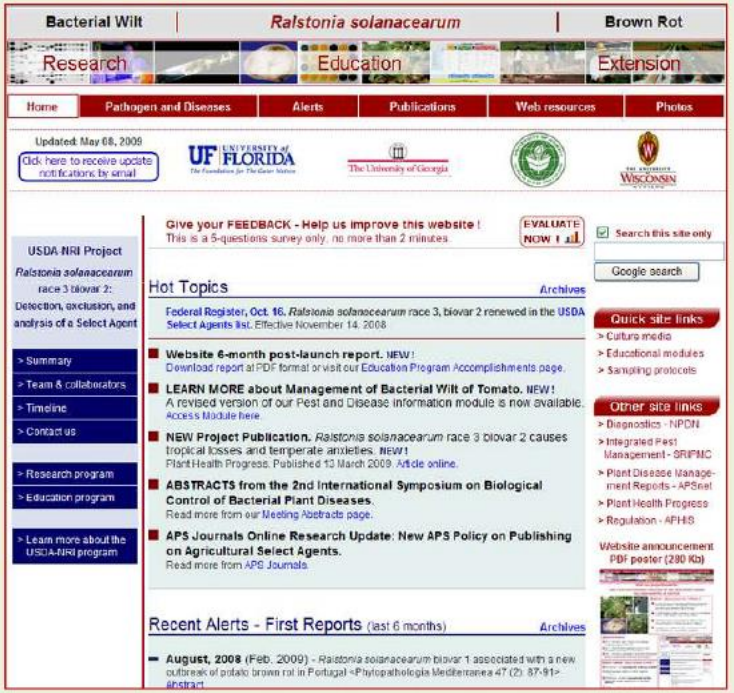
Ralstonia/Bacterial wilt dedicated website

http://plantpath.ifas.ufl.edu/rsol/

Educational resources

Ralstonia / Bacterial wilt dedicated website: http://plantpath.ifas.ufl.edu/rsol/

- ✓ Pest and disease management guides
- ✓ Project description, accomplishments
- ✓ Real time pest alerts and first reports worldwide
- ✓ Protocols, book references and journal articles database
- ✓ Web resources
- ✓ Photo galleries
- ✓ Access to Ralstonia-L mailing list



This website is supported by the USDA-NRI Program (2007-2011).

Ralstonia solanacearum/Brown rot-Bacterial wilt website

http://plantpath.ifas.ufl.edu/rsol/

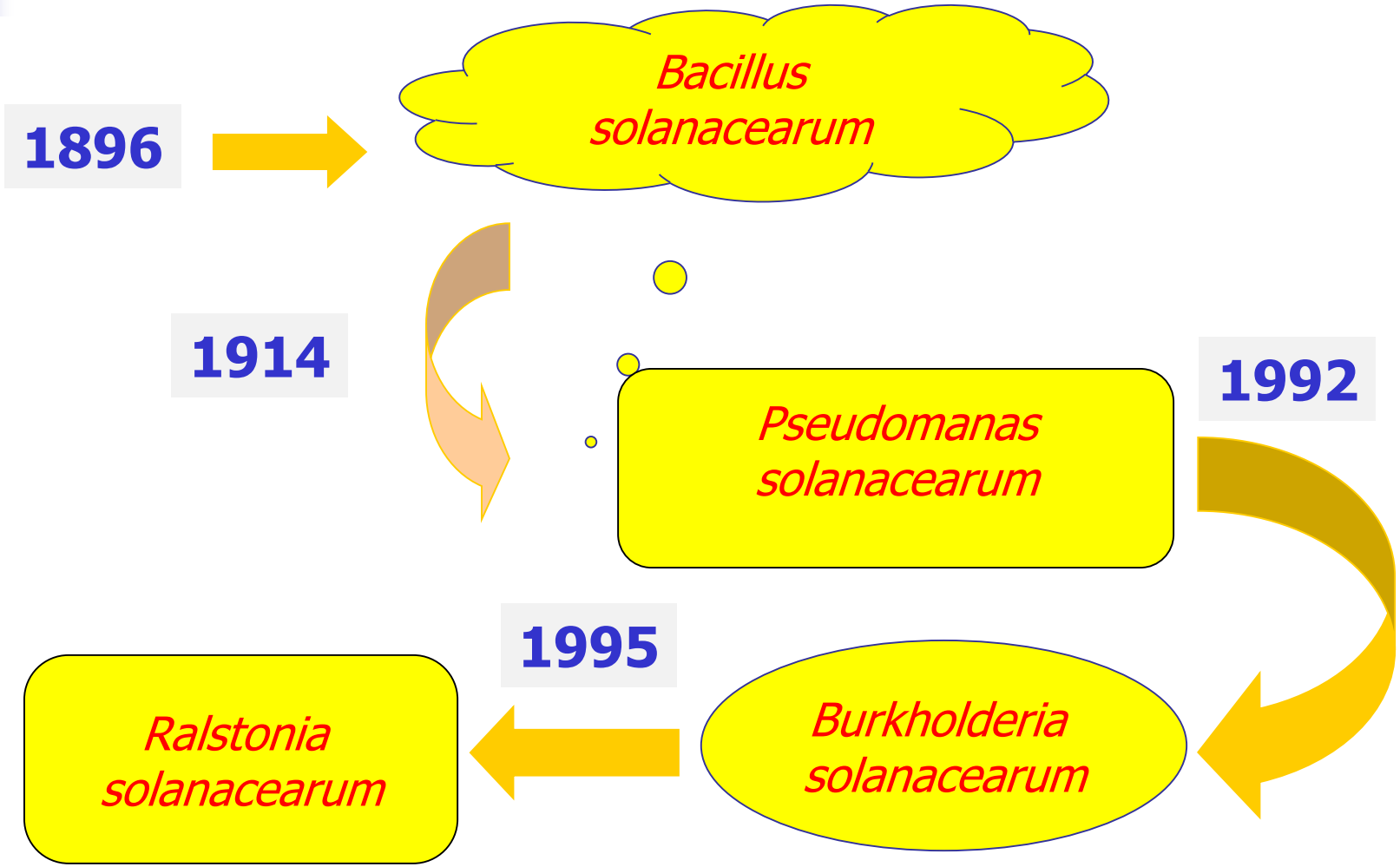
Last Updated: Feb. 23, 2011

Domain: Bacteria
Phylum: Proteobacteria

- Class: Betaproteobacteria
  - Order: Burkholderiales
    - Family: Comamonadaceae
      - Genus: *Acidovorax*
    - Family: Burkholderiaceae
      - Genus: *Burkholderia*
    - Family: *Ralstoniaceae*
      - Genus: *Ralstonia*
    - Family: ----------
      - Genus: *Xylophilus*

J.P. Euzéby,2021

Bacterial nomenclature



The genus *Ralstonia*
The complex genus

- 15 species were recognized within the genus *Ralstonia*.
- Seven of these are as follows:

1. *R. insidiosa*
2. *R. mannitolilytica*
3. *R. pickettii*
4. *R. pseudosolanacearum*
5. *R. eutropha*
6. *R. syzygii*
7. *R. solanacearum*.

- *R. solanacearum* is a genetically diverse soil borne pathogen with a wide host range. More than 450 plant species worldwide. It is a highly polyphagous bacterium.

Safni *et al.*,2014; Prior *et al.*, 2016; Gutarra *et al.*,2017

The genus *Ralstonia*

The complex genus

Three species and four phylotypes were determined

- Whole genome sequence data of 225 strains were used to classify strains based on average nucleotide identity (ANI) and multilocus sequence analysis (MLSA).
- Based on the ANI score (>95%), 191 out of 192(99.5%) RSSC strains(*Ralstonia solanacearum* species complex) could be grouped into the three species:

1. *R. solanacearum*,
2. *R. pseudosolanacearum*, and
3. *R. syzygii*, and

- into the four phylotypes within the RSSC (I,II, III, and IV).

Krum *et al.*,2021

Ralstonia solanacearum species complex

4 Phylotype were determined

- The *Ralstonia solanacearum* species complex consists of four phylogenetically distinct major lineages, named phylotypes.
- Each phylotype contains strains primarily isolated from specific geographic areas:

1. Phylotype I strains are from Asia;
2. Phylotype II are from the Americas;
3. Phylotype III are from Africa, and
4. Phylotype IV are from Indonesia, Japan, Australia, and the Philippines.

Pathogen diversity

Ralstonia solanacearum species complex

4 Phylotype and 23 sequevar were determined

Collection name	Species	Phylotype	Host	Origin	GenBank
GMI1000	*Ralstonia pseudosolanacearum*	I	*Lycopersicon esculentum*	French Guyana	GCA_000009125.1
NCPPB325^{T}	*R. solanacearum*	IIA	*Lycopersicon esculentum*	USA	De novo assembly
PD2673	*R. solanacearum*	IIB	*Solanum tuberosum*	Netherlands	De novo assembly
LMG9673^{T}	*R. pseudosolanacearum*	III	*Pelargonium capitatum*	Réunion	De novo assembly
PSI07	*R. syzygii*	IV	*Lycopersicon esculentum*	Indonesia	GCA_000283475.1
AU12–08	*R. insidiosa*	NA	*Homo sapiens*	Australia	GCA_000442475.1
NCTC10894	*R. mannitolilytica*	NA	*Homo sapiens*	Japan	GCA_900455575.1
ATCC 27511^{T}	*R. pickettii*	NA	*Homo sapiens*	USA	GCA_000743455.1

1.NA not applicable
2.^{T}- type strain of the given species

Krum *et al.*,2021

Diseases caused by plant pathogenic *Ralstonia* spp.

- Polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex. The Phylotype II strains belong to the species *R. solanacearum*, Phylotype IV strains to *R. syzygii* and Phylotypes I and III to *R. pseudosolanacearum*.

Ralstonia solanacearum	**Extremely broad host range throughout the world, including >450 host species representing 54 plant families including potato, tomato, tobacco, banana, peanut, Eucalyptus, some trees and shrubs. Moko disease of banana: *R. solanacearum* (race 2, biovar 1)**
R. pseudosolanacearum	**Bacterial wilt of Eucalyptus**
R. syzygii	
R. syzygii* subsp. *syzygii	**Sumatra disease of cloves**
R. syzygii* subsp. *indonesiensis	**Bacterial wilt disease on a wide range of host plants**
R. syzygii* subsp. *celebensis	**Banana blood disease**

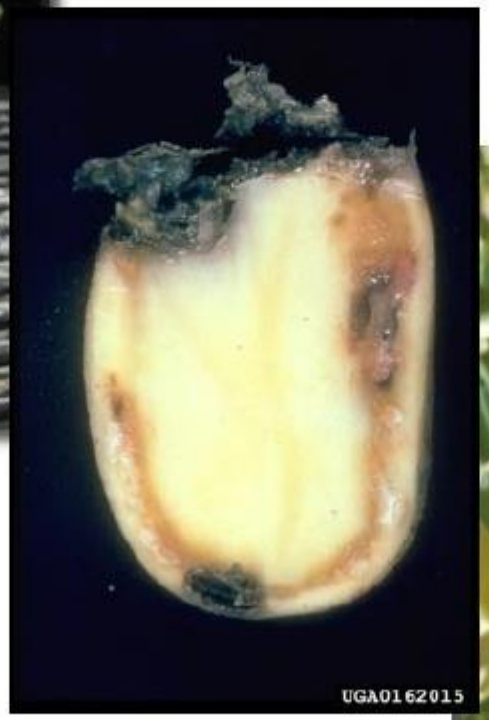
Wicker *et al.*,2007; Euzéby,2021; Safni *et al.*,2018; Paudel *et al.*,2020

Ralstonia solanacearum:

Southern Bacterial Wilt of Solanaceous Plants
Moko Disease of Bananas and Plantain

Bacterial steaming

Geranium wilt: race 3
www.agf.gov.bc.ca

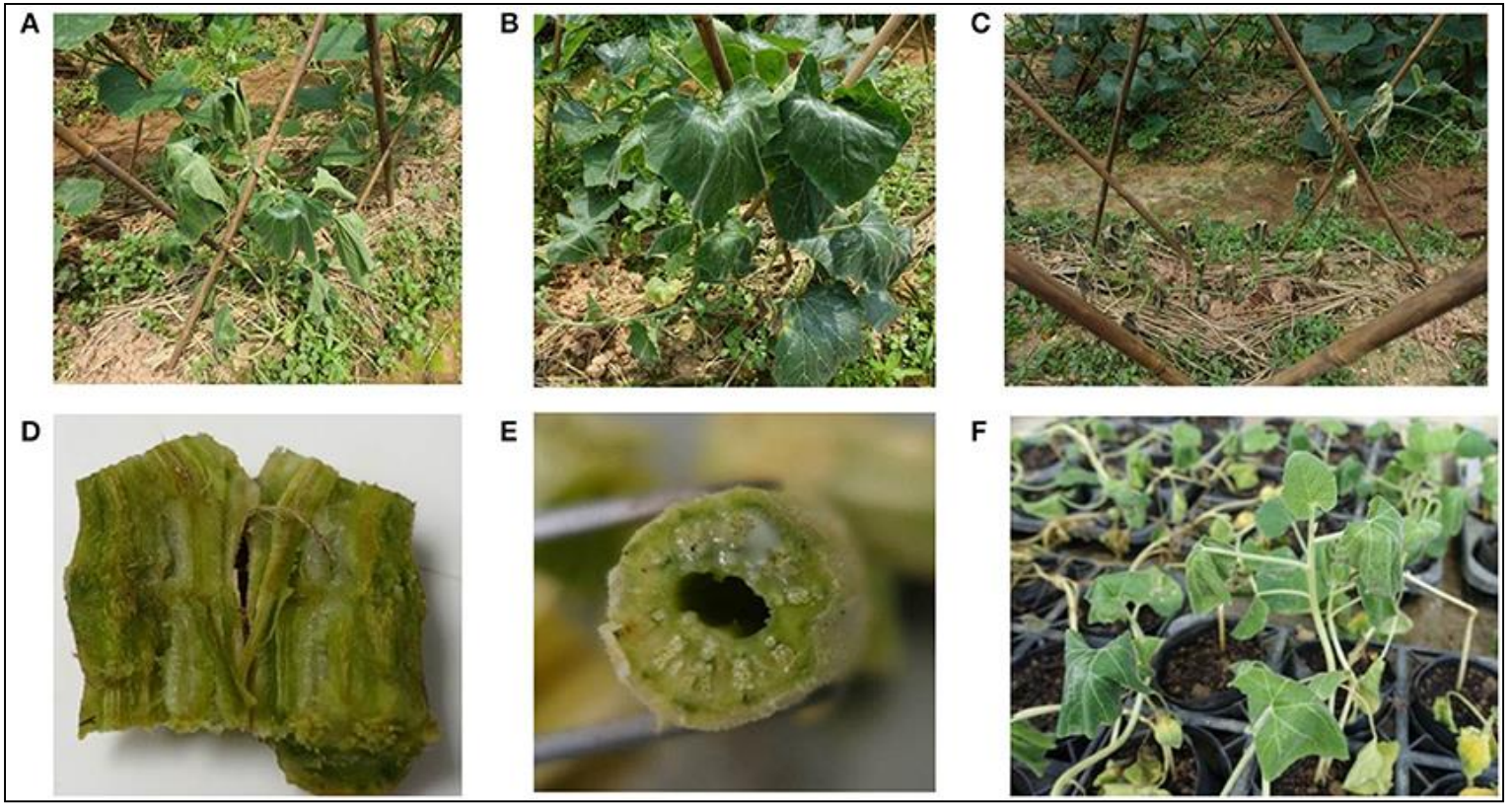


Potato brown rot

Moko Disease, www.tpp.uq.edu.au

Bacterial wilt of *Cucurbita maxima*

Ralstonia solanacearum (race 1)



She *et al.*,2017

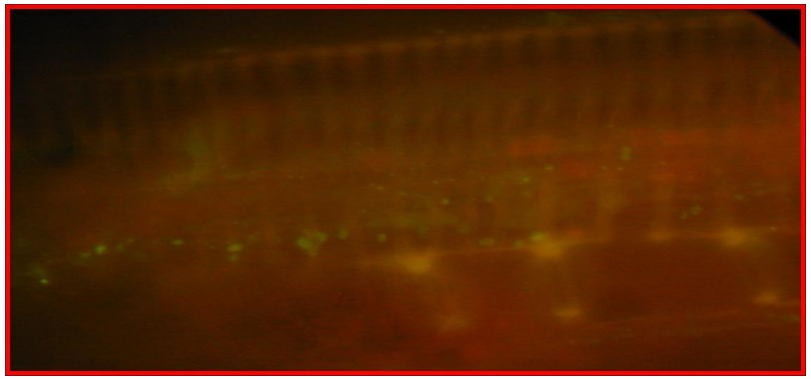
Bacterial wilt (BW) of potato
Ralstonia solanacearum (race 3)

- Bacterial ooze exuding from eyes of potato tuber infected by *R. solanacearum*.

Champoiseau and Allen,2009

Bacterial wilt (BW) of potato
Ralstonia solanacearum (race 3)

- **Amazing pathogen:**
- Phytobacteria *Ralstonia solanacearum* (green cells) inside potato vascular tissue.



Tahzima,2009

Bacterial Wilt (BW) of Banana

Moko/Bugtok disease

Ralstonia solanacearum (race 2, biovar 1)

- Moko disease of banana.
- Moko disease is observed in the commercial banana plantations.
- The name Bugtok is used when wilt symptoms, caused by *Ralstonia solanacearum*, appear on cooking bananas (ABB) in the Philippines.
- Infected bananas have hard pulps.

Blomme *et al.*,2017;..

Banana blood disease

Blood disease bacterium(BDB)

Ralstonia syzygii subsp. *celebesensis*

- The symptoms of blood disease (BDB) are similar to those of moko disease, caused by race 2 of *Ralstonia solanacearum*.
- But, colonies of the BD bacterium are smaller than those of *R. solanacearum* and are non-fluidal.

1. The name *Ralstonia haywardii* subspecies *celebensis* subsp. nov., was proposed by Remenant *et al.*,2011.
2. But now it is reclassified as *Ralstonia syzygii* subsp. *celebesensis* subsp. nov.

Plant with dead leaves

Blood disease bacterium

Ralstonia syzygii subsp. *celebesensis*

Liberato,2007; Plant Heath Australia,2006

Sumatra disease of clove

Ralstonia syzygii subsp. *syzygii*

a) Field infection of Sumatra Disease of Clove caused by *Ralstonia syzygii* subsp. *syzygii* in Magelang, Central Java, Indonesia.

b) Infected twig.

c) Horizontal section of infected twig.

d) Bacterial ooze oozing from the infected twig section.

- Reprinted with permission from Bambang Trianom.

Safni *et al.*,2018

Bacterial wilt of potato
Ralstonia syzygii subsp. *indonesiensis*

- *Ralstonia syzygii* subsp. *indonesiensis* causes disease in a number of solanaceous plants such as cloves in Indonesia and other countries in Asia.

Potato infected by *Ralstonia syzygii* subsp. *indonesiensis* in Magelang, Central Java, Indonesia.

Safni *et al.*,2018

Description and biochemical characteristics

Ralstonia solanacearum species complex

- Gram-negative rods with a polar tuft of flagella.
- Non-fluorescent but diffusible brown pigment often produced.
- Polyhydroxybutyrate(PHB) is accumulated.
- Levan not formed from sucrose;
- Gelatin hydrolysis negative or weak; starch and esculin not hydrolysed;
- Nitrate reduced by nearly all strains, many produce gas (denitrification);
- Oxidative metabolism of glucose only;
- No growth at 4° or 40°C.

OEPP/EPPO Bulletin,2004; Álvarez *et al.*,2010

Description and biochemical characteristics

Ralstonia solanacearum species complex

- Oxidase and catalase positive; arginine dihydrolase, lecithinase (egg yolk) and lipase (tween 80) negative.
- Most strains produce tyrosinase, the main exceptions being those isolated from the family *Musaceae*.
- Growth in 1% NaCl broth but little or no growth in 2% NaCl.
- Growth is weak at pH 8 with no growth at pH 4 or 9.
- This species belongs to rRNA group II.
- It is readily distinguished from other members of the group by failure to grow at 40°C.

OEPP/EPPO Bulletin,2004

Differention of two *Ralstonia* spp., *Ralstonia* (ex. *Pseudomonas*) *syzygii* and the blood disease bacterium of banana[1]

	P. syzygii	BD Bacterium	*R. solanacearum*	*R. pickettii*
Colonies on TTC medium	tenacious, minute	viscid, <5 mm	Fluidal, >5 mm	ND[2]
Motility	-	-	v	+
Growth at 37°C	-	+	+	+
Growth at 41°C	-	-	-	+
NaCl tolerance	<1%	<1.5%	<2.0%	ND
Nitrite from nitrate	v	-	+	+
Gas from nitrate	-	-	v	+
Tobacco HR	v	+	+[3]	ND
Plant pathogenicity and host associations	Clove	Banana	*Solanaceae*, *Musaceae*, etc.	Bacteraemia in humans; intracellular growth in *Acanthamoeba* spp

[1] Based in part on Eden-Green (11).
[2] ND, not determined.
[3] Systemic infection with stains from tobacco.

1. *Ralstonia syzygii* subsp. *celebesensis.*

R. syzygii is non-motile and does not grow on TSA (tryptic soy agar).

Schaad *et al.*,2001

Differential biochemical characteristics of some non-fluorescent plant pathogenic bacteria *Ralstonia solanacearum, Acidovorax, Burkholderia* and *Pseudomonas*

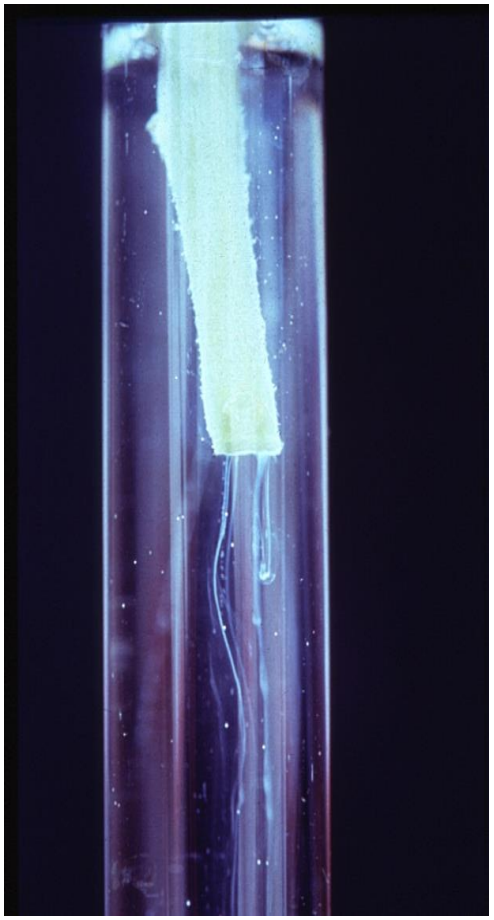
Test	*Ralstonia solanacearum*	*Burkholderia cepacia*	*Burkholderia gladioli*	*Burkholderia caryophylli*	*Pseudomonas corrugata*	*Acidovorax avenae*
Diffusible pigment	+	+	+	+	–	–
Oxidase	–	+	V	+	+	+
Arginine dihydrolase	–	–	–	+	–	–
Nitrate reduction	–	–	–	+	+	+
Growth at 41 °C	–	V	V	+	–	+
Oxidation of:						
Galactose	+/V	+	+	–		
Glycerol	+ W	–	–	+ W		
Mannose	+/V	–	+	–		
Utilization of:						
Cellobiose	V	+	+	V	–	
Trehalose	V	V	+	+		
D-Arabinose	–	+	+	+	–	–
D-Tartrate	–/V	–/W	+	–	V	
Mannitol	V	+	+	+		+
Sorbitol	V	+	+	+		+
L-Rhamnose	–	–	–	–	–	
Levulinate	V	+	–/W	–		
Sucrose	+	+	+	+		–
Glucose	+	+	+	+	+	+
Benzoate	V	–/V	+	–		
n-Propanol	+	+	–	+ W	–	–
β-Alanine	V	–	–	–	–	+
Betaine	–	+	+	+		
L-Arginine	–	+	+	+		
L-Lysine	–	+	+	V		
Heptanoate	–	+	+	–		
D-Fucose	–	+	+	+		
D-Raffinose	–	V	–	+		

+ = positive reaction; – = negative reaction; V = variable; W = weak.

OEPP/EPPO Bulletin,2004

Bacterial ooze exuding from freshly cut stem tissue placed in water *Ralstonia solanacearum*

- Streaming test to observe bacteria oozing from an infected tomato stem in water.
- Note the white milky strand of bacterial cells.



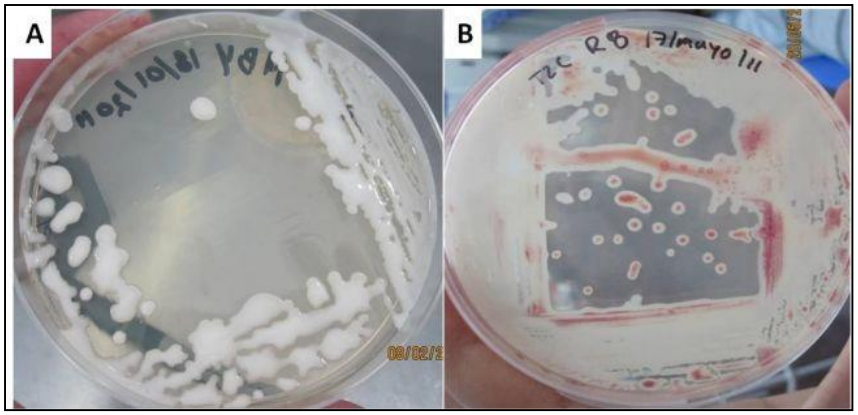
Colony morphology of bacteria isolated from tomato plants showing wilt symptoms

Ralstonia solanacearum

A. Bacteria growing on a Petri dish with NBY+glucose 2.5% alone, and

B. on same media with TZC/TTC added.

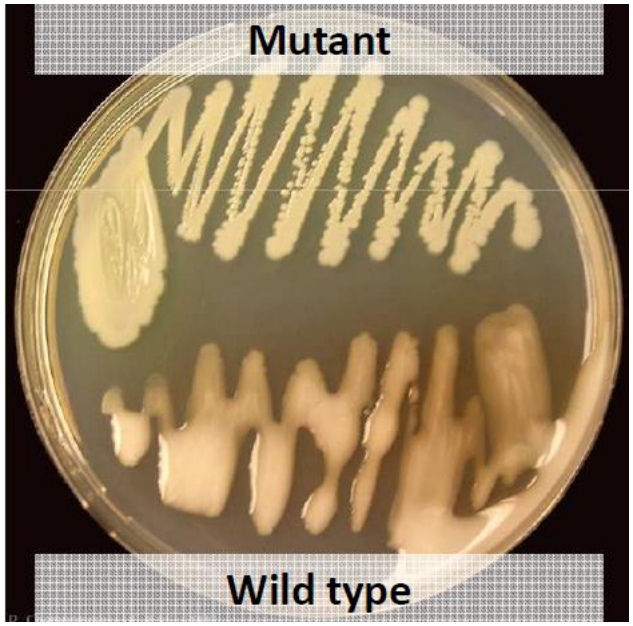
- Colonies were mucous, opaque, pleomorphic, convex, with red centre and whitish periphery in TZC, compatible with the culture features of *R. solanacearum*.



Hernández-Romano *et al.*,2012

Virulent and non-virulent colonies of *R. solanacearum* on TZC

EPS-producing colonies can be differentiated from any red, EPS-negative colonies that might be present



Cultures of virulent (bottom) and non-virulent on TZC agar medium (top) colonies of *R. solanacearum*.

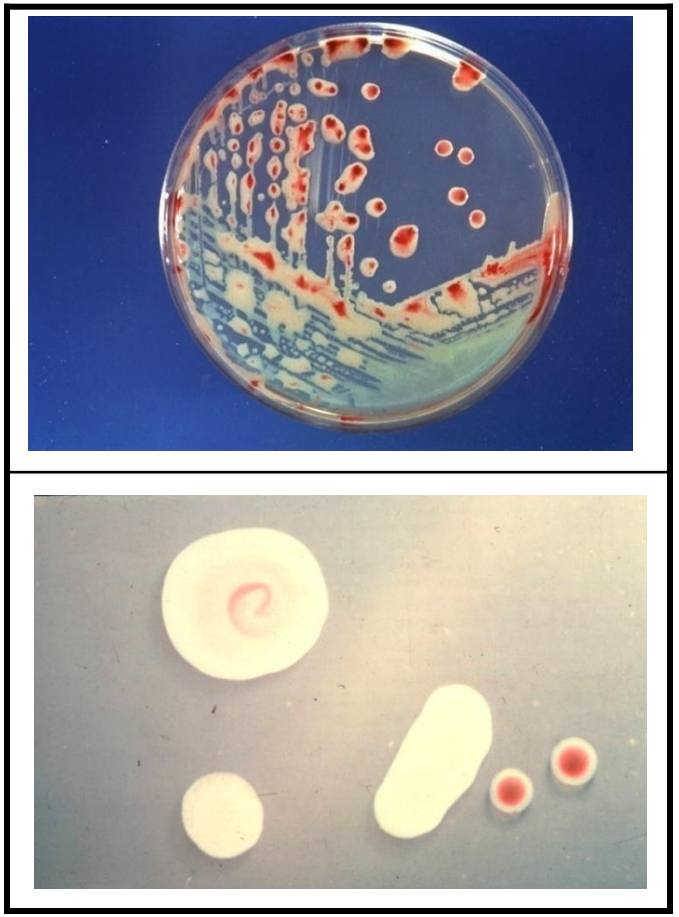
Culture of *R. solanacearum* on TZC agar medium.

Champoiseau,2009; Schaad *et al.*,2001

Colony variants of *Ralstonia solanacearum* on TTC medium

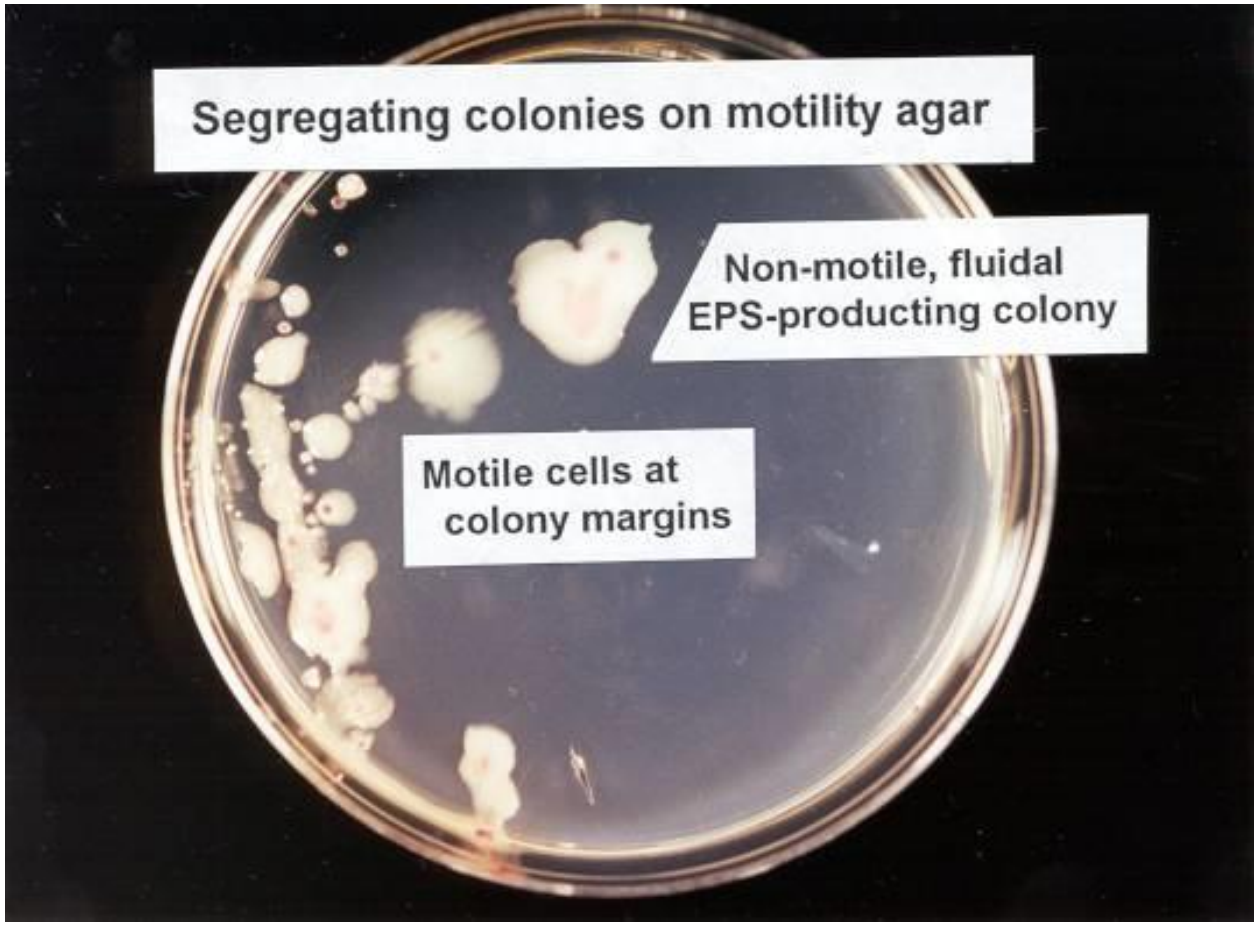
Virulent colonies

- Virulent colonies of *R. solanacearum* on TTC (TZC) agar medium are large, elevated, fluidal, and either entirely white or with a pale red center.
- Avirulent mutant colonies are butyrous, deep-red often with a bluish border.



Ralstonia solanacearum

Segregation of motile and non-motile cells



Pathogen diversity

Ralstonia solanacearum species complex

Biovars/races/phylotypes/sequevars

- Characterizations that are commonly carried out are based on:

1. host range (race),
2. region of origin (phylotype),
3. ability to utilize carbon sources (biovar), and
4. variation of egl gene sequences (sequevar/ subspecies groups).

Nurdikan *et al.*,2022

Pathogen diversity

A phylotype-specific multiplex-PCR

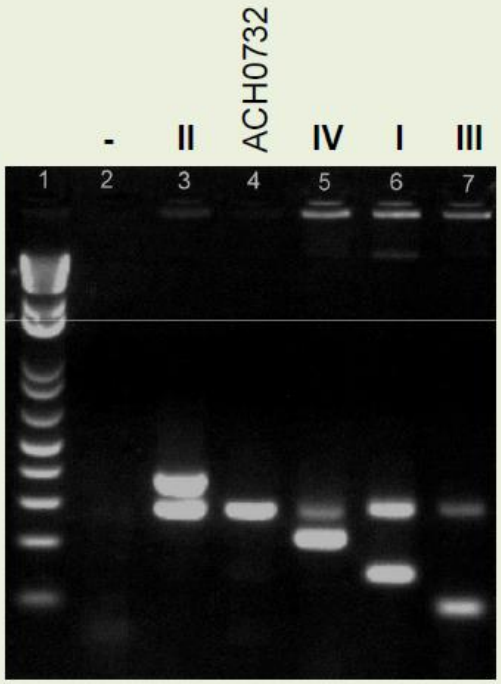
Phylotype and sequevar determination

- Step 4: Identification of phylotype/sequevar

Sub-species level

Multiplex PCR (Fegan and Prior, 2005)

PCR-Phylotype		
Nmult:21:1F	CGTTGATGAGGCGCGCAATTT	Phylotype I (Asiaticum)
Nmult:21:2F	AAGTTATGGACGGTGGAAGTC	Phylotype II (Americanum)
Nmult:22:InF	ATTGCCAAGACGAGAGAAGTA	Phylotype IV (tropical)
Nmult:23:AF	ATTACGAGAGCAATCGAAAGATT	Phylotype III (Africain)
Nmult:22:RR	TCGCTTGACCCTATAACGAGTA	Amorce reverse unique
759R	GTCGCCGTCAACTCACTTTCC	Marqueur interne
760F	GTCGCCGTCAGCAATGCGGAATCG	*R. solanacearum*



Sequevar: sequencing and analysis of partial endoglucanase (*egl*) gene

Champoiseau,2009

Pathogen diversity

Ralstonia solanacearum

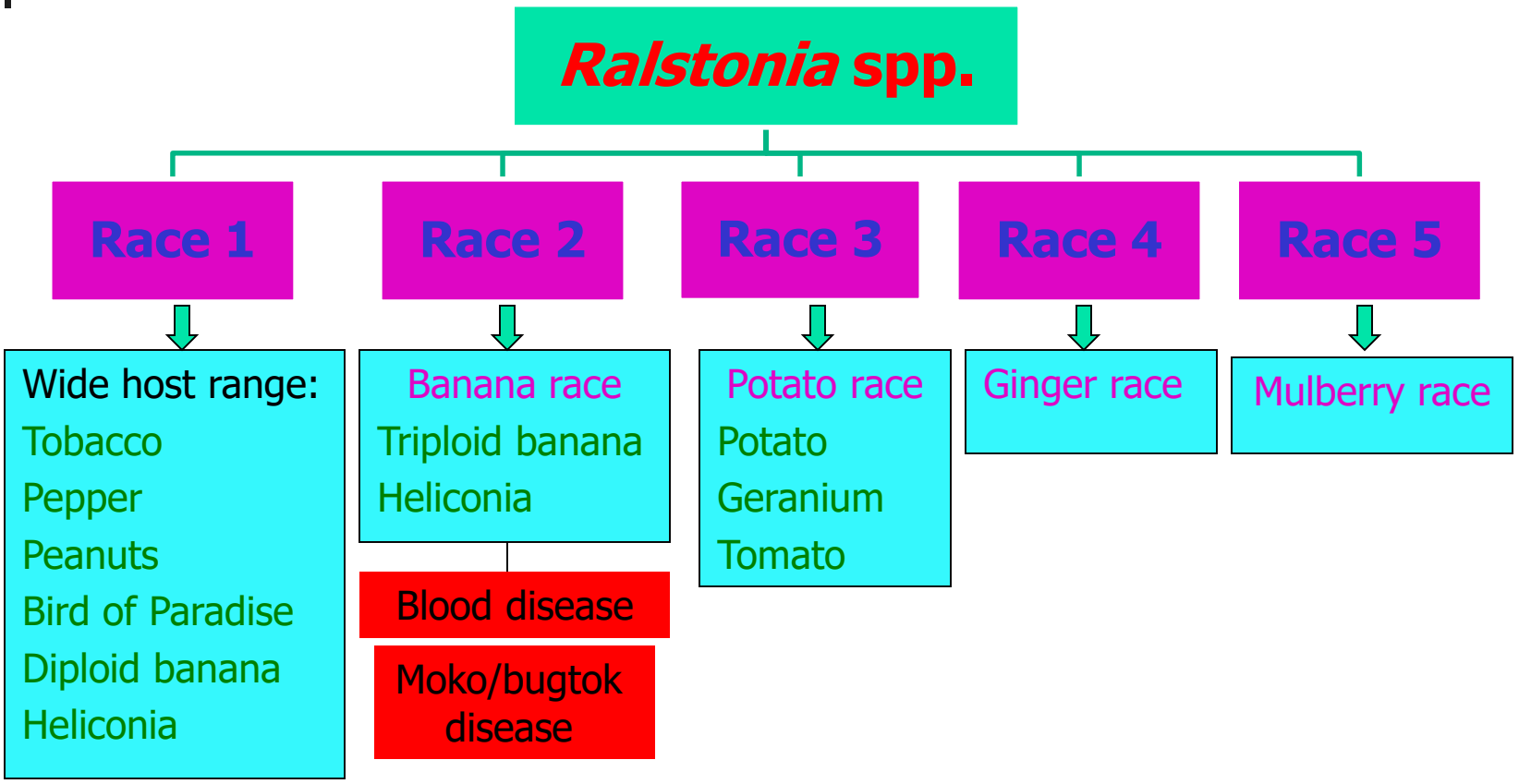
Proposed races

- Based on pathogenicity on different hosts, the strains were separated into five races.

1. Race 1 has a very wide host range and is known as the solanaceous race.
2. Race 2 as the Musa race
3. Race 3 as the potato race
4. Race 4 as the ginger race
5. Races 5 as the mulberry race

Pathogen diversity

Race system



Race: Population of pathogen isolates that have the same virulence. Moko/bugtok disease are caused by Phylotype II (race 2 biovar 1) strains, whereas, the banana blood disease bacterium caused by Phylotype IV strains (*R. syzygi* subspecies *celebensis*).

Race determination in *Ralstonia solanacearum** Janse,1991

Race	1	2	3
Reaction in:			
Tomato/aubergine	Wilting	No reaction	Wilting
Tobacco cv. White Burley plants (stem inoculation)	Wilting	No reaction	No reaction
Tobacco cv. White Burley leaves (hypersensitivity test)	Necrosis (48 h) and wilting (7-8 days)	HR (12-24 h)	Chlorosis (2-8 days)
Musa acuminata	No reaction	Wilting	No reaction

*Race 4, pathogenic to ginger and a few other hosts
Race 5, pathogenic to mulberry only, not included.

The race and biovar classifications do not correspond, except that race 3 is generally equivalent to biovar 2.

OEPP/EPPO Bulletin,2004;..

Characteristics of races and their relationship to biovars of *R. solanacearum*

Typical race 3 strains are sometimes referred to as biovar 2A. New race 3 strains from the Amazon basin have been placed in a new biovar, designed as 2T or N2 (their relation to races is unclear).

Race	Biovar	Geographical Distribution	Primary hosts
1	1,3,4	Asia, Africa, Australia, Americas. Race 1 infect tobacco in Granville County(Granville wilt (tobacco).	Wide (tobacco, tomato, solanceous and non-solanaceous weeds, diploid bananas, groundnut, potato, pepper, eggplant, olive, ginger, strawberry, geranium, Eucalyptus, other plants…)
2	1	Caribbean, Brazil, Philippines	Triploid bananas, other *Musa* spp.
3	2(or 2A)*	Worldwide except United States and Canada	Potato and tomato
4	4	Australia, China, Hawaii, India, Japan, Mauritius, South Asia	Ginger
	3	India	Unknown
5	5	China	Mulberry tree

Denny and Hayward,2001; Daughtrey,2003; USDA,2008

Identification of the biovar

Biovar determination

- Biovars of the strains were determined following the protocol described by Hayward, 1964 and Hayward *et al.*, 1989 using the basal medium ($NH_4H_2PO_4$ 1.0 g, KCl 0.2 g, $MgSO_4.7H_2O$ 0.2 g, Difco bacto peptone 1.0 g, Agar 3.0 g, and Bromothymol blue 0.03 g per liter) containing 1% of each type of sugar (lactose, maltose, mannitol and sorbitol, D (+) trehalose or 1% of L (+) tartrate).
- An inoculum suspension (O.D. + 0.05 at 600 nm) of each strain was prepared in sterile distilled water from 2-day-old cultures.
- For the determination of biovars 40 µl of bacterial suspension was added in the microtiter plate containing 170 µl of the basal medium and for the phenotypes differentiation (2A and 2T) 200 µl of the suspension was added to 3 ml the basal medium without agar contained in test tubes.
- The sealed tubes and plates were incubated at 30°C for 6 days and the color reaction was visually evaluated.

Gutarra *et al.*,2017

Identification of the biovar

Biovar system

- Based on their ability to acidify media containing one each of three disaccharides or three sugar alcohols, the strains were differentiated into six biovars.
- **Produce acid from three disaccharides:**
- Maltose, lactose, and cellobiose.
- **Oxidize three hexose alcohols:**
- Mannitol, sorbitol, and dulcitol.
- Typical race 3 strains (Pelargonium strains) are sometimes referred as biovar 2A (R3bv2).
- A tropical variant of biovar 2 (N2 or 2T), now referred to as the sixth biovar.

There is no strict correlation between races and biovars, except that race 3 strains are usually in biovar 2. The biovar 2T is isolated from potato in Peru. The difference between biovar 2 and biovar 2T strains is that biovar 2 strains are less metabolically active than biovar 2T strains.

Differentiation of *R. solanacearum* biovars biovars 1-5

Test	Biovars				
Utilization of:	**1**	**2**	**3**	**4**	**5**
Dextrose	+	+	+	+	+
Mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	-
Dulcitol	-	-	+	+	-
Trehalose	+	-	+	+	+
Oxidation of[1]:					
Lactose	-	+	+	-	+
Maltose	-	+	+	-	+
(+) cellobiose	-	+	+	-	+
Nitrite from nitrate	+	+	+	+	+
Gas from nitrate	-	-	+	+	+

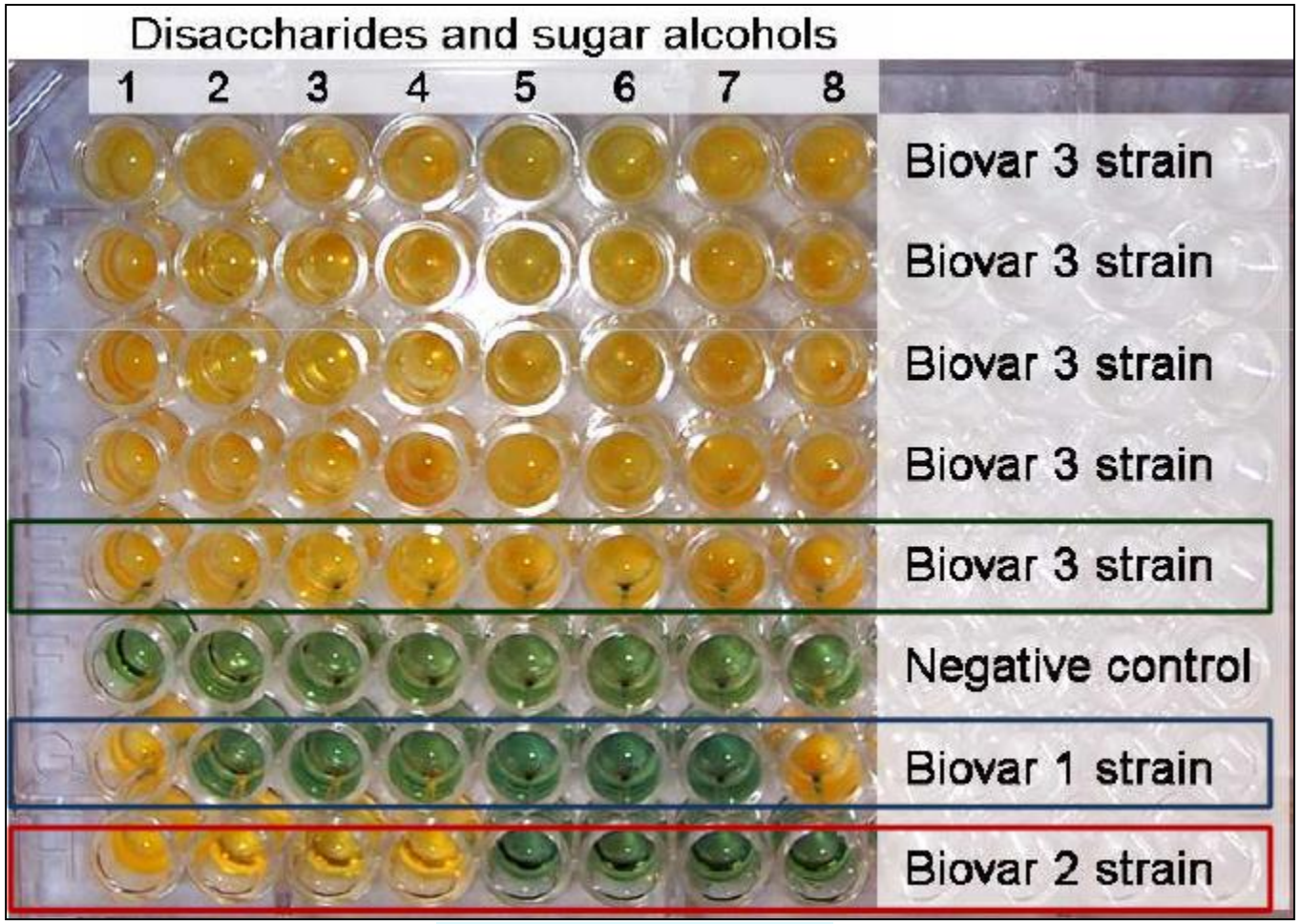
Schaad *et al.*,2001

+, 80% or more strains positive; -, 80% or more strains negative.
[I] Disaccharides are oxidized to bionic acids, but are not utilized as a source of carbon and energy.
A tropical variant of biovar 2 (N2 or 2T), now referred to as the sixth biovar.
Most strains produce nitrite from nitrate, except those of biovar 2.

Identification of the biovar

Biovar test

Biovar 3 is positive for all tests



Champoiseau,2009

Pathogen diversity

PCR-RLFP analysis

Phylogenetic grouping

- Based on PCR-RFLP banding patterns, the species into 46 multi-locus genotypes, which clustered into two major groups.
- These divisions correlated with the geographical origin of the strains:

1. **The 'Asiaticum' division(division I strains)** comprises biovars 3, 4 and 5 strains. These are metabolically more versatile biovars.
2. **The 'Americanum' division(division II strains)** contains biovar 1, 2 and 2T strains.
3. *R. solanacearum* strains with wide host range are found in **both divisions**.

Cook *et al.*,1989; Schaad *et al.*,2001

RLFP analysis

Phylogenetic grouping

Division I strains: mostly isolated from Asia ("Asiaticum")
Division II strains mostly isolated from America ("Americanum")

Race	Host range	Geographical Distribution	Biovar	RFLP Division
1	Wide	Asia, Australia, Americas	3, 4	I II
2	Banana, other *Musa* spp.	Caribbeans, Brazil, Philippines	1	II
3	Primarily potato	Worldwide	2[1]	II
4	Ginger	Asia	3, 4	I
5	Mulberry	China	5	I

Typical race 3 strains are sometimes referred as biovar 2A. Strains from Amazon basin gave been placed in a new biovar, designated by various authors as 2T or N2.

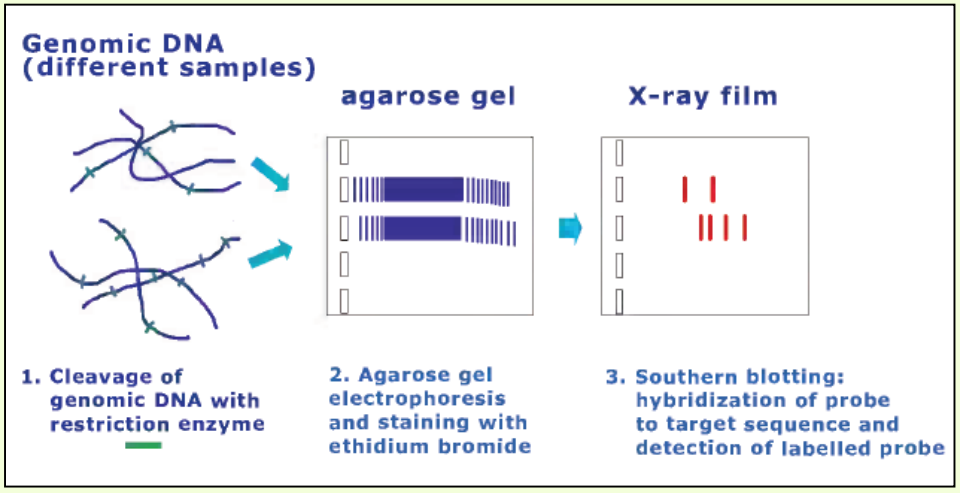
Bacterial wilt of pepper is caused predominantly by biovars 1 and 3 of *R. solanacearum*.

Schaad *et al.*,2001

RFLP analysis

Restriction fragment length polymorphisms

- In RFLP analysis, the amplified DNA fragments considered to be specific to *R. solanacearum* (single fragment 281 bp) were double digested with *Hae*III and *Msp*I restriction endonucleases.
- *Msp*I and *Hae*III recognize and cleave at CCGG and CCG sites, respectively. The resulting restriction fragments are separated according to their lengths by gel electrophoresis.



Janse,2006;..

Ralstonia solanacearum species complex

4 Phylotypes were determined

- The *Ralstonia solanacearum* species complex consists of four phylogenetically distinct major lineages, named phylotypes.
- Each phylotype contains strains primarily isolated from specific geographic areas:

1. Phylotype I strains are from Asia;
2. Phylotype II are from the Americas;
3. Phylotype III are from Africa, and
4. Phylotype IV are from Indonesia, Japan, Australia, and the Philippines.

Pathogen diversity

Ralstonia solanacearum species complex

4 Phylotypes were determined

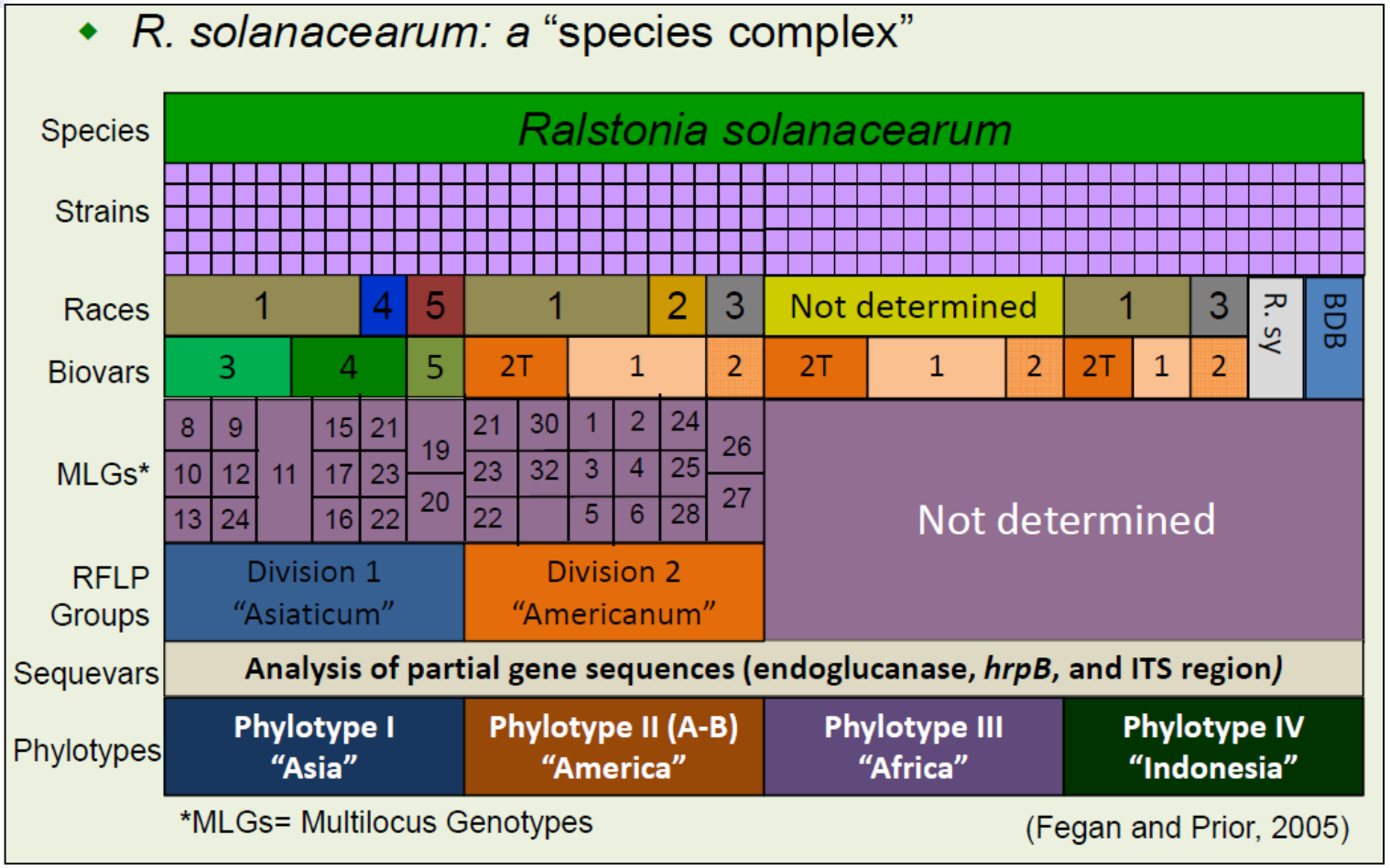
- Reclassification based on phylogenetic analysis divides *Ralstonia solanacearum* species complex (RSSC) into:

1. *R. pseudosolanacearum* (phylotypes I and III),
2. *R. solanacearum* (phylotypes IIa and IIb),
3. *R. syzygii* subsp. *syzygii*, *R. syzygii* subsp. *indonesiensis*, and *R. syzygii* subsp.*celebensis* (phylotype IV).

Safni *et al.*,2014

Pathogen diversity

Biovars/races/phylotypes/sequevars



Champoiseau,2009

Pathogen diversity

Each species, phylotype has different number of sequevars

- *Ralstonia solanacearum* phylotype I strains from Mayotte selected for the virulence test against tomato accessions under controlled conditions.

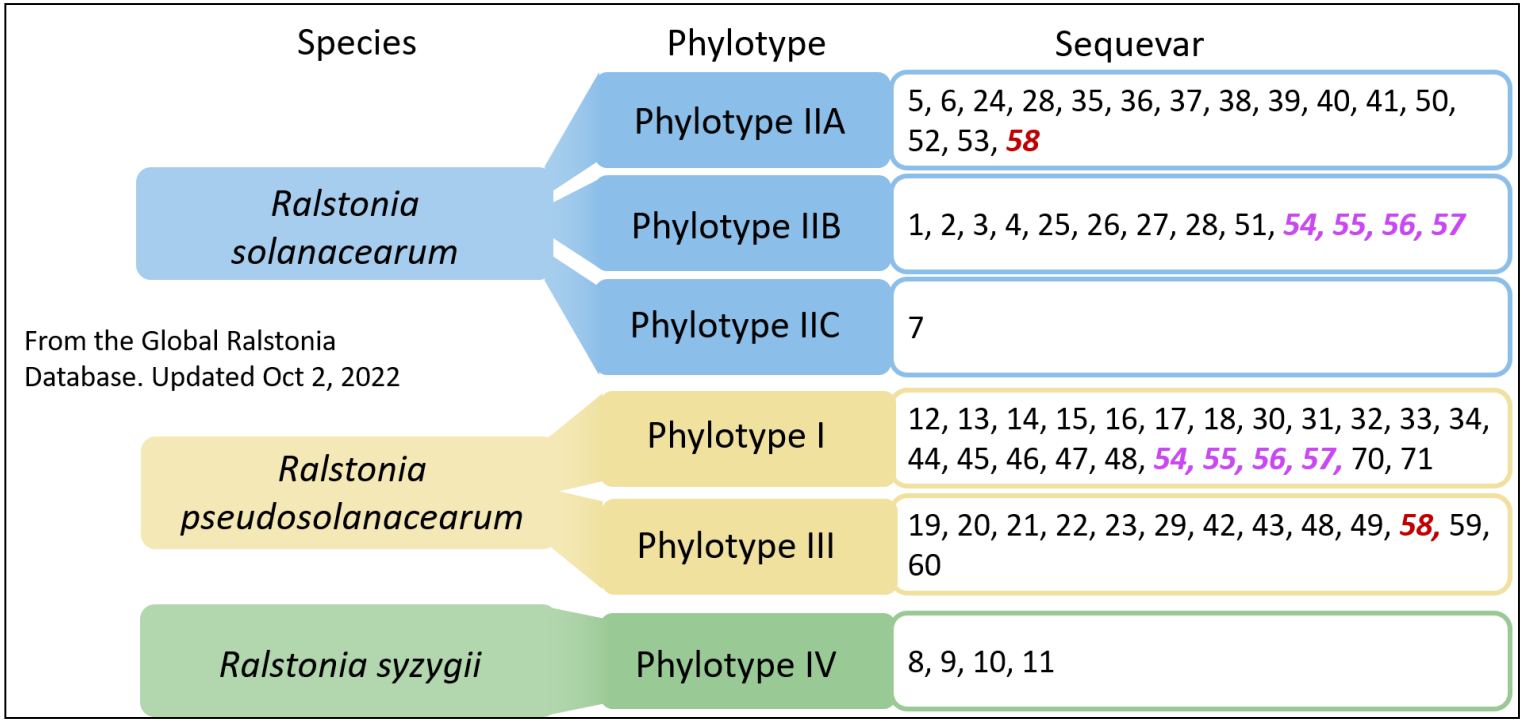
Strains	Location (area)	Isolation host	Species	Sequevar
RUN2170	Coconi (C)	Eggplant	*Solanum melongena*	31
RUN2108	Dembeni (SE)	Tomato	*Solanum lycopersicum*	31
RUN2083	M'romouhou (SE)	Hot pepper	*Capsicum annuum*	18
RUN2150	Miangani (N)	Tomato	*S. lycopersicum*	18
RUN2127	Combani (C)	Sweet pepper	*C. annuum*	46
RUN2146	Miangani (N)	Tomato	*S. lycopersicum*	46
RUN2143	Mitséni (N)	Sweet pepper	*C. annuum*	15
RUN2140	Mitséni (N)	Eggplant	*S. melongena*	15

The phylotypes are subdivided into sequevars based on sequence variation in the endoglucanase (*egl*) partial gene.

Chesneau *et al.*,2017

Pathogen diversity

Each species, phylotype has different number of sequevars



The sequevars highlighted in red and purple text have conflicting reports in the literature.

Chipman *et al.*,2020

Pathogen diversity

A phylotype-specific multiplex-PCR

Phylotype and sequevar determination

- *Ralstonia pseudosolanacearum* phylotypes were identified based on the method of Sagar *et al*.,2014 and Cho *et al*.,2018 with multiplex PCR using different primers
- List of primers for identification of phylotypes and sequevar of *Ralstonia solanacearum*.

Primer	Sequence	Target	Description
Nmult:21:1 F	5′-CGTTGATGAGGCGCGCAATTT-3′	144 bp	Phylotipe I (Asiaticum)
Nmult:21:2 F	5′-AAGTTA TGGACGGTGGAAGTC-3′	372 bp	Phylotipe II (Americanum)
Nmult:22:InF	5′-ATTGCCAAGACGAGAGAAGTA-3′	213 bp	Phylotipe IV (Tropical)
Nmult:23:AF	5′-ATTACGAGAGCAATC GAAAGATT-3′	91 bp	Phylotipe III (African)
Nmult:22:RR	5′-TCGCTTGACCCTATAACGAGTA-3′		Amorce reverse unique
759 R	5′-GTCGCCGTCAACTCACTTTCC-3′	280 bp	Specific primer for *Ralstonia*
760 F	5′-GTCGCCGTCAGCAATGCGGAATCG-3′		*solanacearum*
ENDO-F	5′-ATGCATGCCGCTGGTCGCCGC-3′	750 bp	*egl* gene
ENDO-R	5′-GCGTTGCCCGGCACGAACACC-3′		

Phylotype was determined by multiplex PCR using a set of phylotype-specific primers Nmult:21:1F, Nmult:21:2F, Nmult:22:InF, Nmult:23:AF, and Nmult21:RR.
Each phylotype is further divided into sequevars. Sequevar identification was carried out with ENDO-F and ENDO-R primers (Ji *et al*.,2007) targeting the egl gene region.

Nurdika *et al*.,2022;..

Pathogen diversity

A phylotype-specific multiplex-PCR

Phylotype and sequevar determination

- A phylotype-specific multiplex-PCR was carried out.

1. PCR amplification of a 750-bp region of the *egl* (endoglucanase)gene, a secondary determinant for pathogenicity, was performed by using the primer pair:

- Endo-F (5'ATGCATGCCGCTGGTCGCCGC-3') and
- Endo-R (5'-GCGTTGCCCGGCACGAACACC-3').

1. PCR amplification of the *hrpB* gene was performed with the primer pair:

- RShrpBf (5TGCCATGCTGGGAAACATCT-3)&
- RShrpBr (5-GGGGGCTTCGTTGAACTGC-3).

See also nucleic acid-based methods

Xu *et al.*,2009

Pathogen diversity

A phylotype-specific multiplex-PCR

Phylotype and sequevar determination

- This new classification scheme broadly reflects:

1. The ancestral relationships, and
2. Geographical origins of the strains.

- Primer sequences used in PCR:
- *hrpB* (regulator of pathogenicity-related gene function), *egl* (endoglucanase gene) and *fliC* (or flagellin gene).

Gene	Primer designation[a]	Primer sequence (5 ' ⟶ 3')	Annealing temperature
hrpB	RShrpBF RShrpBR	TGCCATGCTGGGAAACATCT GGGGGCTTCGTTGAACTGC	64
egl	EglF EglR	AAATCCAGATATCGAATTGCCAA GCGTGCCGTACCAGTTCTG	57
fliC	Rsol_fliCF Rsol_fliCR	GAACGCCAACGGTGCGAACT GGCGGCCTTCAGGGAGGTC	63

Patrice,2001; Denny,2006; Mikhail *et al.*,2012

Pathogen diversity

DNA extraction and identification of strains

Phylotype and sequevar determination

- For DNA extraction, the bacteria were streaked on MKM without TZC and the plates incubated at 30°C for 2 days. Then one colony was suspended in 100 µl of sterile NFW, boiled for 10 min and kept at -20°C prior to use.
- The taxonomic identity of *R. solanacearum* was verified with primers 759/760 (Opina *et al.*,1997). PCR amplification was performed in a total volume of 15 µl containing 1X of PCR Buffer, 2.5 mM $MgCl_2$, 0.2 µM each dNTP, 0.2 µM of each primer, 0.3 U of GoTaqG2 Flexi DNA polymerase (PROMEGA) and 1 µl DNA template.
- Amplification was performed in an Applied Biosystem Veriti thermocycler as follows: an initial denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 17 s, and a final extension step at 72°C for 5 min. PCR products (10 µl) were analyzed by electrophoresis through 1% (w/v) agarose gels with 0.01 µl /ml GelRed™ 10,000X (Biotium) and photographed under UV light in a The Chemidoc™ MP Photodocumentation System (BIO-RAD).
- Fragments were compared with a 1 Kb Plus marker ladder. A positive identification was based on the presence of a 282 bp amplicon.

Gutarra *et al.*,2017

Pathogen diversity
A phylotype-specific multiplex-PCR
Phylotype identification

- Phylotype was determined by multiplex PCR using a set of phylotype-specific primers Nmult:21:1F, Nmult:21:2F, Nmult:22:InF, Nmult:23:AF, and Nmult21:RR (Fegan and Prior,2005).
- Amplification was carried out in a total volume of 15 µl containing 1 X PCR buffer, 2.5 mM MgCl2, 0.2 mM of each dNTP, 0.2 µM of each primer, 0.3 U of Go*Taq G2 Flexi* DNA polymerase (PROMEGA) and 1 µl DNA template. Amplifications were performed in an Applied Biosystem Veriti thermocycler as follows: an initial denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 23 s, and a final extension step at 72°C for 5 min.
- PCR products (10 µl) were analyzed by electrophoresis through 1% (w/v) agarose gels with 0.01 µl /ml GelRed™ 10,000X (Biotium) and photographed under UV light in The Chemidoc™ MP Photodocumentation System (BIO-RAD).
- DNA template from strains CIP-277 (phylotype I), CIP-435 (phylotype II), and CIP-358 (phylotype III) were used as positive amplification controls. The size of the amplified fragments was estimated by comparison with a 1 Kb Plus marker ladder.

Gutarra *et al.*,2017

Pathogen diversity
A phylotype-specific multiplex-PCR
Sequevar identification

- PCR amplification of a 750-bp region of the *egl* gene was done using the primer pair Endo-F (5-ATGCATGCCGCTGGTCGCCGC-3) and Endo-R (5-GCGTTGCCCGGCACGAACACC-3) Fegan *et al.*,1998; Fegan and Prior,2005).
- Amplification was carried out in a total volume of 75 µl containing PCR buffer, 1.25 mM MgCl2, and 0.2 mM of each dNTP, 0.2 µM of each primer, 1.5 U of Go*Taq G2 Flexi* DNA polymerase (PROMEGA) and 3 µl DNA template. PCR was performed using an Applied Biosystem Veriti thermocycler. Amplifications were performed with an initial denaturation step at 95°C for 2 min, followed by 28 cycles of denaturation at 95°C for 30 s, annealing at 70°C for 30 s, extension at 72°C for 50 s, and a final extension step at 72°C for 5 min.
- PCR products (5 µl) were checked by electrophoresis through 1% (w/v) agarose gels with 0.01 µl /ml GelRed™ 10,000X (Biotium) and photographed under UV light in The Chemidoc™ MP Photodocumentation System (BIO-RAD). The size of the amplified fragments was estimated by comparison with a 1 Kb Plus marker ladder. Then PCR products were purified and sequenced by Macrogen services (Kumchun-ku, Seoul, Korea) using Endo-F and Endo-R primers. The raw sequences were assembled and aligned together with reference sequences of *R. solanacearum* strains obtained from NCBI sequence database. The DNA sequences of the isolates were assigned accession numbers MF461731 – MF461866 in the NCBI sequence database. The phylogenetic analysis was done based on partial sequences using MEGA7 (Kumar *et al.*, 2016), phylogenetic trees were constructed from the genetic distance data by the Neighbor-Joining (NJ) and Maximum Likelihood (ML) method using the algorithm of Jukes–Cantor with 1000 bootstrap resampling of the data to test the tree topologies.

Gutarra *et al.*,2017

Pathogen diversity

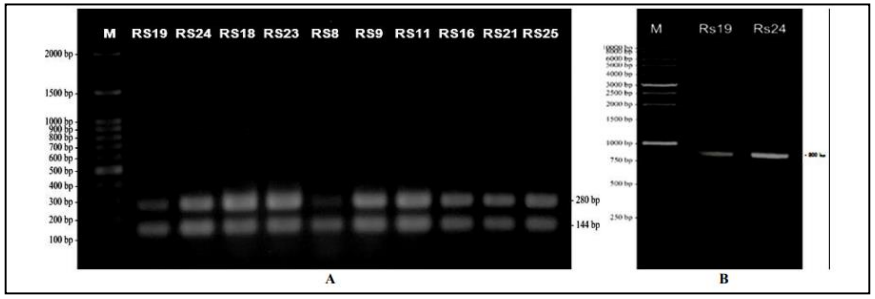
A phylotype-specific multiplex-PCR

Sequevar identification

- Visualization of DNA amplicons of *Ralstonia pseudosolanacearum*:

A. Identification of phylotype by multiplex PCR method, 2 DNA bands with size 144 bp and 280 bp appear,

B. Amplification of endoglucanase gene region, DNA band with the size around 800 bp appear.



The phylotypes are subdivided into sequevars based on sequence variation in the endoglucanase (*egl*) partial gene.

Nurdikan *et al.*,2022;..

Diagnostic methods

Serological methods

- These methods have thresholds of detection that range from 10^3-10^4 bacteria/ml of sample.
- Each serological method has different strengths, but they all suffer from problems with either specificity, sensitivity or both.
- Serological methods also have the disadvantage that they do not discriminate between live and dead cells.
- Knowledge that a sample has living *R. solanacearum* is essential before making a decision to quarantine or destroy a shipment of produce.

Denny,2006

Diagnostic methods
Serological methods

- Polyclonal antisera (PAb) usually react more strongly with *R. solanacearum* than do monoclonal antibodies (MAb), but this greater sensitivity is counterbalanced by reduced specificity.
- An EPS-specific monoclonal antibody (Mab) was used to detect low populations of bacteria (e.g. *Ralstonia solanacearum*) from irrigation or drainage water.

Denny, 2006; Peckham et al

Diagnostic methods

Serological methods

Production of a monoclonal (IgG3) Ab 3.H7

Bound all *R. solanacearum* strains tested (109)

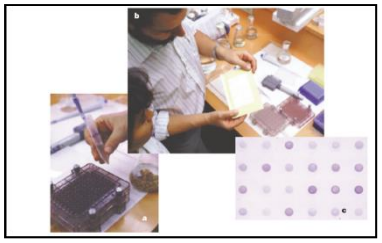
Not R3b2-specific

Not EPS- cells

O.D.		Name	Origin	Hosts
0.7825	R3B2	PS-13	Guatemala	Geranium
0.9925	R3B2	PS-14	Guatemala	Geranium
1.437	R3B2	PS-16	Guatemala	Geranium
0.729	R3B2	PS-17	Guatemala	Geranium
1.593	R3B2	PS-20	Guatemala	Geranium
0.903	R3B2	PS-22	Guatemala	Geranium
0.556	R3B2	UW552	Guatamala	Geranium
0.79	R3B2	UW437	Australia	Tomato
0.565	R3B2	UW491	Colombia	Potato
0.7085	R3B2	UW596	Guatemala	Potato
0.255	R3B2	UW551 EPS-	Kenya	Geranium
1.116	R3B2	UW551 LPS-	Kenya	Geranium

Champoiseau,2009

Serological methods
DIA or DIBA



- Antigens or antibodies are detected using a novel membrane/strips based immunoassay.
- The assay strips/membrane were placed in a substrate solution of chromogenic substrate e.g. 4-chloro-1-naphthol and color development allowed to occur for approximately 30 min at 37°C.
- Dot-immunobinding assay (DIA) provided a better understanding of the interaction between virulent and avirulent isolates of *R. solanacearum*.
- It also provided a simple and rapid test for identification and detection of *R. solanacearum* in infected potato tubers.

Tawfik *et al.*,2008; Duveiller *et al.*,1997;..

Immunodiagnostic assays

Pocket diagnostics

Results in 2 minutes

- Quick serological tests can be used in the field or greenhouse for early identification of the pathogen.
- Most kits use a EPS-specific monoclonal antibody (Mab) to *R. solanacearum* coated onto colored latex beads that are preloaded onto a dipstick or horizontal flow unit.
- Results can be read in several minutes:

1. Positive samples produce two colored bands;
2. Negative samples produce a single control band, and no band indicates a method failure.

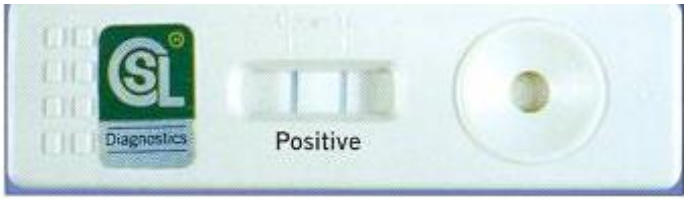
- A rapid agglutination test kit also is available form Neogen Europe.
- However, speed and convenience come at a price, because each test costs $3 to $6 USD.

Denny, 2006; Patrice,2001

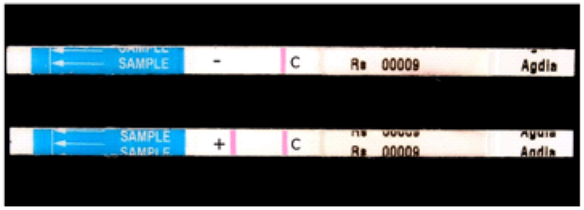
Immunoassay

Pocket diagnostics

Rs BID ELISA and Rs ImmunoStrip



Rs=*R. solanacearum*



Positive samples produce two colored bands;
Negative samples produce a single control band.

Central Science Laboratory, Sand Hutton, UK
www.csl.gov.uk/pocketdiagnostics
Agdia, Inc. Elkhardt, Indiana
www.agdia.com

Serological methods
ELISA, IFA and IFCS tests

- Enzyme-linked immunosorbent assays (ELISA) are still used in many labs, because they are relatively inexpensive, easy, fairly fast, and tolerate foreign material in the sample (e.g., plant tissue, soil).
- Alternatives to ELISA are:

1. Immunofluorescence-antibody staining (IFAS), and
2. Immunofluorescence-colony staining (IFCS).

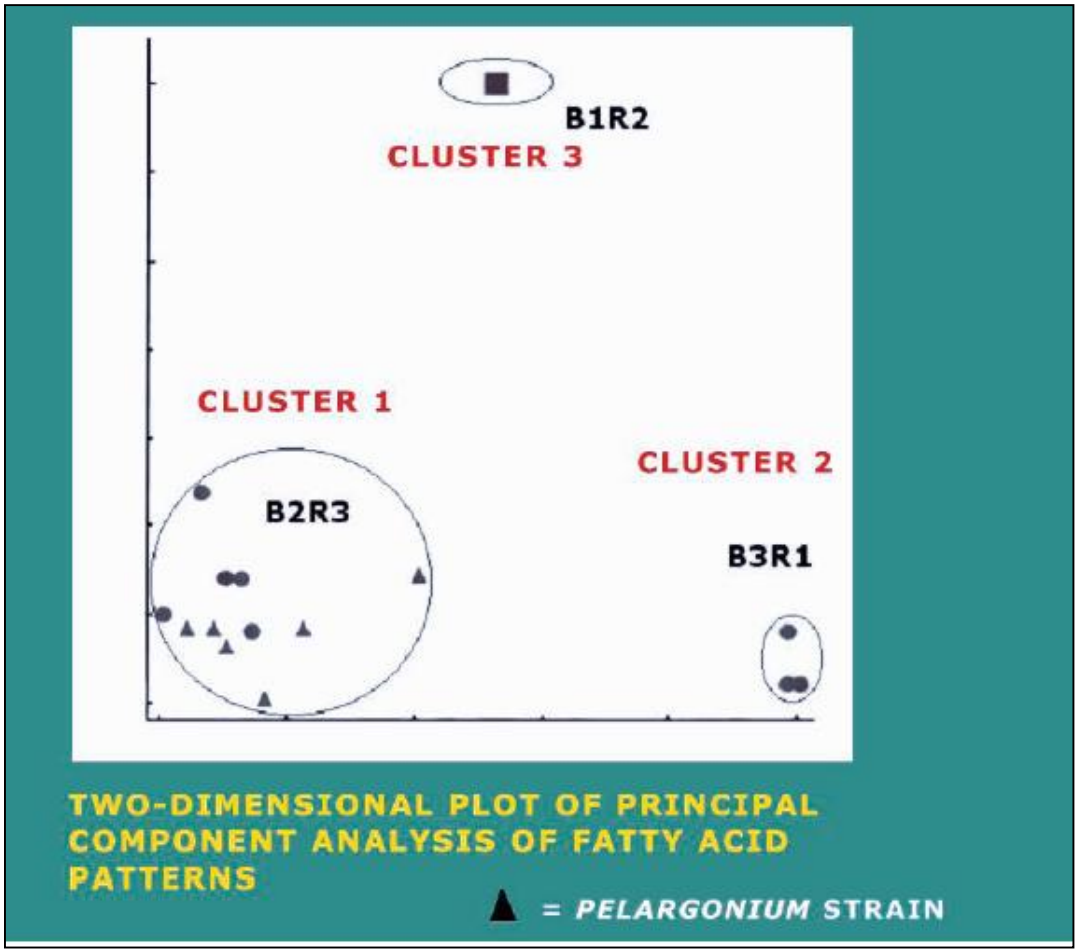
Denny,2006

Phytohormones production

- *Ralstonia solanacearum* synthesize exceptionally high amounts of IAA both in the presence and absence of exogenous tryptophan.
- The auxin (IAA) class regulates various aspects of plant growth and development.
- The pathogen also produces cytokinin, a class of plant hormones that promote cell division.

Fatty acid analysis

Comparison of three races/biovars



B(biovar)/R(race)

Janse,2006

Nucleic acid-based methods

- Many techniques have all been developed to detect *R. solanacearum*:

1. Direct PCR,
2. Nested PCR,
3. Co-operational PCR,
4. Real-time PCR, and
5. Multiplex PCR reactions (See also the classification file).

- When the target sequence is present in multiple copies (e.g., tRNA genes) and reaction conditions are ideal, PCR can detect a single pathogen cell or its equivalent in purified DNA.

Denny,2006

Nucleic acid-based methods

- No significant differences in sensitivity were noted between the different DNA extraction methods, using either commercial kits or the classical CTAB protocol.
- However, a step of pre-enrichment in SMSA broth for 72h before DNA extraction improved the detection level by 10 to 100 fold in soil or water samples.
- This method can be used efficiently to monitor the contamination of the environment by low densities of *Ralstonia solanacearum* and avoid its spread.

Le Roux-Nio,2009

PCR primers useful for *Ralstonia solanacearum* and closely related bacteria

Primer	Primer sequence (5' to 3')	Anneal temp.	No. bases Amplified	Specificity'
Y2 aLII	CCCACTGCTGCCTCCCGTAGGAGT GGGGGTAGCTIGCTACCTGCC		287-288	*R. sol.* + related
759 760	GTCGCCGTCAACTCACTTICC GTCGCCGTCAGCAATGCGGAATCG		281	*R. sol.* + related
PS96-H PS96~	TCACCGAAGCCGAATCCGCGTCCATCAC AAGGTGTCGTCCAGCTCGAACCCGCC		148	*R. sol.*
pehA#3 pebA#6	CAGCAGAACCCGCGCCTGATCCAG ATCGGACTIGATGCGCAGGCCGTI		504	*R. sol.*

Primer pairs Y2IOLII and 759n60 amplify the fragments of the indicated size from *R. solanacearum*, *P. syzygii*, and the BD bacterium, but not from *R. pickettii*; *R. eutropha*, or other bacteria.
Primer pair PS96-Hl-I amplifies DNA only from *R. solanacearum*,
Primer pair pehA#3/#6 amplifies amplifies part of the polygalacturonase gene from all *R solanacearum* tested but not from *R. pickettii*; amplification from *P. syzygii* and BD bacterium were not tested.

Schaad *et al.*,2001

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

Genus *Ralstonia*

Genus *Ralstonia*					
Species/ biovars	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
R. solanacearum	**PS96H/PS96I** Chromosomal DNA (unknown)	Conventional	Bacteria, plant (boiled)	Seal *et al.*, 1992a; Hartung *et al.*, 1998	*Pseudomonas solanacearum*
R. solanacearum	**T3A/T5A** tRNA consensus	Conventional	Bacteria (boiled)	Seal *et al.*, 1992b	*P. solanacearum*
R. solanacearum	**pehA # 3/ pehA # 6** *pehA* gene (polygalacturonase)	Conventional	Bacteria, plant (DNA extraction)	Gillings *et al.*, 1993	*P. solanacearum*
R. solanacearum	**759/760** Genomic DNA (unknown)	BIO	Soil suspensions plated on selective medium (DNA extraction)	Ito *et al.*, 1998	

Palacio-Bielsa *et al.*,2009

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

R. solanacearum	**D1/B** **D2/B** **OLI1/Z** 16S rRNA gene	Conventional	Bacteria, potato tuber (untreated)	Boudazin *et al.*, 1999; van der Wolf *et al.*, 2000	D1/B identify *R. solanacearum* division 1 strains. OLI/Z primers identify *R. solanacearum* division 2 strains.
R. solanacearum	**BP4-R/BP4-L** RAPD fragment	Conventional	Soil (DNA extraction)	Lee and Wang, 2000	
R. solanacearum	**PS-1/PS-2** 16S rRNA gene	Conventional	Potato tubers (DNA extraction)	Pastrik and Maiss, 2000	
R. solanacearum	**OLI-1/Y-2** 16S rRNA gene **OLI-1/OLI-2 + JE2/Y2** 16S rRNA gene	Conventional Nested	Soil suspensions (previously enriched and boiled)	Pradhanang *et al.*, 2000	
R. solanacearum	**Multiplex (generic)** **RS-I/RS-II** (primers) **RS-P** (probe) 16S rRNA gene **Multiplex (biovar 2A)** **B2-1/B2-II** (primers) **B2-P** (probe) 16S rRNA gene **Multiplex (internal control, host)** **RS or B2** **+** **COX-F/COX-R** (primers) **COX-P** (probes) Potato cytochrome oxidase gene	Multiplex real-time (TaqMan) Real-time (TaqMan)	Bacteria, potato tubers extract (boiled)	Weller *et al.*, 2000	
R. solanacearum	**PS-IS-F/PS-IS-R** Insertion sequence (IS*1405*) **PS-IS RA1** **PS-IS-RB1** Flanking regions of IS*1405*b and IS*1405*d	Conventional	Bacteria (untreated)	Lee *et al.*, 2001	Specific detection of *R. solanacearum* race 1.
R. solanacearum	**Rs-1-F/Rs-3-R** 16S-23S rDNA spacer region/ *R. solanacearum* division I **Rs-1-F/Rs-1-R** 16S-23S rDNA spacer region/ *R. solanacearum* division II	Conventional	Bacteria, potato tubers (DNA extraction)	Pastrik *et al.*, 2002	

Palacio-Bielsa *et al.*,2009

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

	NS-5-F/NS-6-R 18S rDNA (host internal control) **Rs-1-F/Rs-1-R+NS-5-F/NS-6-R**	Multiplex			
R. solanacearum	**OLI1/Y2** **OLI1/Z** **OLI1/OLI2** **OLI1/OLI2/JE-2** **(Co-PCR)** 16S rRNA gene	Conventional Co-operative	Bacteria (boiled), water	Caruso *et al.*, 2003	
R. solanacearum	**RSC-F/RSC-R (primers)** **RSC-P (probe)** DNA fragment specific to biovar 2	Real-time (TaqMan)-BIO	Potato tuber extract (boiled)	Ozakman and Schaad, 2003	Race 3, biovar 2 strains are specifically amplified.
R. solanacearum	**RsoLfliC** *fliC* gene (flagellar subunit protein)	Conventional	Bacteria, soil (DNA extraction)	Schönfeld *et al.*, 2003	
R. solanacearum	**OLI-1/Y-2** 16S rRNA gene	Conventional	Pure culture (DNA extraction)	Seal *et al.*, 1993	*P. solanacearum* *Ralstonia syzygii* (*Pseudomonas syzygii*) and Blood Disease Bacterium also amplified.
R. solanacearum	**DIV1F/DIV1R** **DIV2F/DIV2R** **OLI1/BV345** **DIV2F/ITRS** 16S rRNA gene and 16S-23S rRNA region **OLI1+Y2+BV345**	Conventional Multiplex	Bacteria (boiled)	Seal *et al.*, 1993, 1999	Division I and II of *Ralstonia solanacearum* differentiated. *Ralstonia syzygii* (*Pseudomonas syzygii*) and Blood Disease Bacterium also amplified.
R. solanacearum	**RS30/RS31 (external)** **+** **RS30a/RS31a/RS30b/RS31b (internal)** *hrp* genes cluster	Nested	Bacteria (boiled), plant, water and soil (DNA extraction)	Poussier and Luisetti, 2000	*Ralstonia syzygii* and Blood Disease Bacterium also amplified.
R. solanacearum	**RS3/Rs4** *R. solanacearum pehB* gene **XcpM1/XcpM2** *X. c.* pv. *pelargonii* DNA (ERIC) **DG1/DG2** 18S rRNA gene (host internal control)	Multiplex	Bacteria or plant (DNA extraction)	Glick *et al.*, 2002	*Xanthomonas hortorum* pv. *pelargonii* (*X. campestris* pv. *pelargonii*) also amplified.

Palacio-Bielsa *et al.*,2009

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

Blood Disease Bacterium(BDB)

Blood Disease Bacterium (BDB)					
Organism	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
Blood Disease Bacterium (remains unclassified)	**OLI1/Y2** 16S rRNA gene	Conventional	Bacteria (boiled)	Seal *et al.*, 1993	*Ralstonia solanacearum* and *R. syzygii* also amplified.
Blood Disease Bacterium (remains unclassified)	**D2/B**[1] **OLI1/Z** 16S rRNA gene	Conventional	Bacteria (untreated)	Boudazin *et al.*, 1999	

See also BLOs primer section

Palacio-Bielsa *et al.*,2009

Identification of the pathogen using two different PCR primers *Ralstonia solanacearum*

- Identification of the pathogen was done by PCR using two primers pairs:

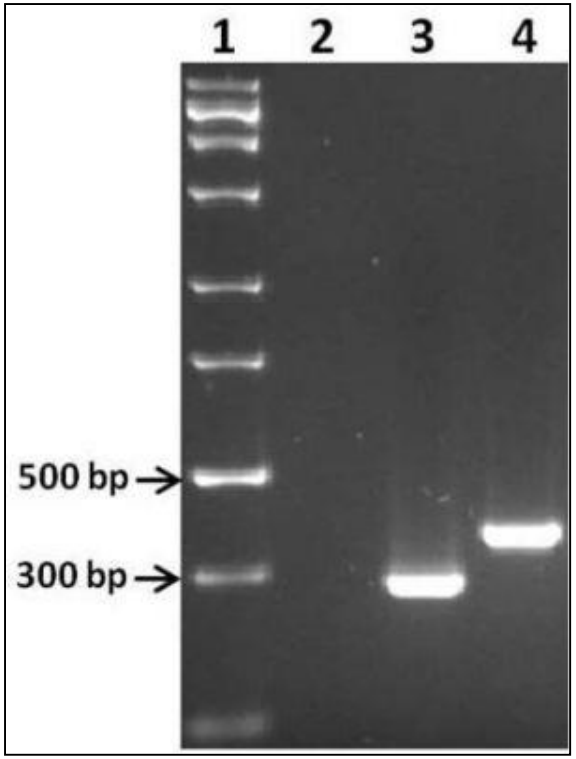
1. 759/760 F(5'-GTCGCCGTCAACTCACTTTCC-3', 5'-GTCGCCGTCAGCAATGCGGAATCG-3'; Opina *et al*.,1997),
2. Nmult21:2F/Nmult22:RR (5'-AAGTTATGGACGGTGGAAGTC-3', 5'-TCGCTTGACCCTATAACGAGTA-3'; Fegan & Prior,2005).

- Each amplification reaction generated 282 bp and 372 bp amplicons, respectively, the first corresponding to the upstream region of *lpxC* gene and used to identify *Ralstonia solanacearum* at the species level;
- The second corresponding to the 16S-23S ITS region specific to phylotype II of *Ralstonia solanacearum*.

Hernández-Romano *et al*.,2012

Identification of the pathogen using two different PCR primers *Ralstonia solanacearum*

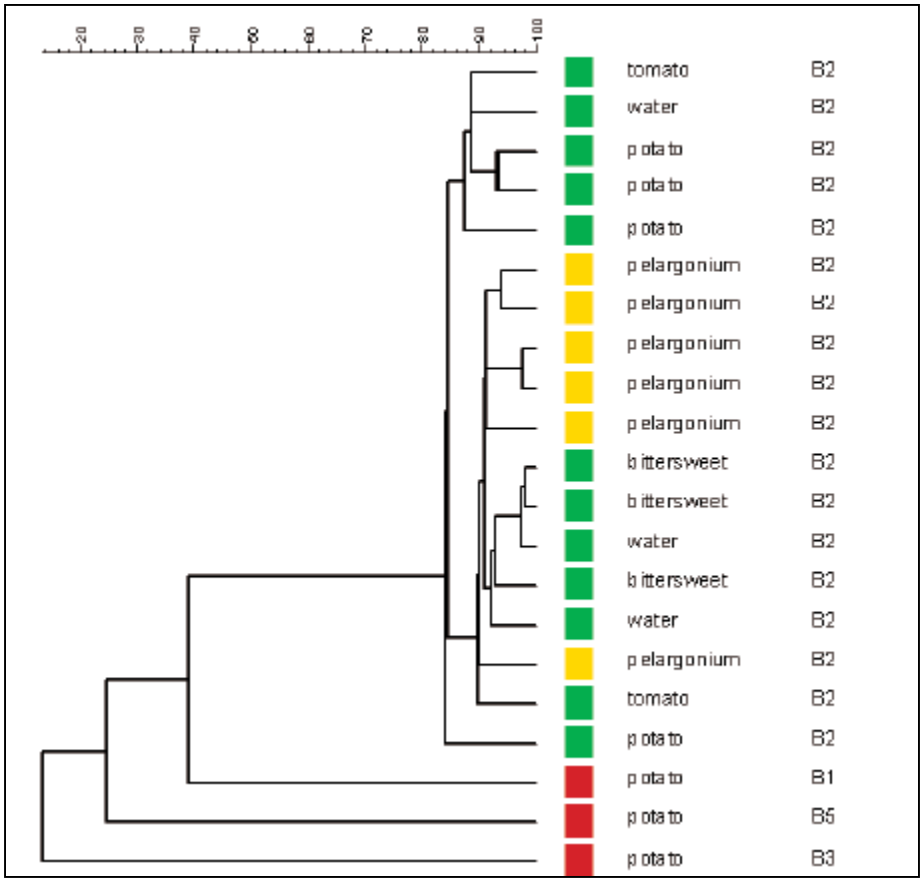
- Identification of *Ralstonia solanacearum* from tomato plants using different PCR primers.
- Lane 1, molecular weight marker, Fermentas SM1373, ladder 1.
- Lane 2, non-template control.
- Lane 3, 282 bp amplicon obtained using primers 759/760.
- Lane 4, 372 bp amplicon obtained using primers Nmult21:2F/Nmult22:RR.



Hernández-Romano *et al.*,2012

rep-PCR
BOX-PCR

- UPGMA dendrogram obtained from BOX-PCR fingerprint.
- *Pelargonium* strains clearly cluster with biovar 2 strains of solanaceous hosts and isolated from surface water.



Janse,2006

Multiplex PCR reaction

To identify of *R. solanacearum* race 2/biovar 1 sequevars (the causal agent of Moko disease)in a single reaction

- All procedures are as Uniplex PCR assays apart from the making of the MasterMix which instead of the one primer pair (2 primers) for the PCR's outlined above contains 5 primer pairs (10 primers) in a multiplex reaction.
- The five primer pairs include:

1. All three sequevar specific PCR primer pairs and
2. An *R. solanacearum*-species specific PCR primer pair, 759/760 (Opina, Tavner *et al*.,1997).

Plant Health Australia,2006

Multiplex PCR reaction

To identify of *R. solanacearum* race 2/biovar 1 sequevars (the causal agent of Moko disease)in a single reaction

- The four genetic groups identified to contain *R. solanacearum* race 2/biovar 1 moko disease causing strains are designated as:
- sequevar 3 (MLG or multilocus genotypes 24),
- sequevar 4 (MLG 25),
- sequevar 6 (MLG 28), and
- sequevar 24.
- A multiplex PCR protocol to identify all of these groups in a single PCR has also been developed.
- This allows the detection of all *R. solanacearum* race 2/biovar 1 strains causing moko disease in a single amplification reaction.

Plant Health Australia,2006

Multiplex PCR reaction

To identify of *R. solanacearum* race 2/biovar 1 sequevars (the causal agent of Moko disease)in a single reaction

- Primer pairs and specificities.

Primer	Primer Sequence	Specificity
SI28F	CGTTCTCCTTGTCAGCGATGG	Sequevar 6 (MLG 28)
SI28R	CCCGTGTGACCCCGATAGC	
MUS20F	CGGGTGGCTGAGACGAATATC	Sequevar 4 (MLG 25)
MUS20R	GCCTTGTCCAGAATCCGAATG	
IS24F	TCGGGCGTAAGAGGCAGAC	Sequevar 3 (MLG 24)
IS24R	GGAGGTGTGCGCCATCAACTG	
VC46F	CTCCTGGGAGTCGGTTGGGTC	Sequevar 24
VC46R	AGGGAACCTAGGCGTGACTG	
759	GTCGCCGTCAACTCACTTTCC	*R. solanacearum*
760	GTCGCCGTCAGCAATGCGGAATCG	

- Preparation of 10x primer mix (containing 2 µM each primer).

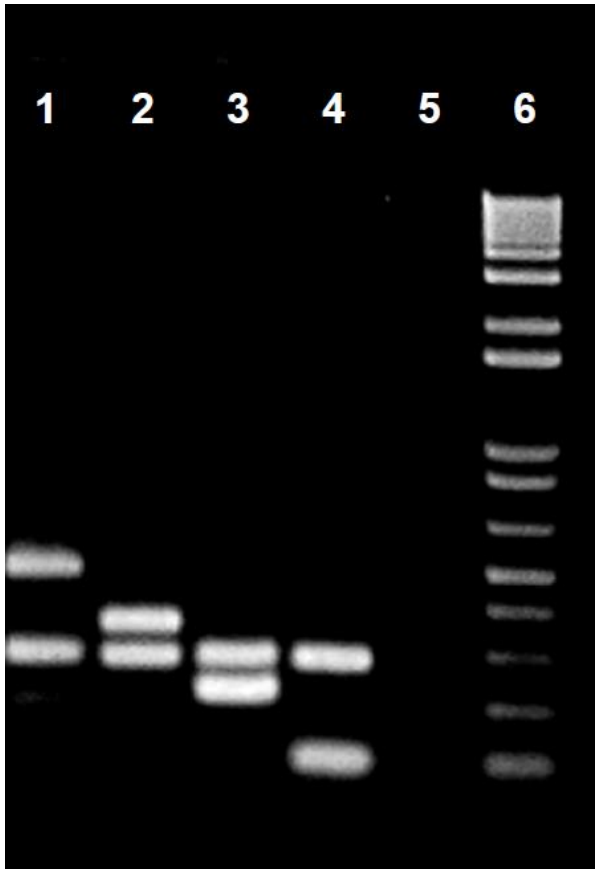
Concentration of primer stock	50 µM (50 pmol/µl)	100 µM (100 pmol/µl)
Each primer (SI28F, SI28R, MUS20F, MUS20R, IS24F, IS24R, VC46F, VC46R)	20 µl	10 µl
Primers 759, 760	10 µl	5 µl
TE buffer (See Chapter 11)	Variable	Variable
Total volume	500 µl	500 µl

Plant Health Australia,2006

Multiplex PCR reaction

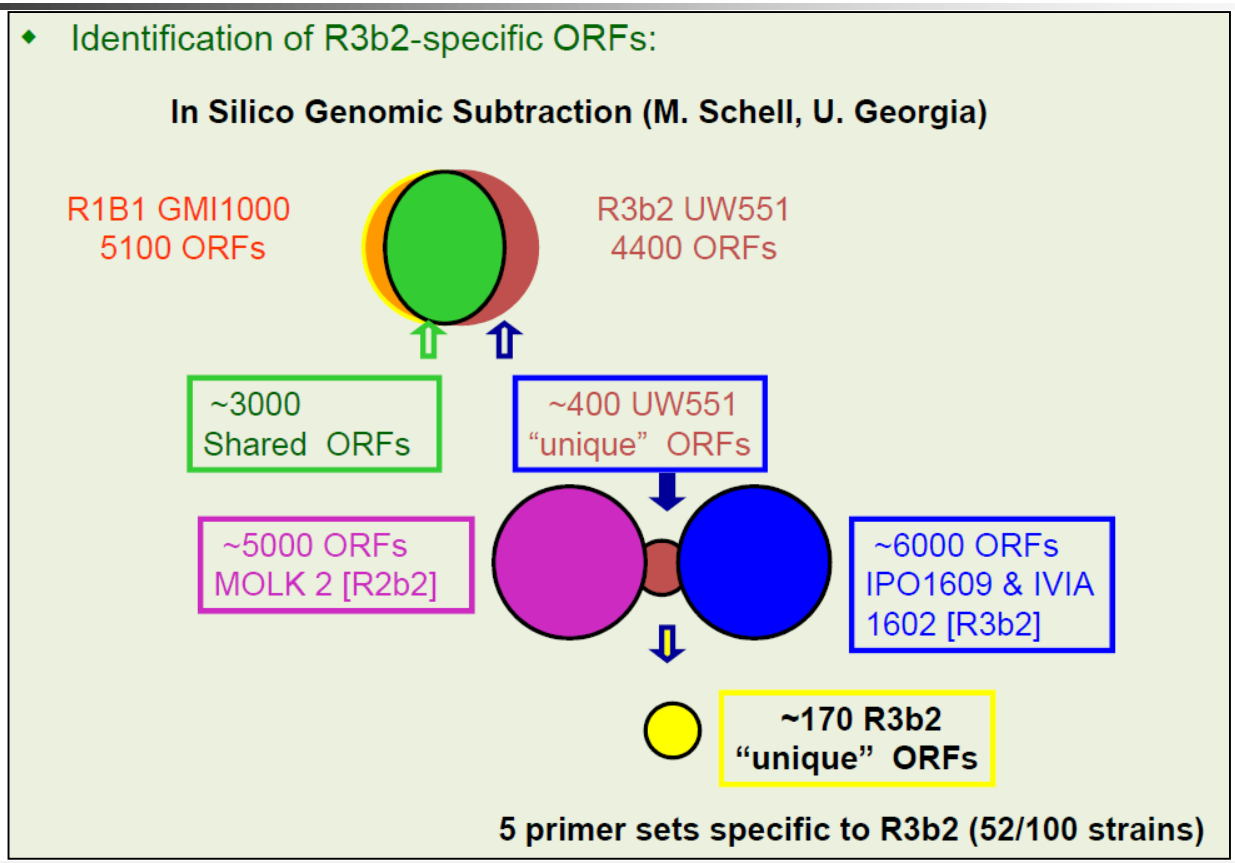
To identify of *R. solanacearum* race 2/biovar 1 sequevars (the causal agent of Moko disease)in a single reaction

- Amplification of *R. solanacearum* race 2/biovar 1 strains with the Moko multiplex PCR.
- Lane 1 – *R. solanacearum* race2/biovar 1 sequevar 3,
- Lane 2 - *R. solanacearum* race2/biovar 1 sequevar 4,
- Lane 3 - *R. solanacearum*
- race2/biovar 1 sequevar 6,
- Lane 4 - *R. solanacearum* race2/biovar 1 sequevar 24,
- Lane 5 negative control and
- Lane 6 molecular weight DNA marker.



Plant Health Australia,2006

Diagnostic methods
Identification of R3b2-specific ORFs
In Silico Genomic Subtraction



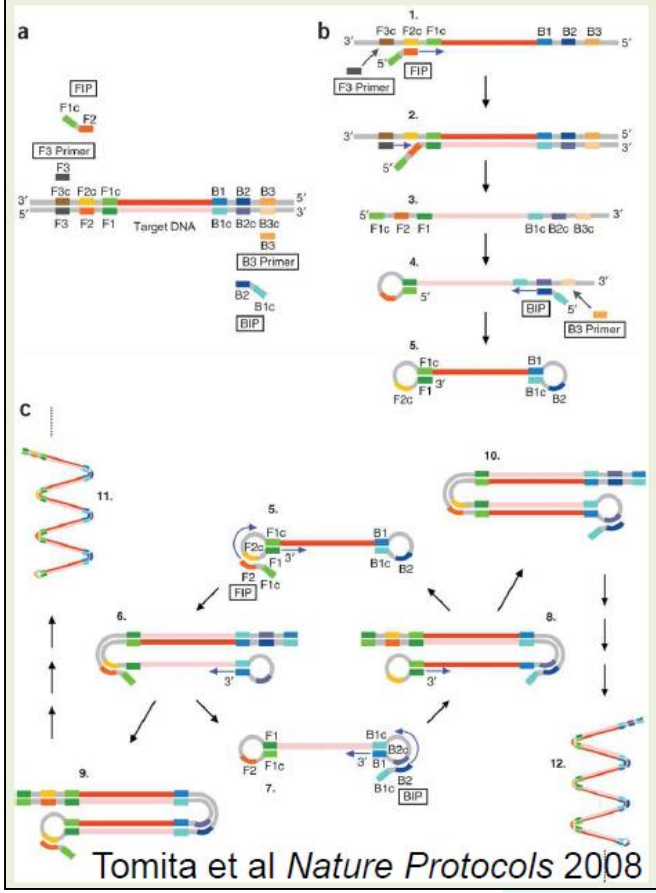
In silico is an expression used to mean "performed on computer or via computer simulation". The term was used to characterize biological experiments carried out entirely in a the computer.

Champoiseau,2009

Diagnostic methods

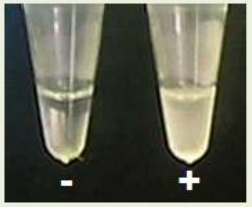
LAMP: Loop-mediated Isothermal Amplification

- LAMP: Loop-mediated isothermal AMPlification



Tomita et al *Nature Protocols* 2008

- **A simple rapid PCR-like way to generate large quantities of DNA**
- Highly specific (3 primer sets)
- Only need 2 temperatures
- No thermocycler required



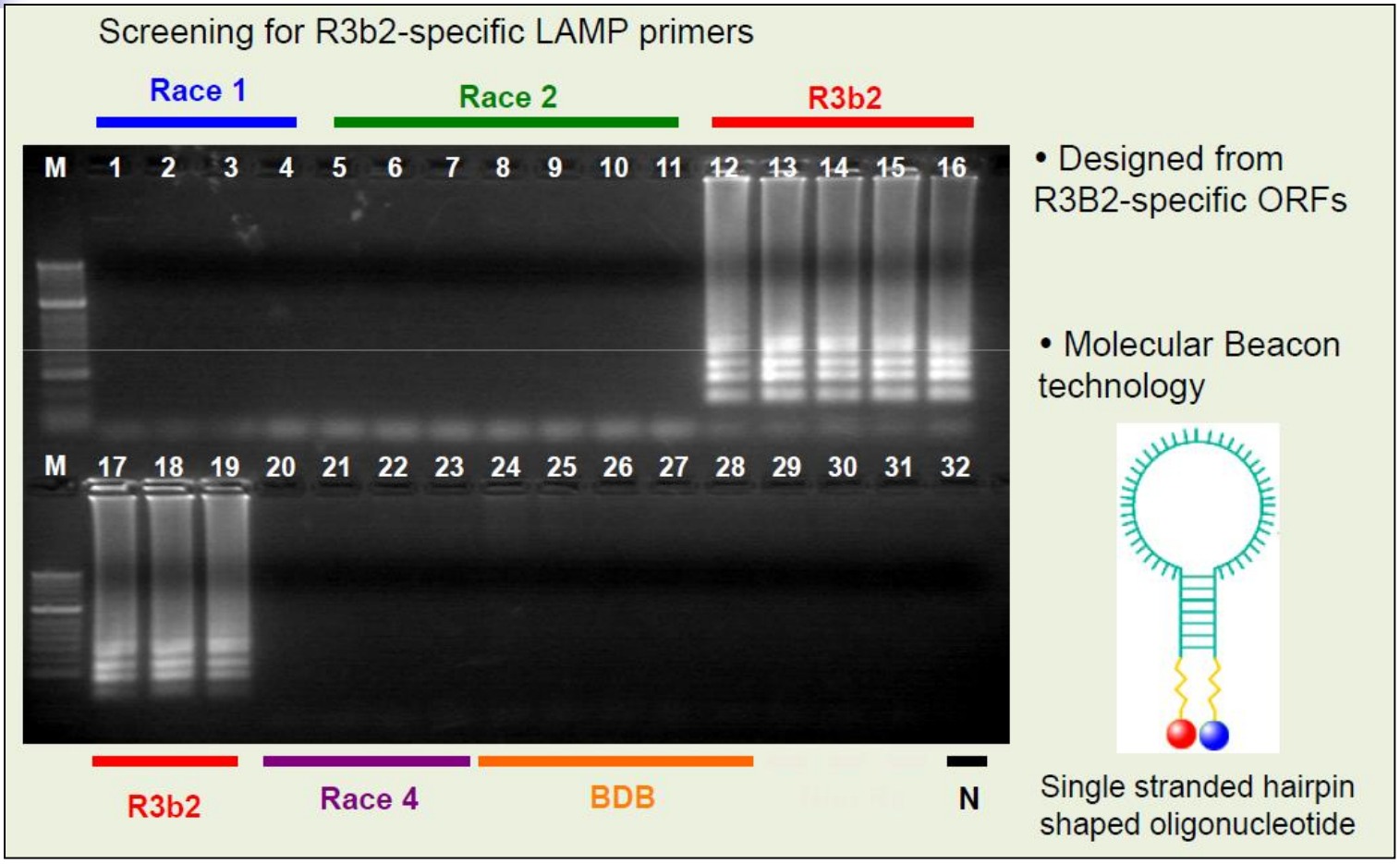
Rapid Visual Assessment

- Sensitive to PCR inhibitors

Champoiseau,2009

Diagnostic methods

LAMP: Loop-mediated isothermal Amplification



Champoiseau,2009

Pathogenicity test
Ralstonia solanacearum

- For plants with large enough stems, the easiest inoculation method is to make a puncture wound into the pith at a leaf axil and apply a drop of inoculum.
- For plants like tomato and eggplant, this is best done by using a disposable tip from a mechanical pipettor to make the wound and then inserting a fresh pipet tip containing 10 or 20µl of inoculum ($1X10^6$ CFU/ml) into the hole.
- Alternatively, a lower leaf can be excised about 0.5 cm from the stem and a droplet of inoculum (e.g., 20µl containing $2x10^5$ cells) deposited on the cut surface of the petiole.

Schaad *et al.*,2001

Pathogenicity test
Ralstonia solanacearum

- Incubate the plants in a greenhouse or growth chamber at 30 to 32°C during the day and 25°C at night (28°C day and 16°C night for the cool temperature race 3 strains from potato), relative humidity> 85%, with 12 h light and 12 h dark periods.
- After the inoculum is taken up by the plant (3 to 10 h or overnight), the pipet tip is removed and the hole that remains is left exposed.
- Keep the plants well watered, but avoid wetting the foliage.
- Wilt symptoms should begin to appear within 4 to 10 days.

Schaad *et al.*,2001

Pathogenicity test
Ralstonia solanacearum

- More natural inoculation methods require that bacteria be applied to plant roots.
- Since these methods require large volumes of inoculum, the bacteria are usually grown in liquid minimal medium, collected by centrifugation and suspended in water.
- Tomato plants can be inoculated by pouring sufficient inoculum onto the soil in the pot containing a young plant (of the same age as for stem inoculation) to give about $1x10^7$ CFU/gram soil.
- Intentionally wounding the roots is not essential, but to improve infection consistency (or to reduce the volume of inoculum needed), it is common to wound some of the roots by drawing a knife through the soil on one side of the plant just before inoculation.
- After inoculation, water plants from the bottom to prevent washing the bacteria out of the soil.

Schaad *et al.*,2001

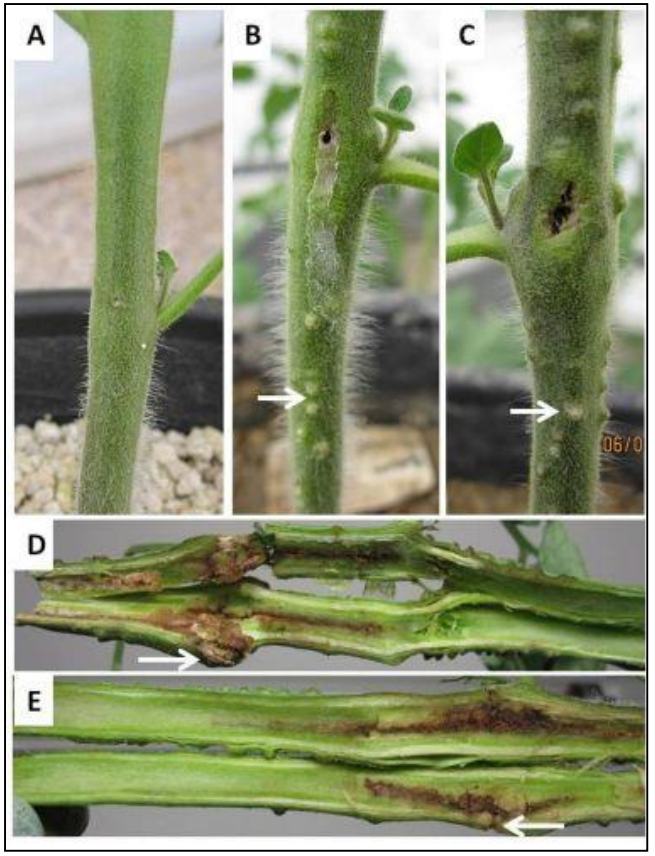
Pathogenicity test on tomato plants *Ralstonia solanacearum*

- Pathogenicity can be determined by inoculating a suspension 10^6 cell ml^{-1}) of a 48-h nutrient agar/YPGA culture into 5-10 susceptible tomato (e.g. cv Moneymaker) or aubergine (e.g. cv Black Beauty) plants at, preferably, the third true leaf stage or slightly older.
- Incubation should be for up to two weeks at 25-28°C under high relative humidity conditions.
- Symptoms are wilting and/or epinasty, chlorosis, sometimes only stunting.
- Colonies can be used for confirmation by IF, PCR or FISH.
- For race determination, also tobacco (e.g. cv. White Burley) and *Musa acuminata* can be inoculated and incubated as described above.
- Symptom development in *M. acuminata* may take longer than two weeks.

OEPP/EPPO Bulletin,2004

Pathogenicity test on tomato plants cv. Ramses

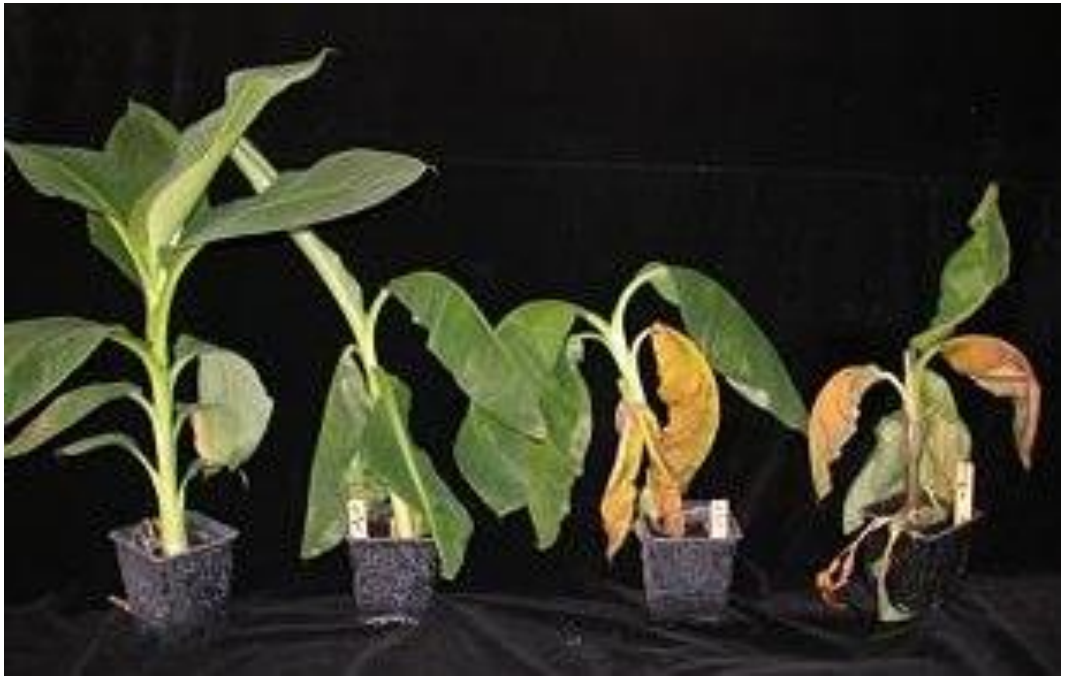
- Control plants: Lesions made with needle without bacteria (A);
- Infected plants showed oozing brown lesions (B, C) and
- The stem showing the emergence of adventitious roots (B, C, arrows);
- Dissection showing bacterial dissemination through the plant xylem with point of inoculation indicated by arrow (pointing towards plant apex) (D, E).



Hernández-Romano *et al.*,2012

Pathogenicity test on banana trees

Evaluation of the disease on a banana tree infected by reference strain of Molk2



Bacterial suspensions were adjusted to an optical density of 0.06 at 660 nm wavelength corresponding to ~10^8 colony-forming units per milliliter (CFU/ml). Molk2 is reference strain of Moko disease.

Genoscope; Sikirou,2017

Bioindicator hosts for detecting *R. solanacearum* ginger race(race 4)

- Bioindicator plant materials (arrows) for detecting *R. solanacearum* race 4:

a) Edible ginger in the field;

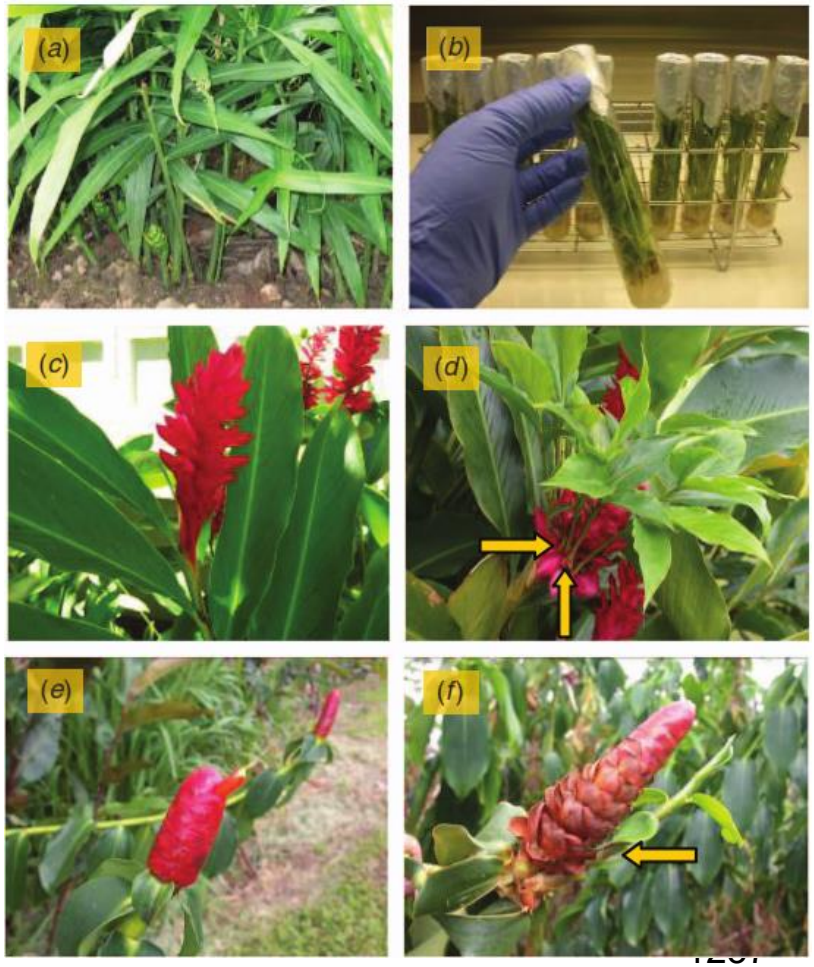
b) Tissue-cultured ginger;

c) Red ginger inflorescence;

d) Micro-sized red ginger plantlets formed from the inflorescence;

e) Spiral ginger inflorescence;

f) Micro-sized spiral ginger plantlet from the inflorescence.



Paret *et al.*,2009

Disease severity and virulence
Inoculation techniques for evaluating resistance to *R. solanacearum*

- To quantify:

1. Relative virulence of bacterial strains, or
2. Resistance of plant cultivars,

- Record:

1. The percentage of leaves wilted (or the number of plants completely wilted) on each plant on a daily basis and then
2. Calculate the average percentage wilt on each day for each treatment.

Schaad *et al.*,2001

Disease severity and virulence

Inoculation techniques for evaluating resistance to *Ralstonia* (ex *Pseudomonas*) *solanacearum*

- The rating system of Winstead and Kelman (1952) has also been widely used.

1. Observe the wilt symptoms developing from 5 to 21 days after inoculation, and
2. record disease ratings using the following scale:

- 1=no symptoms; 2=one leaf wilted; 3=two to three leaves wilted; 4=four or more leaves wilted; 5=whole plant wilted (dead plant).
- Calculate the wilt intensity 21 days after inoculation, using the following formula:

$$I = [L\ (n \times vJ + (V \times N)]\ 100$$

- where I= wilt intensity (%); L= number of plants with respective disease rating; v= disease rating (1,2,3,4 or 5); V= the highest disease rating (5); and N= the number of plants observed.

Schaad *et al.*,2001

Disease severity and virulence
R. solanacearum

- Inocula for pathogenicity tests were adjusted to a density of $1x10^7$ cfu/ml spectrophotometrically to at A_{600}=0.1 (Somodi *et al.*,1992).
- Disease severity was determined on a 1-5 scale:
- 1= no visible symptoms; 2= 1-25% of the plant is wilting; 3= 26-50% wilt, 4= 51-75% wilt and 5= more than 75% wilt.
- Virulence of each strain was rated according to the mean performance of 10 plants from each host after 30 days of inoculation:
- Virulence=1.0; Low virulence= 1.1-2.5; Medium virulence= 2.6-4.0 and High virulence=4.1-5.0 (kelman and Person,1961).

Gunathilake *et al.*,2004

Determination of *R. solanacearum* aggressiveness using hypersensitivity reaction test

- Briefly, tobacco plants (*Nicotiana tabacum*) were first grown on autoclaved soil and maintained in a glasshouse at 18 to 22°C with regular watering. The inoculum was prepared by growing bacterial overnight in liquid nutrient broth medium as earlier described then pelleted by centrifugation. The concentration of cell suspension was adjusted to OD_{600} = 0.1 (~10^8 cfu/mL) using a spectrophotometer.
- Fully expanded leaves from 54 days old plant were infiltrated with a suspension of each *R. solanacearum* isolate using a sterile syringe following the injection technique described by Klement (1963). Sterile distilled water was used as a negative control while an isolate provided by CIP was also included as a positive control.
- Two leaves per plant from a total of 4 plants were infiltrated with each isolate and HR described as necrotic or yellowing areas in the region surrounding an infection point monitored daily for 2 weeks post infiltration. This was scored as described by Shahbaz *et al*. 2015.

Chamedjeu *et al*.,2018

Determination of *R. solanacearum* aggressiveness using hypersensitivity reaction test

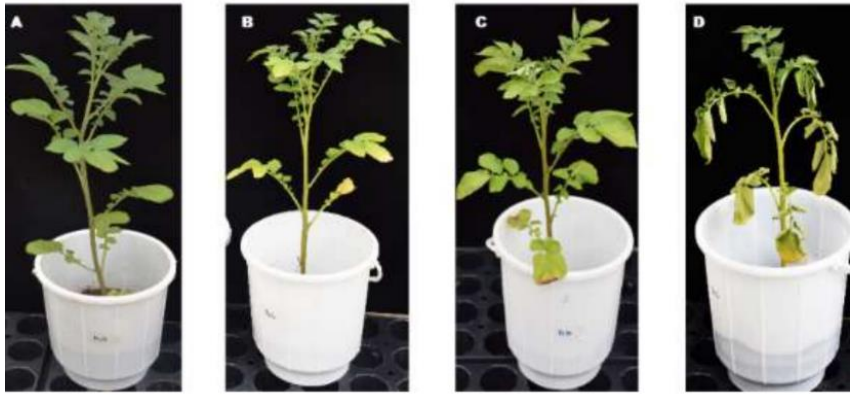
- Different types of symptoms induced on potatoes (cv. Shangi) by the tested *R. solanacearum* strains.

A. No visible symptoms (control with H_2O);

B. Yellowing or chlorotic spots on the leaves;

C. Black streak on the stem and wilting of the upper leaves;

D. Wilting of all leaves and plant death.



Chamedjeu *et al.*,2018

Culture preservation
R. solanacearum

- This pathogen easily loses virulence if repeatedly transferred on agar plates and loses viability if plates are stored at 4°C.
- **Water suspensions:**
- *R. solanacearum* can be stored for several years in deionized water (or tap water) without significant loss of virulence or change in phenotype (Kelman & Person,1961).
- Tap water is a source of useful trace elements and can be used for soil bacteria isolation/culturing. It needs to boil to eliminate chorine which may be slightly toxic for some species.
- **Lyophilizes:**
- *R. solanacearum* tolerates lyophilization very well and some lyophilized cultures known to have remained viable for 30 years.
- **Cryostorage:**
- Freezing (less than -70° C) *R. solanacearum* in a cryoprotectant is the most convenient method of long term storage with minimal phenotypic changes.
- It will also survive long-term at -80°C in liquid culture broth amended to 40% glycerol.

Schaad *et al.*,2001; Champoiseau & Allen,2009

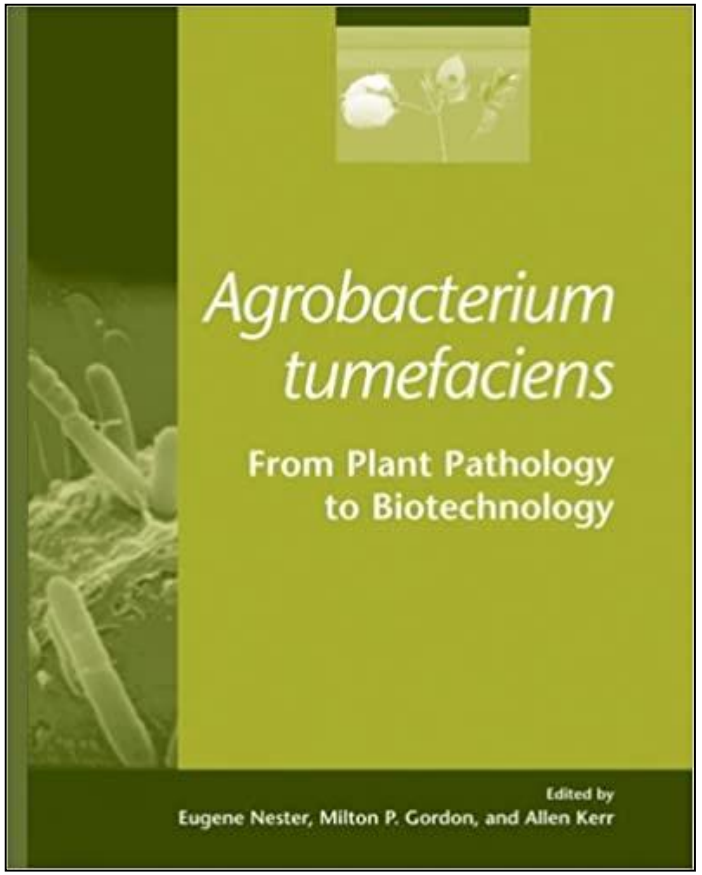
Identification of the bacterial pathogens
Agrobacterium

Disease diagnosis and pathogen diagnostics

Etymology: Gr. n. *rhiza*, a root; Gr. masc. n. *bios*, life; N.L. neut. n. *Rhizobium*, that which lives in a root.
Etymology: Gr. n. *agros*, a field; L. n. *bacterium*, a small rod; N.L. neut. n. *Agrobacterium*, a small field rod.

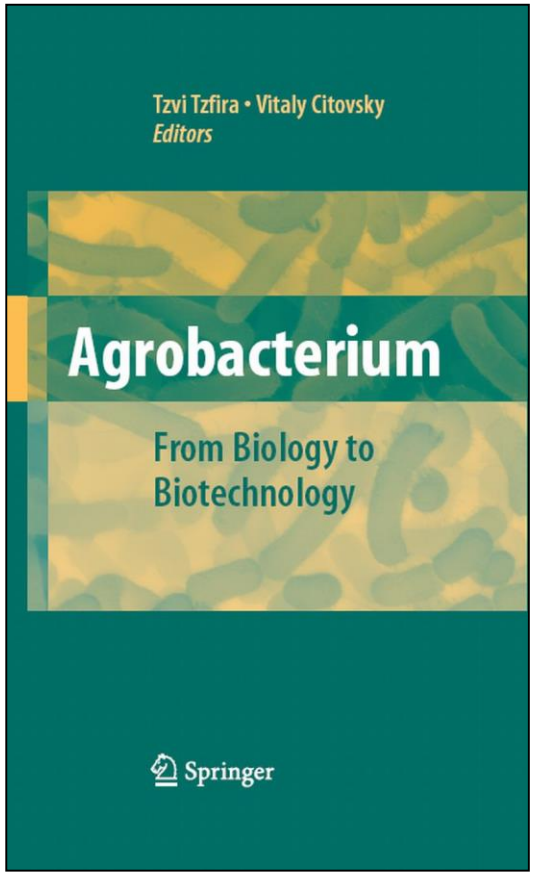
Agrobacterium: From Biology to Biotechnology

- Editors: Eugene Nester, Milton P Gordon, Allen Kerr
- Publisher: Amer Phytopathological Society
- 2005
- 320 pages.



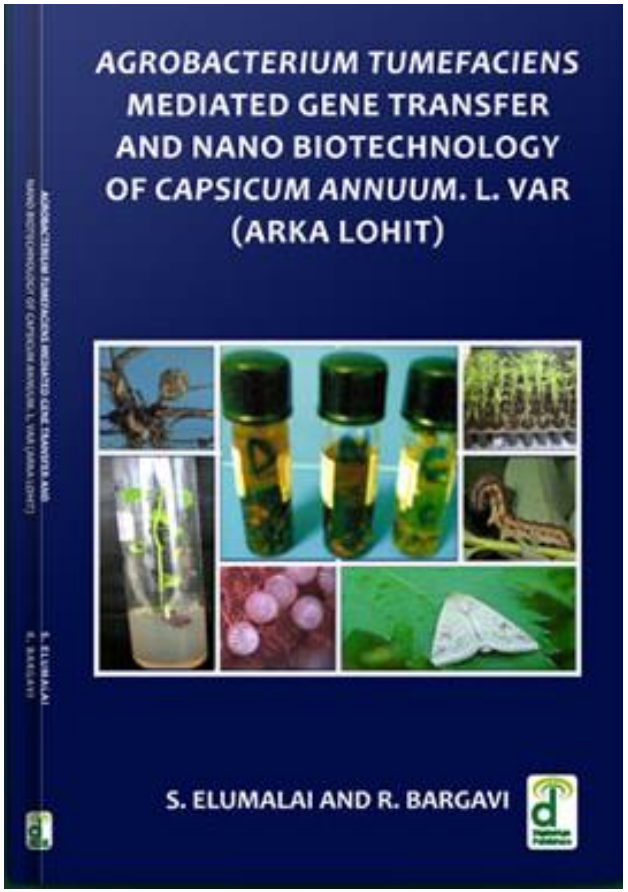
Agrobacterium: From Biology to Biotechnology

- Editors: Tzvi Tzfira and Vitaly Citovsky
- Springer
- 2008
- 784 pages



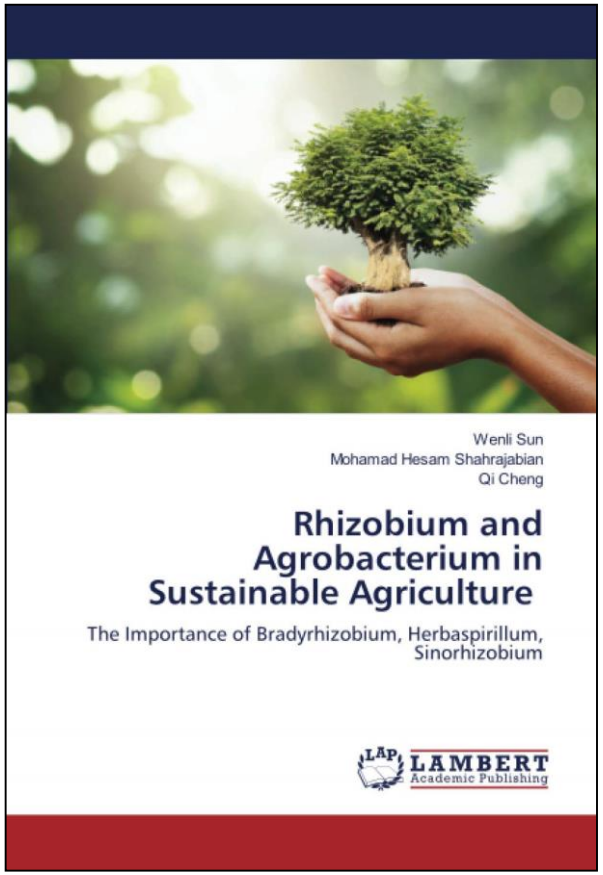
Agrobacterium tumefaciens Mediated Gene Transfer and Nano Biotechnology of *Capsicum annuum*. L.Var (Arka Lohit)

- Author: S. Elumalai and R. Bargavi
- Category-Biotechnology
- Digital Age Publishers (Chennai).
- 2014
- 164 Pages.



Rhizobium and Agrobacterium in Sustainable Agriculture: The Importance of Bradyrhizobium, Herbaspirillum, Sinorhizobium

- Authors: Wenli Sun, Mohamad Hesam Shahrajabian and Qi Cheng.
- LAP LAMBERT Academic Publishing
- 2020
- 52 pages.



Typical disease symptoms
Caused by Rhizobia

Rhizobiaceae

Agrobacterium **species:** ***tumefaciens*** **(crown gall),** ***rhizogenes*** **(hairy root of carrot),** ***vitis*** **(grapevine) or** ***rubi*** **(rasberry/blackberry)**

apple

grapevine

carrot

blackberry

Cuppels Biology 418a

Cuppels,2007

Agrobacterium tumefaciens is about 1.5-3.0 x 0.6-1.0 µm in size. The pathogenic strains contain at least one large plasmid, the tumor- or root-inducing (Ti and Ri, respectively) plasmids.

Proposed Type species

- Proposed Type species: *Agrobacterium tumefaciens* (Smith and Townsend 1907) Conn 1942 (Approved Lists 1980)

https://lpsn.dsmz.de/genus/agrobacterium

Agrobacterium or Rhizobium, which name to use?

Taxonomy and nomenclature within the genus remain controversial

Agrobacterium: resistance to name change

- Currently there is some confusion over whether *Agrobacterium* or *Rhizobium* is the best name to use for these bacteria.
- Although the taxonomic basis for the reclassification of *Agrobacterium* to *Rhizobium* is supported by some bacterial systematists,
- Others — particularity molecular biologists — prefer to use the *Agrobacterium* name with which they are familiar.

Weir,2013

Domain: Bacteria

Phylum: Proteobacteria

Plant pathogenic bacteria

- Class: Alphaproteobacteria

  Order: Rhizobiales

  Family: Rhizobiaceae

  Genera:

  1. *Agrobacterim*
  2. *Allorhizobium*

Note that *Liberibacter* spp. previously known as Rickettsia-like organisms (RLO) or bacteria-like organisms (BLO) are also included in Class Alpha-bacteria-cluster Rhizobiaceae.

Agrobacterium spp.
with a validly published and correct name

Name	Nomenclatural status	Taxonomic status
Agrobacterium vaccinii Puławska *et al.* 2022	validly published under the ICNP	correct name
Agrobacterium tumefaciens (Smith and Townsend 1907) Conn 1942 (Approved Lists 1980)	validly published under the ICNP	correct name
Agrobacterium tomkonis Singh *et al.* 2022	validly published under the ICNP	correct name
Agrobacterium skierniewicense (Puławska *et al.* 2012) Mousavi *et al.* 2016	validly published under the ICNP	correct name
Agrobacterium shirazense Mafakheri *et al.* 2022	validly published under the ICNP	correct name
Agrobacterium salinitolerans Yan *et al.* 2017	validly published under the ICNP	correct name
Agrobacterium rubi (Hildebrand 1940) Starr and Weiss 1943 (Approved Lists 1980)	validly published under the ICNP	correct name
Agrobacterium rosae Kuzmanović *et al.* 2019	validly published under the ICNP	correct name
Agrobacterium rhizogenes (Riker *et al.* 1930) Conn 1942 (Approved Lists 1980)	validly published under the ICNP	correct name
Agrobacterium radiobacter (Beijerinck and van Delden 1902) Conn 1942 (Approved Lists 1980)	validly published under the ICNP	correct name
Agrobacterium pusense (Panday *et al.* 2011) Mousavi *et al.* 2016	validly published under the ICNP	correct name
Agrobacterium nepotum (Puławska *et al.* 2012) Mousavi *et al.* 2016	validly published under the ICNP	correct name
Agrobacterium leguminum Castellano-Hinojosa *et al.* 2021	validly published under the ICNP	correct name
Agrobacterium larrymoorei Bouzar and Jones 2001	validly published under the ICNP	correct name
Agrobacterium fabacearum Delamuta *et al.* 2020	validly published under the ICNP	correct name
Agrobacterium cavarae Flores-Félix *et al.* 2020	validly published under the ICNP	correct name
Agrobacterium burrii Mafakheri *et al.* 2022	validly published under the ICNP	correct name
Agrobacterium arsenijevicii Kuzmanović *et al.* 2019	validly published under the ICNP	correct name

The International Code of Nomenclature of Prokaryotes (ICNP) formerly the International Code of Nomenclature of Bacteria (ICNB) or Bacteriological Code (BC).

https://lpsn.dsmz.de/genus/agrobacterium,2023

Agrobacterium spp.
with a validly published and correct name

	Name	Host plant disease
1	*Agrobacterium arsenijevicii* Kuzmanović et al. 2019	crown gall tumors on raspberry and cherry plum
2	*Agrobacterium cavarae* Flores-Felix et al. 2020	galls in grapevines
3	*Agrobacterium fabacearum* Delamuta et al. 2020	nodules of plants of the family Fabaceae
4	*Agrobacterium larrymoorei* Bouzar and Jones 2001	aerial tumours of *Ficus benjamina*
6	*Agrobacterium radiobacter* (ex. *Agrobacterium tumefaciens*) (Beijerinck and van Delden 1902) Conn 1942 (Approved Lists 1980)	Synonym (*Rhizobium*)
8	*Agrobacterium rosae* Kuzmanović et al. 2019	galls on different agricultural crops
9	*Agrobacterium rubi* (Hildebrand 1940) Starr and Weiss 1943 (Approved Lists 1980)	galls on blackberry

Euzeby,2021

Habitat
Host range


Plants of the Pine Family

- The genus *Agrobacterium* is represented by species that are indistinguishable from members of *Rhizobium* except that they are pathogenic, producing rhizogenic growths or oncogenic galls rather than symbiotic nodules (Young *et al.* 2001a & 2005).
- Both *Agrobacterium* and *Rhizobium* have the unique capacity to induce prolific root formation, nitrogen fixing root nodules and autonomous crown-gall tumors on many higher plants including:

1. Most dicots,
2. Some monocots, and
3. Some gymnosperms(pinaceous gymnosperm hosts).

Vincent,1970; Murugesan *et al.*,2010; Bull *et al.*,2010

Habitat
Host range

- *Agrobacterium* spp. are aerobic soil borne bacteria with worldwide distribution.
- These infect dicotyledonous plant from over 90 different plant families including economically important fruit and nut crops, grapes, ornamental and landscape plants.
- It can affect over 391 plant genera including many species and cultivars within each group.

Vincent,1970; Murugesan *et al.*,2010;Moore,2014

Diseases caused by *Agrobacterium*

The Complex genus: The genus *Agrobacterium* includes plant-associated bacteria and opportunistic human pathogens

***A. radiobacter* (=*A. tumefaciens*)**	**The causative agent of crown gall tumors**
A. rhizogenes	**The causative agent of hairy root disease**
***Allorhizobium vitis* (=*A. vitis*)**	**The causal agent of tumors and necrotic disease on grapevines**
A. rubi	**The causative agent of cane galls on *Rubrus* (blackberry) species**
A. larrymoorei	**Isolated from gall of weeping fig (*Ficus benjamina*)**
A. skierniewicense	**Tumors on chrysanthemum and cherry plum**

Old classification and nomenclature of *Agrobacterium*

Based on biovar and pathogenic characteristics

- In the early 1970s, physiological and biochemical analyses revealed that agrobacteria, could be divided into three biovars.

1. Biovar 1 (*A. tumefaciens*);
2. Biovar 2 (*A. rhizogenes*);
3. Biovar 3 (*A. vitis*).

- Based on their virulence features (kind of plasmids), they were further divided into three strains:

1. Tumorigenic (Ti plasmid);
2. Rhizogenic (Ri plasmid);
3. Nonpathogenic strains (lacking plasmid).

A. tumefaciens strains lacking the plasmid live as rhizosphere-inhabiting bacteria without causing disease.

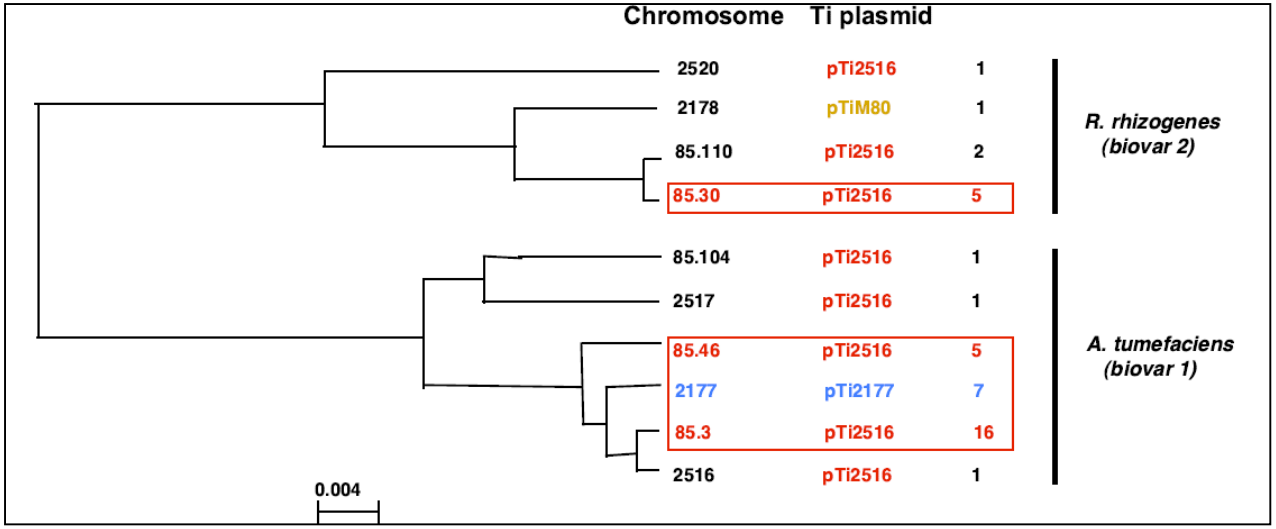
Campillo *et al.*,2012;..

The species complex

Diversity of tumorigenic agrobacteria isolated from poplar crown galls from Orléans

- There is generally more than a single strain involved in crown gall outbreaks at both:

1. Chromosome, and
2. Ti plasmid levels.



A. tumefaciens species complex

Genomovars

***A. tumefaciens* (=*A. radiobacter* =biovar 1) consist of 11 genomovars**

- Taxonomy of "*A. tumefaciens* species complex" (biovar 1) is undergoing substantial reconsiderations because of not only the inhomogeneous nature of the species but also, the isolation of novel strains from various environments.
- So far, more than 10 genomic species "genomospecies" (G; i.e., G1 to G9, G13, and G14) were defined within the A. *tumefaciens* species complex based on DNA-DNA hybridization and amplified fragment length polymorphism (AFLP) analyses.

Genomic species presently called genomovars. Genomovars are phenotypically similar, genotypically distinct groups of strains.

Mafakheri *et al.*,2019

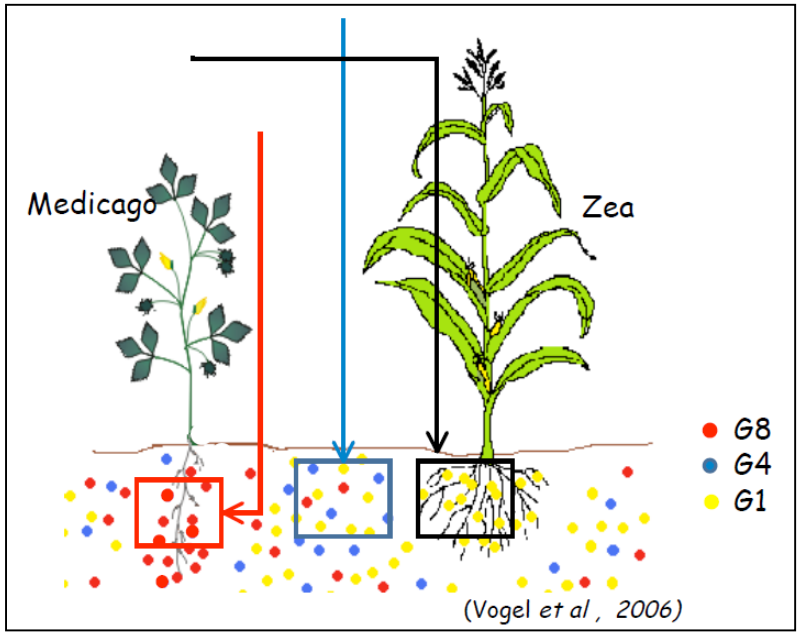
The species complex

Genomic species diversity in *Agrobacterium* spp.

A. tumefaciens (=*A. radiobacter* =biovar 1) consist of more than genomovars

- The species complex *A. tumefaciens* (=*A. radiobacter* =bv.1) consist of many genomovars (G1 to G7, G9, G13. G14 and G15).
- *Agrobacterium* genomovars are differentially selected by plants.

1. G1, G4 and G8 in the same soil;
2. G1 in maize rhizospheres;
3. G8 in medicago rhizospheres.

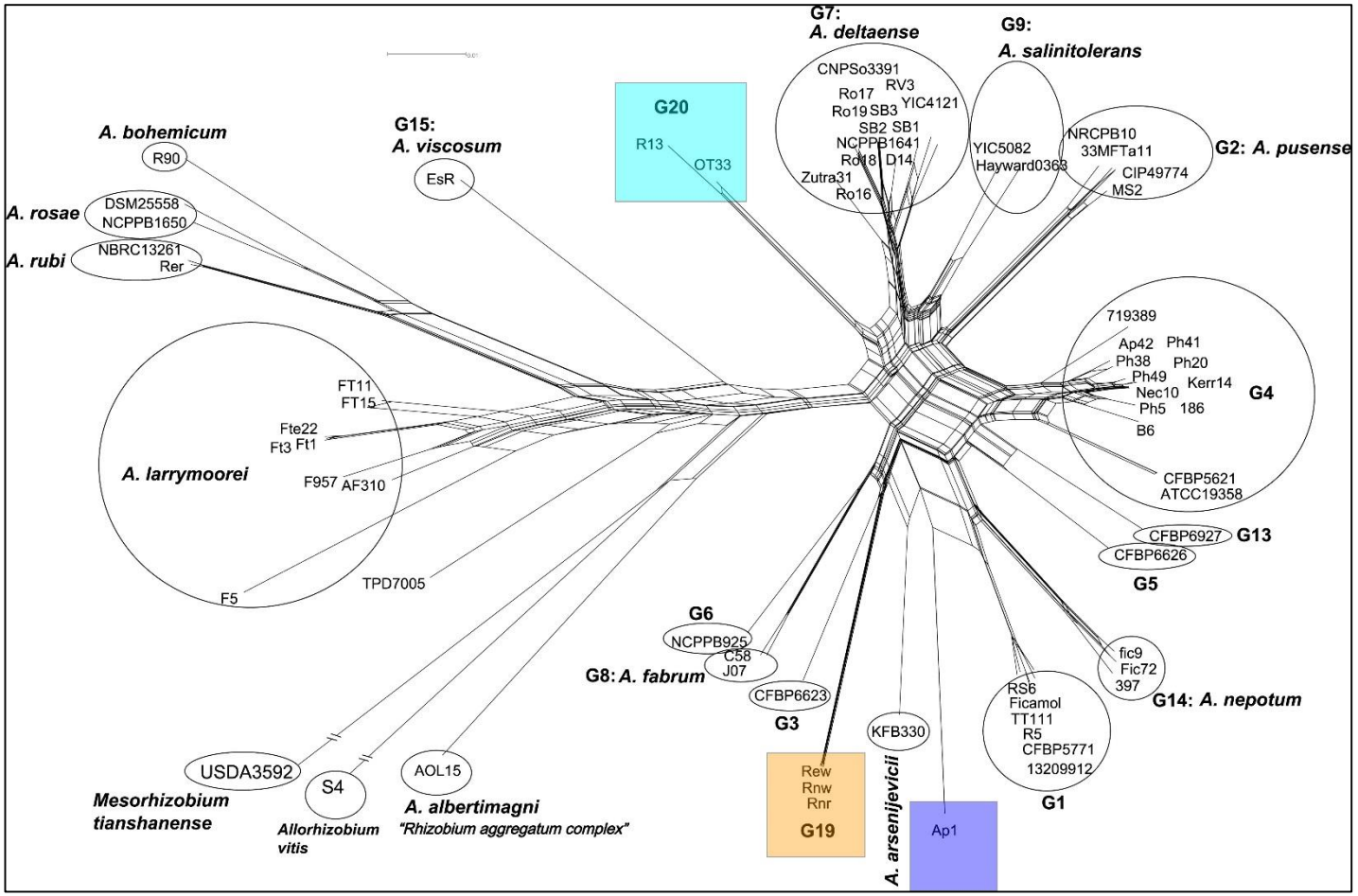


The question is are genomovars ecovars?

Genomovars are phenotypically similar, genotypically distinct groups of strains.

The species complex

Two novel genomospecies within the *A. tumefaciens* species complex were found and we named as G19 and G20.These were isolated from rose and apple plants in Iran



Mafakheri *et al.*,2019

Sampling methods
Agrobacteria

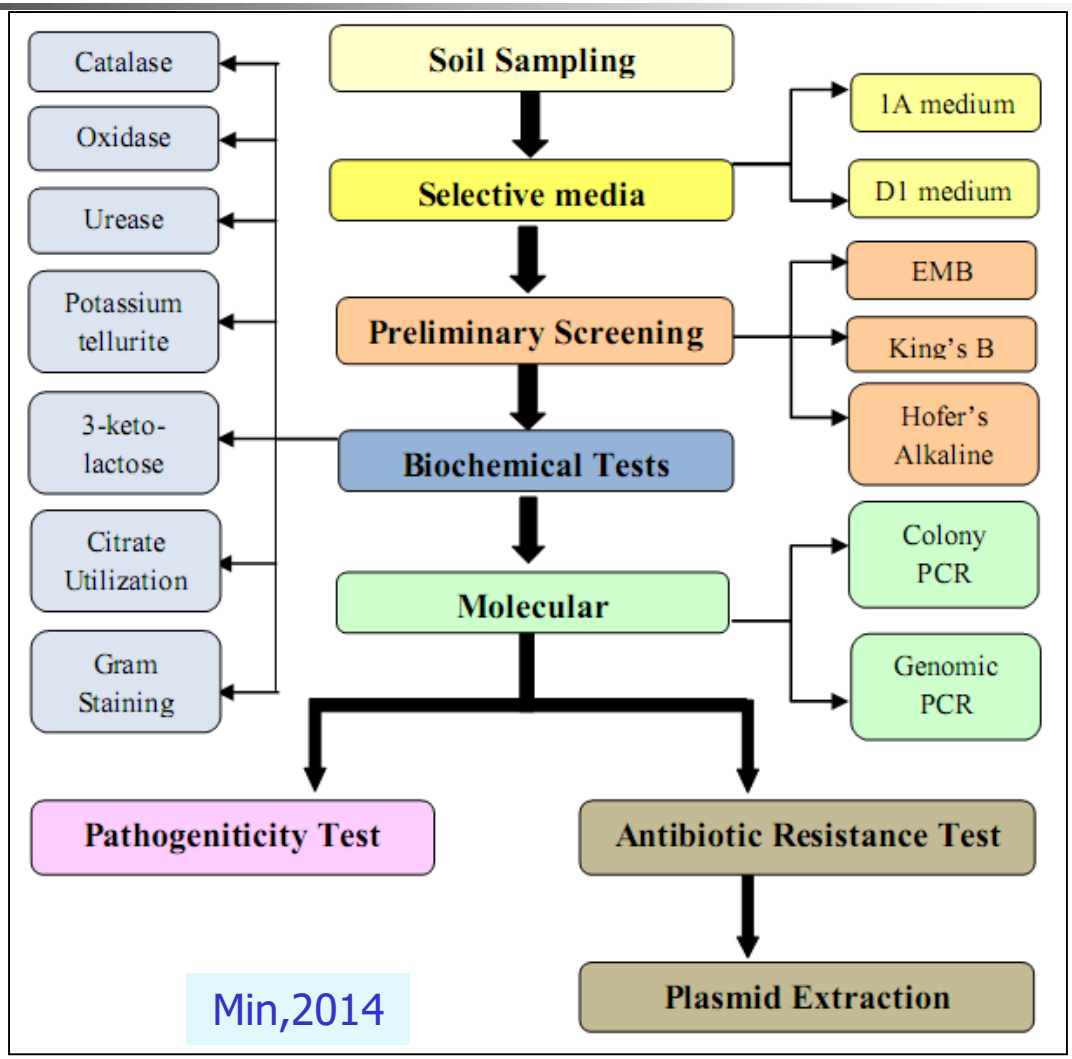
- Agrobacteria can be isolated:

1. From soil obtained from the vicinity of infected plants,
2. From galls formed by the bacteria, or
3. In the case of grapevines, from the xylem sap of infected plants.

- The bacteria are not numerous in older galls and may be easier to isolate from the surrounding soil than from the tumor tissue.

Isolation and characterization of *Agrobacterium* spp.

Experimental Design



1A and D1 media

Tests for distinguishing *Agrobacterium* from *Rhizobium*

Congo red test

Agrobacteria absorbed Congo red, but not rhizobia

- Absorption of congo red was visualized by growing bacteria in YMA supplemented with 0.25 mg L^{-1} congo red.
- Congo red dye is often used to distinguish rhizobia from non-rhizobial strains.
- *Rhizobium* colonies typically do not absorb congo red, and remains white while other bacteria such as agrobacteria absorb it.


FIG. 1: ROOT NODULE

FIG. 2: AGROBACTERIUM SP.

Hahn,1996; Laxmi *et al.*,2018; Soares *et al.*,2020

Tests for distinguishing *Agrobacterium* from *Rhizobium*

Congo red test

Agrobacteria absorbed Congo red, but not rhizobia

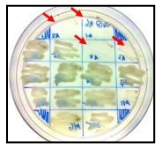
- The strains were grown on either on:

1. Yeast extract mannitol agar(YEMA)/*Agrobacterium* mannitol medium [g/l: tryptone 5; mannitol 5; yeast extract 2.5; L-glutamic acid 1; KH_2PO_4 0.25; NaCl 0.1; $MgSO_4.7H_2O$ 0.1; biotin 10 µl (0.1 mg/ml stock); pH 7.0; agar 15], or
2. Yeast mannitol (YM) medium (10g/L mannitol, 0.4 g/L yeast extract, 0.5 g/L K_2HPO_4, 0.2 g/L $MgSO_4$, 0.1 g/L NaCl, Agar,15 g/L, pH 6.8-7.0) supplemented with congo red.

Tests for distinguishing *Agrobacterium* from *Rhizobium*

Hofer's alkaline broth test

Higher pH suppress the rhizobial growth



- Agrobacteria grow at higher pH levels (Hofer's alkaline broth at pH 11), while rhizobia are unable to do so.
- Therefore, growing bacterial isolates on an alkaline medium (K_2HPO_4, 0.5 g; $MgSO_4$, 0.2 g; NaCl, 0.1 g; $CaCO_3$, 0.05 g; yeast extract, 1.0 g; mannitol, 10 g; and water, 1000, ml; pH adjusted to 11.0 by adding approximately 28 ml of N NaOH and 1 ml of 1.6% thymol blue or 0.016 g phenolphthalein) would serve as a useful criterion for distinguishing the two allied genera.

Gaur and Sen,1981;Min,2014

Isolation
Culture media

- Agrobacteria grow readily in culture on complex or defined media.

1. Nutrient agar(NA) with or without 0.5%yeast extract,
2. NASA medium (add to 1000 ml of autoclaved nutrient agar(NA): 10 ml of Cellenite (0.5g/100ml water) and 10 ml of Cycloheximide (actidione) (0.5g/100ml water).
3. Yeast mannitol agar will support the growth of most strains.

- Some strains require B vitamins for growth, usually 0.2 mg/liter each of biotin, pantothenic acid and/or nicotinic acid.

The Prokaryotes (chapter 3.1.4), 2006; Goszczynaska *et al.*,2000

Colony morphology

Carbohydrate-containing media

Yeast mannitol agar

- The colonies are generally white or slightly cream or pale pink in color.
- No distinctive pigment is produced.
- Large amounts of extracellular polysaccharide may be produced on carbohydrate-containing media, giving the colonies a watery appearance.

The copious production of slime has dripped from the colony to form a mirror image on the Petri dish lid.

Soil sampling (Serial Dilution)
A. tumefaciens from field soil

- One gram samples of field soil were used to inoculate six 100-mL batches of a mineral medium (Schlegel *et al.*,1961) containing 2% m/v glucose as a carbon (C) source and 0.1% (m/v) HWIN(hot-water-insoluble methylene urea nitrogen) as a sole N source.
- Cultures were grown aerobically in an incubator shaker (130 rev/min) at 28°C for 3 weeks, after which 1.0 ml of each suspension was transferred into 100 ml of fresh medium.

Koivunen *et al.*,2004

Soil sampling (Serial Dilution)
A. tumefaciens from field soil

- All the samples were processed within 24 h.
- A sterilized soil auger was used to obtain moist soil samples from plant rhizosphere, at 8-10 cm beneath the surface soil.
- Fresh samples were kept in sterilized containers and tightly sealed with parafilm.
- An mount 2.0 g of sample of bulk soil were macerated in 20.0 g of sterile distilled water (sdH_2O) and vortexed at high speed (18000-22000 rpm) for 30 min serial dilution ranged from 10^{-1} to 10^{-5} was performed.
- Subsequently 100 µL of diluted slurry was then plated on the two semi-selective media, 1A and D1 media.
- Colonies were examined and enumerated 48 h post plating.

Min,2014

Isolation

A. rhizogenes from legume root nodules

- The nodules were kept immersed in 0.1% acidified mercuric chloride solution for 5 min. and washed repeatedly with sterile distilled water.
- Then they were immersed in 70% ethyl alcohol.
- This treatment was followed by repeated washing with sterile distilled water.
- These sterilized root nodules were crushed simply with pestle and mortar and extracted with sterile distilled water.

Murugesan *et al.*,2010

Isolation

A. rhizogenes from legume root nodules

Yeast extract mannitol agar (YEMA)

- The root nodule extract was serially diluted up to 10^{-9} with sterile distilled water and 1 ml of diluted sample was inoculated into sterile Petri plates and poured with the sterilized yeast extract mannitol agar (YEMA).
- Plates were incubated at 28°C for 2 to 3 days.
- This medium allowed both *Agrobacterium* and *Rhizobium* to grow and develop.

Murugesan *et al.*,2010

Isolation
Culture media
Potassium tellurite test

- Samples of bulk soil, roots, shoots or tumorigenic tissues are crushed and macerated in 1 ml of sterile distilled water for at least 30 min.
- The suspension can be directly streaked or dilution can be plated onto appropriate selective agar media (based on the ability to use specific compounds as carbon and nitrogen sources and to resist toxic compounds).
- Inoculated agar media are incubated 2-3 days at 28°C.
- After isolation, typical agrobacterium colonies (e.g. black colonies due to incorporation of tellurite (K_2TeO_3) in the colony) must be purified at least twice.

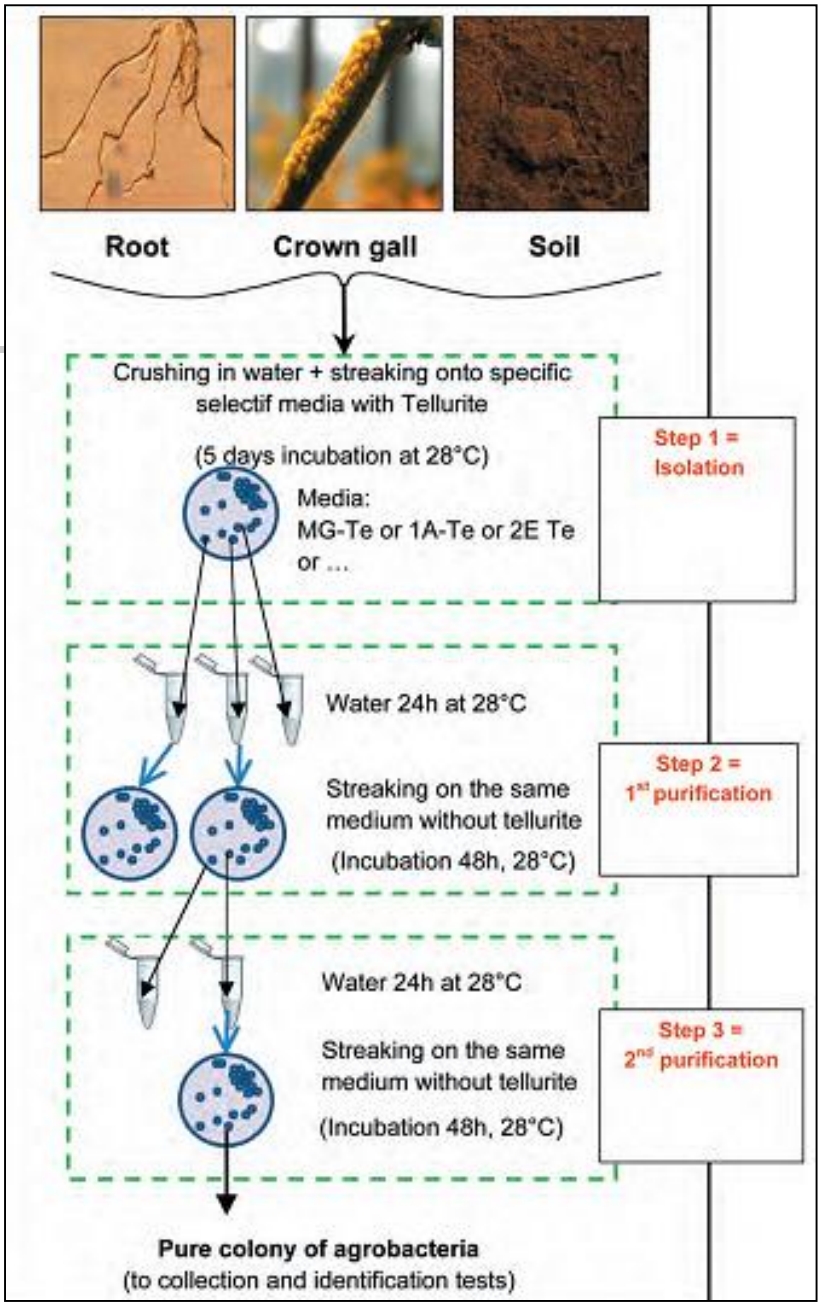
Campillo *et al.*,2012

Isolation

Culture media

Potassium tellurite test

Flow diagram for obtaining a pure culture of agrobacteria.
At least two purification steps are necessary before performing identification with other tests.

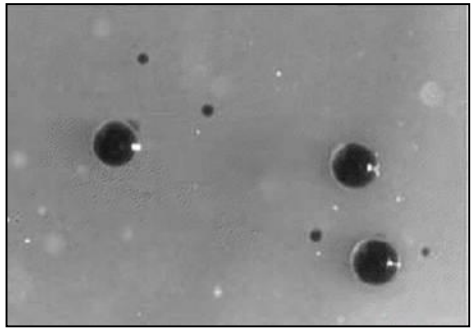


Campillo *et al.*,2012

Isolation

Culture media

Potassium tellurite test



- Nearly all Agrobacteria are able to tolerate with the toxicity of tellurite metal (200 mg/L).
- On media amended with tellurite, colonies are black.
- Potassium tellurite test is a rapid method of testing the ability of isolates to initiate the reduction of potassium tellurite ions.
- *A. tumefaciens* are known to be resistant to the toxicity of tellurite ions.
- Positive reactions were evident by the formation of black deposit in the colonies.

Campillo *et al.*,2012; Min,2014

Isolation

Culture media

Potassium tellurite test

- Per 1 liter deionized or distilled water:
- D-mannitol 5 g,
- L-glutamic acid 2 g,
- KH_2PO_4 0.5 g,
- NaCl 0.2 g,
- $MgSO_4.7H_2O$ 0.2 g,
- Yeast extract 0.5 g,
- Potassium tellurite 0.2 g,
- Agar 15 g.

Isolation

Culture media

Potassium tellurite test

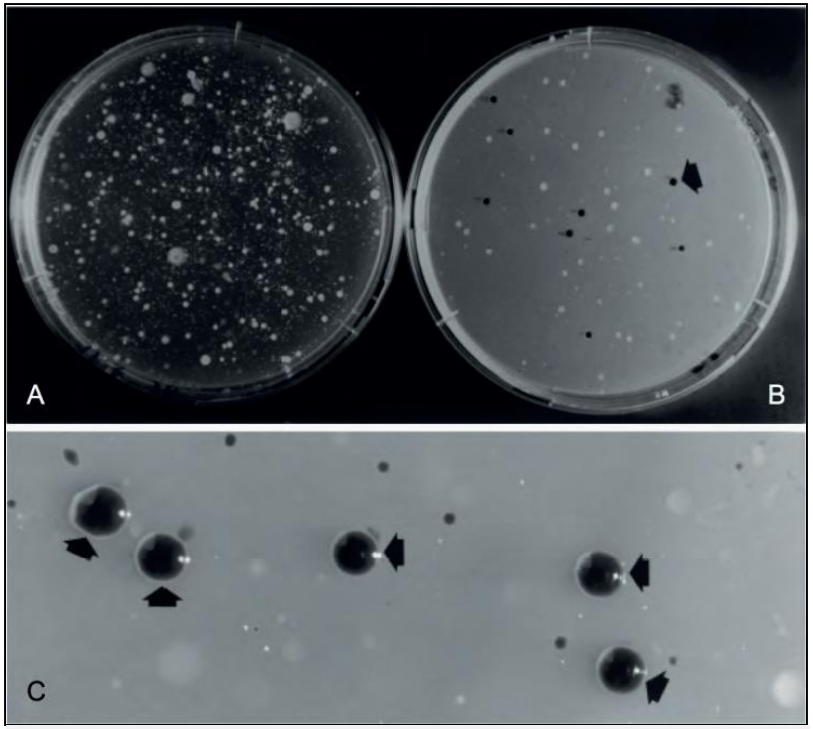
- Plating of a 10^{-1} dilution soil suspension on:

A. 1A medium, or

B. 1A medium amended with 60 ppm of tellurite(K_2TeO_3).

C. Enlarged (magnification, 30X) typical black colonies of *A. tumefaciens* on the amended medium.

- Arrows point to agrobacterial colonies (Mougel *et al.*, 2001).



Nearly all Agrobacteria are able to tolerate with the toxicity of tellurite metal (200 mg/L). Colonies have circular morphology and are black in color with a metallic shine.

Campillo *et al.*,2012;..

Agrobacterium biotypes

Sodium selenite tolerance

Selenite-reducing isolates

- The reduction of selenite by *Agrobacterium tumefaciens*.
- Sodium selenite agar (Difco) and Hendrickson *et al.*,1934 medium were incorporated with toxic sodium selenite(Na_2SeO_3) at 0.1 g increments from 0.1 to 1.0 g/l added both before and after autoclaving.
- In comparison with the biotype 1 isolates (*Agrobacterium tumefaciens*), biotype 2 isolates (*A. tumefaciens*, *A. radiobacter* and *A. rhizogenes*) are differed in utilization of sodium selenite.

The coloration of red-orange (selenite) is due to the reduction of selenite to the amorphous form of elemental selenium within the cells, one potential way to detoxify the oxidizing agent.

Spiers,1979;Min,2014; Josuha Collins

D1 medium

Semi-selective medium for *Agrobaterium* spp.

- D1 medium was specifically developed for plant-pathogenic bacteria using active constituents such as lithium chloride, which are known to affect the permeability of bacterial membranes to permit the growth of agrobacteria in the meantime, restrict the growth of all other microbes.
- 1A medium later was found to be twice more effective than D1 medium in the isolation of *A. tumefaciens* strains.

Kado and Heskett,1970; Min,2014

D1 medium

Semi-selective medium for *Agrobaterium* spp.

- Mannitol 15 g/L
- LiCl 6 g
- $NaNO_3$ 5 g
- $Ca(NO_3)_2.4H_2O$ 20 mg
- K_2HPO_4 2 g
- $MgSO_4.7H_2O$ 0.2 g
- Bromothymol blue 0.1 g
- Agar 15 g
- pH=7.2
- The medium after autoclaving appear dark blue. The circular, convex and glistening colonies appear light blue then turn dark olive green.

Kado and Heskett,1970

Modified D1 medium(D-1M)

Selective for *A. tumefaciens* and *A. rhizogenes*

- Modified D1(D-1) medium:
- Cellobiose 5 g
- NH_4Cl 1 g
- Na_2HPO_4 1 g
- K_2HPO_4 1 g
- $MgSO_4.7H_2O$ 3 g
- Malachite green 10 mg
- Agar 15 g
- $D.H_2O$ 1 L
- pH=7
- Initially the circular, convex and glistening colonies appear light blue then turn dark olive green.

Perry and Kado,1982

Medium 1A

Semi-selective medium

Selective for *Agrobacterium tumefaciens* species complex (biovar 1), *A. fabrum*, *A. rubi* and *A. larrymoorei*

- *Agrobacterium tumefaciens* species complex (biovar 1), *A. fabrum* (biovar 1), *A. rubi* and *A. larrymoorei* can be isolated on semi-selective Schroth medium (Schroth *et al.*, 1965) or semi-selective medium 1A (Brisbane and Kerr, 1983).
- Selectivity of medium 1A is based on the ability of above mentioned agrobacteria to specifically use arabitol as sole carbon source:
- L-arabitol 3.04 g, NH_4NO_3 0.16 g, KH_2PO_4 0.54 g, K_2HPO_4 1.04 g, $MgSO_4.7H_2O$ 0.25 g, sodium taurocholate 0.29 g, 2 ml of crystal violet 0.1% (w/v) aqueous, agar 15 g.
- After sterilization add 1 ml of cycloheximide 2% and potassium tellurite 0.08 g.

Campillo *et al.*,2012;Bouzar *et al.*,1996

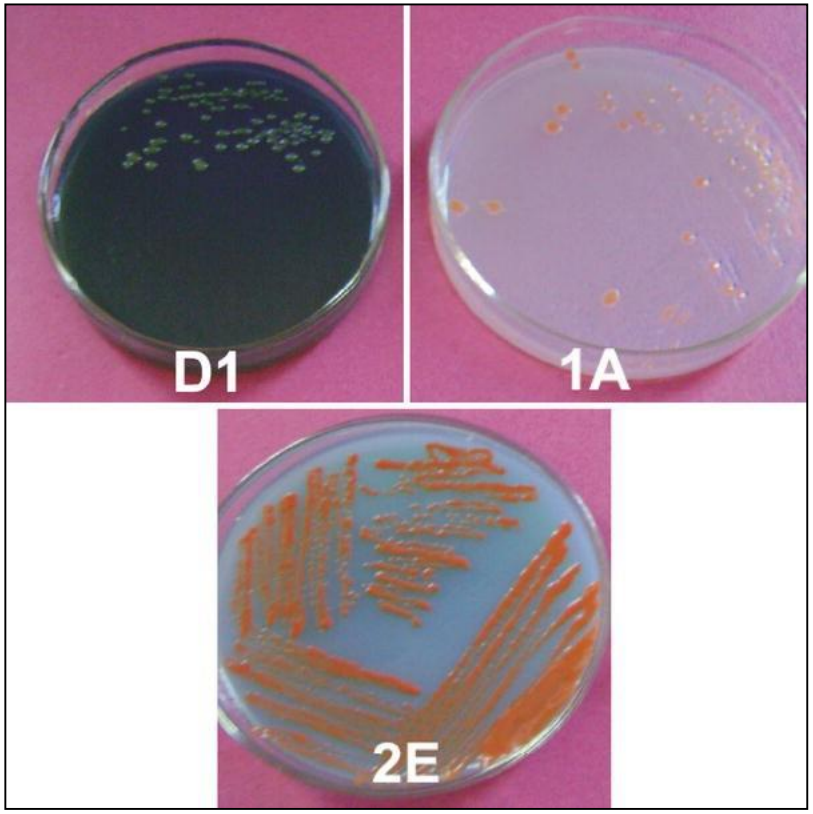
Medium 1A
Semi-selective medium

- The gall should be washed or surface sterilized using 20% household bleach, and rinsed several times in sterile water.
- Cut a few samples from different parts of the white tissue of the gall, and further divide samples into small pieces.
- Place these pieces into a culture tube containing sterile distilled water or buffer, vortex and allow to stand for at least 30 minutes.
- Using an inoculating loop, streak this suspension on Medium 1A (Schaad *et al.*,2001), and incubate at 25-27°C.
- Agrobacterial colonies growing on 1A and 2E media develop an orange-brown to red-brown pigmentation.

Collins,2001;Moguel *et al.*,2001;..

Medium 1A
Semi-selective medium

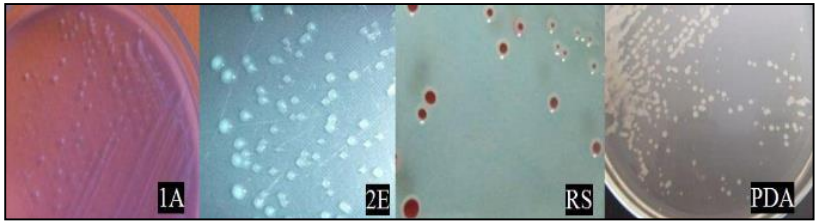
- Agrobacterial colonies growing on 1A and 2E media develop an orange-brown to red-brown pigmentation.



Gupta *et al.*,2013

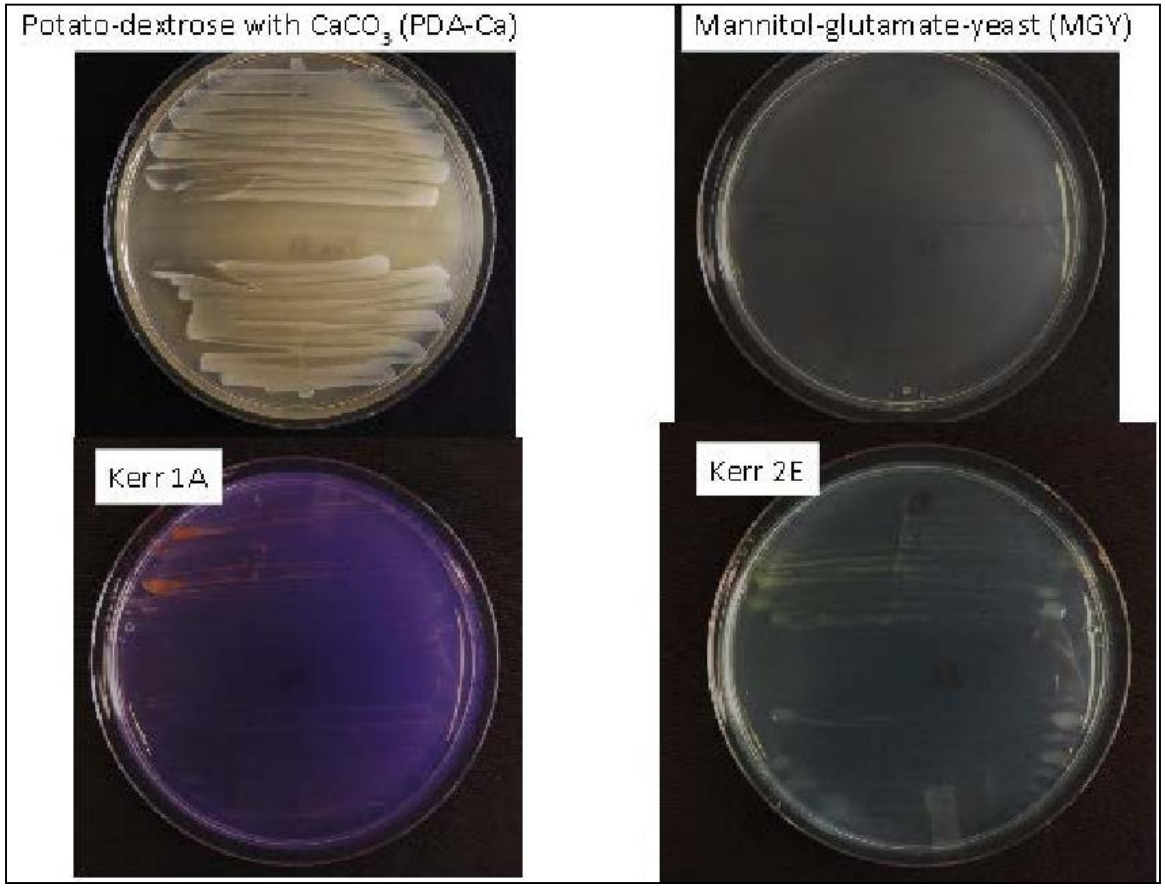
Medium 1A
Semi-selective medium

- On 1A-medium, the colonies were convex, slightly mucoid and had faint purple color with transparent margins.
- On 2E-medium, Colonies were similar to that present on 1A medium but the color was green.
- On RS-medium, colonies were convex and slightly mucoid with red centers and white margins.
- By streaking the resembling Agrobacterium colonies on PDA medium, almost isolates exhibited typical growth of Agrobacterium (convex, glistening, circular with an entire edge, and white to beige in color) after 48 hr at 28°C.



Tolba and Soliman,2014

Four different culture media
1A, 2E, RS-medium and PDA+Ca
Agrobacterium spp.



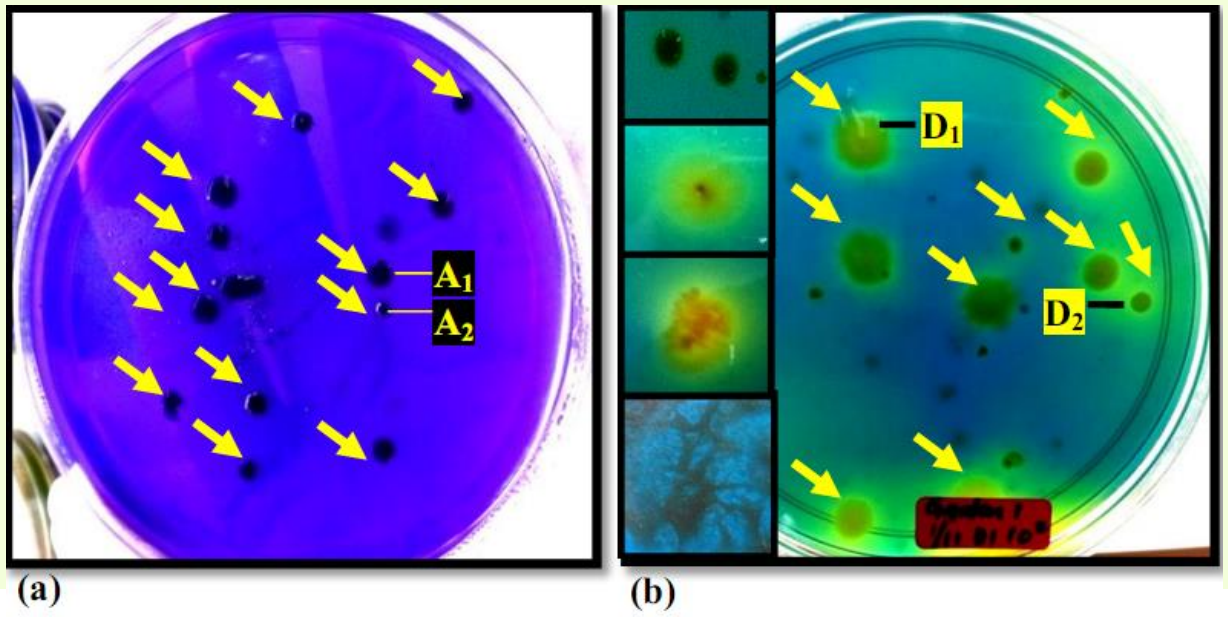
Ain Khalib,2020

Typical agrobacterial colonies on different semi-selective media

D1 medium and 1A medium

- Plating 10^{-1} dilution slurry on 1A medium. Arrows indicated agrobacterial colonies.

a) Plating 10^{-2} dilution slurry on D1 medium built colonies with different morphology. Arrows indicated agrobacterial colonies.



Min,2014

Medium 2E(New and Kerr medium)

Semi selective for *A. rhizogenes*

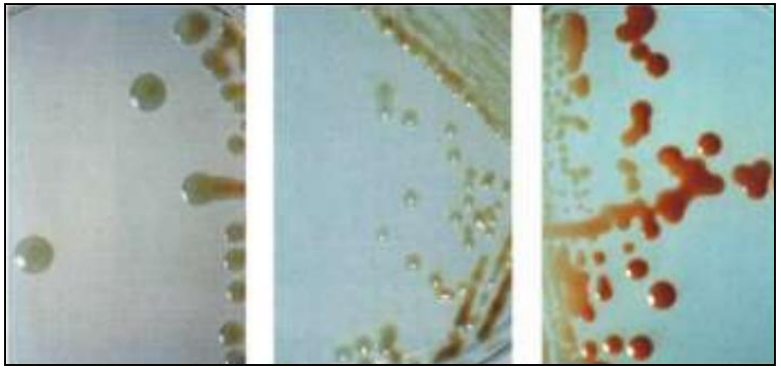
***A. rhizogenes* (biovar 2) uses specifically erythritol as sole carbon source**

- *A. rhizogenes* (biovar 2) can be isolated on two semi-selective media New and Kerr medium (New and Kerr, 1971) or medium 2E (Brisbane and Kerr, 1983).
- Both media, based on the specific ability of biovar 2 strains to use erythritol as sole carbon source, are highly selective for biovar 2.
- Colonies develop slowly on New and Kerr medium whereas 2E medium allows faster growth: erythritol 3.05 g, NH_4NO_3 0.16 g, KH_2PO_4 0.54 g, K_2HPO_4 1.04 g, $MgSO_4.7H_2O$ 0.25 g, sodium taurocholate 0.29 g, 1 ml of yeast extract 1% (w/v) aqueous, 5 ml of malachite green 0.1% (w/v), agar 15 g.

Campillo *et al.*,2012; Emna *et al.*,2013

Medium 2E(New and Kerr medium)
Semi-selective medium for *A. rhizogenes*

- Range colonies of *Agrobacterium rhizogenes* on semi-selective 2E medium. Colony morphology shows convex, circular and smooth characteristics, while its colour becomes non-pigmented to light beige on a nutrient agar plate.



Schaad *et al.*,2001; Shinga,2019

3DG and Roy and Sasser media

Selective for *Agrobacterium vitis* (biovar 3) and *A. larrymoorei*

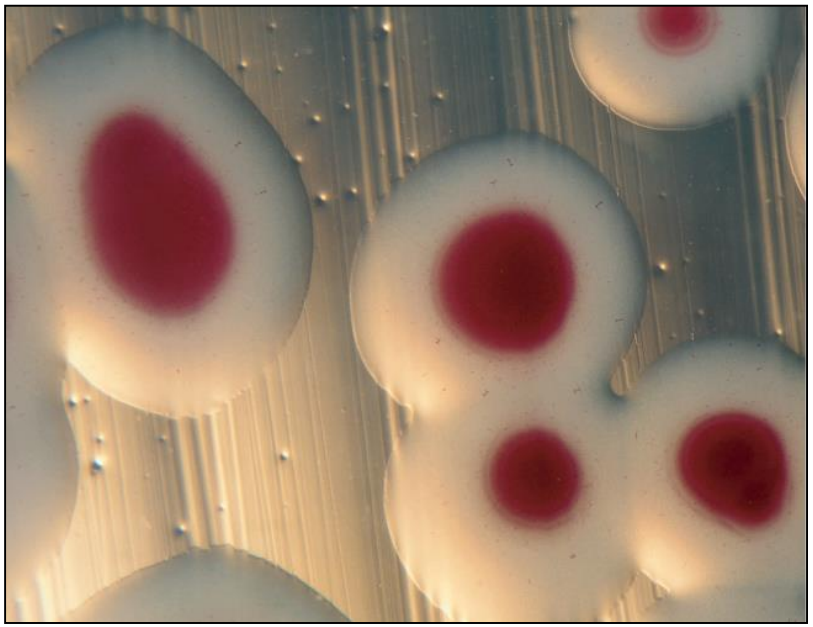
***A. vitis* (biovar 3) and *A. larrymoorei* specifically use L-tartrate as sole carbon source**

- *Allorhizobium vitis* (=*A. vitis*) and *A. larrymoorei* can be isolated on medium 3DG (Brisbane and Kerr,1983) or Roy and Sasser medium (Roy and Sasser,1983).
- Both media are based on specific sodium L-tartrate utilization by biovar 3 strains as sole carbon source.
- Roy and Sasser medium is most commonly used:
- adonitol 4 g, H_3BO_3 1 g, yeast extract 0.14 g, $MgSO_4.7H_2O$ 0.2 g, KH_2PO_4 0.7 g, K_2HPO_4 0.9 g, NaCl 0.2 g, agar 20 g. Triphenyltetrazolium chloride 0.8 g and 1 ml of cycloheximide 2% (w/v) are added after sterilization.

Campillo *et al.*,2012;..

Colony morphology
Roy and Sasser semi-selective medium

- Colonies of *Rhizobium* (*Agrobacterium vitis*) on semi-selective Roy and Sasser(RS) medium.
- Colonies have a typical pinkish white (with red centre) color on this medium.



Pánková and Krejzar,2002-04

Characterization of Agrobacterium Isolates
Common and specific tests

- *Agrobacterium* isolates was characterized based on cultural, biochemical and physiological characteristics such as:

1. Congo red test,
2. Hofer's alkaline broth test
3. Growth in glucose peptone agar
4. Reaction of litmus milk
5. Staining of poly α-hydroxybutyrate (PHB).

- Also some specific tests were used:

1. Growth on potato dextrose agar (PDA),
2. 3-ketolactose test,
3. Sodium chloride tolerance test,
4. Growth and pigmentation in ferric ammonium citrate broth test, and
5. Citrate utilization test.

Murugesan *et al.*,2010

Biochemical test for *Agrobacterium* spp.
Glucose utilization
An aerobe

- Strictly aerobic.
- It is an obligately aerobic organism and thus, growing with proper aeration is critical(Morton and Fuqua,2013).
- Some strains are capable of anaerobic respiration in the presence of nitrate.

Note: Agrobacteria are aerobic bacteria but the test for it is not listed in any of the diagnostic tables.

Traits used for identification of formerly biovars of *Agrobacterium*

Characteristic	Biovar 1	Biovar 2	Biovar 3	*A. rubi*
Growth on selective medium 1A[a]	Yes			
2E[b]		Yes		
RS[c]				Yes
Growth factor requirements	None	Biotin	Biotin, some strains	Biotin, pantothenic acid, nicotinic acid
3-Ketolactose production	Most strains	No	No	No
Growth on 2% NaCl	Yes	No	Yes	Yes
Growth at 37°C	Yes	No	No	Yes
Acid production from mannitol	Yes	Yes	Yes	Yes
Adonitol	Yes	Yes	Yes	Yes
Erythritol	No	Yes	No	No
Dulcitol	Yes	Yes	No	No
Melizitose	Yes	No	No	No
Ethanol	Yes	No	No	No
Arabitol	Yes	No	No	No
Alkali production from tartrate	No	Yes	Yes	No

Data from Kerr (1986).

The Prokaryotes (chapter 3.1.4),2006

Biovar 1= *A. tumefaciens* and *A. radiobacter*
Biovar 2= *A. tumefaciens*, *A. radiobacter* and *A. rhizogenes*
Biovar 3= *Allorhizobium vitis* (=*A. vitis*)

Major phenotypic characteristics differentiating species of the genus *Agrobacterium*

Diagnostic test	*A. tumefaciens*	*A. rhizogenes*	*A. vitis*
3-Ketolactose production	+	-	V
Growth in 2% NaCl	+	-	+
Growth at 35°C	+	V	V
Action on litmus milk	ALK	AC	ALK
Acid from:			
Erythritol	-	+	-
Melezitose	+	-	-
Alkali from:			
Malonic acid	-	+	+
L-tartaric acid	-	+	+
Mucic acid	-	+	-
Ferric ammonium citrate	+	-	-
Oxidase reaction	+	V	V
Citrate utilization	V	+	+

+, 80% or more strains positive; V, between 21-79% of strains positive; -, 80% or more strains negative; ALK, alkaline; AC, acid.

All biovars produce acid from mannitol and adonitol.
Allorhizobium vitis (=*A. vitis*) strains can produce alkali from tartrate.

Schaad *et al.*,2001

Tests to differentiate *Agrobacterium* spp.

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.

Goszczynaska *et al.*,2000

Simplified method for putative classification of *Agrobacterium* species

Diagnostic Test	*A. tumefaciens*	*A. rhizogenes*	*A. vitis*
3-ketolactose production	+	-	-
Acid-clearing on PDA plus $CaC0_3$	-	+	-
Motility at pH 7.0	+	+	-
Pectolytic at pH 4.5	-	-	+

+, 80% or more strains positive; V, between 21-79% of strains positive; -, 80% or more strains negative.

Allorhizobium vitis (=*A. vitis*) causes both crown gall and root decay of grapes. A major virulence factor of this pathogen is polygalacturonase, an enzyme that degrades pectin components of the xylem cell wall. Disruption of the polygalacturonase gene results in a mutant that is less pathogenic and produces significantly fewer root lesions on grapevines.

Schaad *et al.*,2001

Phenotypic characteristics of *Agrobacterium tumefaciens*

Characteristic	Response
Gram stain	–
Motility at room temperature	+
Production of:	
Catalase	+
Oxidase	+
β-Ketolactose	+
β-Galactosidase	+
Utilization of:	
N_2 as a nitrogen source	–
Methylene urea as a carbon source	–
Lactose	+
Mannitol	+
Starch	–
Growth on:	
MacConkey agar	+
Luria–Bertani agar	+
Ferric ammonium citrate	+
Growth at:	
28 °C	+
35 °C	+
Fluorescence on Kings B agar	–
Resistance to:	
Kanamycin (30 $\mu g \cdot mL^{-1}$)	–
Cefuroxime (30 $\mu g \cdot mL^{-1}$)	–
Penicillin G (10 IU)	+
Tetracycline (30 $\mu g \cdot mL^{-1}$)	+

Koivunen *et al.*,2004

Origin and pathological, biochemical and molecular traits of *Agrobacterium* spp. strains isolated from grapevine tumours

Plant sample code	Province	No. of strains	Strain code	Species (biovar)	Tumour induction			Tartrate utilization	Opine utilization		16S rRNA gene sequence type[a]	PCR profile				
					Tomato	Tobacco	Grape		Octopine	Nopaline		pehA	virA	tmr	VirB/G	VCF/VCR
2739	Albacete	3	IVIA 2739-16[b]	*A. vitis* (3)	+	+	+	+	+	+	Bv.3 (C)	+	+	+	–	–
			IVIA 2739-17													
			IVIA 2739-18													
		2	IVIA 2739-29[c]	*A. vitis* (3)	+	+	+	+	–	–	Bv.3 (A)	+	–	–	–	–
			IVIA 2739-35													
2979	Albacete	1	IVIA 2979-10[d]	*A. vitis* (3)	+	+	+	+	–	–	Bv.3 (A)	+	–	–	–	–
3105	Albacete	4	IVIA 3105-8a	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (B)	+	+	+	–	–
			IVIA 3105-9b													
			IVIA 3105-10a													
			IVIA 3105-5c (25)[e]													
3112	Albacete	1	IVIA 3112-A2-2a[f]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (B)	+	+	+	–	–
1698	Badajoz	3	IVIA 1698-2b-2[g]	*A. rhizogenes* (2)	+	+	+	+	–	+	Bv.2	–	–	+	+	+
			IVIA 1698-2b-3													
			IVIA 1698-2b-6													
2709	Ciudad Real	8	IVIA 2709-1a-1-1	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (B)	+	+	+	–	–
			IVIA 2709-1a-1-2													
			IVIA 2709-2a-4-1[h]													
			IVIA 2709-2b-2													
			IVIA 2709-2c-4-2													
			IVIA 2709-2d-1													
			IVIA 2709-2d-2													
			IVIA 2709-2d-3-1													
		2	IVIA 2709-2b-1-2[i]	*A. tumefaciens* (1)	–	–	+	+	+	–	Bv.1	–	+	+	–	–
			IVIA 2709-2b-2-1													
103/05	Ciudad Real	1	CITA 103/05-Av4[j]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (A)	+	–	–	–	–
		1	CITA 103/05-Av5	*A. vitis* (3)	+	+	+	+	–	–	Bv.3 (A)	+	–	–	–	–
2680	Huesca	1	IVIA 2680-2-a3[k]	*A. vitis* (3)	+	+	+	+	–	–	Bv.3 (A)	+	–	–	–	–
6/04	Huesca	1	CITA 6/04-Av2	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (B)	+	+	+	–	–
3001	Madrid	3	IVIA 3001-2-G[l]	*A. vitis* (3)	+	+	+	+	–	–	Bv.3 (A)	+	–	–	–	–
			IVIA 3001-3-1-Ha													
			IVIA 3001-3-2-Ha													
		3	IVIA 3001-3-Ba[m]	*A. vitis* (3)	–	+	+	+	+	–	Bv.3 (B)	+	+	+	–	–
			IVIA 3001-6-6-Aa													
			IVIA 3001-6-7-Aa													

Palacio-Bielsa *et al.*,2009

Biochemical tests

3-ketolactose test

Most *A. tumefaciens* strains utilize lactose

- Specific test for detection of Agrobacteria.
- Most *A. tumefaciens* strains utilize lactose to form a reduced product, ketolactose, through the enzyme ketolactase.
- *A. rhizogenes* and some strains of *Allorhizobium vitis* (=*A. vitis*) strains fail to produce 3-ketolactose.
- Benedict's reagent is prepared is poured over agar medium containing lactose (10 g/litre).
- The formation of yellow coloration due to CU_2O indicates the presence of agrobacteria.

Biochemical tests

3-ketolactose test

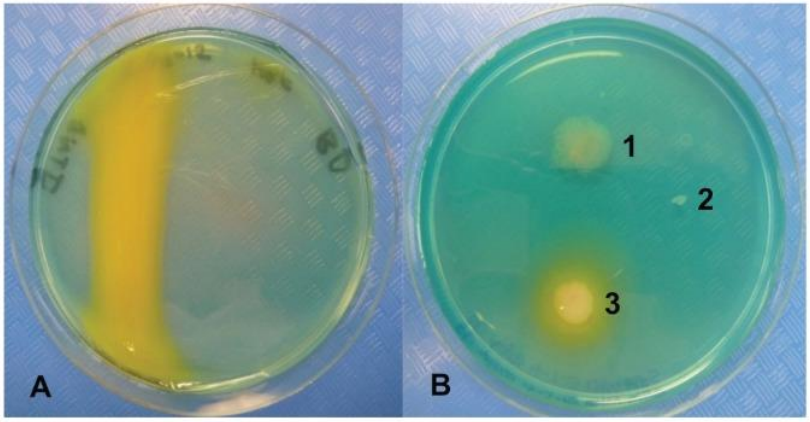
Most *A. tumefaciens* strains utilize lactose

- Test results for 3-ketolactose.

A. Positive control, *A. tumefaciens* EHA105.

B. 1, 2: negative isolates; 3: positive isolate.

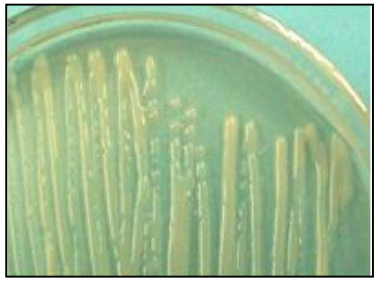
- The formation of yellow coloration due to CU_2O indicates the presence of agrobacteria.



Finer *et al.*,2016

Colony characteristics

Acid-clearing on PDA plus $CaCO_3$



A. vitis on PDA

- On PDA+$CaCO_3$(PDA-Ca), *Agrobacterium* strains grow well, and their colonies are distinguishable from other species.
- Prepare from Difco dehydrated PDA as recommended by manufacturer and supplement with 0.5% $CaCO_3$.
- Acid production on PDA-Ca was observed as clearing zones in the agar beneath the bacterial growth (Schaad *et al.*, 2001).

Schaad *et al.*,2001; Tolba and Zaki,2011, Ain Khalib,2020

Colony characteristics

Acid-clearing on PDA plus $CaCO_3$

- Growth of two isolates from tissue culture galls, Tcg-2s (top half of plate) and Tcg-3s (bottom half of plate), on media PDA-Ca, MGY, IA and 2E, six days after streaking and incubation at 27°C.

Ain Khalib,2020

Determination of specific carbon sources metabolism

- Determination of specific carbon sources metabolism can be made either by:

1. growing the strain on minimal media supplemented with a carbon source (utilization) or,
2. by measuring alkali/acid production (Ayers *et al*., 1919; Keane *et al*., 1970; Kersters and De Ley, 1984; for a detailed protocol see Cubero and López, 2004).

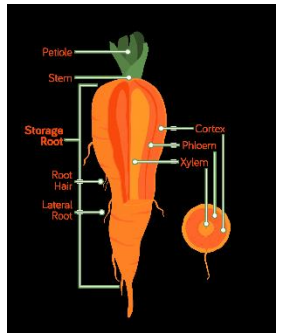
Campillo *et al*.,2012

Colony characteristics
R. skierniewicense

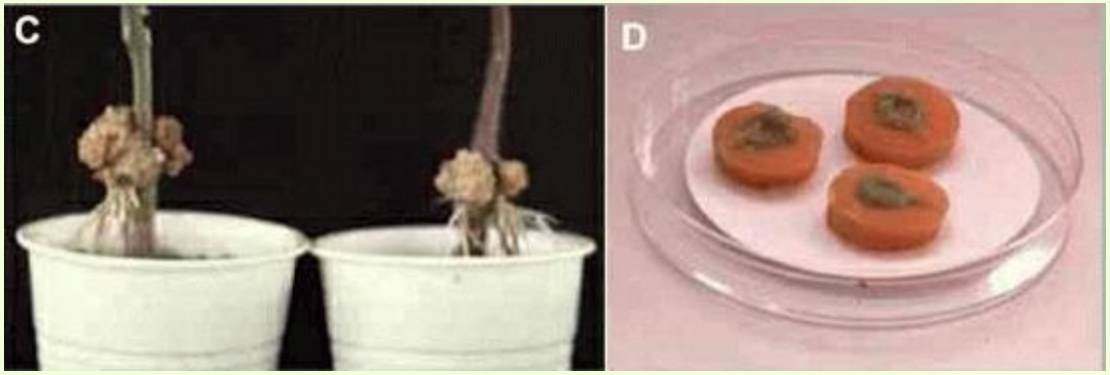
- 3-ketolactose for *R. skierniewicense* isolated from gall on chrysanthemum is negative but grows and produces a reddish brown pellicle at the surface of media containing ferric amonium citrate.
- It can be differentiated from closely related species by its inability to utilize L-fucose and by its ability to utilize ß -hydroxybutyric acid (Pulawska *et al.*, 2012).

Campillo *et al.*,2012

Virulence assays on carrot root disks
Carrot disc and tomato tests



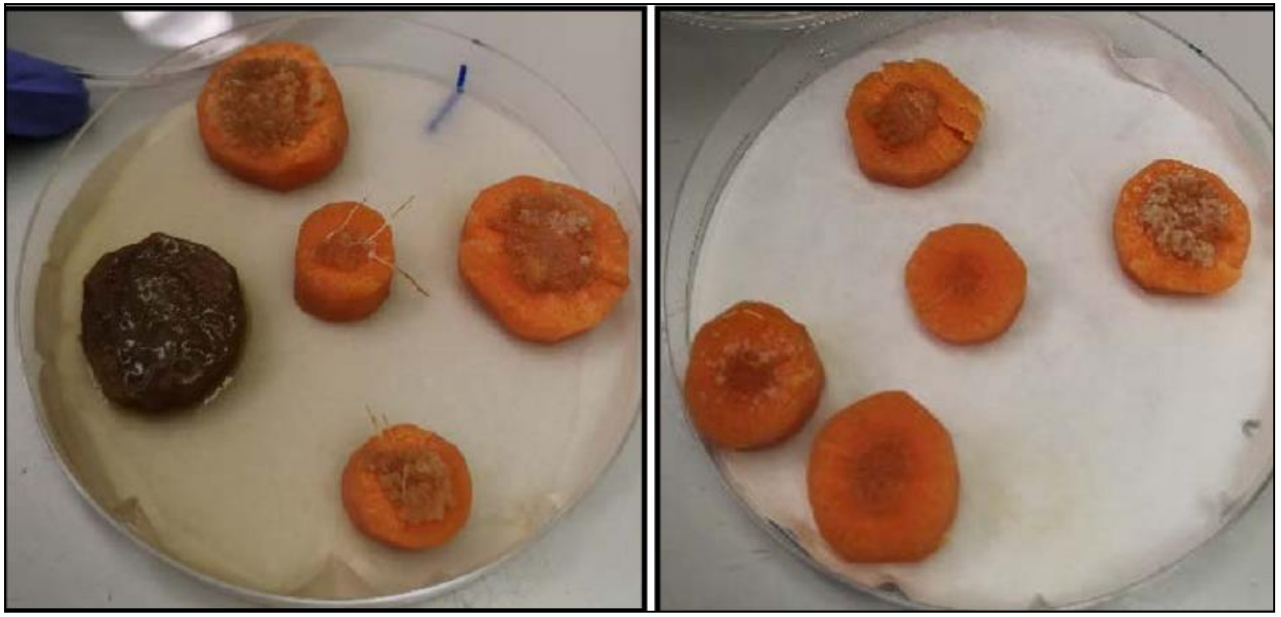
- **Figure C** shows the bases of two young tomato plants where a drop of *A. tumefaciens* bacterial suspension was placed on the stem and a pin prick was then made into the stem at this point. The photograph was taken 5 weeks later (Jim Deacon).
- **Figure D** shows another laboratory assay, where bacterial suspension was added to the surface of freshly cut carrot disks. Suspensions of *A. tumefaciens* on LB broth medium were standardized to 10^7 CFU/ml as determined by an absorbance value of 0.96 ± 0.02 at 600 nm.
- After 2 weeks the young tomato plants galls (green-coloured) developed from the meristematic tissues around the central vascular system.



Jim Deacon

Virulence assays on carrot root disks
Carrot disc assay

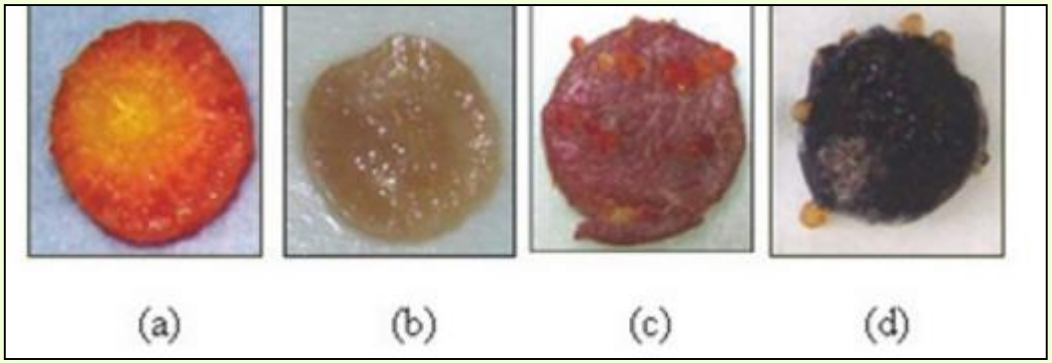
- IAA producing bacteria obtained from blueberry stems; callus and root growth was observed on carrot discs inoculated on the basal surfaces with virD2-negative bacteria from field galls.



Ain Khalib,2020

Virulence assays on different disks
Carrot, radish, beet and potato disc assay

- Phytopathogenicity test on different discs: (a) carrot (b) radish (c) beet (d) potato.
- The crown gall formation was observed after 8 days for both beet (d) and carrot (a) discs without any staining need.
- Meanwhile, the potato discs displayed the galls in 21 days after lugol's iodine staining. As a consequence the potato discs have a high risk of contamination and cost more for bioassay tests. The same conclusion was observed for radish discs with a higher risk of contamination which oblige the repetition of the test many times to get result.



Trigui *et al.*,2013

Pathogenicity Test
Potato disc bioassay

- Red skin potato (*Solanum tuberosum* L., Solanaceae) were sterilized with 0.1% mercuric chloride solution($HgCl_2$) for 10 minutes and thoroughly washed with autoclaved distilled water. Potato discs (5 mm x 8 mm) were made with cork borer and placed on water agar (2%) plates (10 discs per plate). *Agrobacterium* culture (2 ml) mixed with 50 µl each of 10, 100 and 1000 ppm of extract of *Fagonia cretica* (prepared in DMSO) was applied on the surface of each disc of respective concentration. Petri plates were then placed at 28°C for 21 days.
- After 21 days, the discs were stained with Lugol's solution (10% KI and 5% I2) for 30 minutes and then observed under dissecting microscope.
- Numbers of tumors per disc were counted and percent inhibition for each concentration was determined by the formula given below.

$$\text{Percent inhibition} = 100 - \frac{\text{Average number of tumors of sample}}{\text{Average number of tumors of control}} \times 100$$

Hussain *et al.*,2007

Pathogenicity Test
Potato disc bioassay

- Figure shows tumor forming ability of selected isolates on potato disc.

A. AtAh0116,
B. AtTg0117,
C. AtTa0112,
D. AtAc0114,
E. AtSl0105, and
F. AtRc0107.

Soriful Islam *et al.*,2010

Agrocin 84 sensitivity test

- Production of the antiagrobacterial antibiotic agrocin 84, which is coded for by the agrocinogenic plasmid pAgK84, is a key component in the process of biocontrol by strain K84.
- Strain K84 also produces:
- A second antiagrobacterial substance called agrocin 434.
- A third antibiotic-like substance named ALS84, which inhibits many tumorigenic *Agrobacterium* strains *in vitro*.

Penyalver *et al.*,2001

Agrocin sensitivity test

Agrobacterium radiobacter strain K84 against *A. tumefaciens*

- A bacteriocin of *Agrobacterium radiobacter* is used in biocontrol of the plant pathogenic, tumour-producing bacterium *A. tumefaciens*.
- Figure H. Agar plate stab-inoculated in the centre with *A. radiobacter* strain K84 and incubated for 24 hours before the bacterium was killed with chloroform vapour.
- Then a top layer of cooled agar containing *A. tumefaciens* was poured over the plate (the overlay plate technique).
- Growth of the pathogen is inhibited in a wide zone (arrowheads) around the spot where strain K84 had grown.

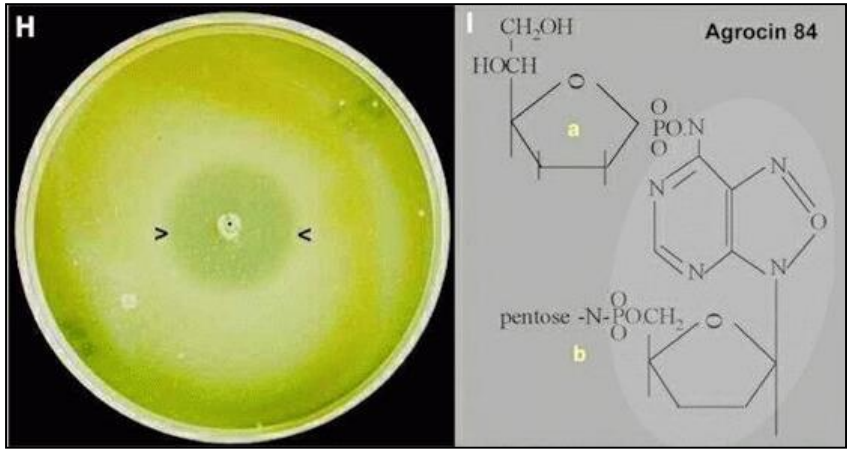


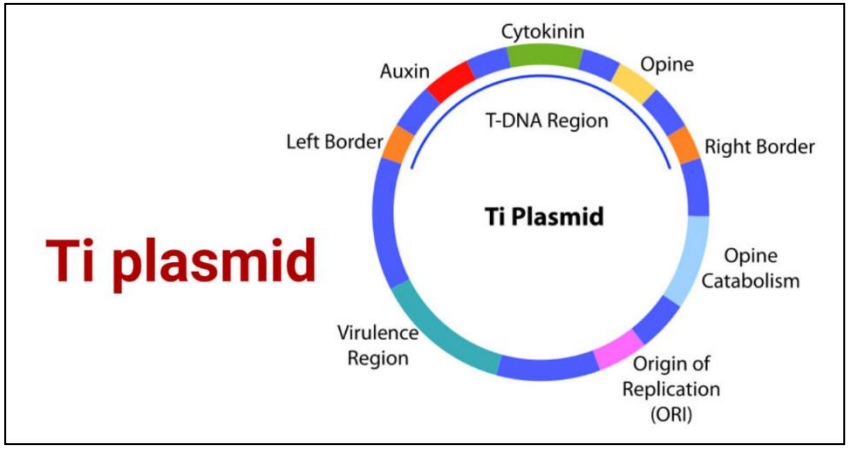
Figure I. Structure of agrocin 84.

Jim Deacon

Mechanism of pathogenicity

Plasmid borne pathogenicity factors

- *A. vitis* -Ti plasmid strains cause crown gall of grape.
- *A. rubi* -Ti plasmid strains cause cane gall of some *Rubus* spp.
- *A. larrymoorei* -Ti plasmid strains cause tumours/crown gall of *Ficus benjamina*.



It is around 200 kb large.
Ti plasmid has three important regions.

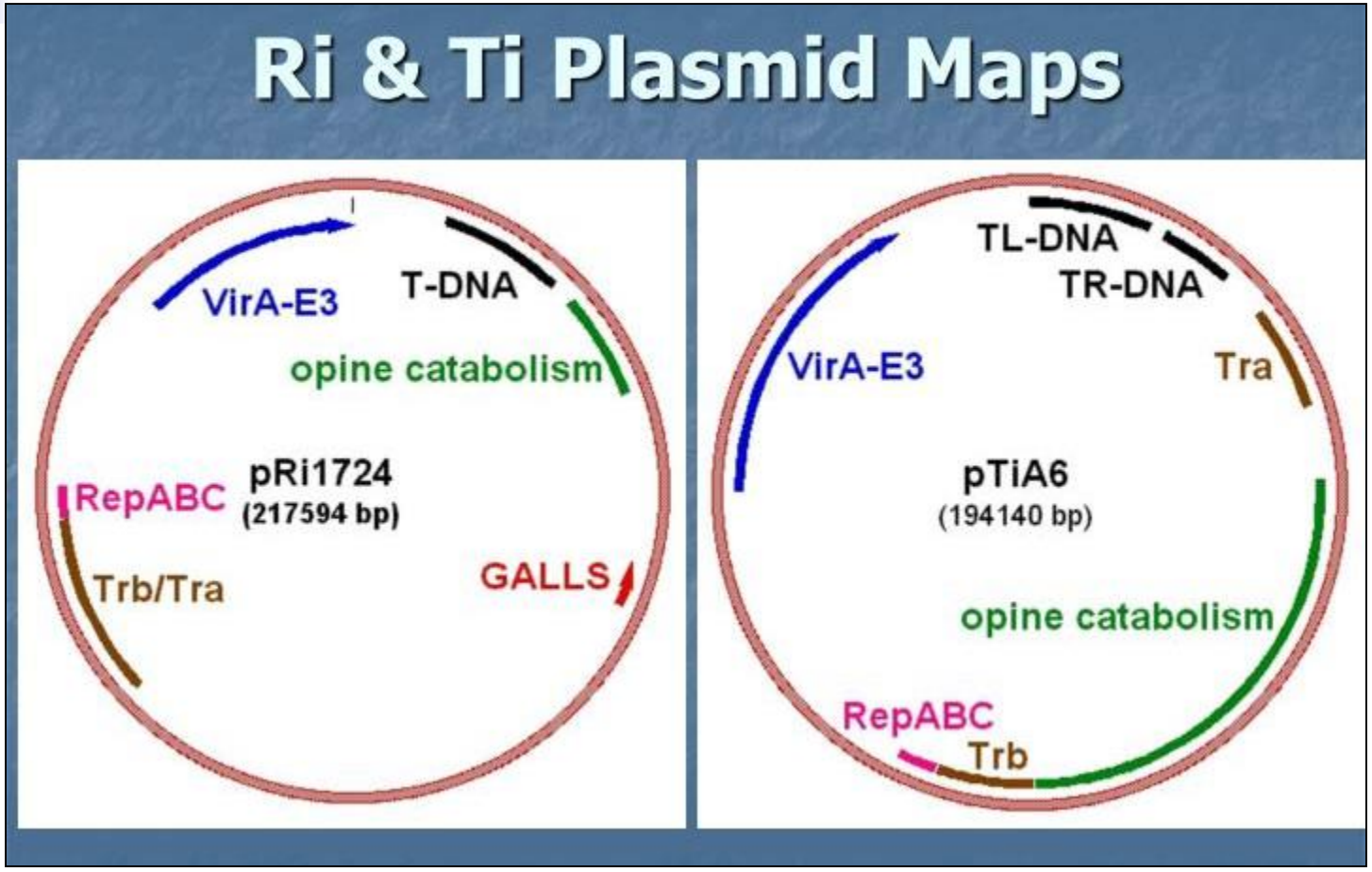
Mechanism of pathogenicity

Plasmid borne pathogenicity factors

Characterstics	Ti Plasmid	Ri Plamis
T-DNA	One copy	Either One or Two
Opine Catabolizing gene	as octopine, nopaline, succinamopine and leucinopine	agropine type or Mannopine type
Virulence region	One copy	One copy
Disease	Tumor Inducing	Hairy root inducing
Bacterial Species	*Rhizobium radiobacter* (*A. tumefaciens*)	*A. rhizogenes*

Mechanism of pathogenicity

Plasmid borne pathogenicity factors



Kennedy,2014

Mechanism of pathogenicity
Plasmid borne pathogenicity factors

- In laboratory conditions it is also possible to cure *Agrobacterium* or *Rhizobium* and then introduce the plasmid of the other organism.

1. Introduction of the T_i plasmid into *Rhizobium* causes this to form galls;
2. Introduction of the *Sym* plasmid into *Agrobacterium* causes it to form nodule-like structures, although they are not fully functional.

Mechanism of pathogenicity

Plasmid borne pathogenicity factors

- However, at some conditions such as growing the bacterium near its maximum temperature about 30˚C *Agrobacterium* or *Rhizobium* plasmid is lost and pathogenicity (of *Agrobacterium*) or nodule-forming ability (of *Rhizobium*) also is lost.
- However, loss of the plasmid does not affect bacterial growth in culture.
- The plasmid-free strains are entirely functional bacteria.

Mechanism of pathogenicity

Plasmid borne pathogenicity factors

Indole-3-acetic acid (IAA)

- The tumorous growth was viewed as a plant cancer and a search was made for the tumour inducing principle, TIP.
- Plasmid borne pathogenicity factors:
- Two main types of plasmid Ri (hairy root) and Ti (crown gall).
- A portion of the plasmid, containing the genes for **auxin and cytokinin synthesis**, is transferred to the host plant and stably incorporated into its genome.
- *Agrobacterium*, with the genes for auxin and cytokinin synthesis deleted and replaced with genes of interest, is now routinely used to transform plants.

Rhizobium strains to produce nitrogen-fixing nodules are contained .on a large plasmid termed the *Sym* (symbiotic) plasmid.

Mechanism of pathogenicity

Plasmid borne pathogenicity factors

Indole-3-acetic acid (IAA)

- The disease is associated with indole-3-acetic-acid (IAA) production.
- Isolates from field galls that caused gall-like callus growth in inoculation sites on tomato were tested for production of IAA.
- The isolates were cultured in three replicate test tubes containing KB broth amended with 0.2 mg ml^{-1} L- tryptophan at 200 r.p.m. for 48 hr at 27°C.
- A 1.4 ml sample of the broth culture was placed in an eppendorf tube and cells were pelleted by centrifugation (5 min at 14,000 rpm).
- A 1 ml sample of the culture supernatant was transferred to a glass test tube and combined with 2 ml of reagent (2% 0.5 M FeCl 3 in 35% perchloric acid) (Gordon and Weber,1951). The samples were incubated at room temperature for 30 min, and OD_{530}nm was measured using a spectrophotometer (Thermo Spectronic 20+). Non-inoculated broth plus reagent was used as a reference.
- Cultures of a known IAA-producer (*Pantoea vagans* C9-1) and a known non-producer of IAA (*Pseudomonas fluorescens* A506) were included as controls (Smits *et al.*, 2011). Each isolate was tested twice with similar results.

T-DNA auxin genes: such as indole-3-acetamide hydrolase gene (iaaH).

Ain Khalib,2020;..

Mechanism of pathogenicity

Plasmid borne pathogenicity factors

Indole-3-acetic acid (IAA)

- Indole-3-acetic acid (IAA) production of field gall isolates measured using colorimetric assay of Gordon and Weber (1951). Data are averages from at least two assays for each isolate

Sample ID	Average quantity(± 1 standard deviation) of IAA (µg/ml) produced in culture
A506[a]	0.13 ± 0.03
C9-1[b]	0.44 ± 0.03
1Ab	0.25 ± 0.08
1Bb	0.27 ± 0.04
1Da	0.30 ± 0.06
2A	0.35 ± 0.05
2Bb	0.10 ± 0.05
2D	0.19 ± 0.05

[a]Non-producing IAA bacteria as a negative control. Values equal or below the value measured on this isolate are considered non-significant.
[b]IAA producing bacteria as a positive control. Values below the value measured on [a] are considered significant.

Ain Khalib,2020;..

A. tumefaciens infection process

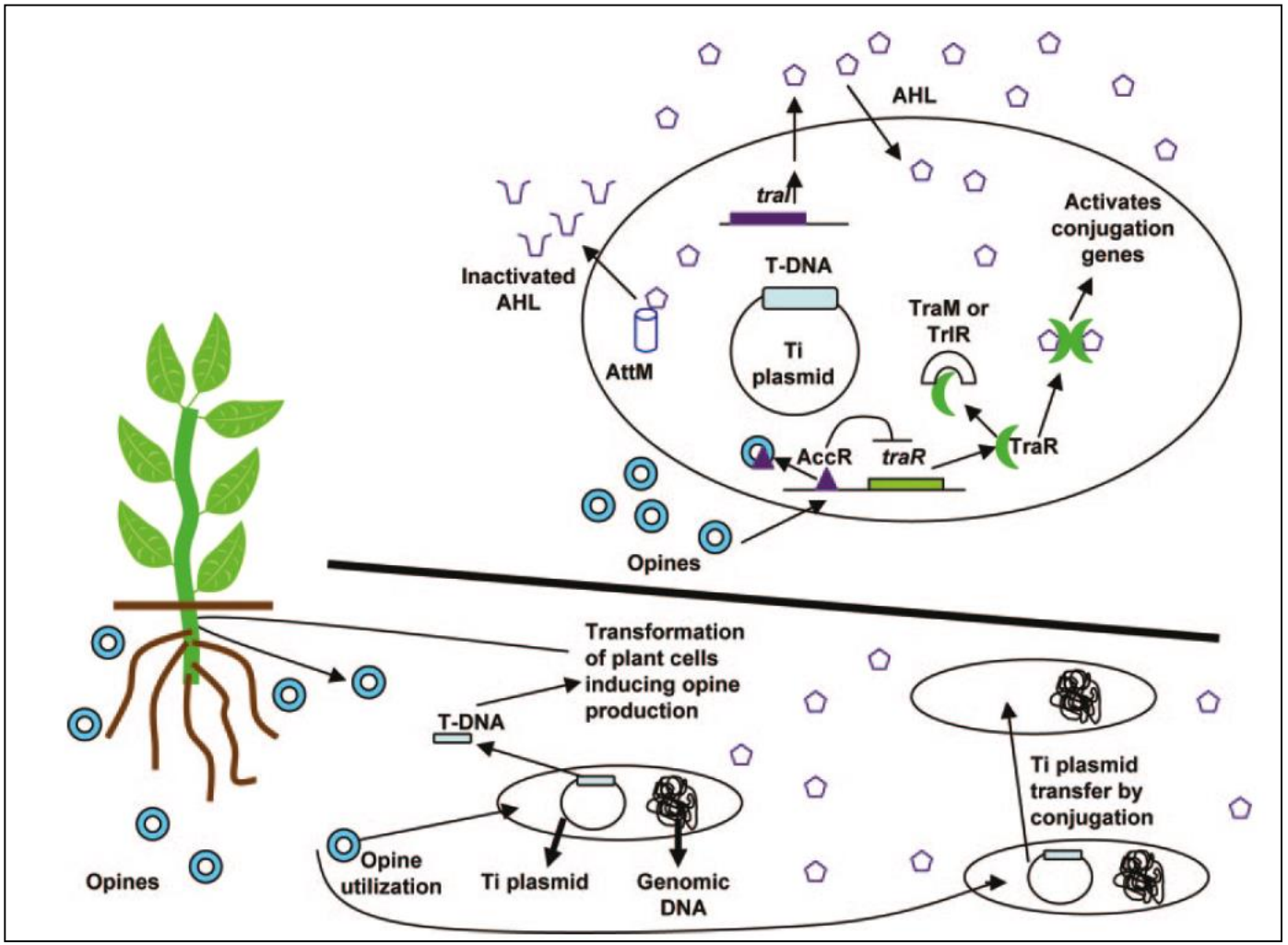
Quorum-sensing cross talk between *A. tumefaciens* and its host plant

- The transfer and integration of bacterial T-DNA into plant cells result in tumorogenesis, leading to crown gall disease in plants.
- The tumor cells directed by the bacterial DNA produce and release opines, which are metabolized by the *A. tumefaciens* present in the soil around the plant roots.
- The conjugal transfer of the Ti plasmid among the rapidly proliferating *A. tumefaciens* in soil is regulated by bacterial quorum sensing as well as by plant-produced opines.

González and Keshavan,2009

A. tumefaciens infection process

Quorum-sensing cross talk between *A. tumefaciens* and its host plant



Infection process

Systemic nature of the bacterial disease

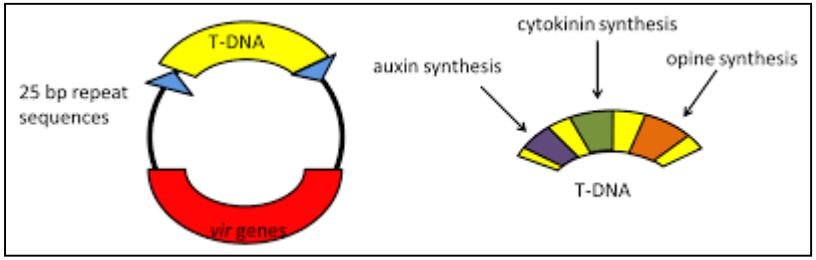
- Some of the critical steps involved in the crown gall disease and subsequent plant transformation by *A. tumefaciens* are enlisted as follows:

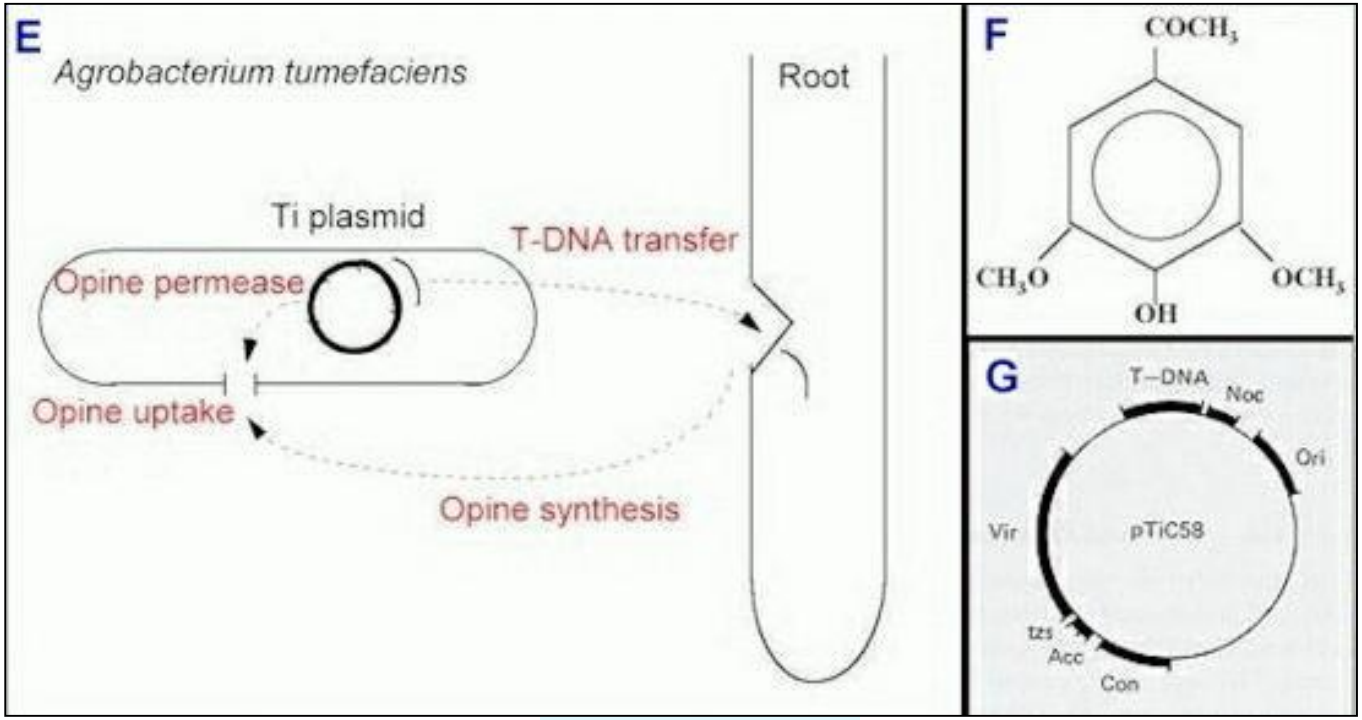
1. Chemotaxis and activation of bacterial virulence;
2. Bacterial attachment;
3. T-DNA processing and transport into plant cell;
4. Nuclear import, integration of the T-DNA into the plant genome and expression of oncogenes.

Anand and Mysore,2006

Infection process

Chemotaxies toward acetosyringone, a specific phenolic compound



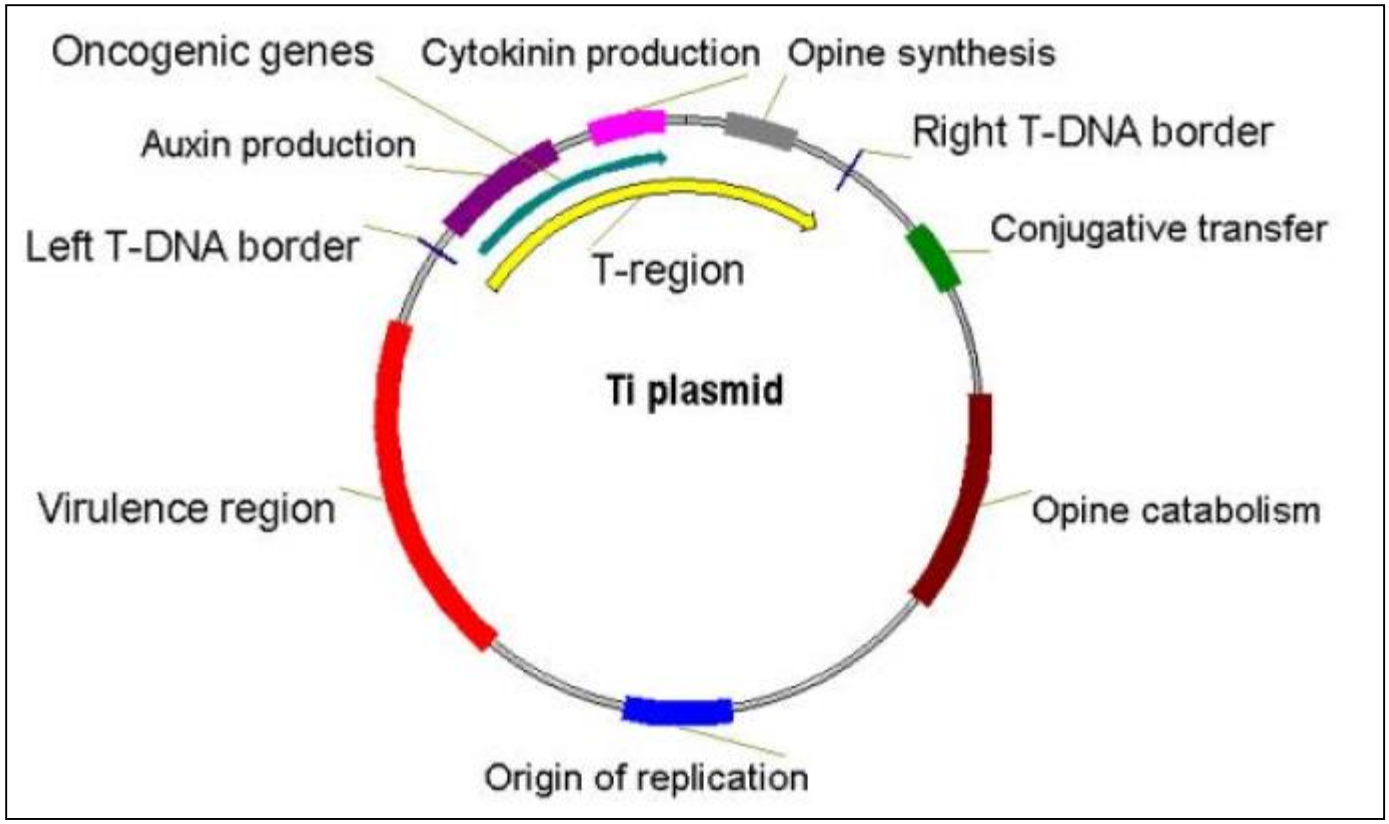


Acetosyringone

Ti plasmid

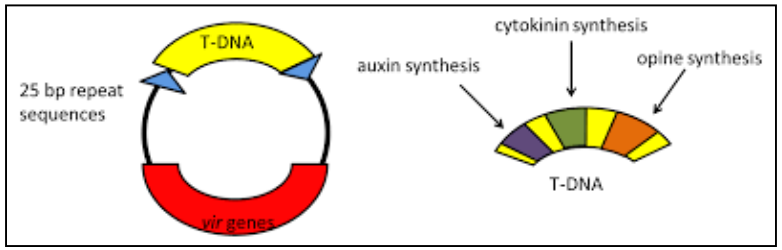
Jim Deacon;..

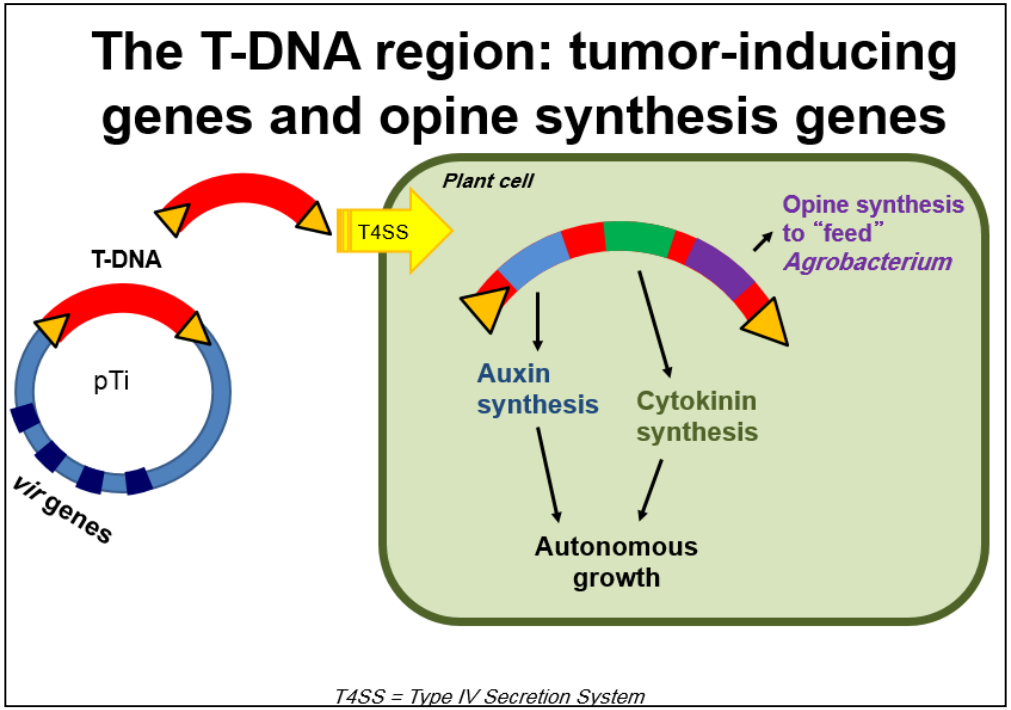
Structure of tumor-inducing plasmid
Ti plasmid



An example of Selfish Genes.
A. tumefaciens **genetically engineers plants to make specialized food for it.**

Structure of tumor-inducing plasmid
Ti plasmid

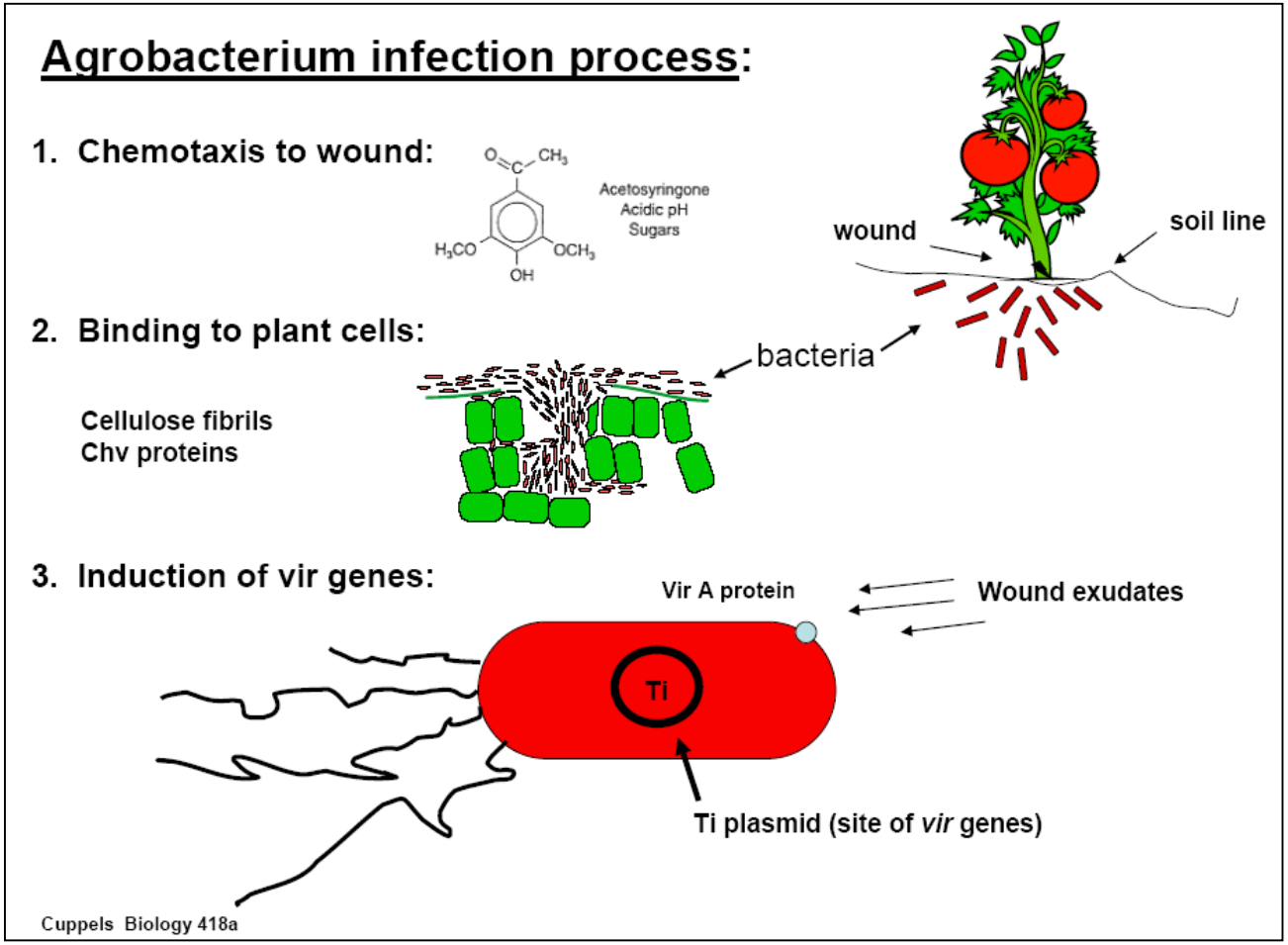




American Society of Plant Biologists,2014

Infection process

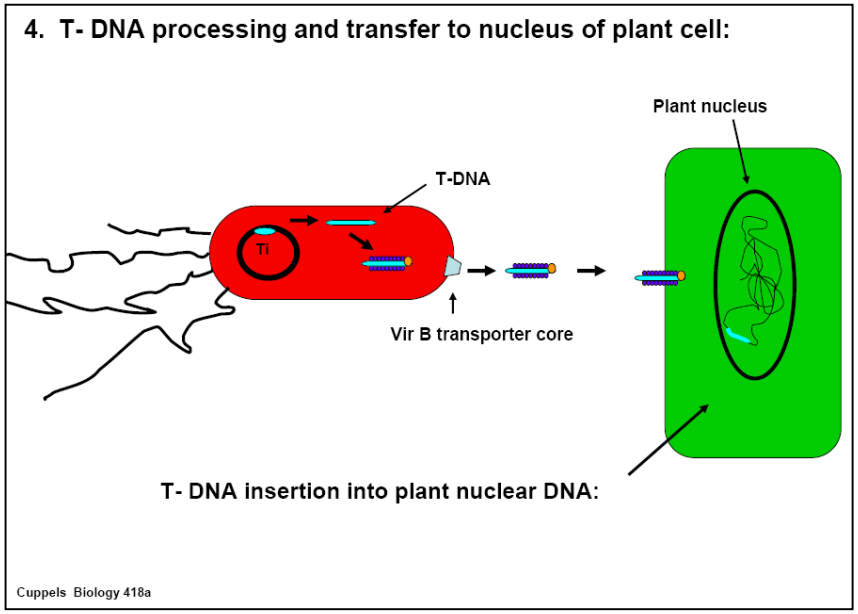
T-DNA transfer into plants



Cuppels,2007

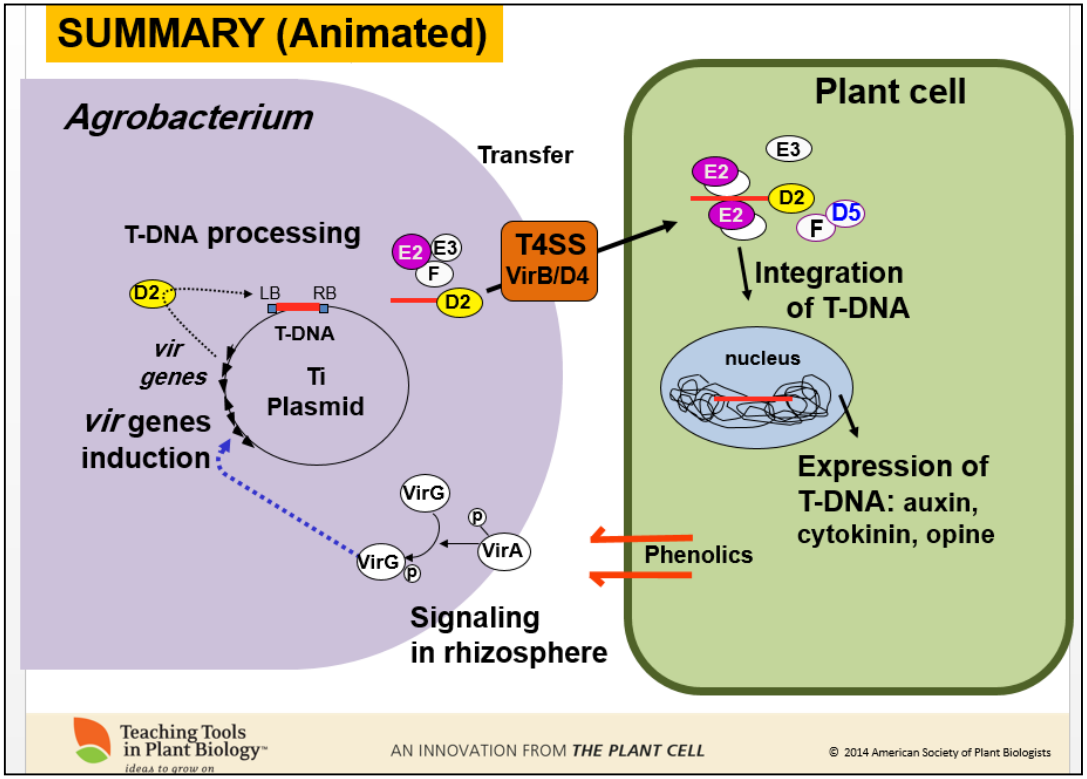
Infection process
T-DNA transfer into plants

- In *A. tumefaciens* the T-DNA is not delivered as naked DNA but is bound to at least one protein, the VirD2.
- In next step, VirE2 interacts with the VirD2/T-DNA complex (mature T-complex), mediating delivery of the T-DNA to the host cell nucleus and resulting in crown gall tumor formation.



Infection process

T-DNA transfer into plants



Model of T-DNA movement through the plant cell, and the proteins and structures with which it interacts. This model assumes that the T-strand/VirD2/VirE2 T-complex exists in a plant cell as hypothesized. Sub-cellular movement of T-complexes and/or Virulence effector proteins may utilize the actin cytoskeleton, as shown.

Opines
The novel plant metabolites

- All these compounds can be used by the bacterium as the sole carbon and energy source, and because they are absent from normal plants they provide *Agrobacterium* with a unique food source that other bacteria cannot use.
- These low-molecular-weight molecules composed of:

1. An amino acid, and
2. A keto acid or a sugar.

Although opines are known as highly specific nutrients for agrobacteria, they can also be used by some other microbes like fluorescent pseudomonads, coryneform bacteria and even by fungal species belonging to the *Cylindrocarpon* and *Fusarium* genera.
Because of this and the fact that opine synthesis and/or utilization genes occupy only relatively small parts of the large Ti/Ri plasmids, the opine classification is considered unreliable and excluded (Otten *et al.*,2006).

Opines
The novel plant metabolites

- The process by which *A. tumefaciens* transforms plant cells to produce opines has been termed genetic colonization of the plant.
- The genetic colonization theory leads to the opine concept, proposed by Tempé *et al.*,1979, which states that the opines are the chemical mediators of the interaction between *Agrobacterium* spp. and its plant host.
- Crown gall tumors induced by the classic nopaline-type *Agrobacterium tumefaciens* strain C58 synthesize and secrete two families of tumor metabolites:

1. Agrocinopines A and B, and
2. Nopaline.

Kim *et al.*,2008

Opines

Opines are low molecular weight compounds found in plant crown gall tumors or hairy root tumors produced by parasitic bacteria of the genus *Agrobacterium*

- During agrobacterial infection of susceptible plants, a copy of the T region in a tumor-inducing plasmid (Ti plasmid), called the T-DNA, is transferred from the bacterium to the plant where it becomes integrated into the chromosome.
- Expression of the genes on the integrated T-DNA results in:

1. the tumorous phenotype, and also
2. the production of unusual low-molecular-weight carbon compounds that belong to a large group of plant tumor-specific metabolites collectively called opine.

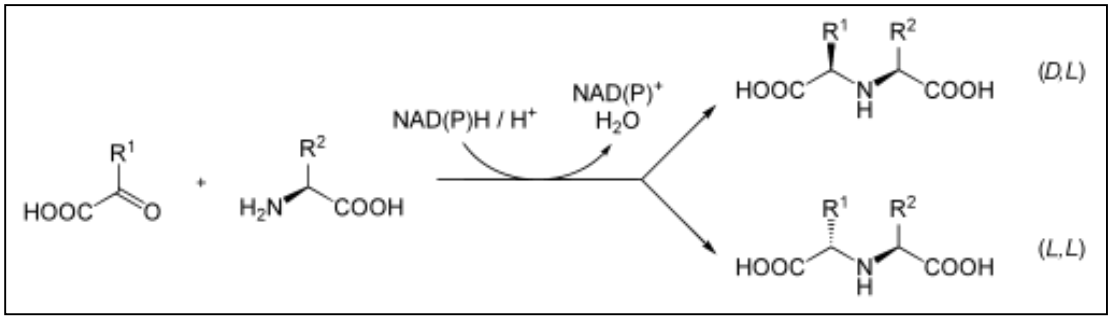
Kim *et al.*,2008

Opines

The novel plant metabolites

Opine biosynthesis

- Opine biosynthesis is catalyzed by specific enzymes encoded by genes contained in a small segment o fDNA (known as the T-DNA, for 'transfer DNA'), which is part of the Ti plasmid, inserted by the bacterium into the plant genome.
- Each strain of *Agrobacterium* induces and catabolizes a specific set of opines.
- There are at least 30 different opines described so far.

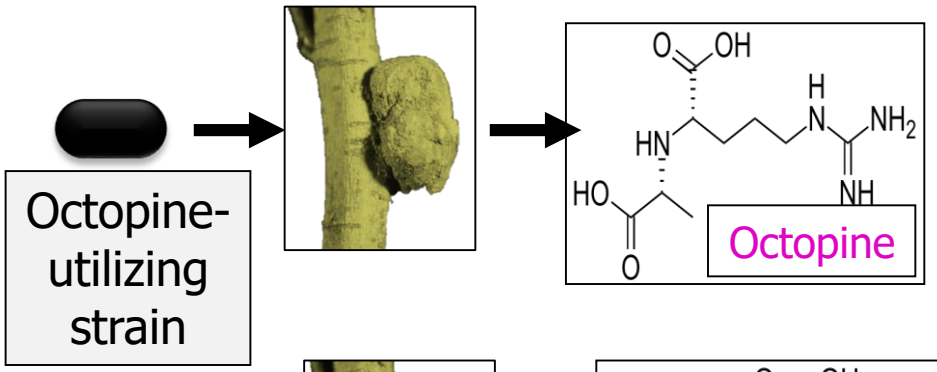


Wikipedia,2018

Opines

The novel plant metabolites

The type of opine is determined by the bacterium, not the plant.



Questions raised:

- What are these compounds?
- Do they cause the tumors?
- How and why do the bacteria cause the plants to make opines?

Opines

Opines are low molecular weight compounds found in plant crown gall tumors or hairy root tumors produced by parasitic bacteria of the genus *Agrobacterium*

- Opines (an amino acid, and a keto acid or a sugar) serve as carbon and/or nitrogen sources for the tumor-inducing bacterium.
- Opines not only provide a growth substrate but probably are also involved in conjugal Ti plasmid exchange and chemotaxsis.
- More than 20 different opines have been identified in crown galls and hairy roots, but only a few subset of them are encoded by the T-DNA of any one *Agrobacterium* strain.

Keto acids are organic compounds that contain a carboxylic acid group and a ketone group ().

Anand and Mysore,2006,..

Opines

The unique amino acid derivatives

More than 20 different opines have been identified

- So far, octopine, nopaline, succinamopine, agropine, agropine/mannopine, mannopine, chrysopine/succinamopine, chrysopine/nopaline, cucumopine/mikimopine, octopine/cucumopine and vitopine strains and plasmids have been identified.
- Vitopine-type agrobacteria form a distinct group within the genus *Agrobacterium*.
- A null-type category has been proposed for cases in which no opine could be detected.

Strain	Characteristics
A. tumefaciens A348	octopine pTi
A. tumefaciens C58	nopaline pTi
Agrobacterium vitis At6	octopine pTi
Agrobacterium vitis* S4	vitopine pTi
Agrobacterium vitis F2/5	avirulent strain

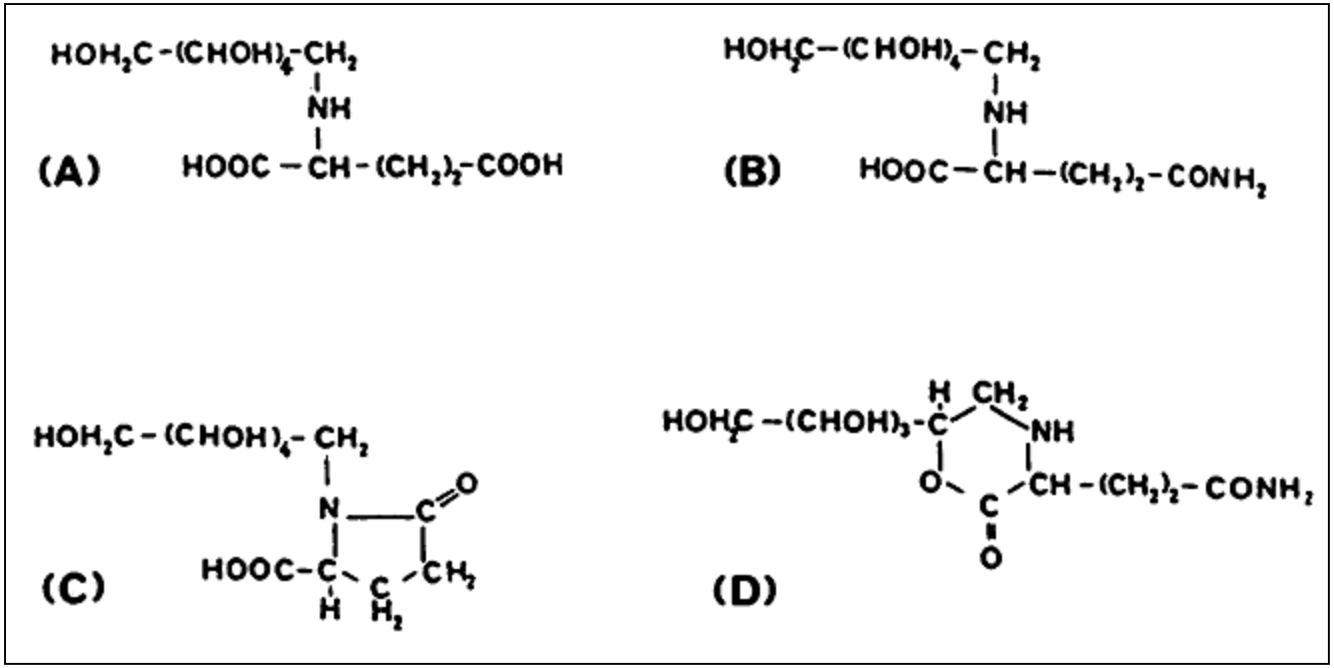
* Now *Allorhizobium vitis*

Otten *et al.*,2006;Bini *et al.*,2008

Opines

Structural formulas of the mannityl opines

- Agropine (AGR), mannopine (MOP), mannopinic acid (MOA), and agropinic acid (AGA).



Dessaux *et al.*,1988

Opines

Opine utilization

- Octopine [N-2-(D-1-carboxyethyl)- L-arginine] and nopaline [N-2-(D-1,3-dicarboxypropyl)-L-arginine] were tested for purity by paper electrophoresis and used as a sole source of both carbon and nitrogen.
- Growth at 28°C was scored at 3 and 7 days.

Perry and Kado,1994

Opines
Determination of opine utilization

- LB medium was used as the rich medium.
- Nitrogen-free minimal medium used to grow *Agrobacterium* spp.
- Unless stated otherwise, this medium was supplemented as follows: octopine or mannityl opines as sole carbon sources, 10 mM; sucrose, glucose, mannitol, or mannose (MAN), 2.0 g/liter; ammonium sulfate, 1.0 glliter. When required, a mixture of neomycin (10 ug/ml) and kanamycin (40 ug/ml) was added to this medium.
- Bacto-Agar was incorporated at a final concentration of 1.6% to solidify the medium. For nutritional studies, Noble agar (final concentration, 1.5%; Difco) was used in place of Bacto-Agar.
- All cultures were incubated at 28°C, and liquid cultures were incubated with agitation to ensure sufficient aeration.

Dessaux *et al.*,1988

Opines
Determination of opine utilization

- Determination of opine utilization and media preparation were based on Canfield *et al*.,1991 method.
- Bacterial suspensions (10^8 cfu/ml) were prepared and placed in wells of a 96-well tissue culture plate and then transferred by a multi-prong replicator to media with 5 mM octopine, nopaline, or mannopine as the only source of carbon and nitrogen.
- Growth of bacteria on the opine plates was examined 3 to 7 days later.

Shu-Fen Lu,1994

Opines

Determination of opine utilization

- The strains were inoculated to liquid media and grown at room temperature on a rotary shaker.
- An aqueous bacterial suspension of each strain was prepared with an optical density of 0.16 to 0.18 (about 10^8 cfu/ml) as inoculum.
- An aliquot of 0.3 ml of bacterial suspension was pipetted into 2.7 ml of the opine liquid medium at 0 h (0.D.= 0.01).
- Bacterial growth was measured at 16, 20, 24, and 40 h using a Spectronic 20 colorimeter at 600 nm.
- A strain was rated positive for opine utilization when the optical density of the cell suspension was greater than or equal to 0.2.
- Some strains reached O.D. 0.2 within 20 h; others reached this value by the 40 h reading.

Shu-Fen Lu,1994

Biofilm Assay

Agrobacterium

- Overnight bacterial cultures in ATGN liquid media were diluted with fresh medium to obtain an OD_{600} of 0.04.
- Then, 200 L of diluted culture was placed into 96-well polystyrene or polypropylene microtiter plates and incubated at 28°C.
- After 48 h of incubation, an aliquot of the liquid culture was initially transferred to a new plate, and then OD_{600} was measured.

Table 3. Biofilm formation and pathogenicity of *A. tumefaciens* isolate from rose crown gall.

Strain [a]	Abs [b]. (Mean ± std.)	Gall formed [c] (+, −)	Strain	Abs. (Mean ± std.)	Path (+, −)	Strain	Abs. (Mean ± std.)	Gall formed (+, −)
C58	0.49 ± 0.13	+	RC076	0.11 ± 0.01	−	RC132	0.21 ± 0.04	+
RC002	0.78 ± 0.15	−	RC077	0.10 ± 0.01	−	RC133	0.12 ± 0.01	+
RC003	0.61 ± 0.23	−	RC079	0.13 ± 0.04	−	RC134	0.18 ± 0.05	+
RC004	0.61 ± 0.18	−	RC080	0.12 ± 0.01	−	RC135	0.27 ± 0.01	+
RC005	0.61 ± 0.04	−	RC081	0.23 ± 0.00	−	RC140	0.18 ± 0.03	+
RC006	0.61 ± 0.13	−	RC082	0.32 ± 0.16	−	RC141	0.85 ± 0.15	−
RC007	0.71 ± 0.11	−	RC083	0.09 ± 0.01	−	RC160	0.61 ± 0.11	−

Chandrasekaran *et al.*,2019

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

Genus *Agrobacterium* (*Rhizobium*)

Genus *Agrobacterium*					
Species/biovars	Primer name Target DNA	Variant of PCR Protocol	Sample (treatment)	Reference	Synonyms/observations
Agrobacterium spp.	**tms2F1/tms2R2** **tms2B** *tms2* gene	Semi-nested	Bacteria from soil (DNA extraction)	Pulawska and Sobiczewski, 2005; Sobiczewski *et al.*, 2005	Tumour-inducing strains.
A. tumefaciens	**FGPtmr 530/FGPtmr 701** T-DNA **FGP vir B_{11+12}/FGP vir B 15** Intercistronic *vir* B/G region	Conventional	Bacteria (DNA extraction)	Nesme *et al.*, 1989	
A. tumefaciens	**tms2A/tms2B** pTi *tms2* gene **RBF/RBR** Nopaline type T-DNA **ocsF/ocsR** Octopine type T-DNA **virD2A, virD2C´, virD2E´** *vir* D2 gene	Conventional	Bacteria (boiled)	Tan *et al.*, 2003	

Palacio-Bielsa *et al.*,2009

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A. tumefaciens	**FGP vir B_{11+12}/FGP vir B 15** Intercistronic *vir* B/G region	Conventional	Plant roots (DNA extraction)	Puopolo *et al.*, 2007	
A. radiobacter	**Primers** **rol-F/rol-R** **Probe** **rol-Pr** Ri-plasmid	Real-time (TaqMan)	Bacteria (boiled)	Weller and Stead, 2002	*Agrobacterium* biovar 1
A. vitis	**Tm 4 ipt, IS866, S4 6b/vis** T-DNA	Conventional	Bacteria (boiled)	Schulz *et al.*, 1993	
A. vitis	**virA** *virA* region **6a** *6a* gene **pehA** Pectin enzyme hydrolase gene	Conventional	Bacteria (lysed) or plant tissue (DNA extraction)	Eastwell *et al.*, 1995	
A. vitis	**VCF/VCR** *vir*C gene **PGF/PGR** Polygalacturonase gene **VirE2PF/VirE2PR** *vir*E2 gene **VisF/VisR** pTiS4 vitopine synthase gene	Conventional	Bacteria (lysed)	Szegedi and Bottka, 2002	
A. tumefaciens *A. rhizogenes*	**UF** Universal agrobacteria 23S rRNA gene **BIR** *A. tumefaciens* specific 23S rRNA gene **B2R** *A. rhizogenes* specific 23S rRNA gene **AvR** *A. vitis* specific 23S rRNA gene **ArR** *A. rubi* specific 23S rRNA gene	Multiplex	Bacteria from soil or plant tissue (DNA extraction)	Pulawska *et al.*, 2006	*Agrobacterium* biovar 1 *Agrobacterium* biovar 2

Palacio-Bielsa *et al.*,2009

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

A. tumefaciens *A. vitis*	**Wide 1/Wide 2 (WHR)** T-DNA **Narrow 1/Narrow 2 (NHR)** T-DNA	Conventional	Bacteria (DNA extraction)	Dong *et al.*, 1992	*A. tumefaciens* biovar 1 *A. tumefaciens* biovar 3
A. tumefaciens *A. vitis*	**FGPtmr 530/FGPtmr 701** T-DNA **FGP vir B_{11+12}/FGP vir B 15** Intercistronic *vir* B/G region **VCF/VCR** *vir* C gene	Conventional	Bacteria (boiled) or plant tissue (DNA extraction)	Cubero *et al.*, 1999	
A. tumefaciens *A. vitis*	**VCF/VCR** *vir*C **VisF/VisR** pTiS4 vitopine synthase gene **TF/TR** *6b* gene of *A. vitis* octopine pTis **NF/NR** *6b* gene of *A. vitis* nopaline pTis **ttuCfw/ttuCrev** *A. vitis* tartrate deshydrogenase gene	Conventional	Bacteria (lysed)	Szegedi *et al.*, 2005	
A. vitis *A. radiobacter*	**Ab3-F3/Ab3-R4** *Agrobacterium* and *Rhizobium* 16S rRNA gene **VCF3/VCR3** *vir*C gene	Multiplex	Bacteria (boiled)	Kawaguchi *et al.*, 2005	*A. tumefaciens* biovar 3 (tumorigenic *A. vitis*) *A. radiobacter* biovar 3 (nonpathogenic *A. vitis*)
A. tumefaciens *A. rhizogenes* *A. vitis*	**A, C´, E´** *vir* D2 gene **CYT/CYT´** *ipt* oncogene	Conventional Multiplex	Bacteria (DNA extraction or boiled)	Haas *et al.*, 1995	
A. tumefaciens *A. rhizogenes* *A. vitis*	**VCF/VCR** *vir*C gene	Conventional	Bacteria (cells lysates or DNA extraction)	Sawada *et al.*, 1995	*Agrobacterium* biovar 1 (Ti or Ri plasmid) *Agrobacterium* biovar 2 (Ti or Ri plasmid) *Agrobacterium* biovar 3 (Ti plasmid)
A. tumefaciens *A. rhizogenes*	**VirE2PF/VirE2PR** *vir* E2 gene	Conventional	Bacteria (DNA extraction)	Genov *et al.*, 2006	

Palacio-Bielsa *et al.*,2009

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

A. vitis	**VisF/VisR** Vitopine synthase gene				
A. tumefaciens *A. rhizogenes* *A. vitis* *A. rubi*	**FGPS6, FGPS1509´, FGPL 132´** Chromosomal genes **FGPtmr 530, FGPtmr 701, FGPnos975, FGPnos1236´, FGPvir**A2275, FGP*vir*$B_2$164´ Ti plasmid genes	PCR-RFLP	Bacteria (DNA extraction)	Ponsonnet and Nesme, 1994	*Agrobacterium* biovar 1 *Agrobacterium* biovar 2 *Agrobacterium* biovar 3

Palacio-Bielsa *et al.*,2009

Primers sets for identification of plant pathogenic *Agrobacteria*

PGF/PGR and iaaHF2/iaaHR1 and iaaH-F10/iaaH-R10 primer pairs

- The PGF/PGR primers amplifying the chromosomal polygalacturonase gene *pehA* were used to identify *A. vitis* strains and to distinguish them from *A. tumefaciens*.
- For detection and amplification of diverged *iaaH* genes DNA sequences from *A. tumefaciens* and *A. vitis*, strains were aligned and two conserved regions separated by about 400 bp were chosen for primer design (iaaHF2/iaaHR1 and iaaH-F10/iaaH-R10 primer pairs).

T-DNA auxin genes: such as indole-3-acetamide hydrolase gene (iaaH).

Bini *et al.*,2008

Primers sets for identification of plant pathogenic *Agrobacteria*

Genes codes for auxin and cytokinin

- Expression of three T-DNA genes in the plant causes tumorous growth.
- Two of the T-DNA oncogenes, *iaaM* and *iaaH*, encode tryptophan monooxygenase and indole acetamide hydrolase; these enzymes convert tryptophan to indoleacetic acid, an auxin.
- The third oncogene, *ipt*, encodes an isopentenyl transferase which converts adenosine monophosphate into isopentenyl adenosine, a cytokinin.
- Overproduction of these phytohormone biosynthetic enzymes results in gall formation.

Haas *et al.*,1995

Primers sets for identification of plant pathogenic *Agrobacteria*

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- Two of the T-DNA oncogenes, *iaaM* and *iaaH*, encode tryptophan monooxygenase and indole acetamide hydrolase; these enzymes convert tryptophan to indoleacetic acid, an auxin.
- The third oncogene, *ipt*, encodes an isopentenyl transferase which converts adenosine monophosphate into isopentenyl adenosine, a cytokinin.
- Overproduction of these phytohormone biosynthetic enzymes results in gall formation.

Haas *et al.*,1995

Primers sets for identification of plant pathogenic *Agrobacteria*

Name (forward/ reverse)	Primer specificity	Sequence	Length of amplified fragment	Annealing temperature used during PCR	Reference
Primers used for strain detection and identification					
PGF/ PGR	Polygalacturonase gene of *A. vitis* CG49 (accession number: U73161)	5'GGGGCAGGATGCGTTTTTGAG3' 5'GACGGCACTGGGGCTAAGGAT3'	466 bp	54 °C	Szegedi and Bottka 2002
VirD2A/ VirD2C, or VirD2F	*virD2* gene of *A. tumefaciens* and *A. rhizogenes* (see ref.)	5'ATGCCCGATCGAGCTCAAGT3' 5'TCGTCTGGCTGACTTTCGTCATAA3' 5'CTGACCCAAACATCTCGGCTGCCCA3'	224 bp 338 bp	50 or 54 °C	Haas *et al.* 1995
$VirFF_1$/ $VirFR_2$	*virF* gene of *A. vitis* octopine and nopaline pTi (accession number: AF044200)	5' ATG AGA AAT TCG AGT TTG CAT GAT G 3' 5' TCG TGA TGG GTA TAC GCT ACG 3'	382 bp	60 °C	This work
$VirD2S4F_{716}$/ $VirD2S4R_{1036}$	*virD2* gene of *A. vitis* S4 vitopine pTi (http://agro.vbi.vt.edu)	5' GAC CGC AAA ACC TGC CAG 3' 5' GAG CCT GTA TTG ACG ATG TC 3'	320 bp	60 °C	This work
OCTF/ OCTR	Octopine synthase gene of *A. vitis* pTiTm4 (accession no.: U83987)	5' GAA TAT GAG AAA TCC GTC TCG 3' 5' ACT CAG AGC TCG TGG CCT TG 3'	475 bp	50 or 52 °C	Bini *et al.* unpublished
NOPF/ NOPR	Nopaline synthase gene of *A. vitis* pTiAB4 (accession no.: X77327)	5' GCA AAC GTA AGT GTT GGA TC 3' 5' CAA GCG AAT ACT CGA GAC G 3'	394 bp	50 or 52 °C	Bini *et al.* unpublished
TF/TR	*6b* gene of *A. vitis* octopine Ti plasmid pTiTm4 (accession no.: U83987)	5'TGGCCGAAATTGTTTACTTCCACCC3' 5'CTATG CCGAAAGACGGCTTGACCCT3'	520 bp	58 °C	Szegedi *et al.* 2005
NF/NR	*6b* gene of *A. vitis* nopaline Ti plasmid pTiAB4 (Accession no.: X77327)	5'TTAACCCAAATGAGTACGATGACGA3' 5'TTATTTCGGTACTGGATGATATTAG3'	570 bp	54 °C	Szegedi *et al.* 2005
SF/SR	*6b* gene of *A. vitis* vitopine Ti plasmid pTiS4 (accession no.: M91608)	5'TGGCGGTACCGAGATGGGCTGTTCG3' 5'TTAAGCAGAATTAGGACATGAGCCC3'	620 bp	62 °C	This work
iaaH-F2/ iaaH-R1	*iaaH* gene of *Agrobacterium* T-DNA (see Material and Methods)	5'ACATGCATGAGTTATCGTTTGGAAT3' 5'GCATCAAGGTCATCGTAAAAGTAGGT3'	420 bp	54 °C	This work
iaaH-F10/ iaaH-R10	*iaaH* gene of *Agrobacterium* T-DNA (see Material and Methods)	5'GGAAACATGCATGAGTTATCGTT3' 5'CCACATCAGCATCAAGGTCATC3'	424 bp	54 °C	This work
S4iaaM5/ S4iaaM3	*iaaM* gene of the vitopine Ti plasmid pTiS4 (accession no.: M91609)	5'CGCGTCCCCGTTTACACTA3' 5'CGAGATCGCGCTTCAAGAT3'	800 bp	54 °C	This work
VisF/ VisR	vitopine synthase gene of *A. vitis* vitopine Ti plasmid pTiS4 (accession no.: M91608)	5'CCGGCCACTTCTGCTATCTGA3' 5'CCATTCACCCGTTGCTGTTATT3'	561 bp	54 °C	Szegedi and Bottka 2002
Sequencing primers					
iaaH-p1/ iaaH-end	Forward and reverse primers for sequencing of the *iaaH* gene of *A. vitis* AT1 and AB4	5'GGAAATTCCCTCCAATAATCGC3' 5'CAAGCAGATGTTTTGATTTTGGG3'	-	-	This work
Hend2/ Hend4	Forward and reverse primers for sequencing the 3' part of *iaaH* gene of *A. vitis* AT1 and AB4	5'CTTGGCCTGAAGGATTGACG3' 5'AATTCGTAGTCCCGATGTAGCG3'	-	-	This work
Hinv3/ Hinv4	Reverse and forward primers for sequencing the *iaaH* gene of *A. vitis* AT1 and AB4	CGCAGCAGCCACACCAC CACCGCCGGAATCATAGC	-	-	This work

virD2A/virD2C or virD2F/virFF1

Bini *et al.*,2008

Primers sets for identification of plant pathogenic *Agrobacteria*

Combination of iaaH and iaaM specific primers

- Primer pairs iaaHF2/iaaHR1 and iaaH-F10/iaaH-R10 are specific for the *iaaH* gene of *A. tumefaciens* and *A. vitis* octopine and nopaline strains.
- The S4iaaM5/S4iaaM3 is specific for the *iaaM* gene of *A. vitis* vitopine strains.
- A combination of these iaaH and iaaM specific primers indeed amplified the appropriate region from all laboratory strains tested including pathogenic agrobacteria representing the three opine types of *A. tumefaciens* as well as octopine, nopaline and vitopine strains of *A. vitis*.

Bini *et al.*,2008

Primers sets for identification of plant pathogenic *Agrobacteria*

Combination of iaaH and iaaM specific primers

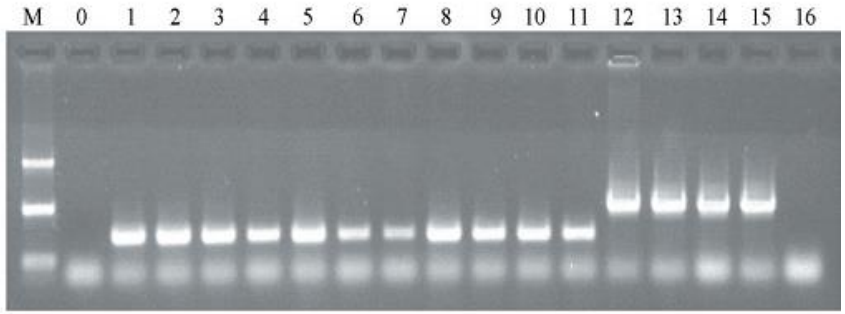
- PCRs with *iaaH-*, *iaaM-* primers were carried out in 25 µl volumes in 1X *Taq* polymerase buffer prepared with 5% (v/v) DMSO, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 200 µM of each dNTP, 1.25 unit of *Taq polymerase* and 1 µl of template DNA prepared in Triton X-100/sodium azide buffer.
- The DNA amplification was started with an initial denaturation at 94°C for 1 min followed by 32 cycles at 92°C for 1 min, 50-62°C for 1 min and 72°C for 1.5 min.
- The reaction was completed with a final elongation step at 72°C for 3 mins.
- Samples were analyzed after electrophoretic separation in ethidium-bromide stained 1.5% (w/v) agarose gels.

Bini *et al.*,2008

Primers sets for identification of plant pathogenic *Agrobacteria*

Combination of iaaH and iaaM specific primers

- PCR analysis of *Agrobacterium* strains with the iaaHF2/iaaH-R1+S4iaaM5/S4iaaM3.
- Order of samples:
- Size markers (M: 1794, 753 and 191 bp), DNA-free control (0), *A. tumefaciens* A348 (1), C58 (2) and A281 (3), *A. vitis* octopine strains AT6 (4), Tm4 (5), AB3 (6) and Zw2 (7), *A. vitis* nopaline strains AT1 (8), AB4 (9), Ni1 (10) and Rr4 (11), *A. vitis* vitopine strains S4 (12), Sz1 (13), NW221 (14) and SF93 (15), and the avirulent A. vitis F2/5 (16).



Bini *et al.*,2008

Small-scale plasmid isolations

Agrobacterium spp.

Medium and buffers

1. Inoculate fresh bacteria in 2 mL of LC medium, and incubate overnight at 29°C with agitation.
2. Centrifuge 0.5 mL of the bacterial culture for 5 min at 12,000g(10355 rpm), and discard the supernatant.
3. Suspend the pellet by vortexing in 100 µL of solution 1. Incubate 10 min at room temperature.
4. Add 200 µL of solution 2, mix by inverting the tube four times, and incubate for 10 min at room temperature.
5. Add 30 µL of solution 3, and mix by brief and gentle vortexing.
6. Immediately add 150 µL of solution 4, mix by inversion, and incubate at -20°C for 15 min.
7. Centrifuge for 5 min at 12,000g, and transfer the supernatant to a fresh 1.5-mL tube.
8. Add 400 µL of phenol-chloroform, and mix by brief vortexing.
9. Centrifuge for 3 min at 12,000g, and transfer the aqueous (top) layer to a 1.5-mL tube.
10. Add 800 µL of ice-cold 96% ethanol, and mix by inversion.
11. Precipitate the DNA by incubation at -70°C for 15 min.
12. Centrifuge for 10 mm at 12,000g, and discard the supernatant.
13. Wash the pellet with 250 µL of 70% ethanol, and dry briefly in a vacuum centrifuge.
14. Dissolve the pellet in 25 µL of dH_2O or TE buffer.
15. Use 5 µL of the DNA solution to check plasmid presence in a 0.6% agarose gel.

Small-scale plasmid isolations

Agrobacterium spp.

Medium and buffers

- LC medium: 10.0 g tryptone, 5.0 g yeast extract, 8.0 g NaCl, distilled water to 1 L. Sterilize by autoclaving for 20 min at 120°C. When indicated, supplement medium with 0.1% glucose.
- Solution 1: 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0, 4 mg/mL lysozyme. Add lysozyme just before use.
- Solution 2: 1% SDS, 0.2N NaOH. This solution must be freshly prepared from stock solutions of 20% SDS and 4N NaOH.
- Solution 3: 1 mL H_2O-saturated phenol plus 15 µL 4N NaOH.
- Solution 4: 3M sodium acetate, adjust to pH 4.8 with acetic acid.
- Phenol-chloroform: 1 vol of 100 mM Tris-HCl, pH 8.0, saturated phenol plus 1 vol of chloroform:isoamylalcohol (24:1).
- TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Dulk-Ras and Hooykaas,1995

PCR for genes related to carbon source utilization

Opines

- PCR primer sets were used to detect genes for agrocinopine, nopaline, and octopine to characterize isolates regarding the utilization of carbon source.
- The sequences of primers used for PCR analysis are listed in the Table.

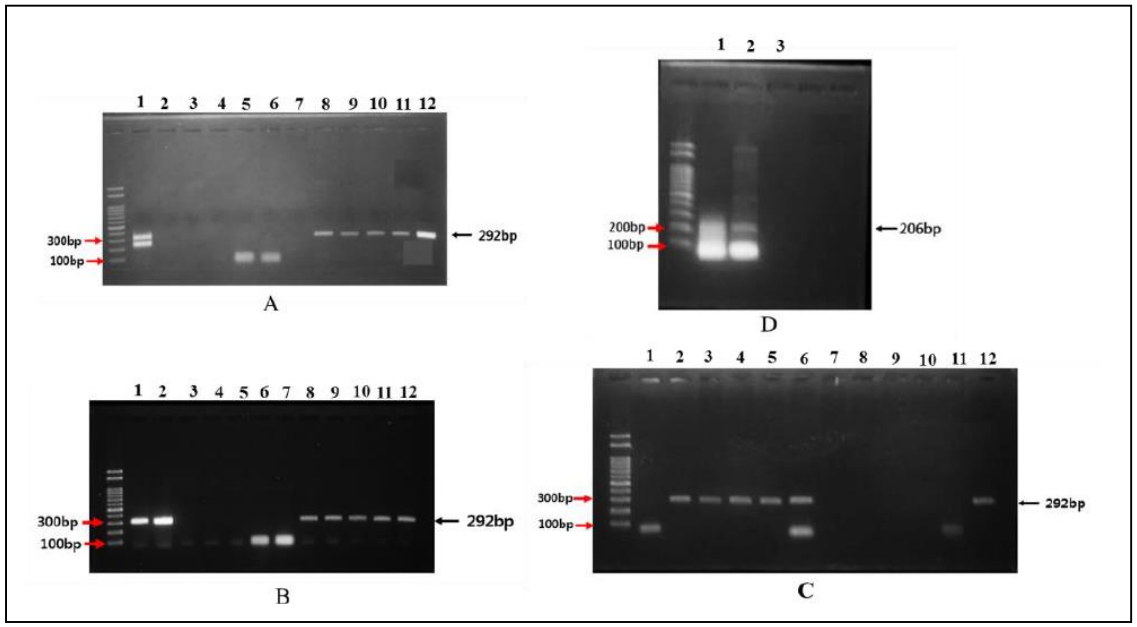
Gene	Primer Name	Primer Sequence
Agrocinopine	ACC-F	5′ AGGAATGAAAATGAACCCTCT 3′
	ACC-R	5′ CTCCGAACTGAACCAACTCCC 3′
Nopaline	RB-F	5′ TGACAGGATATATTGGCGGGTAA 3′
	RB-R	5′ TGCTCCTCCGTCAGGCTTTCCGA 3′
Octopine	OCS-F	5′ ATGGCTAAAGTGGCAATTTTGGG 3′
	OCS-R	5′ TCAGATTGAASTTCGCCAACTCG 3′

Chandrasekaran *et al.*,2019

PCR for genes related to carbon source utilization

Opines

- PCR amplification of gene fragments of agrocinopine, nopaline, and octopine of *Agrobacterium tumefaciens* strains.
  Agrocinopine and nopaline gene fragments were amplified, showing sizes of 292 bp and 206 bp, respectively.



Chandrasekaran *et al.*,2019

PCR detection and identification of *Agrobacterium* spp. fD1/rP2, tmsA/tms2B, A'/C' and TFP_R

- **Specific for tumor-inducing (Ti) plasmid genes**
- virD2 gene located on Ti plasmid (Haas *et al.*,1995).
- All isolates analyzed were tested for the presence of Ti plasmid using *virD2* gene specific primers (A' and C') yielding a 224 bp amplicon to confirm their tumorigenic characteristic.
- Other primers were also tested on isolates from previous isolation method such as tms 2F1/R2 and fD1/rP2 (16s rRNA); assist with differentiating between closely related bacterial species.

PCR detection and identification of *Agrobacterium* spp. fD1/rP2, tmsA/tms2B, A'/C' and TFP_R

- Genomic DNA of isolates that were PCR-positive for *virD2* was used for PCR assays to amplify 16S rRNA (Weisburg *et al.*, 1991) and *flaA* (Kuzmanovic *et al.*, 2019).

Primer names	Target	Amplicon Size (bp)	Sequence (5'-3')	Annealing temperature (°C)	Source
fD1	16S		AGAGTTTGATCCTGGCTCAG		Weisburg *et*
rP2	rRNA	~1400	ACGGCTACCTTGTTACGACTT	55	*al.* 1991
tms2F1			TTTCAGCTGCTAGGGCCACATCAG		Puławska *et*
tms2R2	*tms2*	458	TCGCCATGGAAACGCCGGAGTAGG	60	*al.* 2004
A'			ATGCCCGATCGAGCTCAAGT		Haas *et al.*
C'	*virD2*	224	TCGTCTGGCTGACTTTCGTCATAA	55	1995
			GTTTGAAAGTCGCTTCCGCATCC		Kuzmanović,
TFP_R	*flaA*	304	CTCAGCATGTTGACGCCGTTG	62	*et al.* 2019

A'/C' primers targeting the virD2 gene located on Ti plasmid (Haas *et al.*, 1995) was used. TFP_F and TFP_R primers targeting chromosomal gene flaA specific for R. tumorigenes-like bacteria.

Ain Khalib,2020;..

Primers sets for identification of plant pathogenic *Agrobacteria*

Specific for tumor-inducing (Ti) plasmid genes

- Most molecular methods for detection of tumorigenic isolates target tumorigenicity genes on a conserved vir region on the pTi.
- Sawada *et al.*,1995 developed VCF/VCR primers that target pTi-encoded virC1 and virC2 genes.
- Suzaki *et al.*,2004 improved the specificity of the primers (VCF3/VCR3) for pathogenic *Agrobacterium* strains from apple seedlings, but the primers work on *A. vitis* strains as well (Kumagai and Fabritius, 2008).

Lamovšek *et al.*,2014

Primers sets for identification of plant pathogenic *Agrobacteria*

Specific for tumor-inducing (Ti) plasmid genes

- Bacterial strains isolated from blackberry were subjected to PCR analysis using primers specific for tumor-inducing (Ti) plasmid genes:

1. *virC* (VCF3/VCR3),
2. *virD2* (A/C'),
3. *ipt* (CYT/CYT'), and
4. *tms2* (tms2F1/tms2R2).

- By using PCR, presence of virC, virD2, ipt and tms2 genes was detected in all four strains studied, indicating that they carry the Ti plasmid required for plant tumorigenic ability.

Kuzmanović *et al.*,2018

Primers sets for identification of plant pathogenic *Agrobacteria* Primers A'/C' and E' and VCF/VCR

- Primers from Haas *et al.*,1995 have also been used in both conventional and real-time PCR for detection of *A. tumefaciens* in soil (Sudarshana *et al.*,2006), achieving a sensitivity of 200 and 20 CFU g^{-1} soil, respectively.

Primer A: 5'-ATG CCC GAT CGA GCT CAA GT-3' Primer C': 5'-TCG TCT GGC TGA CTT TCG TCA TAA-3' Primer E': 5'-CCT GAC CCA AAC ATC TCG GCT GCC CA-3' These oligonucleotides were used in two different pairs to produce PCR products of 338 base pairs (bp) (A-E') and 224 bp (A-C').	Haas et al.
Primer VCF: 5Õ-ATCATTTGTAGCGACT-3Õ Primer VCR: 5Õ-AGCTCAAACCTGCTTC-3Õ These primer sets produced a product of 730 bp.	Sawada et al.

Schaad *et al.*,2001

Determination of virulence genes region on Ti-plasmid
VCF3/VCR3 primer pairs

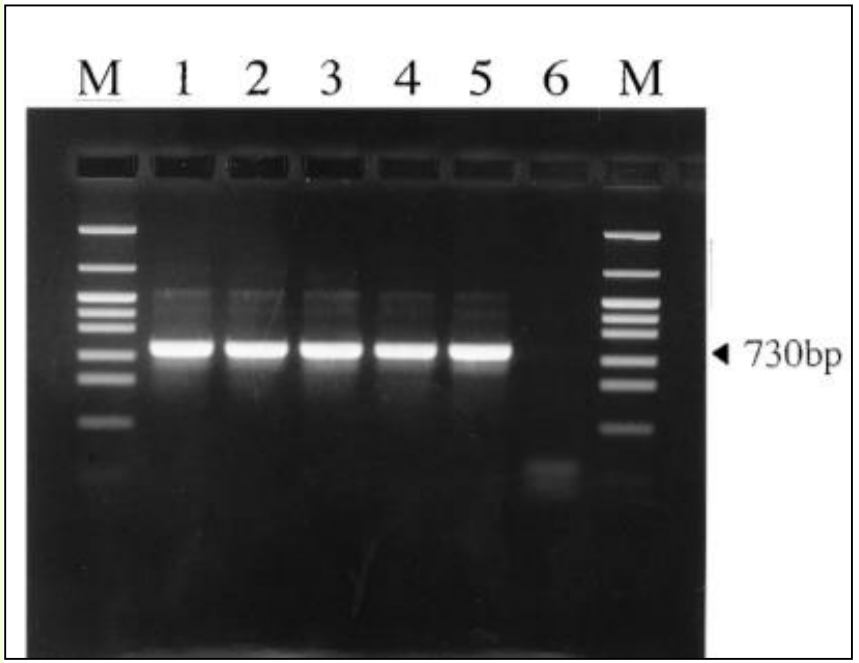
- **pTi detection:**
- A universal primer set (VCF/VCR) for PCR analysis based on the sequences of the *vir*C operon located on Ti and Ri plasmids was designed to detect these plasmids from phytopathogenic *Agrobacterium* strains.
- With the VCF (sequence, 5'-ATCATTTGTAGCGACT-3') and VCR (sequence, 5'-AGCTCAAACCTGCTTC-3') primer set, DNA fragments of 730 bp in length were amplified.

Sawada *et al.*,1995

Primers sets for identification of plant pathogenic *Agrobacteria*

VCF/VCR universal primer pair

- Agarose gel (2%) showing PCR products of 730 bp in length amplified from various *Agrobacterium* strains harboring Ti plasmids, using the universal primer set (VCF/VCR).
- Lanes: M, pHY marker (bands from top to bottom: 4,870, 2,016, 1,360, 1,107, 926, 658, 489, and 267 bp) (Takara); 1, *Agrobacterium* biovar 1 strain ATCC 33970; 2, *Agrobacterium* biovar 2 strain NCPPB 2303; 3, *Agrobacterium* biovar 2 strain MAFF 03-01546; 4, *Agrobacterium* biovar 3 (*A. vitis*) strain G-Ag-27; 5, *A. rubi* IFO 13261T;
- 6, nonpathogenic *Agrobacterium* biovar 1 strain IAM 12048T, which does not harbor a Ti or Ri plasmid (negative control).



All of the 37 pathogenic agrobacteria used in this preliminary experiment produced clear bands of about 730 bp in length with C1 and C2 as the forward and reverse primers, respectively.

Sawada *et al.*,1995

Determination of virulence genes region on Ti-plasmid
VCF3/VCR3 primer pair

- The genes located on the transmittable Ti-plasmid are useful markers for the detection of *A. tumefaciens*.
- As proposed by Kawaguchi *et al.*,2005 the presence of Ti-plasmid within agrobacteria can be detected through the combination of
- VCF3 (5'-GGC GGGCGYGCYGAAAGRAARACYT-3') and VCR3 (5'- CGAGATTGCGTGCTTGTA GA-3') primers.
- These primers were designed on *virC1-C2* genes with an annealing temperature of 57°C.

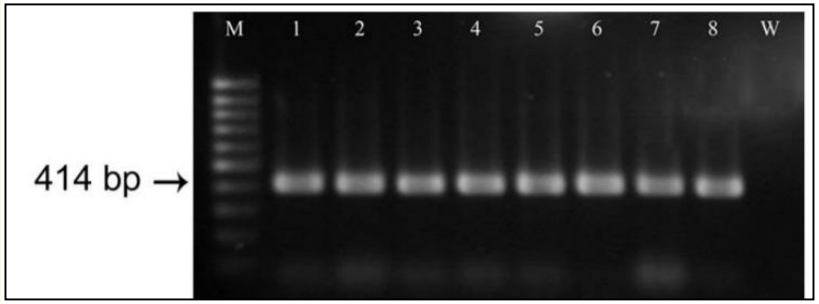
Note: This primer pair VCF3/VCR3 is improved the specificity of the primers VCF/VCR primers designed by Sawada *et al.*,1995.

Campillo *et al.*,2012;Min,2014; Lamovšek *et al.*,2014

Determination of virulence genes region on Ti-plasmid

VCF3/VCR3 primer pair

- *vir*C primer pair amplified 414-bp product from all studied and control strains.
- Lane M, marker (MassRuler Low Range DNA Ladder, Fermentas, Lithuania);
- Lane 1 - KFB 096/C58 (*A. tumefaciens* control strain);
- Lane 2 - KFB 098/ A4 (*A. rhizogenes* control strain);
- Lane 3 - KFB 099/S4 (*A. vitis* control strain);
- Lane 4-8 - bacterial strains used in this study;
- Lane W – SDW (negative control).

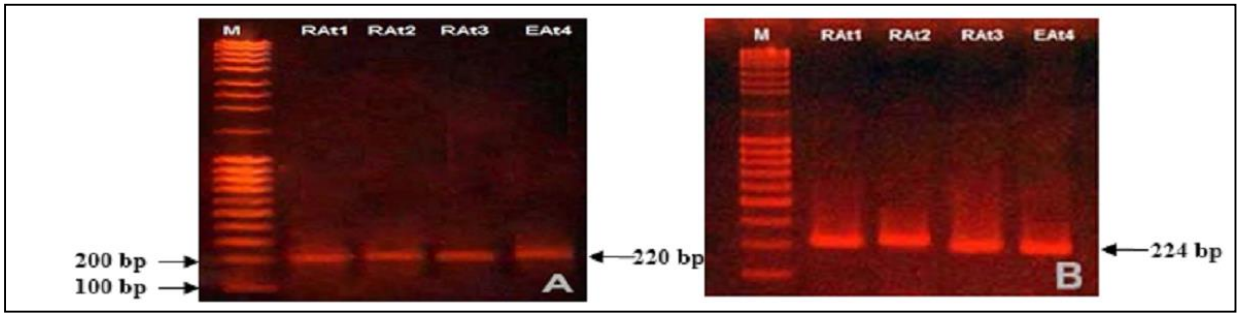


Tumorigenic (Ti) plasmid was detected in all strains by PCR using primers designed to amplify the *vir*C pathogenicity gene, producing a 414-bp PCR product.
VCF3 (5′ – GGC GGG CGY GCY GAA AGR AAR ACY T – 3′), and
VCR3 (5′ – AAG AAC GYG GNA TGT TGC ATC TYA C – 3′)

Kuzmanović *et al.*,2012

Primers sets for identification of plant pathogenic *Agrobacteria* P0/P6, virD2A/virD2C and tmsA/tms2B

Primer name (forward/reverse)	Primer specificity	Nucleotide sequence (5` to 3`)	Length of amplified fragment (bp)
tms2A/ tms2B	*tms2* gene of the tumor inducing (Ti) on the Ti plasmid.	CGCCACACAGGGCTGGGGGTAGGC GGAGCAGTGCCGGGTGCCTCGGGA	220
virD2A/ virD2C	*virD2* gene of the virulence (vir) region on the Ti plasmid.	ATGCCCGATCGAGCTCAAGT TCGTCTGGCTGACTTTCGTCAT	224
P0 (F)/ P6 (R)	16S rRNA gene	GAAGAGTTTGATCCTGGCTCAG CTACGGCTACCTTGTTACGA	1550

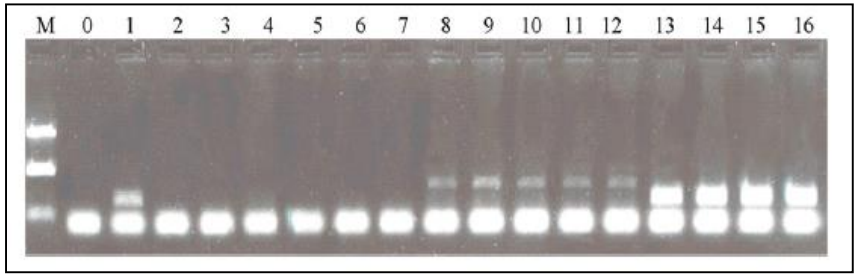


Amplified products resulted from the use of the two specific primers: tms2A/tms2B, (A); virD2A/virD2C (B); M: a 100 bp DNA marker ladder.

Younis *et al.*,2006

Primers sets for identification of plant pathogenic *Agrobacterium vitis* virD2A/virD2F primer pair

- PCR analysis of *Agrobacterium vitis* with virD2A/virD2F primers.
- **M**: size markers (1794, 753 and 191 bp), 0: DNA-free sample,
- **1**: *A. tumefaciens* A6 DNA (positive control), **2**: *A. vitis* F2/5 (avirulent control), **3** to **7**: *A. vitis* AT6, Tm4, AB3, Zw2 and B10/7 (octopine strains), **8** to **12**: *A. vitis* AT1, AT66, N1, AB4 and CG49 (nopaline strains), **13** to **16**: *A. vitis* S4, Sz1, NW221 and SF93 (vitopine) strains yielding a specific amplification product.



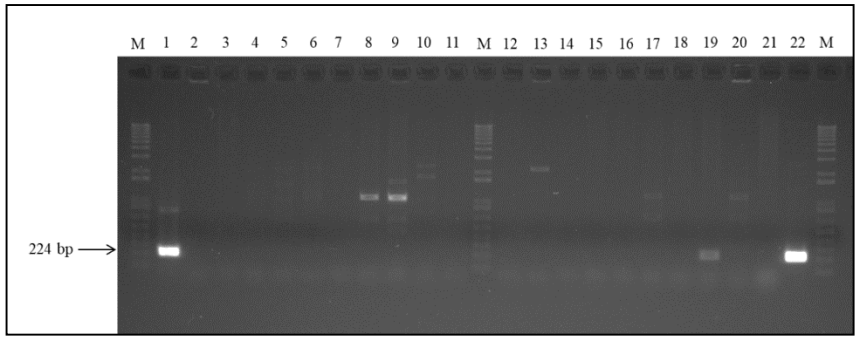
When the annealing temperature was increased to 54°C we could only detect *A. tumefaciens* (lane 1) and vitopine strains of *A. vitis* (lanes 13-16) with virD2A/virD2F primers.

The common band present in all samples is a non-specific product probably generated by primer annealing.

Bini *et al.*,2008

Primers sets for identification of plant pathogenic *Agrobacterium vitis* virD2A/virD2C primer pair

- Electrophoresis gel showing PCR products obtained in *virD2* (A/C) PCR reaction with isolates from field galls;
- Lane 1, A5A2 Red 1 (positive control);
- Lanes 2-18, field isolates;
- Lane 19, 6b*2e;
- Lanes 20-21, field isolates;
- Lane 22, C58 (*A. tumefaciens* positive control);
- M, 1 Kb+ marker.



A 224 bp product is generated from the amplification via virD2A/virD2C(A'/C') primers. The virD2 primer pair appears to be universal for all pathogenic *Agrobacterium* specie.

Ain Khalib,2020

Determination of virulence genes region on Ti-plasmid

F14-*vir* /F749-*vir* primer pair

- Alternately, cooperation of
- F14-*vir* (5'-GAACGTGTTTCAACGGTTCA-3' and
- F749-vir (5'-GCTAGCTTGGAAGATCGCC-3') primers, which work at an annealing temperature of 57°C, are designed to detect the type of residents plasmids associated in the host *Agrobacterium* (Nesme *et al.*,1989).

Campillo *et al.*,2012;Min,2014

Identification of plant pathogenic *Agrobacteria*

Polygalacturonase gene amplification(*peh*A)

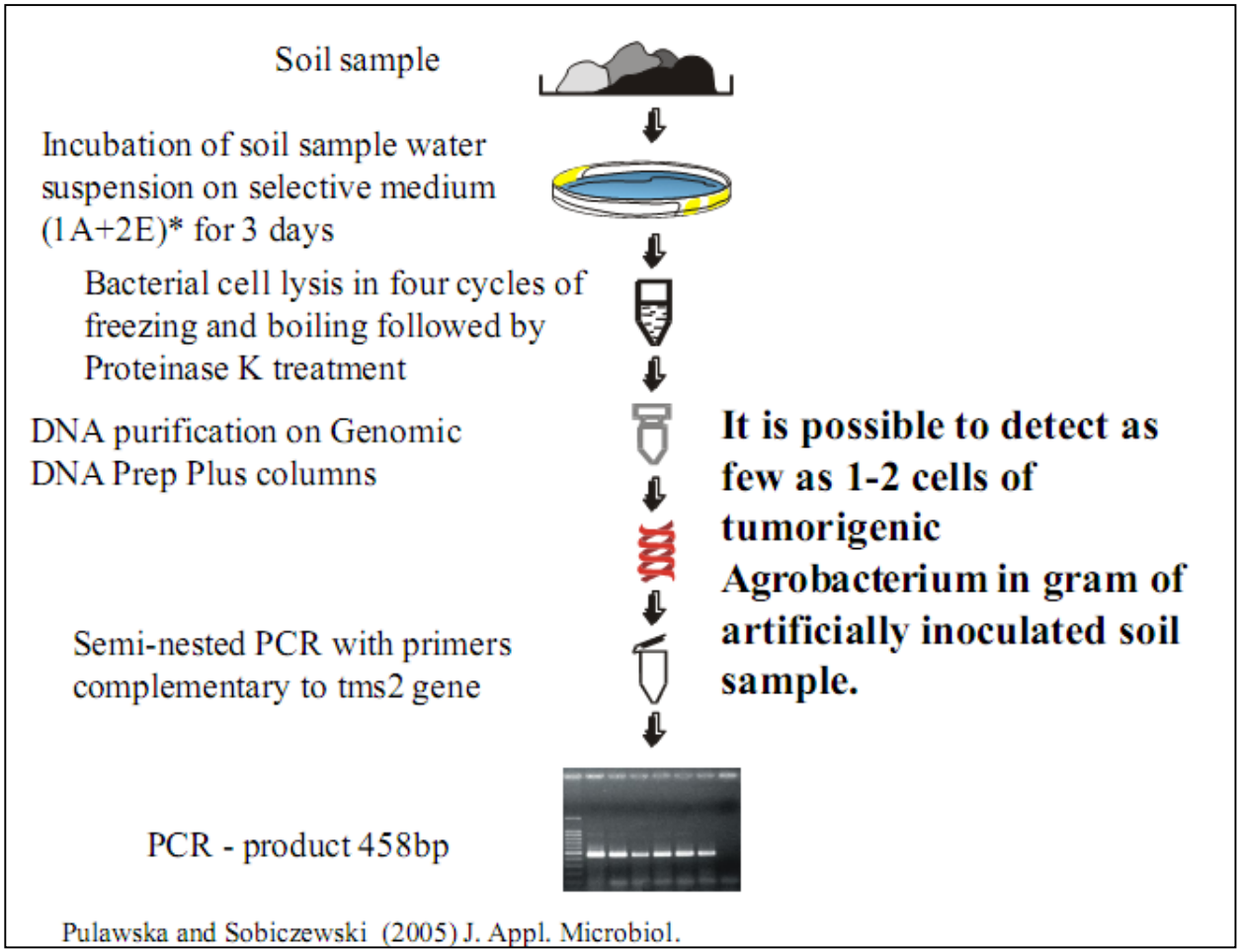
- The *peh*A PCR was performed in a total volume of 25 µl applying the protocol of Eastwell *et al.*, 1995.
- For the PCR reaction, 1 µl of bacterial DNA template was used for PCR amplification in 1× PCR Buffer (Promega), 2.0 mM $MgCl_2$, 0.1 µM each *peh*A primer, 0.2 mM dNTPs, 0.25 U GoTaq Flexi DNA Polymerase.
- pehAF 5'-CGATGGCGGCGAGGATTT-3'
- pehAR 5'-ATCGGGCGTGAAACAAGT-3'
- The thermal cycler was programmed for an initial denaturation at 95°C for 3 min followed by 40 cycles of amplification (95°C for 1 min, 55°C for 1 min, 72°C for 1.5 min) with 5 min of final elongation at 72°C.
- The amplified fragments of 205 bp were visualized on 2% agarose gel.

Note: This primer pair is different from the PGF/PGR primers which also amplify the chromosomal polygalacturonase gene *pehA*.

Lamovšek *et al.*,2014

Standard PCR or semi-nested PCR

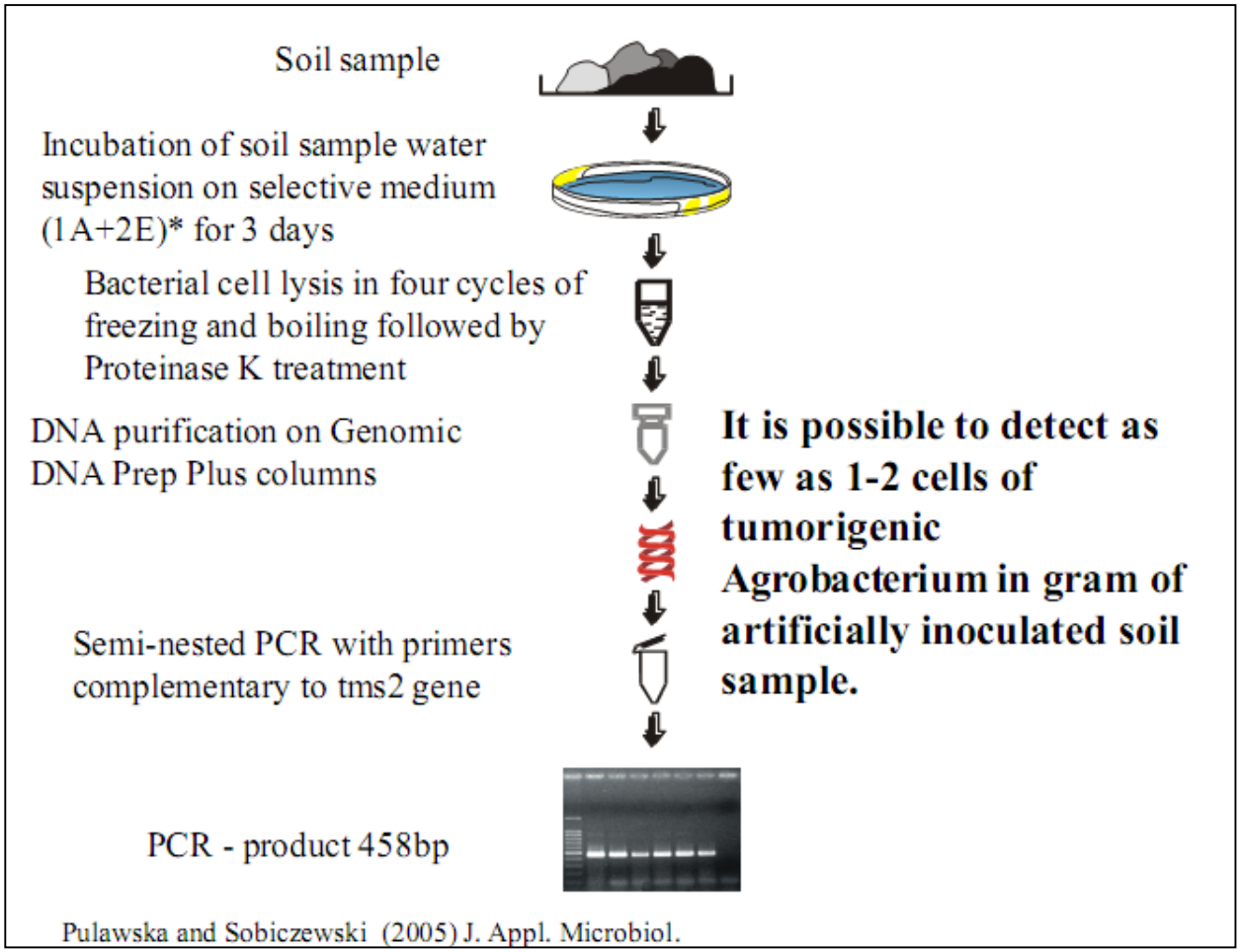
Semi-nested PCR with primers tms2F1/tms2R2/tms2B amplifies target DNA(*tms2* gene)



Puławska and Sobiczewski,2005

Standard PCR or semi-nested PCR

Semi-nested PCR with primers tms2F1/tms2R2/tms2B amplifies target DNA(*tms2* gene)

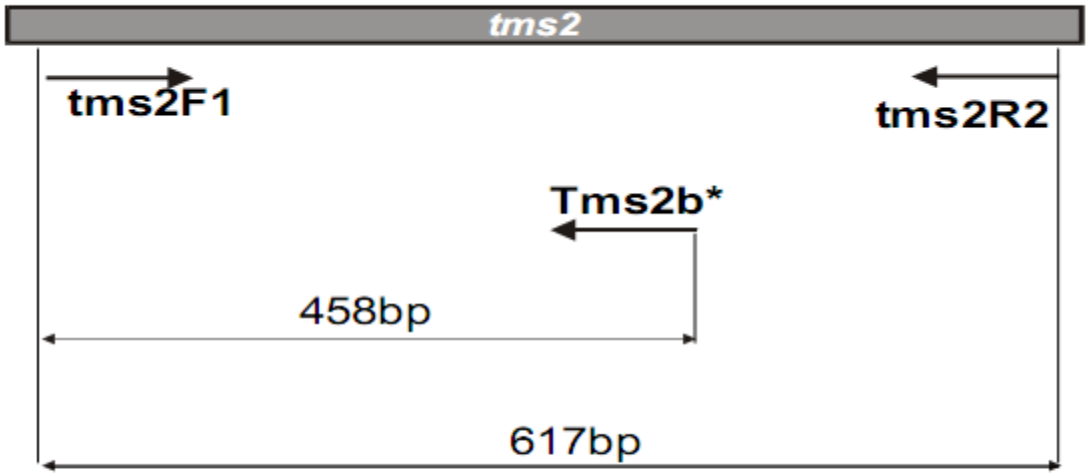


Puławska and Sobiczewski,2005

Standard PCR or semi-nested PCR

Semi-nested PCR with primers tms2F1/tms2R2/tms2B amplifies target DNA(*tms2* gene)

Identification of tumorigenic Agrobacterium strains



out of 118 phytopathogenic and saprophytic bacteria only tumorigenic Agrobacterium strains produced specific PCR products

* Sachadyn & Kur (1997) Acta Microbiol. Polonica

Puławska and Sobiczewski,2005

Standard PCR or semi-nested PCR

Determination of oncogenes through semi-nested PCR method

- Once the presence of plasmid species is identified, semi-nested PCR can be carried out for the rapid determination of tumorigenic agrobacteria in soil.
- Primers:
- tms2F1 (5′-TTTCAGCTGCTAGGGCCACATCAG-3′) and
- tms2R2 (5′-TCGCCATGGAAACGCCGGAGTAGG-3′) can be used in standard PCR or in semi-nested PCR combined with primer tms2B (5′-GGAGCACTGCCGGGTGCCTCGGGA-3′) of Sachadyn and Kur (1997).

Campillo *et al.*,2012;..

Standard PCR or semi-nested PCR

Determination of oncogenes through semi-nested PCR method

- *tms2* gene within the T-DNA fragment was the target for tms2F1 (5′-TTTCAGCTGCTAGGGCCACATCAG-3′), tms2R2 (5′-TCGCCATGGAAACG-CCGGAGTAGG-3′) and tms2B (5′-GGAGCACTGCCGGGTGCCTCGGGA-3′) primers.
- The coupled of tms2F1 and tms2R2 bind to the complementary *tms2* gene sequence and resulted in a 617 bp product during the first round of semi-nested PCR.
- Subsequently, a 458 bp product is generated from the amplification via tms2F1 and tms2B primers.

Campillo *et al.*,2012;Min,2014

Standard PCR or semi-nested PCR

Semi-nested PCR with primers tms2F1/tms2R2/tms2B amplifies target DNA(*tms2* gene)

- Amplification conditions are: PCR buffer (10 mmol l^{-1} Tris-HCl, pH 9.0, 50 mmol l^{-1} KCl, 0.1% Triton X-100) with 1.5 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} of each dNTP, 1 µmol l^{-1} of each primer and 1U of thermostable DNA polymerase (Promega, USA).
- Amplification is done with initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, extension at 72°C for 1.5 min and a final extension step for 10 min.

Campillo *et al.*,2012

PCR detection and identification of tumorigenic agrobacteria

23S rDNA-based multiplex PCR

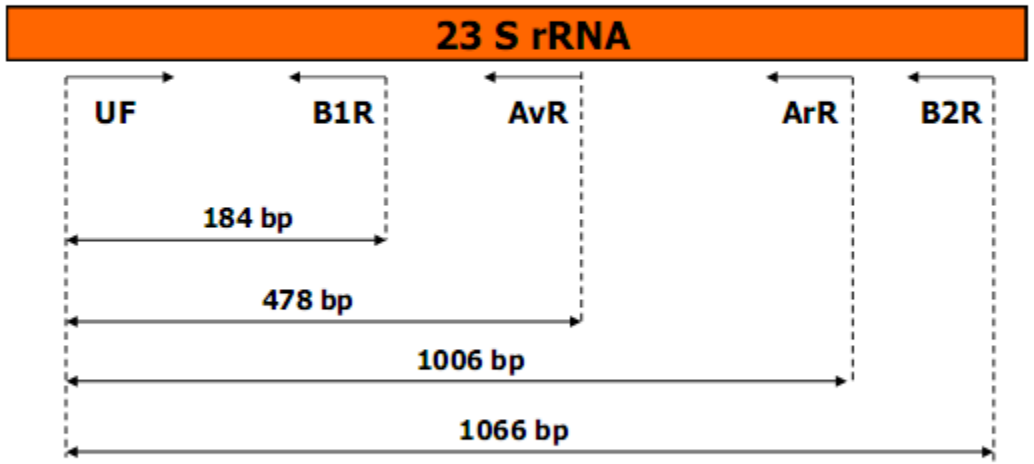
- A multiplex PCR has been designed to aid in identification and differentiation of *A. tumefaciens*, *A. rhizogenes*, *A. rubi* and *A. vitis* (Pulawska *et al.*,2006).
- Five primer pairs were designed on the basis of the nucleotide sequence of the 23S rDNA.
- One of them is universal for all agrobacteria, whereas the remaining four are species-specific.
- Differentiation between biovar 2 and the others requires the use of restriction analysis of the PCR product and the protocol has only been used for strain identification.

López *et al.*,2010

PCR detection and identification of tumorigenic agrobacteria

23S rDNA-based multiplex PCR

Identification of biovar 1, 2 Agrobacterium and A. vitis with multiplex PCR



Pulawska et al. (2006) Syst. Appl. Microbiol.

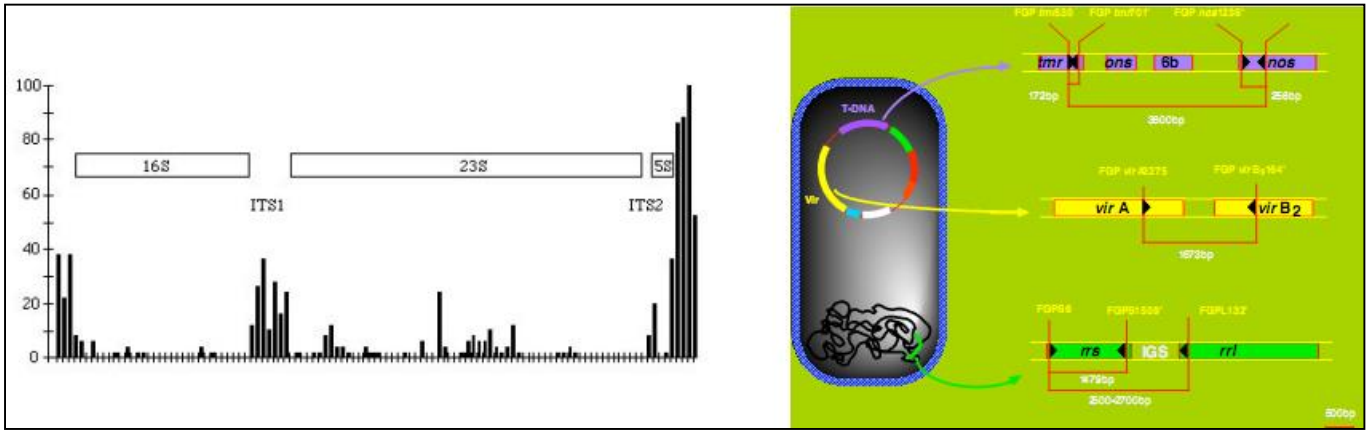
Puławska and Sobiczewski,2005

PCR detection and identification of tumorigenic agrobacteria

PCR primers designed based on chromosome (16S+ITS), or Ti plasmids

- 16S+16S-23S-ITS regions useful at the infraspecific level.
- PCR primers designed to type:

1. Chromosome (16S+ITS), or
2. Ti plasmids (vir or T-DNA conserved regions).



PCR detection and identification of tumorigenic agrobacteria
A multiplex PCR

- The genetic diversity was evaluated for these isolates by comparing DNA samples using multiplex PCR with three specific primer pairs.
- The isolates were segregated into three main groups:

1. The first group that is isolated carry octopine type Ti plasmids;
2. The second group that is isolated carry vitopine Ti plasmids and,
3. The third group that is isolated carry both octopine and vitopine type Ti plasmids.

Tolba and Zaki,2011

Primers sets for identification of plant pathogenic *Agrobacteria* 23S rDNA-based multiplex PCR

- *Agrobacterium* species/biovar specific primers based on 23S rDNA sequences (Pulawska *et al.*,2006).
- This multiplex PCR consists of:

1. primers designed on the 23S rRNA gene sequence, i.e. one universal forward primer and,
2. four taxon-specific (*A. rubi*, *A. vitis* and *Agrobacterium* biovars 1 and 2) reverse primers (Table).

- The primers developed for the identification of *A. vitis*, *A. rubi* or *Agrobacterium* biovar 1 yield amplicons of 478 bp, 1,006 bp and 184 bp, respectively.

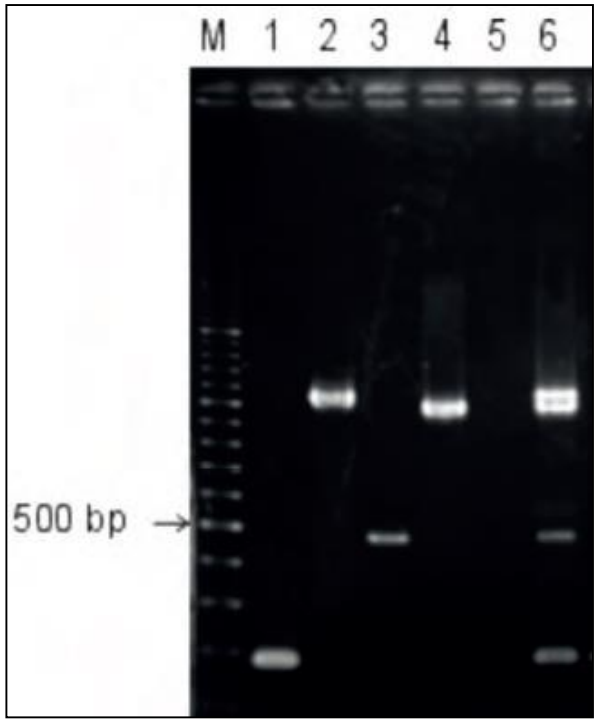
Name of primer	Target position*	Sequence (5' – 3')	Specific for:
UF f	171 – 193	GTAAGAAGCGAACGCAGGGAACT	universal
B1R r	338 – 360	GACAATGACTGTTCTACGCGTAA	biovar 1
B2R r	1207 – 1230	TCCGATACCTCCAGGGCCCCTCACA	biovar 2
AvR r	640 – 662	AACTAACTCAATCGCGCTATTAAC	*A. vitis*
ArR r	1150-1173	AAAACAGCCACTACGACTGTCTT	*A. rubi*

* *E. coli* position numbering
f –forward; r – reverse

Campillo *et al.*,2012

Primers sets for identification of plant pathogenic *Agrobacteria* 23S rDNA-based multiplex PCR

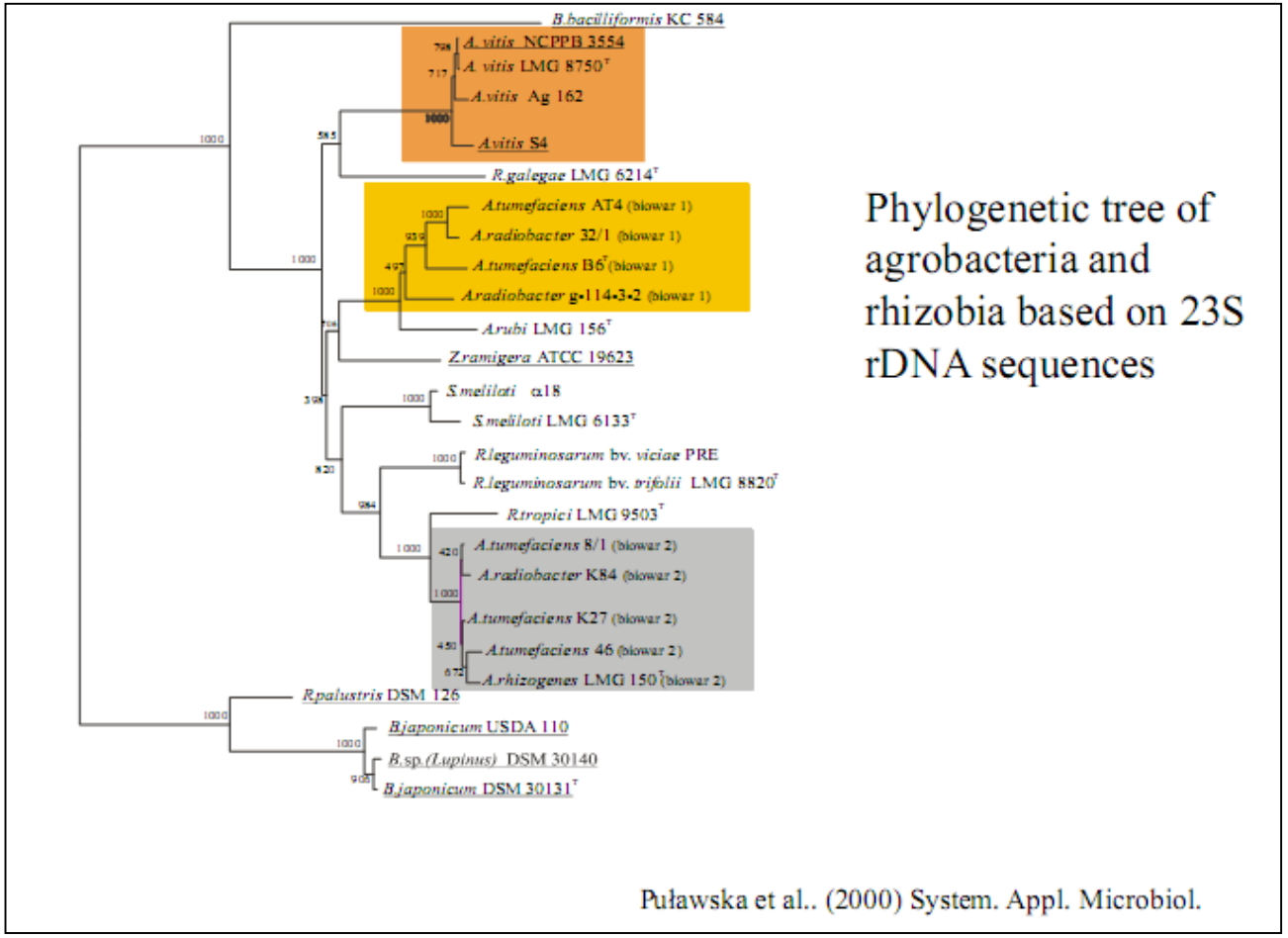
- Electrophoresis gel showing PCR products obtained in a multiplex PCR with DNA of the following *Agrobacterium* strains:
- lane 1, B6 (*A. tumefaciens* (= *A. radiobacter* = biovar 1); lane 2, LMG 150 (*A. rhizogenes* (=bv. 2); lane 3, LMG 8750 (*A. vitis*); lane 4, LMG 156 (*A. rubi*); lane 5, LMG 21410 (*A. larrymoorei*); lane 6, mixture of DNA of strains B6, LMG 150, LMG 8750, LMG 156 and LMG 21410; lane M, molecular weight marker 100 bp ladder (Pulawska *et al.*, 2006).



Campillo *et al.*,2012

PCR detection and identification of tumorigenic agrobacteria

23S rDNA-based multiplex PCR



Puławska and Sobiczewski,2005

Primers sets for identification of plant pathogenic *Agrobacteria* 23S rDNA-based multiplex PCR

- The multiplex PCR was performed in a 15 µl reaction volume applying the protocol of Pulawska *et al.*,2006.
- All reactions were performed in 1× PCR buffer (Promega), 1.5 mM $MgCl_2$, 1 µM each primer (UF, B1R, B2R and AvR), 0.2 mM dNTPs and 1.0 U GoTaq Flexi DNAPolymerase (Promega).
- The amplification conditions comprised an initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 67°C for 1 min, extension at 72°C for 1.5 min and a final extension step at 72°C for 10 min.
- The amplified PCR fragments were visualized on a 2 % agarose gel.
- Strains belonging to *A. tumefaciens* gave a 184 bp product and those belonging to *A. vitis* gave a 478 bp product.

Lamovšek *et al.*,2014

Primers sets for identification of plant pathogenic *Agrobacteria*
23S rDNA-based multiplex PCR

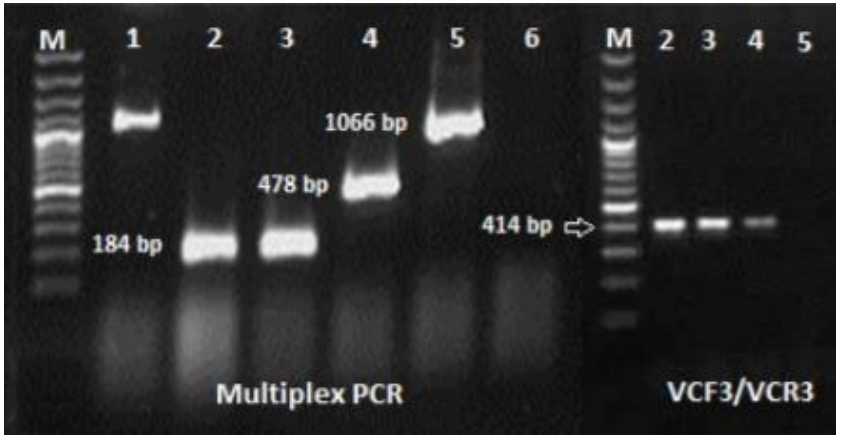
- Primers pair sequences used in our study:
- UF f 5′-GTAAGAAGCGAACGCAGGGAACT-3′
- B1R r 5′-GACAATGACTGTTCTACGCGTAA-3′
- B2R r 5′-TCCGATACCTCCAGGGCCCCTCACA-3′
- AvR r 5′-AACTAACTCAATCGCGCTATTAAC-3′
- VCF3 5′-GGCGGGCGYGCYGAAAGRAARACYT-3′

Lamovšek *et al.*,2014

Primers sets for identification of plant pathogenic *Agrobacteria* 23S rDNA-based multiplex PCR

- Agarose gel electrophoresis of diagnostic fragments from multiplex and VCF3/VCR3 PCR;
- M (ladder),

1. (unknown soil isolate),
2. (grapevine isolate),
3. (C58, A. tumefaciens),
4. (339-26, *A. vitis*),
5. (K84, *R. rhizogenes*),
6. Water(negative control).



Strains belonging to *A. tumefaciens* gave a 184 bp product and those belonging to *A. vitis* gave a 478 bp product. The amplified fragments of 414 bp were visualized on 2% agarose gel.

Lamovšek *et al.*,2014

PCR detection and identification of tumorigenic agrobacteria
A multiplex PCR

- The multiplex PCR was conducted with three different primer pairs that amplify characteristic fragment sizes from genes from *A. vitis*.

1. The PGF/PGR primers amplifying the chromosomal polygalacturonase gene *pehA* (Szegedi and Bottka, 2002) were used to identify *A. vitis* isolates and to distinguish them from *A. tumefaciens* (Table).
2. The opine types of each isolate were determined in parallel using primers designed according to the octopine, nopaline and vitopine synthase gene sequences of *A. vitis*.

- Specific primers VIRFF1/VIRFR2 and VIRD2S4F716/VIRD2S4R1036 designed by Bini *et al.*,2008 on sequences of octopine, nopaline and vitopine synthase genes of *A. vitis* were used.

Tolba and Zaki,2011

PCR detection and identification of tumorigenic agrobacteria
A multiplex PCR

- PCR amplification of the target sequences was performed in a reaction volume of 25 µl containing 1x buffer, 3 mM $MgCl_2$, 200 µM each dNTP, 0.3 µM for primers $VIRFF_1$/$VIRFR_2$ and $VIRD2S4F_{716}$/$VIRD2S4R_{1036}$, 0.4 µM for primers PGF-PGR, 5% DMSO, 0.02 U/µl DNA polymerase (GoTaq Flexi DNA polymerase, PROMEGA) and 5 µl of template DNA prepared in 0.1% Tween-20.
- PCR experiments were performed in a 9700 Perkin–Elmer thermal cycler according to the following conditions:
- pre-denaturation 94°C 1 min; 40 x 94, 60, 72°C 1 min; final extension 72°C 5 min.

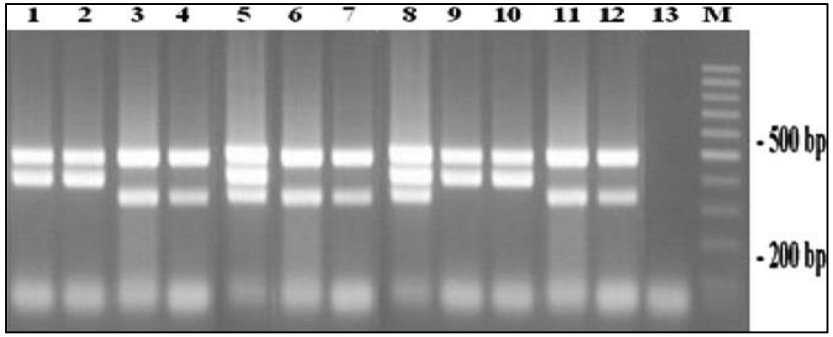
Tolba and Zaki,2011

Multiplex PCR with primer pairs PGF/PGR (466 bp), $VIRFF_1/VIRFR_2$ (382 bp) $VIRD2S4F_{716}/VIRD2S4R_{1036}$ (320 bp) using pure cultures of *A. vitis*

- Lane 1: isolate Av1(octopine); lane 2: isolate Av2 (octopine); lane 3: isolate Av3 (vitopine); lane 4: Av43 (vitopine); lane 5: Av5 (octopine and vitopine); lane 6: Av6 (vitopine); lane 7: Av7 (vitopine); lane 8: Av8 (octopine and vitopine); lane 9: Av9 (octopine); lane10 Av10 (octopine); lane 11 Av11(vitopine); lane12 Av12(vitopine);
- lane 13: H_2O, negative control.
- M: 100 bp ladder, PROMEGA.

Name (forward/reverse)	Primer specificity	Sequence	Length of amplified fragment (bp)
PGF/ PGR	*pehA* Polygalacturonase gene	5′ GGGGCAGGATGCGTTTTTGAG 3′ 5′ GACGGCACTGGGGCTAAGGAT 3′	466
$VIRFF_1/VIRFR_2$	*virF* gene of *A. vitis* octopine and nopaline pTi	5′ ATG AGA AAT TCG AGT TTG CAT GAT G 3′ 5′ TCG TGA TGG GTA TAC GCT ACG 3′	382
$VIRD2S4F_{716}/VIRD2S4R_{1036}$	*virD2* gene of *A. vitis* vitopine pTi	5′ GAC CGC AAA ACC TGC CAG 3′ 5′ GAG CCT GTA TTG ACG ATG TC 3′	320

All primers were synthesized by UCDNA Services, University of Calgary, Albena, Canada.



Tolba and Zaki,2011

Primers sets for identification of plant pathogenic *Agrobacteria*

Based on multilocus sequence typing (MLST) analysis

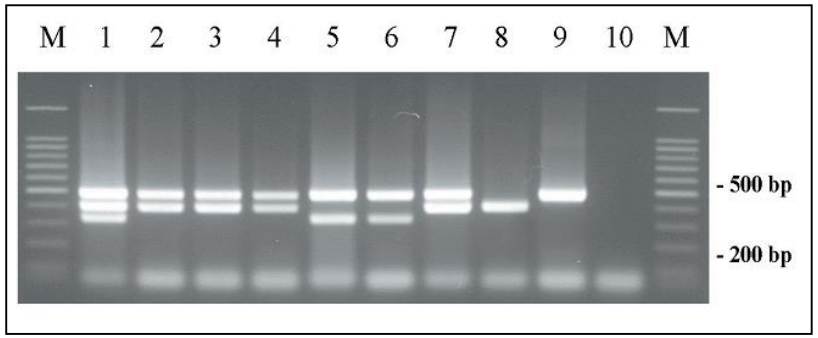
- Reproducible detection of all the tested pathogens in a single reaction was only possible **with multiplex PCR using mixtures of virulence-, or oncogene specific primers**.
- A primer combination including *peh*A, *vir*F and virD2 gene-specific oligonucleotides amplified the corresponding fragments from nearly all strains included and distinguished *A. vitis* and *A. tumefaciens* strains carrying carrying octopine or nopaline pTis and *A. vitis* vitopine strains.

Bini *et al.*,2008

Primers sets for identification of plant pathogenic *Agrobacteria*

Based on multilocus sequence typing (MLST) analysis

- Multiplex PCR with primer pairs $VIRFF_1$/$VIRFR_2$+$VIRD2S4F_{716}$/$VIRD2S4R_{1036}$ (382 bp and 320 bp, respectively), PGF/PGR (466 bp) using pure cultures of *Agrobacterium vitis* and *Agrobacterium tumefaciens*.
- Lane 1: *A. vitis* IPV-BO 5372 (octopine and vitopine); lane 2: *A. vitis* Tm4 (octopine); lane 3: *A. vitis AB4* (nopaline); lane 4 : *A. vitis* CG49 (nopaline); lane 5: *A. vitis* IPV-BO 5159 (vitopine); lane 6: *A. vitis* FC/14 (vitopine); lane 7: *A. vitis* CG102 (octopine); lane 8: *A. tumefaciens* Ach5 (octopine); lane 9 : *A. vitis* F2/5 (non tumorigenic); lane 10: H_2O, negative control.
- M: 100 bp ladder, PROMEGA.



Bini *et al.*,2008

Genetic diversity in *Agrobacterium* species populations

Based on multilocus sequence typing (MLST) analysis

- MLST or multilocus sequence-based analysis (MLSA) is a technique in molecular biology for the typing of multiple loci.
- Usually seven housekeeping genes were used for gene amplification and sequencing.
- Seven genes encoding housekeeping proteins involved in:
- Transcription (*rpoB*),
- Stress response (*dnaK and groEL*),
- Amino acid biosynthesis (*glnA and trpE*), and
- Energy metabolism (*atpD* and *zwf*) were selected from the complete genome sequence of *A. tumefaciens* C58.

Aujoulat *et al.*,2011

Quorum sensing

PCR analysis of *Agrobacterium vitis* DNAs with avsI-F2/avsI-R2 specific primers responsible for long-chain AHL production in *A. vitis*

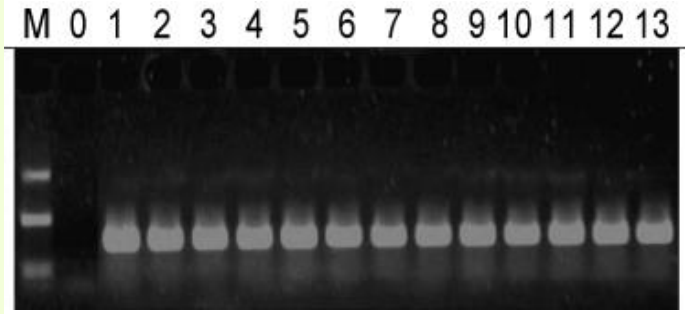
- To determine if an *avsI* homolog, responsible for long-chain AHL production in *A. vitis* strain F2/5, is present in the *A. vitis* strains, avsI-specific primers were used with total DNA of various *A. vitis* strains in PCR assays.
- DNA was prepared from overnight bacterial cultures using the Triton/sodium-azide lysis method (Abolmaaty *et al.*,2000).
- Primers avsI-F2 (5'-AGCCGACATAAGCAGACGCAACAG-3') and
- avsI-R2 (5'-CGAAACATCCGCTCCAAAAACAC-3') designed on the basis of *A. vitis* S4 sequence data (Hao & Burr, 2006) were used for experiments.
- These oligos yield a 447-bp amplification product.

Lowe *et al.*,2009

Quorum sensing

PCR analysis of *Agrobacterium vitis* DNAs with avsI-F2/avsI-R2 specific primers responsible for long-chain AHL production in *A. vitis*

- The predicted *avsI* homologous amplicon of 447 bp was detected in all octopine-, nopaline-, and vitopine-type strains analyzed.
- This is consistent with the detection of a long-chain AHL spot(s) with biosensor NTL4 after TLC separation.
- All tested strains of *A. vitis* produced the expected amplicon of the *avsI* gene region.
- M, size markers (1794, 753 and 191 bp),
- 0, DNA-free control
- Lanes 1–13 are *A. vitis* AT6, Tm4, AB3, Zw2, AT1, AB4, Ni1, Rr4, S4, Sz1, NW221, SF93 and F2/5 in the same order.
- *Agrobacterium vitis* S4 (lane 9) and F2/5 (lane 13) strains were used as controls (Hao & Burr, 2006).



Lowe *et al.*,2009

Genetic diversity in *Agrobacterium* species populations

Based on multilocus sequence typing (MLST) analysis

Primers used for gene amplification and sequencing

Locus	Function	Putative gene product	Locus position[a]	Gene size (bp)	Primers[b]	Primer sequence 5′–3′	Sequence length (bp)
atpD	Energy metabolism	F_0-F_1 ATP synthase subunit beta	2604717	1,454	**800F**	GGCCAGGACGTTCTGTTCTT	465
					1350R	CTTGAAGCCCTTGATCGTGT	
dnaK	Stress response	Heat shock protein, 70 kDa	126205	1,901	**720F**	GAAGACTTCGACATGCGTCT	480
					1400R	GCCGAGCAGCTTGTTGTC	
glnA	Amino acid biosynthesis	Glutamine synthetase	196923	1,358	**144F**	GTCATGTTCGACGGCTCCT	474
					1340R	CGCATGACTTCCTGCATCT	
					900R	CCTTGGCATGCTTGATGAT	
groEL	Stress response	Heat shock protein, 60 kDa	676328	1,634	**100F**	GTGGTGATCAGCAGCGAAG	504
					1240R	AGGCCAAGGCCAAGAAGAT	
					760R	CTGGAAGACATCGCCATCCT	
rpoB	Transcription	Beta subunit RNA polymerase	1927198	4,136	**2040F**	GAAAACGACGACGCCAAC	534
					3150R	TGGACCTTTTCGACCTTGTC	
					2718R	GCGCAGAAGCTTTTCTTCC	
trpE	Amino acid biosynthesis	Anthranilate synthase	2262145	2,189	**890F**	CGCCCTATTCCTTCTTCATC	510
					2090R	ATCGATTCCGGGTGGAACT	
					1630R	GAAATAATTCGCCAGCGTGT	
zwf	Pentose phosphate pathway	Glucose-6-phosphate 1-dehydrogenase	585849	1,475	**530F**	AGATCTTCCGCATCGACCA	384[c]
					950R	CTTGATGGCGACGAAGGTT	

[a] Gene start codon position on the circular chromosome sequence of *A. tumefaciens* C58 (accession number AE007869).
[b] F, forward primer; R, reverse primer. Primers in boldface were used for gene sequencing in both directions. The primer denominations correspond to their hybridization regions in the gene according to the complete genome sequence of *A. tumefaciens* C58.
[c] The size of the *zwf* sequences of the two strains of *A. vitis* was 381 bp.

Aujoulat *et al.*,2011

Host test

For *Agrobacterium tumefaciens*

- All the *Agrobacterium* isolates was confirmed by the pathogenicity test i.e., hairy from different root formation. To perform this:

1. Use the original host where this is possible.
2. If this impractical use several hosts.
3. Tomato, marigold, sunflower, *Kalanchoe* sp. and chrysanthemum are useful and easily cultivated.
4. Inoculate either by stabbing stems or leaves with a needle through a droplet of bacterial suspensions containing ca. 10^8 cells/ml.
5. Gall usually develop within 2 weeks.
6. Observe for gall symptoms for upto 28 days.

Plant Bacteriology Manual,2008;..

Pathogenicity test

- Agrobacteria do produce tumors (or hairy roots) when inoculated onto test plants.
- The test plants most often used are:

1. Tomato,
2. Sunflower,
3. *Datura* spp.,
4. *Kalanchoë daigremontiana* (also called *Bryophyllum*),
5. Tobacco, and
6. *Nicotiana glauca.*

- These plants respond relatively readily and rapidly to inoculation of *Agrobacterium* strains by producing tumors in as few as 10 days.

Pathogenicity test

Crown gall disease of tobacco

Tumorigenicity tests on carrot disc and squash fruits

- The pathogenicity of the isolates was evaluated on:

1. carrot (*Daucas carota* L.) disc, and
2. squash (*Cucurbita pepo* var. *italica* L.) fruits.

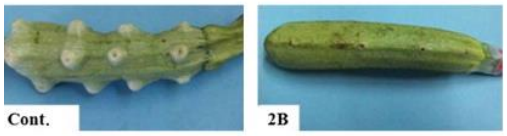
- The tested isolates grown on NA at 28°C for 48 h were suspended in SDW to a final concentration of 10^7 CFU mL^{-1}.
- SDW served as a negative control treatment.
- The carrot and squash fruits were washed, dried, and then disinfected with 70% ethanol before being artificially inoculated in line with the methods described by Ryder *et al.*,1985 and Tolba and Soliman,2013, respectively.
- The inoculated samples were stored in plastic boxes on filter paper at 25°C for three weeks. Tumor formation was considered a positive reaction.

Iličić *et al.*,2013

Pathogenicity test

Tumorigenicity tests on squash fruits by Tolba and Soliman,2013 method

- Uniform squash fruits were stabbed by toothpick to make holes at 12 sites distributed over three rows (four sites/row) per fruit.
- Into each hole, 2 µl of *A. tumefaciens* cell suspension or saline solution (negative control) was pipetted.
- After the suspension was absorbed into the wound, the treated fruits were maintained in plastic containers with transparent plastic covers and kept at 27 ± 2°C in growth chamber until galls measuring were assessed.
- Number and size of formed galls were recorded after 10 days.



Positive control (left) against negative control (right).

Pathogenicity test

Crown gall disease of tobacco

Tumorigenicity tests on tobacco plant

- Selected tobacco isolates were also tested for pathogenicity to *N. tabacum* hybrid PVH2310.
- A bacterial suspension (at a final concentration of 10^8 CFU mL^{-1}) was prepared from cultures grown on NA at 28°C for 48 h.
- The stems of 1-month-old tobacco plants grown in an air-conditioned greenhouse at a constant temperature of 28°C were inoculated by the needle prick method.
- Three plants were inoculated with each strain, and the experiment was repeated twice.
- Upon symptom development, pathogenicity was evaluated based on tumor formation at the inoculation site.
- Reisolations were performed on D1 medium as soon as symptoms were observed.

Iličić *et al.*,2013

Pathogenicity test

Crown gall disease of tobacco

Tumorigenicity tests on carrot disc, squash fruits and tobacco tree

- Pathogenicity of *A. tumefaciens* isolate DA21 on:

a. carrot discs;

b. squash fruit;

c. tobacco plant.

1. Positive results on the carrot discs and squash fruits were observed 12 to 16 days after inoculation.
2. Symptoms associated with crown gall of tobacco plants, emerged 21 to 25 days after inoculation.

Iličić *et al.*,2013

Pathogenicity test

Agroinfiltration of *Nicotiana benthamiana*

Agroinfiltration is a method in plant biology to induce transient expression of genes in a plant or to produce a desired protein

- A single piece of leave was infiltrated with four components at different spot, an *Agrobacterium* isolate (S1D1), Green Fluorescence Protein (GFP), *Agrobacterium tumefaciens* C58 (positive control), A1 buffer without inoculum (negative control) were also included.
- The infiltration was done by using syringe without needle.
- The samples were injected to approximately 4 cm^2 at the abaxial site of leaves.
- Fingers were placed at the opposite surface of injection spot in order to exert some pressure that enhances the efficiency of infiltration.
- The infiltrated leaves were observed for bulge at 4 days after agroinfiltration (Wydro,2006).

Min,2014

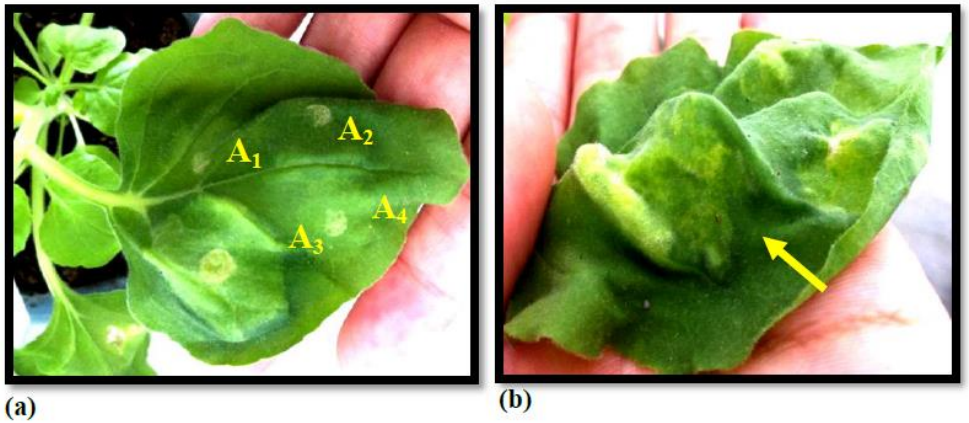
Pathogenicity test

Agroinfiltration of *Nicotiana benthamiana*

Agroinfiltration is a method in plant biology to induce transient expression of genes in a plant or to produce a desired protein

a) Tobacco leave was divided into four injection sites. A_1=S1D1, A_2=GFP, A_3=Positive control *A. tumefaciens* C58), A_4=Negative control (A1 buffer with no inoculum)

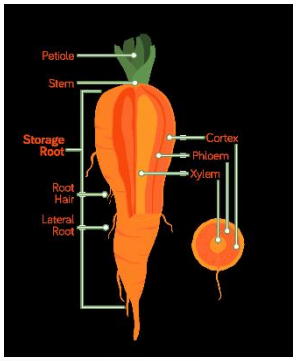
- *A. tumefaciens* C58 caused abnormal cell proliferation depending on its original injection site (arrow).

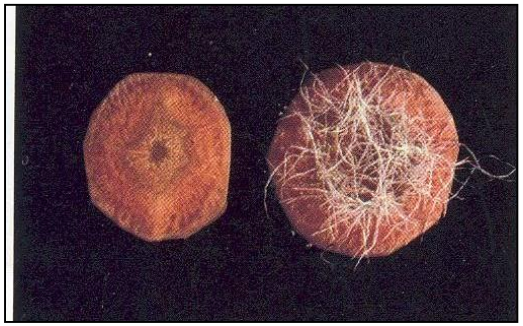


Min,2014

Pathogenicity tests

Carrot disc and tomato tests

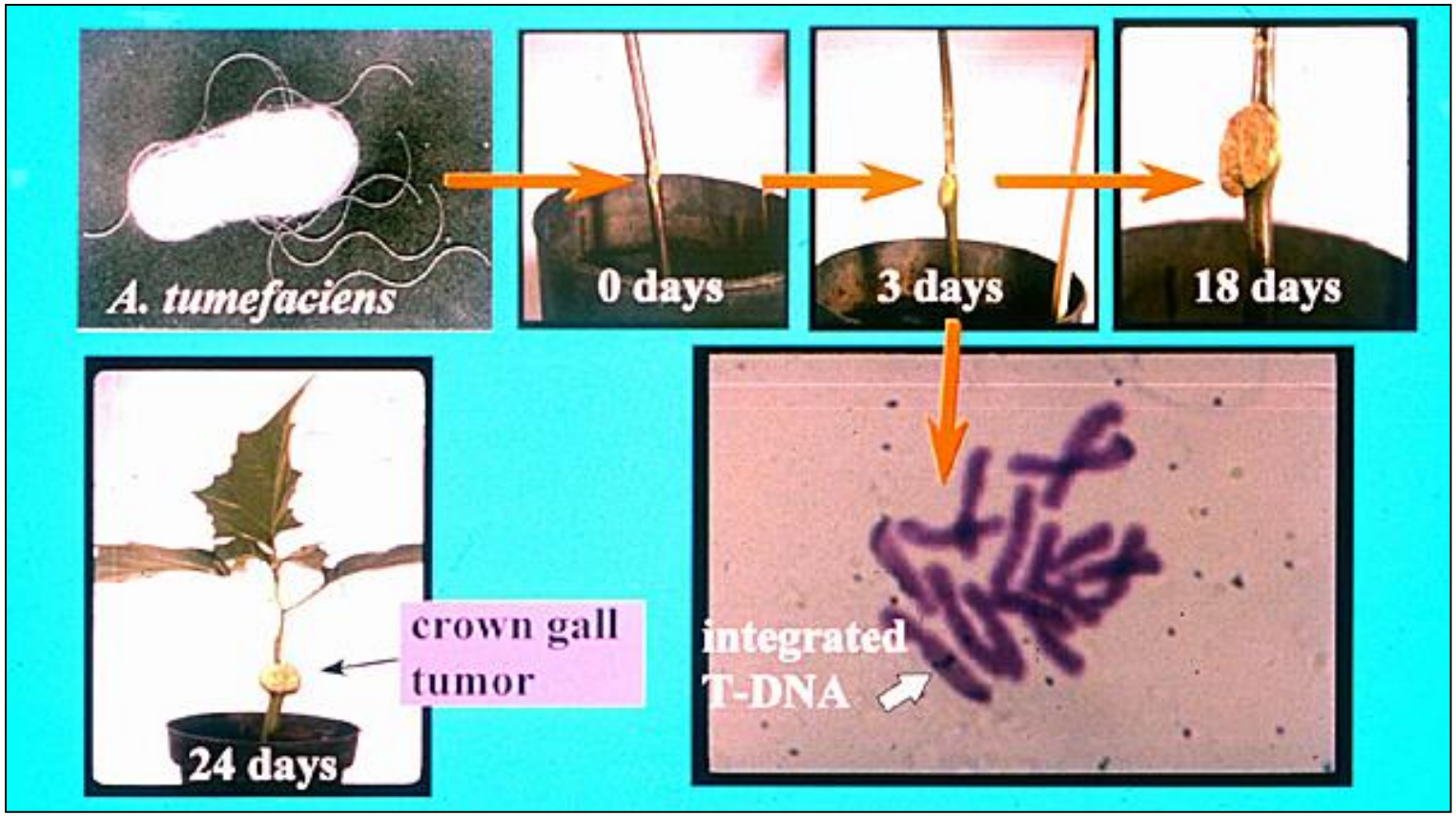


		
Gall induced on tomato stem by *A. tumefaciens*	**Hairy roots induced on carrot root disc by *R. rhizogenes***	**Gall formation on a carrot slice inoculated with *A. tumefaciens***

Alsup,2004;...

Pathogenicity test

Development of crown gall *Datura stramonium* (Jimson weed) experimentally inoculated with *A. tumefaciens* after 2-3 weeks



Kado,2002

Pathogenicity test
Tobacco plant

- The stem of a tobacco plant wounded at two places and inoculated with *A. tumefaciens*.
- The tumors are shown at 6 weeks after inoculation.

The Prokaryotes (chapter 3.1.4),2006

Pathogenicity test on

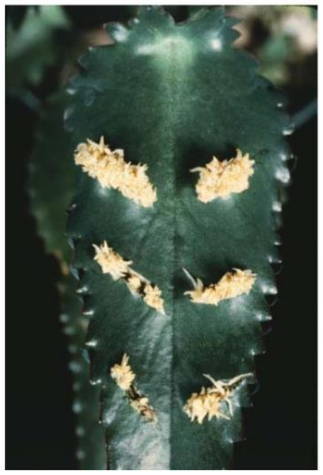
Bryophyllum daigremontiana or mother of thousands

1. A leaf of *Bryophyllum daigremontiana* inoculated with *A. tumefaciens*.

- The site on the back right was inoculated with a strain lacking the Ti plasmid.
- Tumors are shown after 4 weeks growth.

2. A leaf of *Bryophyllum daigremontiana* inoculated with *A. rhizogenes*.

- Note that the roots formed at the wound sites are branching and ageotropic.
- The leaf is shown 5 weeks after inoculation.



Pathogenicity test

With mixed cultures of *A. rhizogenes* and *Rhizobium* sp. and inhibition of *A. rhizogenes*

- Pathogenicity test was conducted by seed inoculation technique.
- When *Agrobacterium* alone was inoculated into the host species, there was the formation of proliferate and multi-branched secondary roots from all species tested.
- On the other hand, when *Agrobacterium* along with *Rhizobium* were inoculated, there were not much branched secondary roots in any of the host species tested, instead the formation of nodules was observed.
- It was noted when *Rhizobium* present along with *Agrobacterium*, it inhibits the formation of hairy roots in the host species.

Murugesan *et al.*,2010

Pathogenicity test

On a stem of a tomato plant and sunflower seedlings

- Virulence assays (adapted from Watson *et al.*, 1975) can be conducted on stems of 3-week-old tomato plants or 7-day-old sunflower seedlings.
- Suspension of agrobacteria (OD_{600}nm 0.1 in H_2O) are prepared from overnight cultures in rich growth media (LB or YPG broth).
- Each tomato or sunflower stem is incised (length = 4 cm on tomato and 1 cm on sunflower; depth = 1 mm) with a scalpel or a sterile needle and 20 µl (*ca.* 1×10^6 CFU) are deposited into the scarification.
- On sunflower, incisions are made on the basal part of the stem so as to cover the inoculated wound with soil.
- Infected plants are incubated in a climatized greenhouse at 20°C with a night/day photoperiod of 16h/8h and 75% relative humidity.
- Number and size of tumors per incision are counted up to 28 days postinoculation.

Campillo *et al.*,2012

Pathogenicity test

On a stem of a tomato plant

- Crown gall formation on a stem of a tomato plant infected with wild-type *A. tumefaciens*.

Storage of Agrobacterium strains

- Bacterial isolates can be stored as a mixture of glycerol-liquid culture (*ca.* 10^{10} CFU.ml^{-1}) (1:4 volume rate) at -78 to -82°C.
- If possible, a backup stock should be stored in a separate freezer.
- Periodic check on viability, contamination and pathogenicity is advisable.

Campillo *et al.*,2012

Identification of the bacterial pathogens *Xylophilus*

Disease diagnosis and pathogen diagnostics

Domain: Bacteria
Phylum: Proteobacteria

- Class: Betaproteobacteria
  - Order: Burkholderiales
    - Family: Burkholderiaceae
      - Genus: *Burkholderia*
    - Family: Comamonadaceae
      - Genus: *Acidovorax*
    - Family: *Ralstoniaceae*
      - Genus: *Ralstonia*
    - Family: ----------
      - Genus: *Xylophilus*

J.P. Euzéby,2010

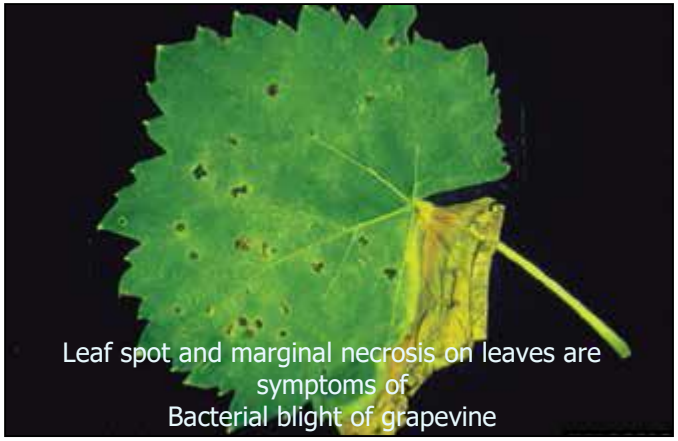
The geuns *Xylophilus*

- *Xylophilus* is a genus of slow-growing, yellow pigmented bacteria belonging to the family Comamonadaceae in the β subclass of the Proteobacteria.
- It comprises a single species, *Xylophilus ampelinus* (synonym: *Xanthomonas ampelina*) a phytopathogenic bacterium that, so far, has been isolated in different parts of the world only from diseased grapevines affected with bacterial necrosis and canker.

Willems *et al.*,1987. Transfer of *Xanthomonas ampelina* Panagopoulos 1969 to a new genus, *Xylophilus* gen. nov., as *Xylophilus ampelinus* (Panagopoulos 1969) comb. nov. *Int. J. Syst. Bacteriol.*, 37, 422-430.

Bacterial blight of grapevine

Xylophilus ampelinus



Leaf spot and marginal necrosis on leaves are symptoms of Bacterial blight of grapevine

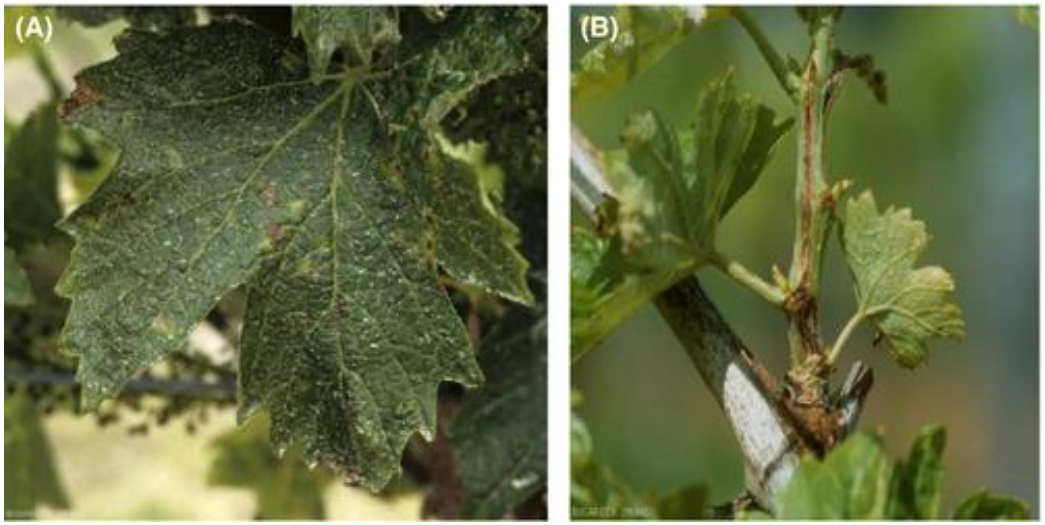
Cup shaped leaves are a symptom of Bacterial blight of grapevine

Necrotic canker on developing grapevine shoot

Cankers will darken, crack and spread throughout the grapevine stem

Bacterial blight of grapevine
Xylophilus ampelinus

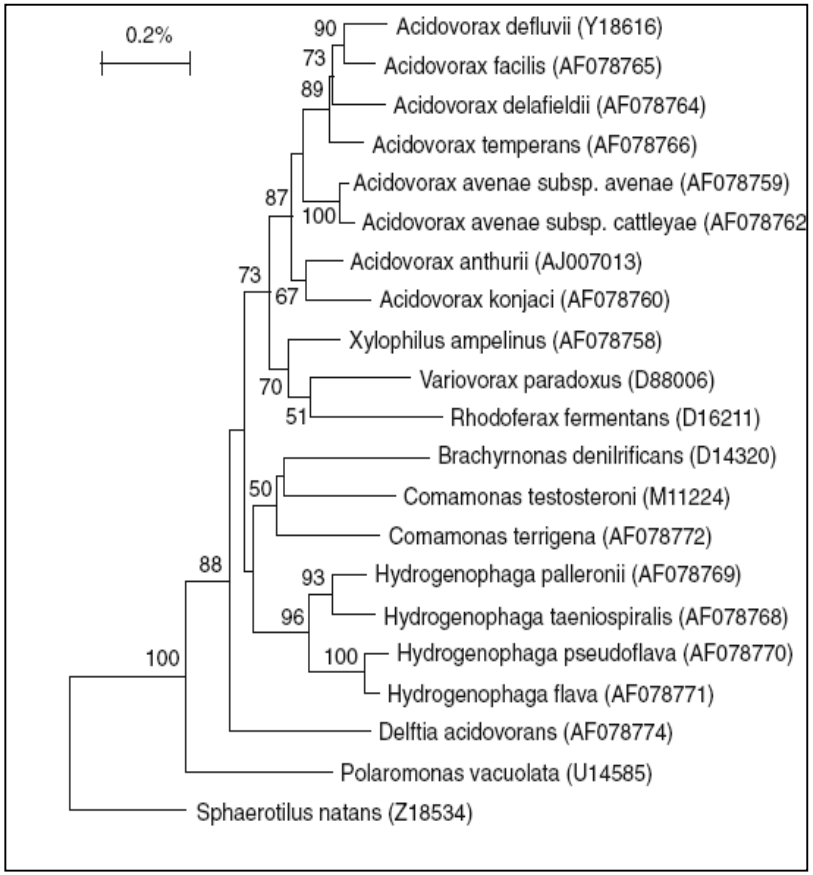
- Symptoms of *Xylophilus ampelinus* on grapevine showing (A) symptoms on leaf and (B) symptoms on shoots.
- Photo: INRA ephyitia, http://ephytia.inra.fr.



EPPO,2018

Taxonomy
Xylophilus ampelinus

- Dendrogram obtained by neighbor-joining analysis of a distance matrix of 16S rDNA sequences, showing the position of *Xylophilus* among other members of the Comamonadaceae.
- Accession numbers are given in parentheses.



The Prokaryotes (chapter 3.3.24),2006

Habitat

- *Xylophilus ampelinus*, formerly *Bacillus vitovorus* Bacc. and *Xanthomonas ampelina* is responsible for the bacterial necrosis of grapevine called "maladie d'Oléron".
- This disease is responsible for progressive destruction of grape vine (*Vitis vinifera*) shoots, leading to their death.
- *X. ampelinus* causes necrosis and canker disease of grapevines only.
- No really resistant cultivars have been detected so far.
- The capability of *X. ampelinus* to survive for several years inside plants without inducing symptom development may result in a latency period which depends on many factors including climatic conditions.

Grall and Manceau,2003

Isolation
Nutrient agar (NA)

- Strains can be isolated throughout the year (Panagopoulos, 1969), although isolation from infected material collected in hot and dry periods may be difficult.
- The most common isolation sources are small pieces of infected wood, taken aseptically from diseased vines and soaked for 20 minutes in sterile water.
- The resulting bacterial suspension is plated out onto nutrient agar.
- *X. ampelinus* grows poorly on YDC or NBY but much better on NA.
- After 5 to 6 days, small pale-yellow colonies will appear which, after 8 to 10 days, can attain a diameter of 0.4 to 0.6 mm.

Isolation

Solid (YPGA) and in liquid (YPG) media

- *X. ampelinus* strains were routinely grown at 25°C on YPGA medium (7 g of yeast extract, 7 g of Bacto-Peptone, 7 g of glucose, and 7 g of agar per liter [pH 7.2]).
- Bacterial growth was monitored on solid (YPGA) and in liquid (YPG) media supplemented with increasing kanamycin concentrations (from 0 to 120 mg per liter).
- Although *X. ampelinus* is yellow but it is easily differentiated from Xanthomonads:

1. 10-12 days inoculation are required for colonies of *X. ampelinus* to reach 1 mm diameter.
2. No mucoid colonies on GYCA medium.
3. *X. ampelinus* has distinctive electronic absorption and chemical properties of the pigments, suggesting that this species does not belong in the genus *Xanthomonas* (Starr *et al.*,1997).

Characteristics of the genus *Xylophilus*

Characteristics

- The bacterium is:
- Gram negative, rod shaped.
- Aerobic.
- On medium containing 1% yeast extract, 2% D-galactose,2% $CaCO_3$ and 2% agar the colonies are yellow, and a brown diffusible pigment is produced(Holt *et al.*,1994).
- Urease positive (all xanthomonads are Urease negative).
- It produce acid from arabinose and galactose but not from flucose or maltose.
- Esculin, starch, casein, arbutin (a glucoside, carbon source), Tween 80, nitrate and gelatin are negative.
- Oxidase negative.
- Potato soft rot test is negative.
- Produce H_2S from cysteine.
- Growth is very slow in the presence of 0.01-0.02% TTC.

Characteristics of the genus *Xylophilus* Biochemical and protein profiles

- *Xylophilus ampelinus* strains have a strictly chemoorganotrophic metabolism.
- They use only a limited number of carbohydrates, organic acids and amino acids for growth.
- Of a total of 60 substrates tested, growth was recorded only on D-glucose, D-galactose, L-glutamic acid, Nasuccinate, Na-fumarate, K, Na-tartrate, Na-Lmalate, Na3-citrate and Ca-gluconate.
- *Xylophilus* strains generally show very little variation.
- Even isolates originating from different geographical regions showed highly similar whole-cell protein SDS-PAGE patterns and little variation in enzymatic features, as tested with API ZYM systems (Willems *et al.*,1987).

The Prokaryotes (chapter 3.3.24),2006

Differentiation of plant pathogenic *Acidovorax* and *Xylophilus* from other genera of *Comomonadaceae*

Characteristics of the genus *Xylophilus*

Characteristic	*Acidovorax*	*Xylophilus*	*Comomonas*	*Hydrogenophaga*	*Variovorax*
Cell morphology	Rods	Rods	Rods to spirilla	Rods	Rods
Flagella	Polar, 1 to 2	Polar, 1	Bipolar,1 to 5	Polar, 1	peritrichous
Yellow insoluble pigment on nutrient agar	-	+	-	+	+
Growth at 41°C	+	-	+	+	+
Carbon sources utilized for growth:					
Adonitol or L-arabitol	-	-	-	-	+
D-glucose	+	-	-	+	+
Glycolate	-	ND	$+^D$	+	+
L-mandelate	-	ND	$+^D$	+	$+^D$
L-tyrosine	+	ND	$+^D$	$+^D$	-
Isolated from infected plant	+	+	-	-	-
Hypersensitive reaction	+	ND	-	-	-

+, 80% or more strains positive; $+^D$, 80% or more strains positive but delayed; -, 80% or more strains negative; ND, not determined.

Schaad *et al.*,2001

Biochemical characteristics for *X. ampelinus*

Characteristics	Results
Gram's reaction	–
Catalase test	+
Kovac's oxidase	–
Urease production	+
H_2S production from cysteine	–
Utilization as carbon sources:	
Citrate	+
Fumarate	+
Malate	+
DL-tartrate	+
Acid production from:	
L-arabinose	+
D-galactose	+
Glucose	–
Sucrose	–
Lactose	–
Utilization of asparagine as the sole source of carbon and nitrogen	+

EPPO Bulletin,2009

Diagnostic method
Immunofluorescent staining

- Serological tests with specific antisera are used for rapid identification of the pathogen.
- **Preparation of antiserum**
- A polyclonal antiserum against *X. ampelinus* was raised in rabbits as follows:
- Three subcutaneous injections of 1.2 ml of a suspension of living bacteria (approximately 10^8 cells/ml) mixed with 1.2 ml of Freund's incomplete adjuvant were performed at 5-day intervals.
- When the titer was high enough (1/1,500), the rabbits were bled.
- The antiserum was mixed with 50% glycerol and stored at -20°C.
- Its specificity was then tested against a large collection of plant-associated bacteria and taxonomically related bacteria.
- The antiserum was specific and reacted with all the *X. ampelinus* strains tested.

Diagnostic method
Indirect immunofluorescence

- **Detection of bacteria**
- Bacteria were detected by immunofluorescent staining.
- The anti-*X. ampelinus* polyclonal serum was used as a primary antibody for indirect immunofluorescence analysis.
- A goat anti-rabbit immunoglobulin G-fluorescein isothiocyanate conjugate was used as a secondary antibody.
- Microscopic observations were done with an Olympus BH2 microscope under UV light with a 455-nm filter (filter EY455) at a magnification of ×1,000.

Diagnostic method
Polymerase chain reaction (PCR)

- A polymerase chain reaction (PCR) identification test using the specificity of the 16S-23S rDNA spacer sequence was reported (Serfontein *et al.*,1997) but not published in detail.
- The EMBL accession number for the spacer sequence is U76357.

The Prokaryotes (chapter 3.3.24),2006

Diagnostic method

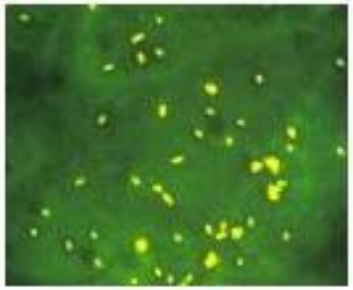
Detection techniques

- Visual inspection

- growth on agar medium

- ELISA

- immunofluorescence



- PCR test

Manceau and Grall,2004

Pathogenicity test
Plant growth conditions

- *V. vinifera* cv. Ugni blanc was used because of its high susceptibility to bacterial necrosis.
- Woody vine shoots were collected each year in January, dipped into water containing 1% Cryptonol for 20 min, dried at room temperature, and stored at 4°C until use.
- One-node cuttings were made, top-covered with wax, and planted in humid sand.
- They were grown at 28 or 24°C under saturated humidity with 16 h of light and 8 h of darkness per day for 2 to 3 weeks.
- When the first leaves emerged, the plantlets were transplanted into individual pots containing a substratum made either of 33.3% compost, 33.3% sand, and 33.3% peat or 50% Irish peat and 50% perlite complemented with 1.75 g of $CaCO_3$ per liter.
- The plants were grown under the environmental conditions required for the experiments.

Pathogenicity test

1. Inoculation by spraying

- Bacterial suspensions of *X. ampelinus* (2.6×10^8 cells/ml) were sprayed onto young plants with seven or eight leaves until runoff occurred.
- Sterile distilled water was sprayed on control plants.
- Throughout the experiments, the plants were grown in a growth chamber with 95% relative humidity at 24°C for 16 h of light and at 18°C for 8 h of darkness.
- Typical leaf spots appeared 10 days after spraying.
- They were first white and then became progressively brown, polygonal, and circled by a yellow halo. The leaf spots spread throughout the foliar parenchyma.
- Typical leaf spots were counted 21 days after spraying.

Pathogenicity test

2. Inoculation by wounding the stem

Stem-cutting method

- Plants of approximately eight foliar stages were used.
- The stems were cut in the middle of the sixth or seventh node, and a 6-µl drop of bacterial suspensions (10^8 cells/ml) was applied to the fresh sections.
- Control plants were inoculated with sterile distilled water.
- The test was conducted in a culture chamber under the conditions required for symptoms development (saturated humidity, 24°C, 16 h of light and 8 h of darkness).
- The recorded number of leaf spots was significantly larger on the plants grown from water-infiltrated cuttings than on those grown from cuttings infiltrated with an *X. ampelinus* suspension.

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

Genus *Xylophilus*

Genus *Xylophylus*					
Species	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
X. ampelinus	**Xamp 1.27A/Xamp 1.27B** **Xamp 1.27A/Xamp 1.27C** **Xamp 1.3A/Xamp 1.3B** Subtractive hybridization	Conventional	Bacteria (DNA extraction)	Manceau *et al.*, 2000	
X. ampelinus	**A1/B1** **S3/S4** **A1/B1** (external primers) **S3/S4** (internal primers) ITS region	Conventional Nested	Bacteria and stem sap (DNA extraction)	Botha *et al.*, 2001	
X. ampelinus	**XATS1/XATS2-Biotin** ITS region	PCR-ELISA	Bacteria (boiled) and bleeding sap (DNA extraction)	Grall *et al.*, 2005	
X. ampelinus	**Xamp 1.27A/Xamp 1.27C** Subtractive hybridization	Conventional	Plant, sap (DNA extraction)	Manceau *et al.*, 2005	
	S3/S4 ITS region **XATS1/XATS2**-Biotin ITS region	PCR-ELISA			
X. ampelinus	**A1/B1** (external primers) **S3/S4** (internal primers) ITS region **Xamp 14F/Xamp 104R (primers)** **Xamp 14F/104 MGB (probe)** Subtractive hybridization	Nested Real-time (TaqMan)	Plant (DNA extraction)	Botha *et al.*, 2001 Dreo *et al.*, 2007	

Palacio-Bielsa *et al.*,2009

Identification of the bacterial pathogens *Xanthomonas*

Disease diagnosis and pathogen diagnostics

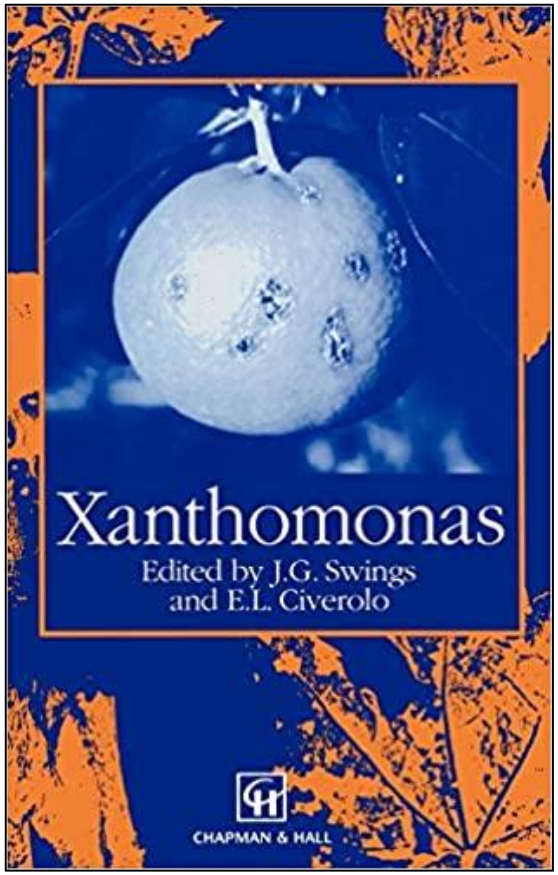
Domain: Bacteria
Phylum: Proteobacteria

- Class: Gammaproteobacteria
  - Order: Pseudomonadales
    - Family: Pseudomonadaceae
      - Genus: *Pseudomonas*
  - Order: Xanthomonadales
    - Family: Xanthomonadaceae
      - Genera: *Xanthomonas* and *Xylella*

Euzéby,2021

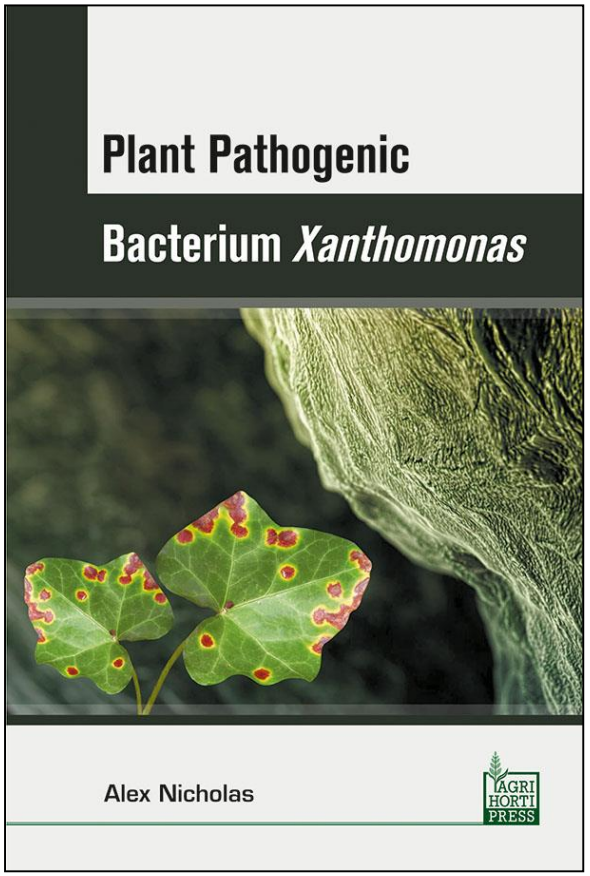
Xanthomonas

- **Xanthomonas**
- Jean Swings and Lucia Civetta
- Springer
- 1993
- 412 pages.



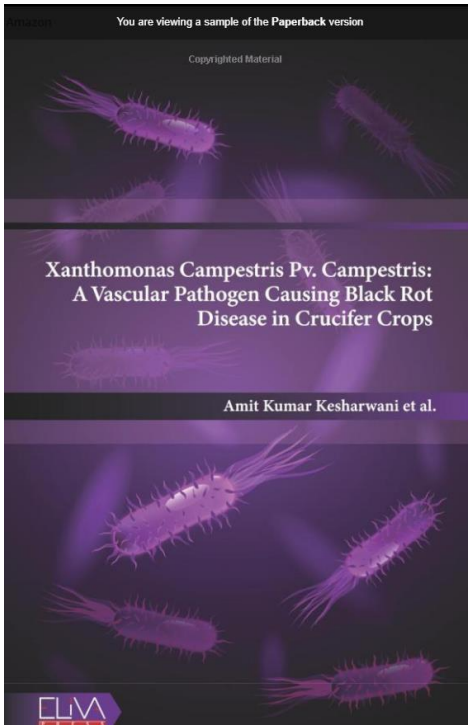
Plant Pathogenic Bacterium *Xanthomonas*

- **Plant Pathogenic Bacterium *Xanthomonas***
- Alex Nicholas
- Agri Horti Press
- 2017
- 252 pages.



Xanthomonas campestris pv. *campestris*: A Vascular Pathogen Causing Black Rot Disease in Crucifer Crops

- by Amit Kumar Kesharwani, Dinesh Singh, Ravinder Pal Singh, Anupama Sharma Avasthi.
- Eliva Press
- 2022
- 43 pages.



Genome structure

- *Xanthomonas* bacteria are composed of a circular chromosome of about five mega bases.
- Each cell also contains two plasmids, with 34 and 65 kilobases.

MicrobeWiki,2006

Xanthomonas genomics and diversity

- Starting with the sequencing of two *Xanthomonas* spp. in the early 2000s, there are now more than 1,400 *Xanthomonas* genomes representing all named *Xanthomonas* spp. publicly available in the National Center for Biotechnology Information (NCBI) database.
- A typical *Xanthomonas* genome is ~5 Mb with a GC content well over 60% and encodes >4,000 genes.
- The exception is *X. albilineans*, which has a reduced genome of ~3.7 Mb. This species has undergone genome erosion with an estimated loss of more than 500 genes, but the drivers of this gene loss are unclear.

Timilsina *et al.*,2020

The family Xanthomonadaceae
Genera *Xanthomonas* and *Xylella*

- The genera *Xanthomonas* and *Xylella* belong to the same family (Xanthomonadaceae) and are made up of several species.
- They possess similar systems and live in the same habitat as *fastidiosa* (citrus stain XF-9a5a) and *X. citri* pv.*citri* (XCC).
- This observation indicates that adaptation to a specific host such as citrus is due to:

1. slight differences in sequence composition, or
2. in the number of genes.

Comparison characteristics of the genera *Xanthomonas* and *Xylella*

Characteristic	*Xanthomonas*	*Frateuria*	*Fulvimonas*	*Luteimonas*	*Lysobacter*	*Nevskia*	*Pseudoxanthomonas*	*Rhodanobacter*	*Schineria*	*Stenotrophomonas*	*Thermomonas*	*Xylella*
Motility	+ (exceptions)	+	+	−	+ (flexing, gliding)	+		−	−	+	V	−
Flagellation	Single, polar	Single, polar	Single, polar	None		Single, polar		None	None	Multiple, polar	Single, polar	None
Cell shape	Rods	Rods	Rods		Rods	Rods		Rods	Rods	Rods	Rods	Rods
Cell size mm)	0.4–0.6 × 1.0–2.9	0.5–0.7 × 0.7–3.5	0.5 × 2		0.2–0.5 × 1.0–15 (sometimes 70)				2–3 × 0.8–0.9	0.5 × 1.5	1.0–12.5 × 0.5–0.75	0.25–0.35 × 0.9–3.5
Temperature optimum	28					20–25	28	30	28–37		37–50	26–28
Oxidase	−	−	+		+			+	V	V+	+	−
Catalase	+	+	+		+			+	+		+	+
Nitrate reduction	−	−		−	−		−	−	−	+	−	−
Aesculin hydrolysis				−			−				−	−
Susceptibility to:												
Ampicillin		+		−			−			−	+	+
Penicillin G		−		−	V−		−			−	+	−
Erythromycin	+	−		−			−			V−	+	+
Kanamycin		+		+			−				+	−
Neomycin		−		V−			+			−	+	
Streptomycin		+		−	V−		−			−	+	−
Quinones	Q8	Q8		Q8			Q8		Q8	Q8	Q8	
Polyamines	Spermine									Spermidine and cadaverine	Spermadine	
Mol% G + C of the DNA	63.3–69.7	62–64	71.5–71.9		65.4–70.1	67–69		68	42	66.1–67.8	67.1–68.7	51–52.4
Ecology	Plant pathogens	Plant associated	Soil; plastic degrader	Biofilter	Soil, freshwater; lysing microbes	Freshwater surfaces	Biofilter	Soil; lindane degrader	Fly larvae	Clinical materials; widespread	Kaolin slurry	Plant xylem

Bergey's Manual of Systematic Bacteriology,2005

Comparison characteristics of the genera *Xanthomonas* and *Xylella*

Characteristic	*Xanthomonas*	*Frateuria*	*Fulvimonas*	*Luteimonas*	*Lysobacter*	*Nevskia*	*Pseudoxanthomonas*	*Rhodanobacter*	*Schineria*	*Stenotrophomonas*	*Thermomonas*	*Xylella*
Major fatty acids:												
$C_{14:0}$									+			
$C_{15:0\ iso}$	+	+	+	+			+			+	+	
$C_{15:0\ anteiso}$	+						+			+		+
16:0							+		+			
16:1	+						+				+	
17:0 iso	+		+	+			+					+
17:1 iso			+	+								+
18:0									+			
18:1												
Major hydroxy fatty acids:												
$C_{11:0\ iso\ 2OH}$							+					
$C_{11:0\ iso\ 3OH}$	+		+				+			+		
$C_{12:0\ 3OH}$	+								+	+		+
$C_{12:0\ iso\ 3OH}$			+									
$C_{13:0\ 2OH}$										+	+	
$C_{13:0\ iso\ 3OH}$	+		+							+		
$C_{14:0\ 3OH}$									+			
Differential fatty acids:												
$C_{11:0\ iso}$	+											
$C_{11:0\ iso\ 2OH}$							+					
$C_{11:0\ iso\ 3OH}$	+											
$C_{12:0\ 2OH}$		+										
$C_{13:0\ iso\ 3OH}$	+											
$C_{17:0\ cyclo}$		+								+		
$C_{18:0\ cyclo}$									+			
Major polar lipids:												
Diphosphatidyl-glycerol	+								+	+		
Phosphatidyl-ethanolamine	+								+	+		
Phosphatidyl-glycerol	+								+	+		
Phosphatidyl-monoethanolamine	+									+		
Phosphatidylserine									+			
Unknown polar lipid	+									+		

*For symbols see standard definitions.

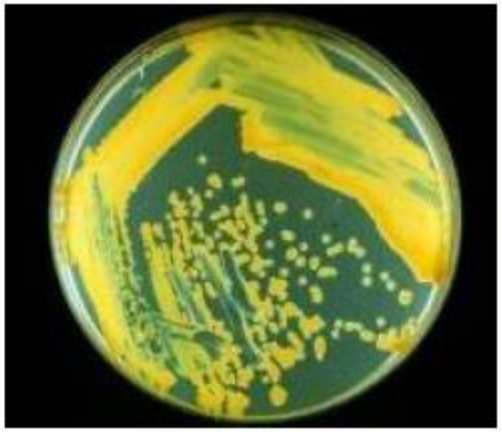
Typical characteristics of xanthomonads

- Cells are straight, Gram-negative rods; mucoid growth on 5% sucrose.
- Obligate aerobe.
- Do not produce poly ß-hydroxybutyrate inclusions.
- Nitrate is not reduced to nitrite except *X. populi*. No denitrification.
- Optimum temperature 25-30°C (*X. populi* will not grow above 23°C).
- Growth is inhibited by 6% NaCl, 30% glucose, 0.01% lead acetate, methyl green, or thionin, and by 0.1% (and usually by 0.02%) triphenyl tetrazolium chloride.
- Colonies usually yellow, smooth and butyrous or viscid.
- Yellow pigments are highly characteristic brominated aryl polyenes (Xanthomonadins);
- Asparagine is not used as sole carbon and nitrogen source.

Typical characteristics of xanthomonads

- Oxidase negative or weakly positive, Catalase positive; Urease not produced. H_2S is usually produced, but not indole, urease and acetoin.
- Starch hydrolysis was positive for certain pathovars of *X. campestris*.
- Grow on calcium lactate but not glutamine.
- Growth factors required for many.
- Mostly plant pathogens (opportunistic or saprophytic xanthomonads exist).
- GC ratio ranges from 63-71%.
- Xanthomonads produce an extracellular polysaccharide, Xanthan. Xanthan gum is stable in applications with a wide range of pH values (2-12).
- Ice nucleation genes- *ina*X present in *X. c. translucens* causes frost damage on wheat.

Phytopathogenic Xanthomonas sp.



Yellow pigment (brominated aryl-polyenes) ▶

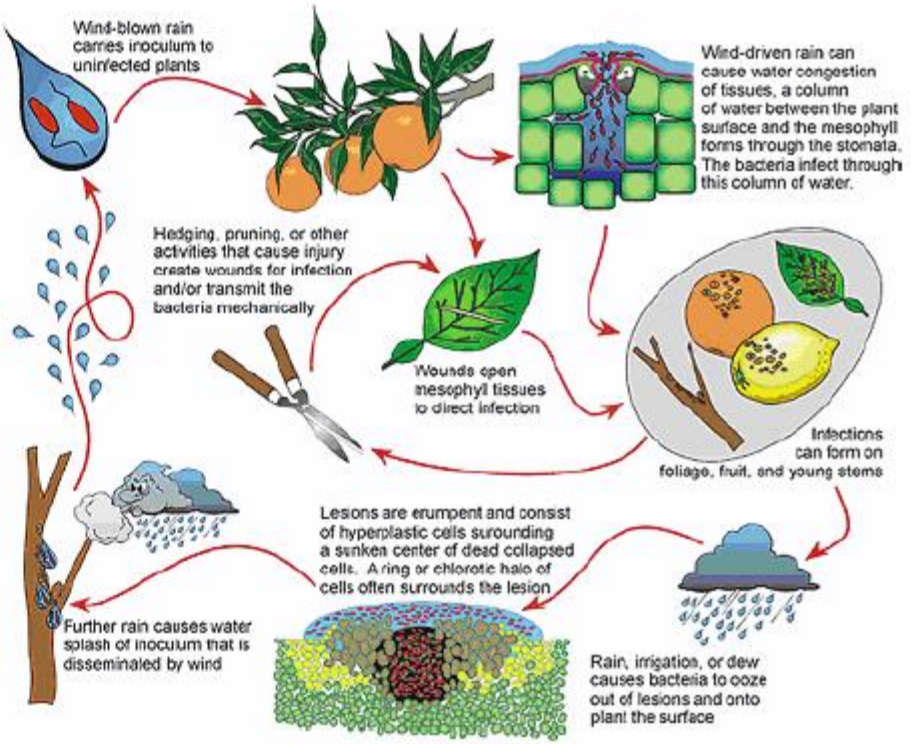
Xanthan gums

Monotrichous flagellation

Host-specific

Example: axonopodis *pv. citri*

X. axonopodis pv. citri
citrus canker pathogen



http://www.apsnet.org/education/lessonsPlantPath/CitrusCanker/

Pigmentation in the genus *Xanthomonas* Atypical characteristics

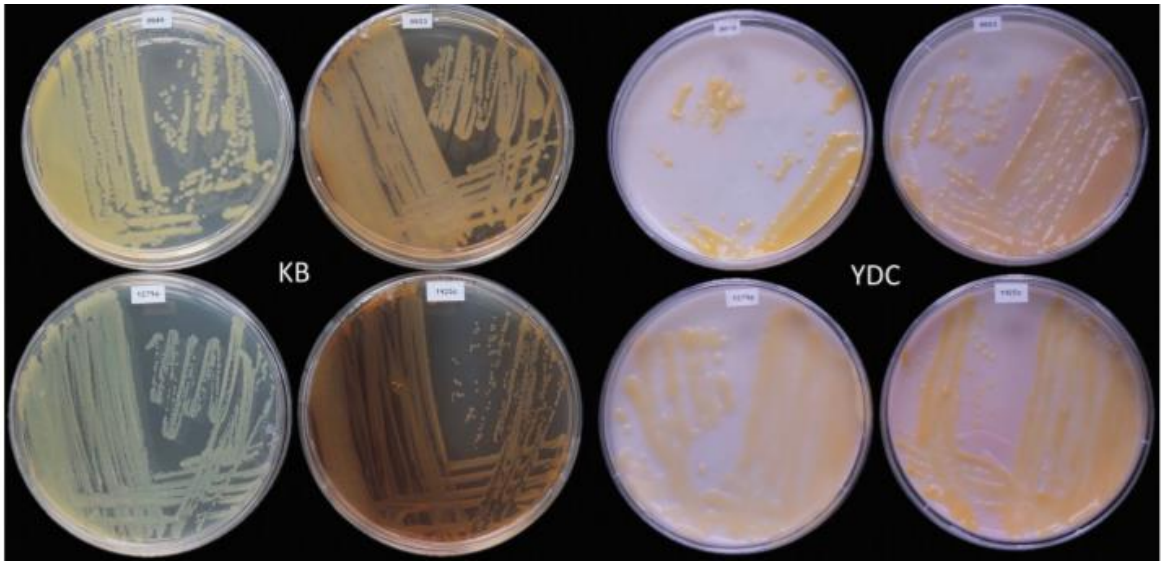
- Pigmentation in the genus *Xanthomonas* is found to be variable with age of culture and nutritional status and sometimes with the organism.
- For instance, a yellow diffusible pigment is produced optimally by *Xanthomonas vesicatoria* on GYCA and SPA than in other media.

GYCA medium (glucose 10.0 g; yeast extract 5.0 g; calcium carbonate 30.0 g; agar 20.0 g; distilled water to 1.0 L).
SPA(sucrose peptone agar) contains (in g L^{-1}): sucrose, 20.0; peptone, 5.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.25 and agar, 15.0, the pH adjusted to 7.2 with NaOH.

A semiselective agar medium (XTS agar) was developed and tested for the isolation of *X.t.* pv. *tanslucens* (Schaad & Forster,1985).

Opara and Odibo,2009;..

Pigmentation in the genus *Xanthomonas*
Atypical characteristics
On KB and YDC media



Xanthomonas floridensis (WHRI 8848), *Xanthomonas nasturtii* (WHRI 8853), *Xanthomonas campestris* pv. *campestris* (WHRI 1279A) and *Xanthomonas phaseoli* (WHRI 1925C) in plates with King's B (KB) agar and yeast dextrose calcium carbonate (YDC) agar media 3 days after sub-culturing

X. floridensis and *X.nasturtii* isolated from leaves of watercress grown in Florida.

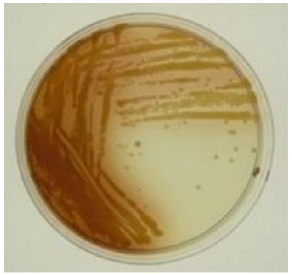
Vicente *et al.*,2017

Pigmentation in the genus *Xanthomonas* Atypical characteristics

- Intensity of the yellow color vary with organism:
- Paler yellows (paler than *X.vesicatoria*):
  - pv. *pruni*
  - pv. *vitians*
- With no yellow pigmentation on GYCA medium or LPGA medium:
  - *X. axonopodis* pv. *pedalii*
  - *X. campestris* pv. *azadirachtae*

Opara and Odibo,2009

Atypical characteristics
Continued



- With brown pigmentation on culture media:
  - *X. phaseoli*
- Colonies with white color:
  - pv. *manihotis*
  - pv. *ricini*
  - pv. *magniferaindica*
- Non-mucoid colonies:
  - *X. albilineans*
- Highly motile:
  - *X. albilineans*

Van den Mooter and Swings,1990; Karavina *et al.*,2011

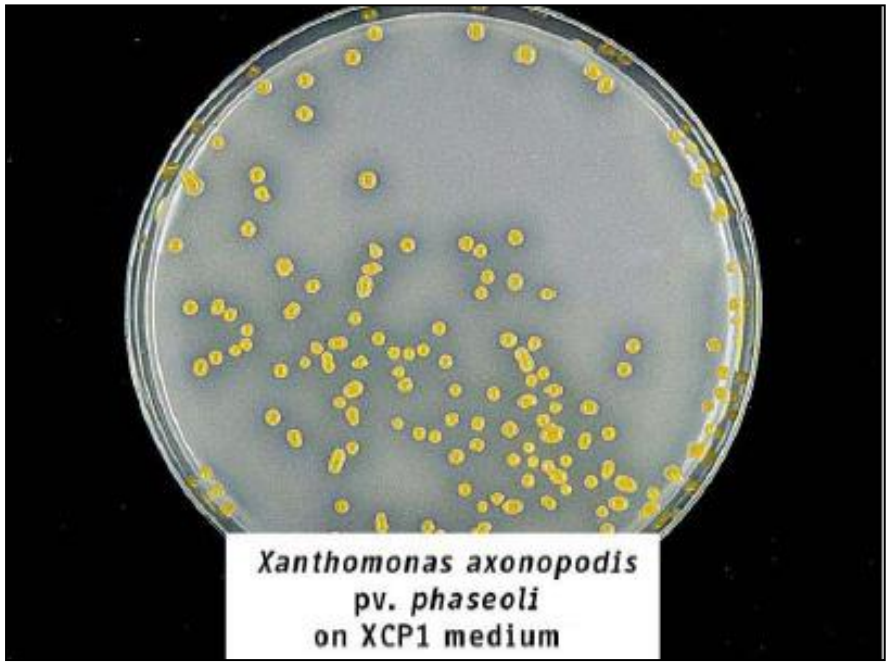
Atypical characteristics
Continued

- No reaction to O/F test:
  - *X. albilineans*
  - *X. axonopodis*
- Slower growth, nonmucoid colonies, amino acid requirement, antibiotic resistance and other phenotypic characteristics:
  - *X. albilineans*
- In particular, *X. albilineans* a xylem-invading bacterium is readily distinguished from other *Xanthomonas* species.

Van den Mooter and Swings,1990;...

Biochemical test
Starch hydrolysis

- Colonies of *X. phaseoli* growing on XCP1 with zones of starch hydrolysis visible around individual colonies.



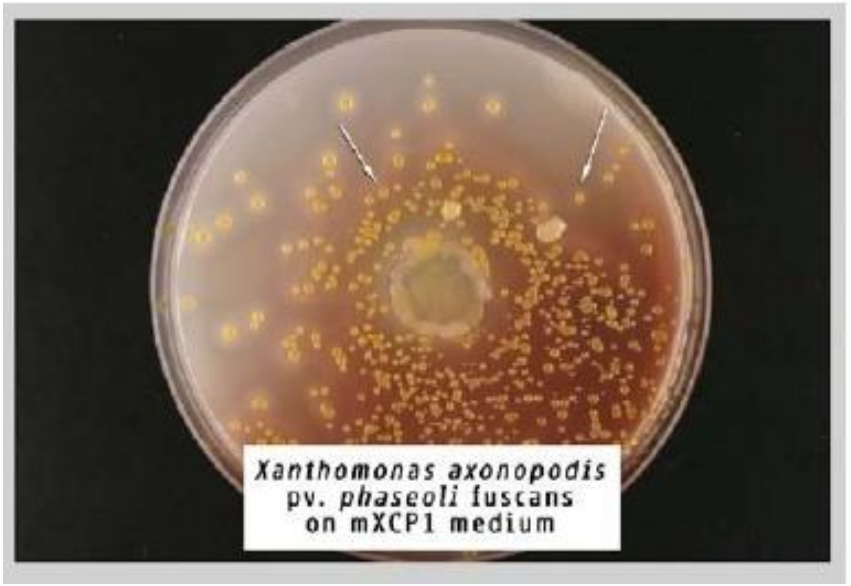
Biochemical test

Casein hydrolysis

- Colonies of *X. phaseoli* growing on MT with zones of casein hydrolysis visible around individual colonies.

Pigmentation
Brown pigment

- The brown pigment (melanin) that develops on *X. phaseoli* (fuscans type) 5 or more days of incubation on the semi-selective media(tyrosine containing agar media).
- This pigment can also be observed on YDC.



Tyrosine containing agar medium

Differentiation of pathovars of *Xanthomonas*

Host specificity and phylogenetic groups

- *Xanthomonas* spp. are subdivided into pathovars on the basis of:

1. host specificity and
2. can also be divided into two main phylogenetic groups based on sequence analysis of 16S rDNA, *gyrB*, *dnaK*, *rpoD*, and *fyuA*.

Ali Shah *et al.*, 2019

Host range
Xanthomonads

- There are at least 32 plant associated Xanthomonas spp., that all together infect at least 400 plant species such as rice, wheat, citrus, tomato, pepper, cabbage, cassava, banana and bean.
- About 124 monocots, and
- 268 dicots.
- The real number of plant species that is susceptible to xanthomonad pathogens might be far greater.
- They do not cause true vascular wilts.

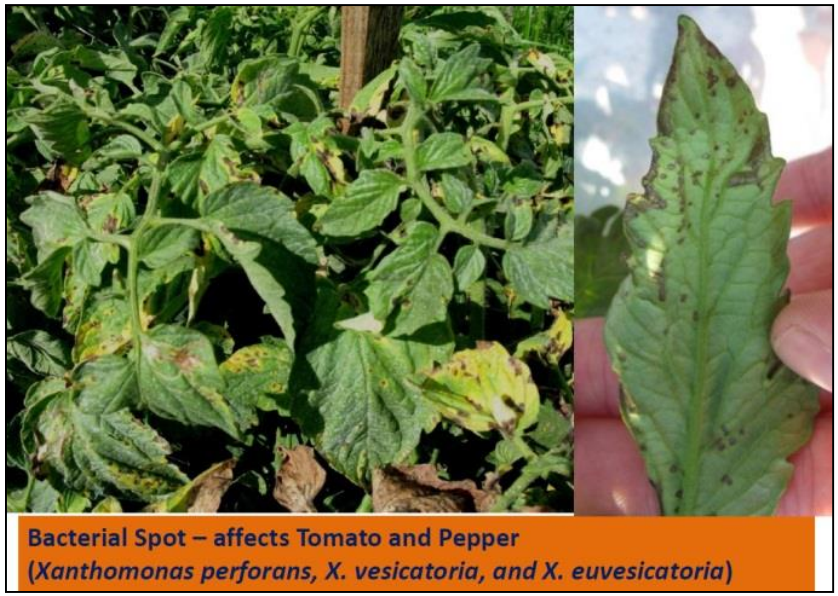
Ali Shah *et al.*,2019; Timilsina *et al.*,2020; Euzeby,2021

Diseases caused by Xanthomonads

Xanthomonas euvesicatoria, X. vesicatoria, X. perforans, and *X. gardneri*

- Tomato/pepper spot can be caused by:

1. *X. euvesicatoria*,
2. *X. vesicatoria*,
3. *X. gardneri*, and
4. *X. perforans.*



Bacterial Spot – affects Tomato and Pepper
(*Xanthomonas perforans, X. vesicatoria, and X. euvesicatoria*)

See more detailed information in the following slides

David Stead;..

Non-pathogenic xanthomonads

- Xanthomonas are primarily known as a group of phytopathogens infecting diverse plants.
- Recent molecular studies reveal existence of novel species and strains of *Xanthomonas* that follow non-pathogenic (NPX) lifestyle.
- Many of these non-pathogenic xanthomonads are frequently isolated from healthy plant materials.
- These non-pathogenic xanthomonads often cannot be classified to existing species.

Non-pathogenic xanthomonads

Non-pathogenic *Xanthomonas* strains associated with healthy citrus plants

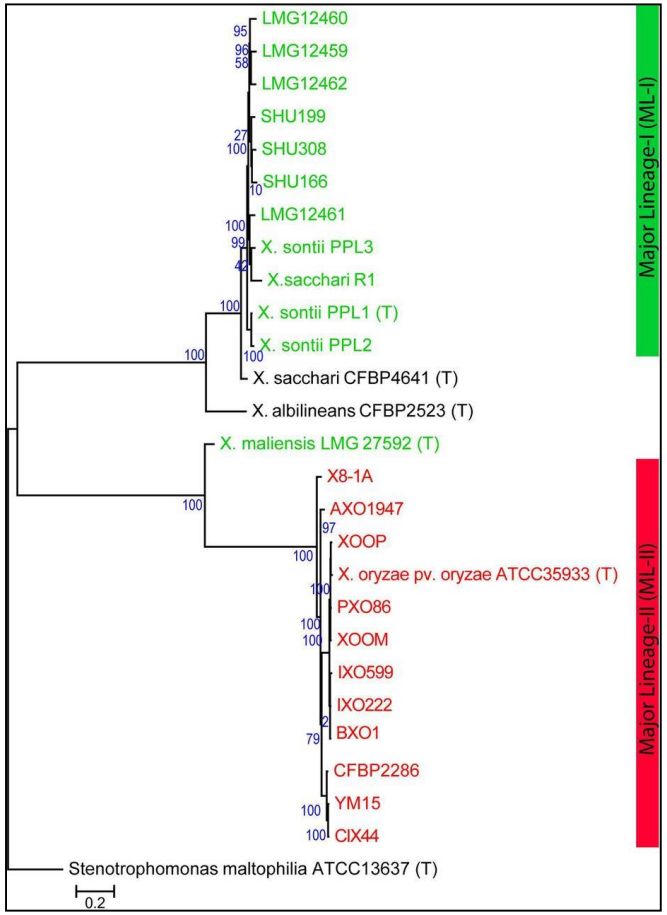
- *Xanthomonas citri* is one of the top phytopathogenic bacteria and is the causal agent of citrus canker.
- Interestingly *Xanthomonas* is also reported to be associated with healthy 60 citrus plants.
- Our investigations have revealed hidden and extreme inter-stain diversity of non-pathogenic Xanthomonas strains from citrus plants warranting further large scale studies.
- The knowledge and genomic resource will be valuable in evolutionary studies exploring its hidden potential and management of pathogenic species.

Bansal *et al.*,2019

Non-pathogenic xanthomonads

Non-pathogenic *Xanthomonas* strains associated with healthy rice plants

- PhyloPhlAn tree of all rice associated strains.
1. Rice non-pathogenic isolates are in green, and
2. pathogenic in red color.
- Here, strains of ML-I and ML-II are designated.
- *Xanthomonas oryzae* is a devastating pathogen of rice worldwide, however, *X. sontii* and *X. maliensis* are its non-pathogenic counterparts from the same host.

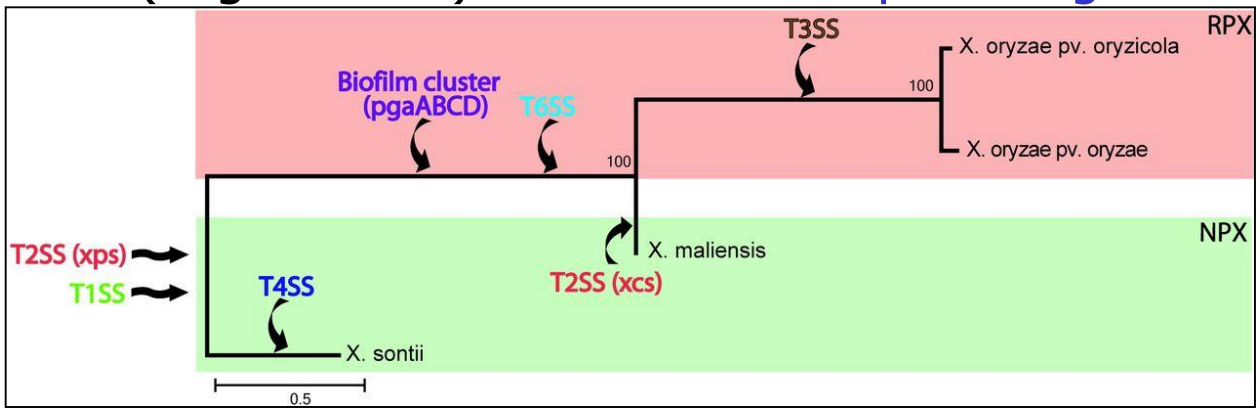


Bansal *et al.*,2019

Pathogenic(RPX) and Non-pathogenic(NPX) xanthomonads

Pattern of acquisition or loss of pathogenicity

- *Xanthomonas* strains are known to have two representative T2SS gene clusters *xps* and *xcs*. Out of these, *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* are equipped with one (*xps*) and *X. campestris* pv. *campestris*, *X. citri* pv. *citri* have both the systems.
- Pattern of **acquisition or loss of pathogenicity** related clusters among *X. oryzae* (in red box) and *X. sontii* and *X. maliensis* (in green box). Black arrows depict the gene flow.



Bansal *et al.*,2019

Xanthomonads
Plant pathogenic spp./pvs.

- **Some validly named species.**
- Species with no pathovar:

1. *X. albilineans*
2. *X. cannabis*
3. *X. cucurbitae*
4. *X. cynarae*
5. *X. euvesicatoria*
6. *X. floridensis*
7. *X. fragariae*
8. *X. gardneri*
9. *X. maliensis*
10. *X. nasturtii*

A pathovar is a bacterial strain or set of strains with the same or similar characteristics, that is differentiated at infrasubspecific level from other strains of the same species or subspecies on the basis of distinctive pathogenicity to one or more plant hosts.

David Stead; Euzeby,2010; Bull *et al.*,2010&2012; An *et al.*,2019

Xanthomonads
Plant pathogenic spp./pvs.
Continued

- **Some validly named species.**
- Species with no pathovar:

1. *X. perforans*
2. *X. prunicola*
3. *X. sacchari*

An *et al.*,2019

Xanthomonads
Plant pathogenic spp./pvs.

- **Some validly named species.**
- Species with multiple pathovars/subspecies:

1. *Xanthomonas alfalafa* (2)
2. *X. arboricola* (5)
3. *X. axonopodis* (42)
4. *X. campestris* (75)
5. *X. citri* (3)
6. *X. dyei* (3)
7. *X. hortorum* (4)
8. *X. oryzae* (2)
9. *X. translucens* (10)

Euzeby,2010; Bull *et al.*,2010 and 2012

List of names and acronyms of *Xanthomonas* strains discussed in this review

- Pathogenic species of *Xanthomonas* show a high degree of:

1. host plant specificity, and
2. species can be further differentiated into pathovars that are defined by characteristic host range and/or tissue specificity,

- invading either:

1. the xylem elements of the vascular system, or
2. the intercellular spaces of the mesophyll parenchyma tissue of the host.

An *et al.*,2019

Xanthomons spp.	Pathovar	Acronym	Disease	Host
X. albilineans			Leaf scald	Sugarcane
X. alfalfae			Bacterial leaf spot	Alfalfa
X. arboricola	*X. arboricola* pv. *pruni*	*Xap*	Bacterial spot	Prunus
	X. arboricola pv. *punicae*	*Xcp*	Leaf blight	Pomegranate (*Punica granatum*)
	X. arboricola pv. *juglandis*	*Xaj*	Walnut blight	Persian (English) walnut (*Juglans regia*)
X. axonopodis	*X. axonopodis* pv. *manihotis*	*Xam*	Bacterial blight	Cassava
X. campestris	*X. campestris* pv. *armoraciae*	*Xca*	Bacterial leaf spot	Horseradish
	X. campestris pv. *campestris*	*Xcc*	Black rot	Brassicaceae
	X. campestris pv. *leersiae*	*Xcl*	Bacterial streak	Perennial grass
	X. campestris pv. *musacearum*		Enset wilt	Banana
	X. campestris pv. *raphani*		Bacterial leaf spot	Brassica oleracea
	X. campestris pv. *vitians*		Bacterial leaf spot	Lettuce

Xanthomons spp.	Pathovar	Acronym	Disease	Host
X. cannabis			Bacterial leaf spot	Cannabis (*Cannabis sativa* L.)
X. citri	*X. citri* pv. *citri*	*Xcci*	Citrus canker	Citrus
	X. citri pv. *fuscans*	*Xcf*	Bacterial blight	Bean (*Phaseolus vulgaris* L.)
	X. citri pv. *glycines*	*Xcg*	Bacterial pustule	Soybean (*Glycine max*)
	X. citri pv. *malvacearum*	*Xcm*	Bacterial blight	Cotton (*Gossypium spec.*)
	X. citri pv. *mangiferaeindicae*	*Xmi*	Bacterial black spot	Mango (*Mangifera indica*)
	X. citri pv. *punicae*	*Xcp*	Leaf blight	Pomegranate (*Punica granatum*)
X. cucurbitae			Bacterial spot	Cucurbits
X. cynarae			Bacterial bract spot	Artichoke (*Cynara scolymus* L.)
X. euvesicatoria	*X. campetris* pv. *vesicatoria*	*Xav*	Bacterial leaf spot	Pepper and tomato

An *et al.*,2019

Xanthomons spp.	Pathovar	Acronym	Disease	Host
X. floridensis			–	Watercress
X. fragariae			Bacterial angular leaf spot	Strawberry
X. gardneri			Bacterial spot	Pepper and tomato
X. maliensis			–	Rice
X. nasturtii			–	Watercress
X. oryzae	*X. oryzae* pv. *oryzae*	*Xoo*	Bacterial blight	Rice
	X. oryzae pv. *oryzicola*	*Xoc*	Bacterial streak	Rice
X. perforans			Bacterial spot	Tomato
X. phaseoli	*X. phaseoli* pv. *phaseoli*	*Xcp*	Bacterial blight	Bean (*Phaseolus vulgaris* L)
X. prunicola			–	nectarine *(Prunus persica* var. *nectarina*) trees

An *et al.*,2019

Xanthomons spp.	Pathovar	Acronym	Disease	Host
X. pseudoalbilineans	Not in NCBI list yet			
X. sacchari			Chlorotic streak disease	Sugarcane
X. translucens	*X. translucens* pv. *translucens*	*Xtt*	Black chaff	Wheat
	X. translucens pv. *undulosa*	*Xtu*	Black chaff	Wheat
X. vasicola	*X. vasicola* pv. *vasculorum*	*Xvv*	Gumming disease	Sugarcane

Note: *Xanthomonas arboricola* pv. *punicae* is currently listed as *X. citri* pv. *punicae* in NCBI taxonomy.

An *et al.*,2019

Diseases caused by Xanthomonads
Species/pathovars

1.	*Xanthomonas albilineans*	Leaf scald of sugarcane
2.	*Xanthomonas alfalfae* (with 2 subspecies)	
2.1	*Xanthomonas alfalfae* subsp. *alfalfae*	Leaf & stem spotting of Trifolium, Medicago
2.2	*Xanthomonas alfalfae* subsp. *citrumelonis*	Xanthomonad pathogens on citrumelo
3.	*Xanthomonas arboricola* (with 5 pathovars)	
3.1	*X. arboricola* pv. *celebensis*	Causes a disease on banana (*Musa* spp.)
3.2	*X. arboricola* pv. *corylina*	Bacterial blight of hazelnut (*Corylus* spp.)
3.3	*X. arboricola* pv. *fragariae* (See also *X . fagariae*)	Bacterial leaf spot of strawberry (*Fragaria vesca*)
3.4	*X. arboricola* pv. *juglandis*	Walnut (*Juglans* spp.) blight
3.5	*X. arboricola* pv. *pruni*	Bacterial blight of stone fruits (plum, peach,..)
4.	*Xanthomonas axonopodis* (with 44 pathovars)	
4.1	*X. axonopodis* pv. *allii*	Bacterial blight of welson onion

Diseases caused by Xanthomonads
Species/pathovars

4.2	*Xanthomonas axonopodis* pv. *anacardii*	Cashew bacterial spot
4.3	*X. axonopodis* pv. *axonopodis*	Attack the *Agropyron* sp., *Axonopus scoparius*, *Axonopus micay*, *Axonopus compressus* and *Axonopus affinis*
4.4	*X. axonopodis* pv. *bauhiniae*	On *Bauhinia racemosa*
4.5	*X. axonopodis* pv. *begoniae*	Leaf spotting & necrosis of begonia (*Begonia* spp.)
4.6	*X. axonopodis* pv. *beticola*	Bacterial pocket of beet (*Beta vulgaris*)
4.7	*X. axonopodis* pv. *biophyti*	On *Biophytum sensitivum*
4.8	*X. axonopodis* pv. *cajani*	Leaf spot and stem canker of pigeon pea (*Cajanus cajan*)
4.9	*X. axonopodis* pv. *cassiae*	Blight of *cassia* sp. (*C. occidentalis*)
4.10	*X. axonopodis* pv. *clitoriae*	On *Clitoria biflora*
4.11	*X. axonopodis* pv. *coracanae*	On *Eleusine coracana*
4.12	*X. axonopodis* pv. *cyamopsidis*	Bacterial blight of Guar (*Cyamopsis tetragonoloba*)
4.13	*X. axonopodis* pv. *desmodii*	bacterial leaf spot of *Desmodium diffusum*

Diseases caused by Xanthomonads
Species/pathovars

4.14	*X. axonopodis* pv. *desmodiigangetici*	Bacterial leaf spot of *Desmodium gangeticum*
4.15	*X. axonopodis* pv. *desmodiilaxiflori*	On *Desmodium laxiflorum*
4.16	*X. axonopodis* pv. *desmodiirotundifolii*	On *Desmodium rotundifolium*
4.17	*X. axonopodis* pv. *dieffenbachiae*	Bacterial leaf spot of dieffenbachia; Leaf & stem necrosis of Anthurium
4.18	*X. axonopodis* pv. *erythrinae*	Leaf spot disease on Erythrina (*Erythrina indica*)
4.19	*X. axonopodis* pv. *fascicularis*	On *Corchorus fascicularis*
4.20	*X. axonopodis* pv. *glycines*	Leaf spot disease on Erythrina (*Erythrina indica*)
4.21	*X. axonopodis* pv. *khayae*	Leaf spot of *Khaya senegalensis*
4.22	*X. axonopodis* pv. *lespedezae*	On *Lespedeza* spp.
4.23	*X. axonopodis* pv. *maculifoliigardeniae*	On *Gardenia* spp. ; *Ixora coccinea*
4.24	*X. axonopodis* pv. *manihotis*	Bacterial blight of cassava (*Manihot* spp.)
4.25	*X. axonopodis* pv. *mangiferaeindicae*	Mango bacterial canker/Black spot of mango
4.26	*X. axonopodis* pv. *martyniicola*	On *Martynia diandra*

Diseases caused by Xanthomonads
Species/pathovars

4.27	*X. axonopodis* pv. *melhusii*	On *Tectona grandis*
4.28	*X. axonopodis* pv. *nakataecorchori*	Bacterial leaf-spot disease of *Corchorus acutangulus*
4.29	*X. axonopodis* pv. *patelii*	On *Crotalaria juncea*
4.30	*X. axonopodis* pv. *pedalii*	Bacterial leaf spot of *Pedalium murex*
4.31	*X. axonopodis* pv. *phyllanthi*	On *Phyllanthus niruri*
4.32	*X. axonopodis* pv. *physalidicola*	On *Physalis alkekengi* var. *francheti*
4.33	*X. axonopodis* pv. *poinsettiicola*	Angular leaf spot of Poinsettia (*Euphorbia pulcherrima*)
4.34	*X. axonopodis* pv. *punicae*	Bacterial leaf spot of pomegranate
4.35	*X. axonopodis* pv. *rhynchosiae*	On *Rhynchosia memnonia*
4.36	*X. axonopodis* pv. *ricini*	Leaf spot and necrosis of castor (*Ricinus communis*)
4.37	*X. axonopodis* pv. *sesbaniae*	Leaf and stem spots and defoliation of *Sesbania aegyptiaca*
4.38	*X. axonopodis* pv. *spondiae*	Syndrome on ambarella (*Spondias dulcis*) and mombin (*S. mombin*)

Diseases caused by Xanthomonads
Species/pathovars

4.39	*X. axonopodis* pv. *tamarindi*	On *Tamarindus indica*
4.40	*X. axonopodis* pv. *vignaeradiatae*	Leaf spot of mungbean (*Vigna radiata*)
4.41	*X. axonopodis* pv. *vignicola*	leaf spot on cowpea (*Vigna unguiculata*) and beans
4.42	*X. axonopodis* pv. *vitians*	Angular leaf spot/necrosis of lettuce
	Xanthomonas bonasiae	Isolated from weeping fig
5.	*Xanthomonas bromi*	Wilting disease on bromegrass (*Bromus* spp.); Sugarcane gummosis
6.	*Xanthomonas campestris* (with 75 pathovars)	Strains of this species cause disease in various crucifers (members of the Brassicaceae)
6.1	*X. campestris* pv. *aberrans*	On *Brassica oleracea* var. *botrytis*
6.2	*X. campestris* pv. *alangii*	On *Alangium lamarckii*
6.3	*X. campestris* pv. *amaranthicola*	Bacterial leaf spot of *Amaranthus* sp.
6.4	*X. campestris* pv. *amorphophalli*	On *Amorphophallus campanulatus*
6.5	*X. campestris* pv. *aracearum*	On *Xanthosoma sagittifolium*

Diseases caused by Xanthomonads

Species/pathovars

6.6	*X. campestris* pv. *arecae*	On *Areca catechu*
6.7	*X. campestris* pv. *argemones*	Bacterial blight of *Argemone mexicana*
6.8	*X. campestris* pv. *armoraciae*	Leaf spot of *Armoracia rusticana*
6.9	*X. campestris* pv. *arracaciae*	On *Arracacia xanthorrhiza*
6.10	*X. campestris* pv. *azadirachtae*	On *Azadirachta indica*
6.11	*X. campestris* pv. *badrii*	On *Xanthium strumarium*
6.12	*X. campestris* pv. *barbareae*	On *Barbarea vulgaris*
6.13	*X. campestris* pv. *beticola*	On *Beta vulgaris*
6.14	*X. campestris* pv. *bilvae*	On *Aegle marmelos*
6.15	*X. campestris* pv. *blepharidis*	On *Blepharis* spp.
6.16	*X. campestris* pv. *boerhaaviae*	On *Boerhaavia repens*
6.17	*X. campestris* pv. *brunneivaginae*	On *Oryza sativa*
6.18	*X. campestris* pv. *campestris*	Black rot of crucifers (*Brassica* spp.)
6.19	*X. campestris* pv. *cannabis*	On *Cannabis sativa*
6.20	*X. campestris* pv. *cannae*	Bacterial disease of ornamental cannas (*Canna* X *generalis*)

Diseases caused by Xanthomonads
Species/pathovars

6.21	*X. campestris* pv. *carissae*	On *Carissa congesta*
6.22	*X. campestris* pv. *centellae*	On *Centella asiatica*
6.23	*X. campestris* pv. *clerodendri*	On *Clerodendron phlomoides,*
6.24	*X. campestris* pv. *convolvuli*	Leaf spot of *Convolvulus arvensis*
6.25	*X. campestris* pv. *coriandri*	On *Coriandrum sativum*
6.26	*X. campestris* pv. *daturae*	Bacterial leaf spot of *Datura metel*
6.27	*X. campestris* pv. *durantae*	On *Duranta repens*
6.28	*X. campestris* pv. *esculenti*	On *Hibiscus esculentus*
6.29	*X. campestris* pv. *euphorbiae*	Bacterial leaf spot of *Eurphorbia* spp.
6. 30	*X. campestris* pv. *fici*	Angular leaf spot of ornamental *Ficus* spp.
6. 31	*X. campestris* pv. *guizotiae*	Leaf spot disease of *Guizotia abyssinica*
6. 32	*X. campestris* pv. *gummisudans*	On *Gladiolus* sp.
6. 33	*X. campestris* pv. *heliotropii*	On *Heliotropium* spp.
6. 34	*X. campestris* pv. *incanae*	Bacterial blight of garden stocks

Diseases caused by Xanthomonads
Species/pathovars

6.35	*X. campestris* pv. *ionidii*	On *Ionidium heterophyllum*
6.36	*X. campestris* pv. *lantanae*	On *Lantana camara* var. *aculeata*
6.37	*X. campestris* pv. *laureliae*	Bacterial disease of pukatea (*Laurelia novae-zelandiae*)
6.38	*X. campestris* pv. *lawsoniae*	On *Lawsonia alba*
6.39	*X. campestris* pv. *leeana*	Bacterial blight of *Leea edgeworthii*
6.40	*X. campestris* pv. *leersiae*	On *Leerisa hexandra*
6.41	*X. campestris* pv. *malloti*	Bacterial leaf (brown) spot of *Mallotus japonicus*
6.42	*X. campestris* pv. *merremiae*	On *Merremia gangetica*
6.43	*X. campestris* pv. *mirabilis*	On *Mirabilis jalapa*
6.44	*X. campestris* pv. *musacearum**	Bacterial wilt of banana (*Musa* spp.) and enset (root crop)bacterial wilt
6.45	*X. campestris* pv. *nigromaculans*	On *Zinnia elegans*
6.46	*Xanthomonas campestris* pv. *obscurae*	On *pomoea obscura*
6.47	*X. campestris* pv. *olitorii*	On *Corchorus olitorius*

Diseases caused by Xanthomonads
Species/pathovars

6.48	*X. campestris* pv. *papavericola*	On *Papaver* spp.
6.49	*X. campestris* pv. *parthenii*	Leaf blight of parthenium
6.50	*X. campestris* pv. *passiflorae*	Greasy lesion on passion fruit plants (*Passiflora edulis*)
6.51	*X. campestris* pv. *paulliniae*	On *Paullinia* spp.
6.52	*X. campestris* pv. *pennamericanum*	Bacterial leaf streak of pearl millet (*Pennisetum americanum*)
6.53	*X. campestris* pv. *phormiicola*	On *Phormium tenax*
6.54	*X. campestris* pv. *physalidis*	On *Physalis* spp.
6.55	*Xanthomonas campestris* pv. *plantaginis*	On Plantain (*Plantago lanceola*)
6.56	*X. campestris* pv. *raphani*	Leaf spot disease of radish
6.57	*X. campestris* pv. *sesami*	Leaf spotting of sesamum (*Sesamum orientale*)
6.58	*X. campestris* pv. *spermacoces*	On *Spermacoce hispida*
6.59	*X. campestris* pv. *silvia*	Bacterial blight of sunflower
6.60	*X. campestris* pv. *syngonii*	Bacterial leaf blight of *Syngonium podophyllum*

Diseases caused by Xanthomonads
Species/pathovars

6.61	*X. campestris* pv. *tardicrescens*	Bacterial leaf blight of Iris (*Iris* spp.)
6.62	*X. campestris* pv. *thespesiae*	leaf-spot disease of *Thespesia populnea*
6.63	*X. campestris* pv. *thirumalacharii*	On *Triumfetta pilosa*
6.64	*X. campestris* pv. *tribuli*	On *Tribulus terrestris*
6.65	*X. campestris* pv. *trichodesmae*	On *Trichodesma zeylanicum*
6.66	*X. campestris* pv. *uppalii*	Pathogenic on *Ipomoea muricata*
6.67	*X. campestris* pv. *vernoniae*	On *Vernonia cinerea*
6.68	*X. campestris* pv. *viegasii*	Bacterial spot and blight of yellow-shrimp (*Pachystachys lutea*)
6.69	*X. campestris* pv. *viticola*	Bacterial canker of grapevine
6.70	*X. campestris* pv. *vitiscarnosae*	On *Vitis carnosa*
6.71	*X. campestris* pv. *vitistrifoliae*	On *Vitis trifolia*
6.72	*X. campestris* pv. *vitiswoodrowii*	On *Vitis woodrowii*
6.73	*X. campestris* pv. *zantedeschiae*	Pathogenic to *Zantedeschia aethiopica*
6.74	*X. campestris* pv. *zingibericola*	Bacterial leaf blight of ginger (*Zingiber officinale*)

Diseases caused by Xanthomonads
Species/pathovars

6.75	*X. campestris* pv. *zinniae*	Leaf blight of cultivated wild rice (*Zinnia elegans*)
7.	*Xanthomonas cassavae*	Leaf spotting of cassava (*Manihot* spp.)
	Xanthomonas cissicola	Xanthomonas cissicola
8.	*Xanthomonas citri* (with 4 pathovars)	
8.1	*Xanthomonas citri* pv. *citri*	Citrus canker
8.2	*Xanthomonas citri* pv. *aurantifolia*	Citrus canker
8.3	*Xanthomonas citri* pv. *eucalyptorum*	Leaf blight on eucalypt plants
8.4	*Xanthomonas citri* pv. *malvacearum*	Bacterial blight of cotton
9.	*Xanthomonas codiaei*	Wilting disease on Codiaeum.
10.	*Xanthomonas cucurbitae*	Leaf & stem spot of cucurbits
11.		
12	*Xanthomonas dyei* (with 3 pathovars)	
12.1	*Xanthomonas dyei* pv. *dysoxyli* Strains previously identified as *Pseudomonas syringae* pv. *dysoxyli*	Greasy lesions in the leaf axils of *Dysoxylum spectabile* (Kohekohe)
12.2	*Xanthomonas dyei* pv. *eucalypti*	Bacterial leaf blight (die-back) of eucalyptus (*Eucalyptus* spp.)

Diseases caused by Xanthomonads
Species/pathovars

12.3	*Xanthomonas dyei* pv. *laureliae*	Bacterial disease of pukatea (*Laurelia novae-zelandiae*)
13	*Xanthomonas euroxanthea*	Pathogenic and non-pathogenic strains of walnut
14.	*Xanthomonas euvesicatoria*	Blister of tomato and pepper
15.	*Xanthomonas floridensis*	*Xanthomonas* associated with watercress production in Florida
16.	*Xanthomonas fragariae* (See also *X. a. fragariae*)	Angular leaf spot of strawberry
17.	*Xanthomonas hortorum* (with 4 pathovars)	
17.1	*Xanthomonas hortorum* pv. *carotae*	Bacterial leaf blight of carrot, carrot scab
17.2	*Xanthomonas hortorum* pv. *hederae*	Bacterial leaf spot of English ivy (*Hedera* spp.)
17.3	*Xanthomonas hortorum* pv. *pelargonii*	Bacterial leaf spot/blight of geranium (*Geranium* spp.; *Pelargonium* spp.)
17.4	*Xanthomonas hortorum* pv. *taraxaci*	On *Taraxacum bicorne*
18.	*Xanthomonas hyacinthi*	Wilting (Yellow) disease of Hyacinths (*Hyacinthus orientalis*)
	Xanthomonas hydrangeae	

Diseases caused by Xanthomonads
Species/pathovars

19.	***Xanthomonas maliensis***	Isolated from rice leaves
20.	***Xanthomonas meloni***	Leaf spot/soft rot of watermelon fruit (*Cucumis melo*)
21.	***Xanthomonas nasturtii***	*Xanthomonas* associated with watercress production in Florida
22.	***Xanthomonas oryzae*** (with 2 pathovars)	
22.1	***Xanthomonas oryzae*** pv. ***oryzae***	Bacterial leaf blight of rice (o*ryza* spp.)
22.2	***Xanthomonas oryzae*** pv. ***oryzicola***	Bacterial leaf streak of rice
23.	***Xanthomonas perforans***	Associated with bacterial spot disease of tomato and pepper
24.	***Xanthomonas phaseoli***	Common bacterial blight of beans
25.	***Xanthomonas pisi***	Root rot of pea (*Pisum sativum*)
26.	***Xanthomonas populi***	Bacterial canker of poplar trees
27.	***Xanthomonas prunicola***	a novel pathogen that affects nectarine (*Prunus persica* var. *nectarina*) trees
28.	***Xanthomonas sacchari***	Sugarcane gummosis
29.	***Xanthomonas theicola***	Canker of tea plants (*Camellia sinensis*)

Diseases caused by Xanthomonads
Species/pathovars

30.	***Xanthomonas translucens* (with 10 pathovars)**	**The strains of this species cause diseases in various members of the Poaceae**
30.1	***Xanthomonas translucens* pv. *arrhenatheri***	**Infects false oatgrass (*Arrhenatherum elatius*)**
30.2	***Xanthomonas translucens* pv. *cerealis***	***Xtc* has a broad host range. Leaf streak of cereal crops**
30.3	***Xanthomonas translucens* pv. *graminis***	**Leaf spot of Timoty grass; *Lolium* spp.**
30.4	***Xanthomonas translucens* pv. *phlei***	**Infects timothy grass (*Phleum pratense*)**
30.5	***Xanthomonas translucens* pv. *phleipratensis***	**Infects timothy grass (*Phleum praterse*), *Agropyron* sp. and *Bromus* sp.**
30.6	***Xanthomonas translucens* pv. *pistaciae***	**Dieback of pistachio**
30.7	***Xanthomonas translucens* pv. *poae***	**Bacterial wilt of Poa (*Poa trivialis*)**
30.8	***Xanthomonas translucens* pv. *secalis***	**Leaf & stem spotting of Secale (*Secale cereale*)**
30.9	***Xanthomonas translucens* pv. *translucens***	**Leaf streak of cereal crops**
30.10	***Xanthomonas translucens* pv. *undulosa***	**Leaf streaks of cereal crops**

The leaf streak disease of cereals such as wheat, barley,.. caused by Xtc, *Xtt* (spikes). and *Xtu*, are called black chaff when on the glumes.

Diseases caused by Xanthomonads

Xanthomonas translucens

translucens" group and graminis group

- Vauterin *et al*., 1992, 1995 proposed the re-establishment of the species *Xanthomonas translucens* including:

1. strains that cause leaf streak on small grains and some grasses (the "translucens" group), and
2. strains that cause bacterial wilt on forage grasses (the "graminis" group).

- This classification and nomenclature system has been supported by recent molecular and whole genome sequence data (Peng *et al*., 2016; Langlois *et al*., 2017; Hersemann *et al*., 2017).
- Genomic sequence data also suggest that the pathovars cerealis could be genetically separated from other translucens and graminis group pathovars.

Sapkota *et al*.,2020

Diseases caused by Xanthomonads
Species/pathovars

31.	***Xanthomonas vasicola*** **(with 3 pathovar)**	
31.1	***Xanthomonas vasicola*** **pv.** ***holcicola***	Leaf striping of Zea (*Sorghum* spp.)
31.2	***Xanthomonas vasicola*** **pv.** ***musacearum***	Bacterial wilt of banana (*Musa* spp.) and enset (*Ensete ventricosum*) bacterial wilt
31.3	***Xanthomonas vasicola*** **pv.** ***vasculorum***	Bacterial leaf streak of corn and gummosis (Yellow slime) of sugarcane.
32.	***Xanthomonas vesicatoria***	Leaf spot of pepper & tomato
32.1	***Xanthomonas youngii***	Crown gall tissues of amaranth (*Amaranthus* sp.)

Compiled from various references including ISPP LISTS, 2004; Bergey's Manuals, Janse,2006; Bull *et al.*,2010 & 2012; Euzeby,2023

Major diseases caused by *Xanthomonas* spp.

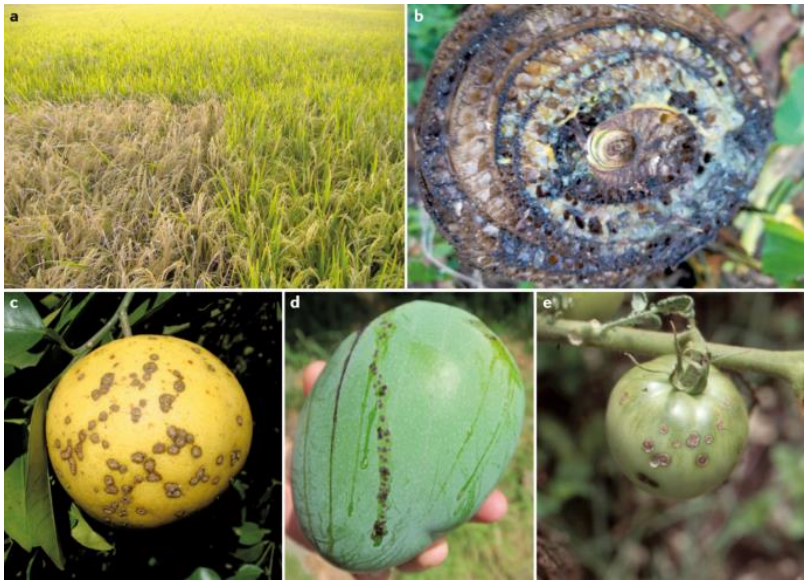
a) *Xanthomonas oryzae* causes bacterial blight in rice.

b) *X. campestris* pv. *musacearum* causes banana *Xanthomonas* wilt, which can lead to extensive oozing.

c) *X. citri* infects citrus and produces unique pustules in the leaf and fruit tissues.

d) *X. a* pv. *mangiferaindicae* causes mango black spot disease.

e) Four *Xanthomonas* spp. are associated with bacterial spot disease in tomato and pepper: *X. cynarae* pv. *gardneri*, *X. euvesicatoria*, *X. perforans* and *X. vesicatoria*.



Timilsina *et al.*,2020

Major diseases caused by *Xanthomonas* spp.

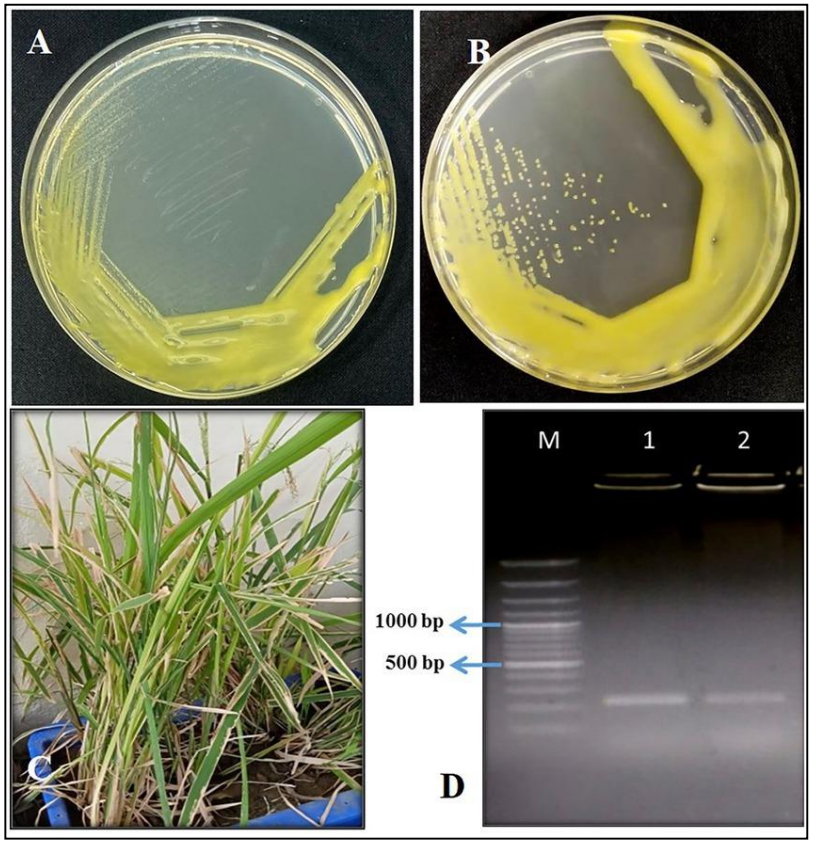
Xanthomonas oryzae pv. *oryzae*

A. Growth of *Xoo* culture straw yellow color smooth entire margin after 3 days;

B. Slimy and glistening colony after 5-day incubation;

C. Pathogenicity testing of *Xoo* isolates in rice pots with symptoms of BLB infection;

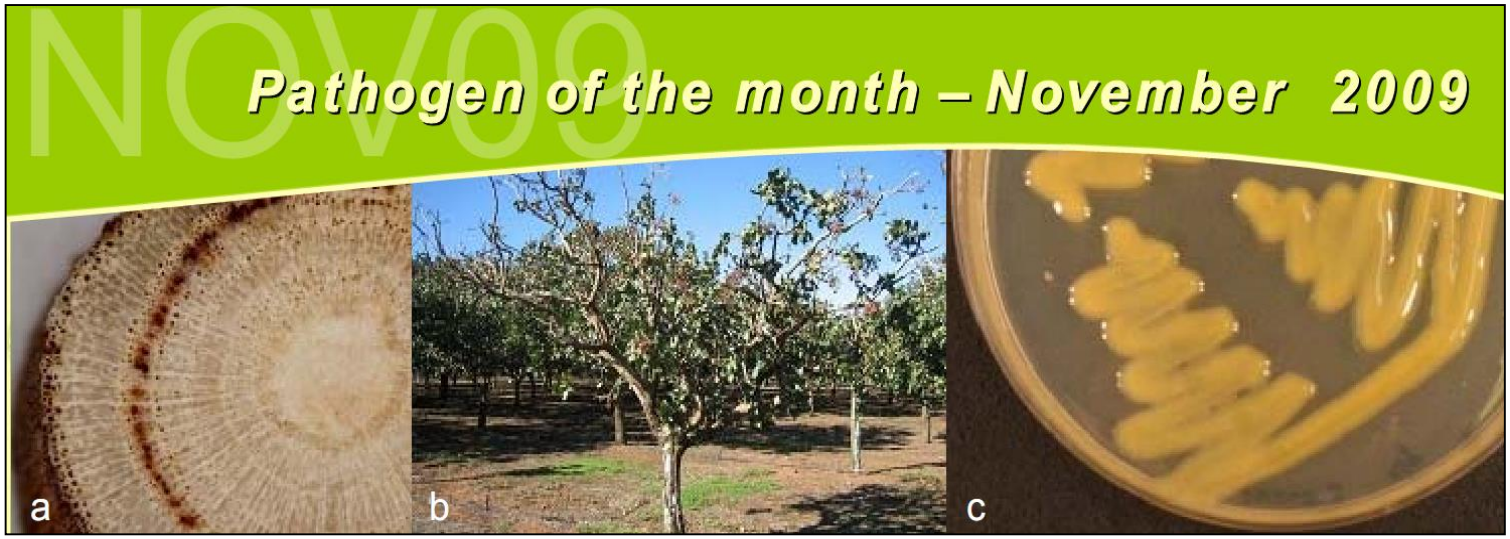
D. Confirmation of *Xoo* by JLXoo-1 primer PCR with captions M: 100 bp Ladder, 1: Xoo culture, 2: Xoo re-isolated after pathogenicity test in the rice pot.



Bacterial leaf blight (BLB)

Jain *et al.*,2023

Dieback or decline of pistachio
Xanthomonas translucens pv. *pistaciae*



Typical xylem staining (a) and dieback (b) associated with *X. translucens* pv. *pistaciae* infection in Pistachio, invading the xylem elements of the vascular system. *X. translucens* pv. *pistaciae* on Sucrose Peptone Agar, SPA (c). Photo credits C. Taylor (a, b) and A. Salowi (c).

Giblot Ducray and Eileen Scott;..

Bacterial blight or black spot of walnut
Xanthomonas arboricola pv. *juglandis*

- Bacteria survive in buds and catkins, in fruit lesions, to a lesser extent in cankers on twigs.
- Dispersal by large walnut aphid *Chromaphis juglandicola* and mites (walnut leaf gall mite, *Eriophyes erineus*).
- Wet weather in spring favors disease.

J.D. Janse

Diseases caused by Xanthomonads
Xanthomonas axonopodis pv. *beticola*

- One pathogen, *X. axonopodis* pv. *beticola* (Bacterial pocket of beet) has been described to cause a hyperplastic or gall type of disease (Brown,1928; Moliszewska *et al*.,2016).

Bacterial blight of cotton

Xanthomonas citri subsp. *malvacearum*

Allen *et al.*,2010

Cassava bacterial blight
Xanthomonas axonopodis pv. *manihotis*

- Cassava (*Manihot esculentum*) showing three different symptoms, caused by one bacterium, *Xanthomonas axonopodis* pv. *manihotis* :

1. Wilting due to systemic infection and blocking of transport vessels by masses of bacteria embedded in slime;
2. Yellowing, due to action of a toxin, and
3. Leaf spots, due to infection through stomata or wounds.

Janse,2006

Bacterial leaf spot

Xanthomonas axonopodis pathovar *vitians*

- Bacterial leaf spot on green leaf lettuce caused by *Xanthomonas axonopodis* pathovar *vitians*.
- (Courtesy S. T. Koike)

Riley *et al.*,2002

Yellow disease of hyacinths

Xanthomonas hyacinthi

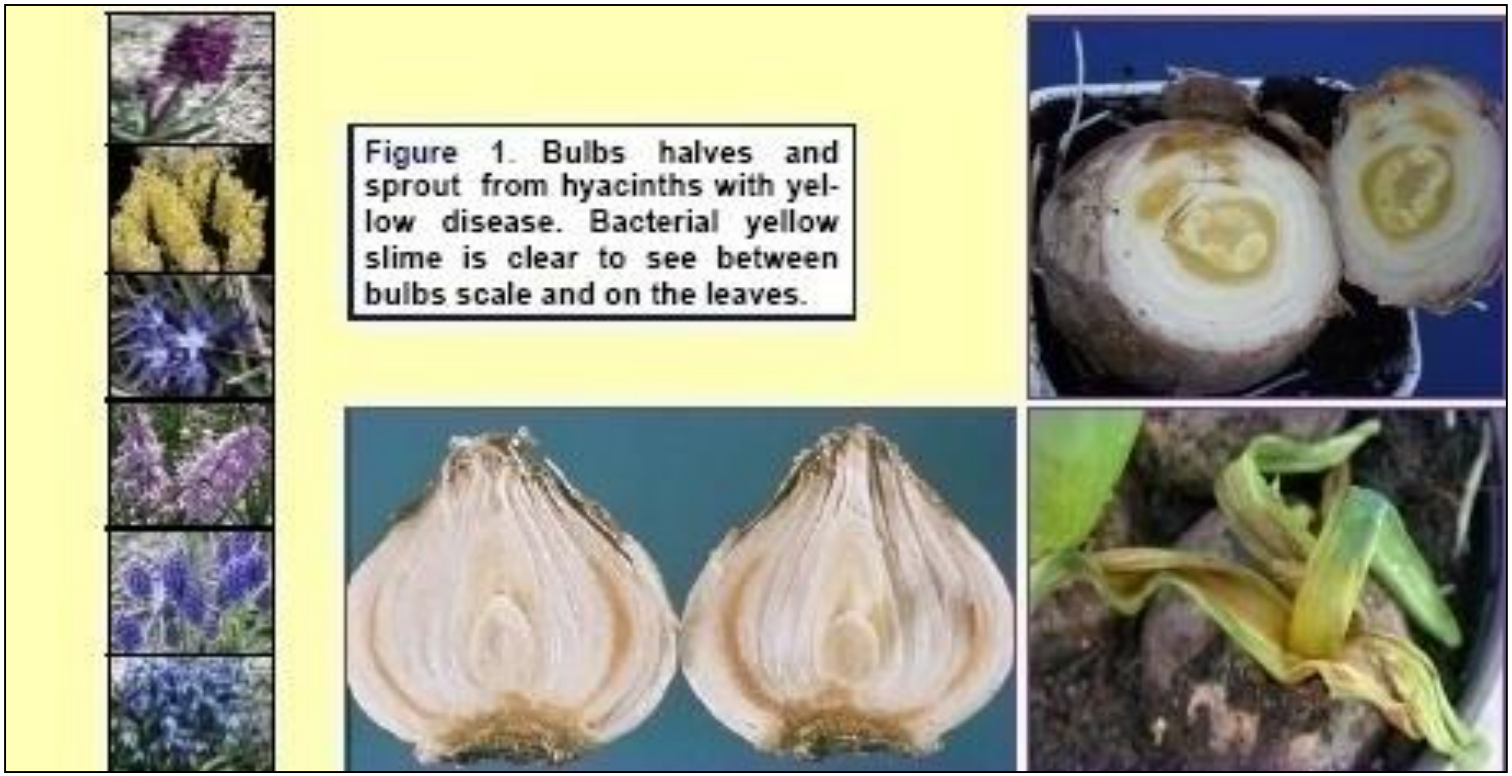


Figure 1. Bulbs halves and sprout from hyacinths with yellow disease. Bacterial yellow slime is clear to see between bulbs scale and on the leaves.

Miglino *et al.*,

Bacterial diseases of strawberry
Two Xanthomonas-caused diseases

- The quarantine bacterium *Xanthomonas fragariae* causes angular leaf spots on strawberry.
- Its population structure was recently found to be divided into four (sub)groups resulting from two distinct main groups.
- *Xanthomonas arboricola* pv. *fragariae* causes bacterial leaf blight, but the bacterium has an unclear virulence status on strawberry.

Gétaz *et al.*,2020

Bacterial diseases of strawberry
Two Xanthomonas-caused diseases

1. *X. arboricola* pv. *fragariae* (bacterial leaf blight of strawberry):

- Only on some cases, induce water-soaked areas along the midribs.
- The presence of bacterial exudate was never observed.

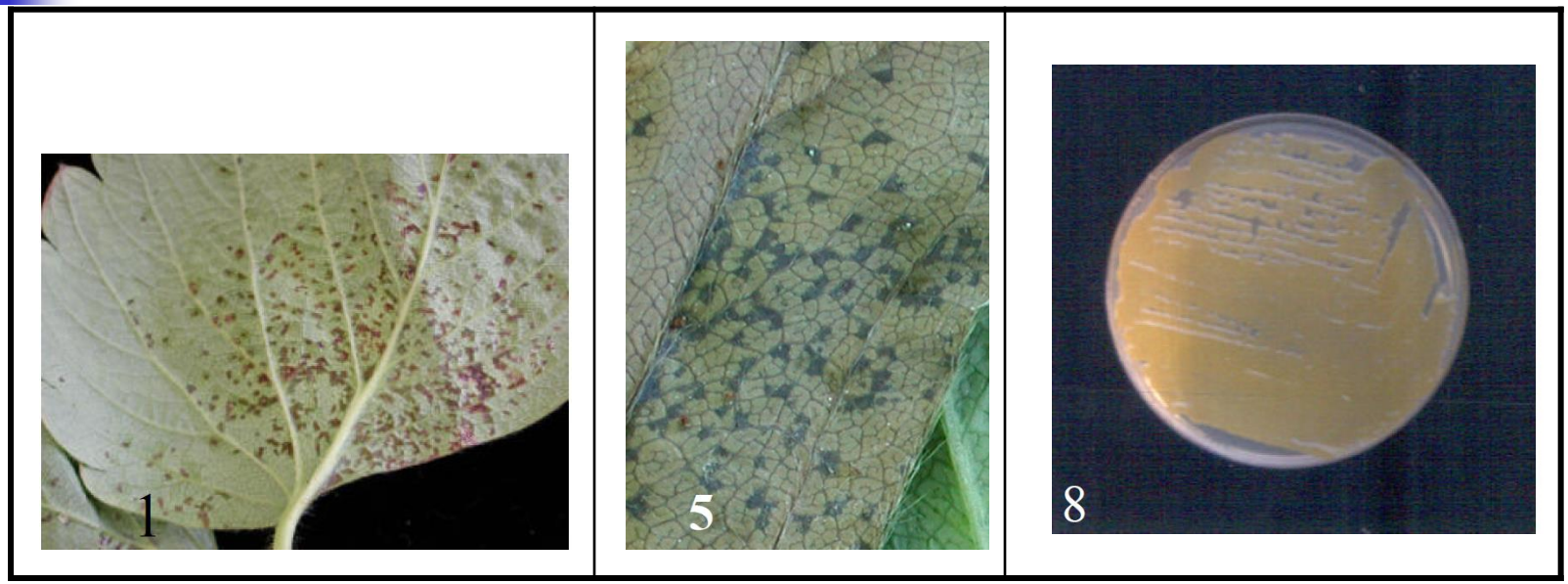
2. *X. fragariae* strains (angular leaf spot of strawberry):

- All induce water-soaked symptoms.
- Samples with young lesions should be examined for the presence of bacterial ooze as this is the best indictor that the cause is bacterial.

Janse,2001;D. Noble

Angular leaf spot of strawberry

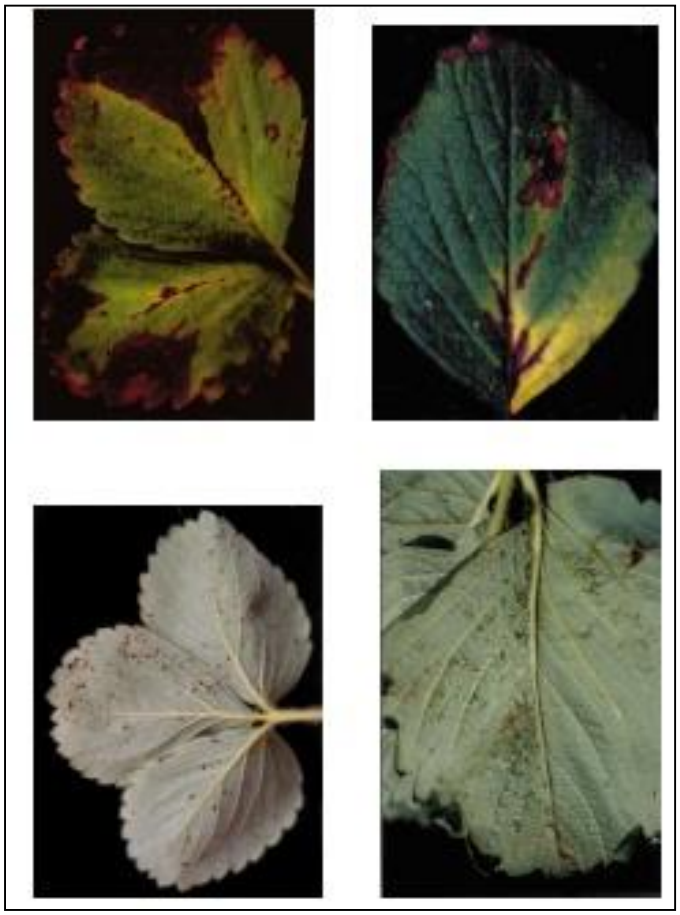
Xanthomonas fragariae



Bacterial leaf blight

Xanthomonas arboricola pv. *fragariae*

- On the lower leaf surface, small, reddish-brown lesions, which were neither watersoaked nor translucent, were observed as the initial stage of the disease (Fig. 1).
- On the upper leaf surface, such lesions appeared as reddish spots.
- The presence of bacterial exduate was never observed.
- After some time the lesions enlarged and became surrounded by a chlorotic halo.
- In some cases along the leaf margin, large brown V-shaped lesions surrounded by a chlorotic halo were also observed (Fig. 2).
- Water soaking, as found with infections of *X. fragariae* (Fig. 3).

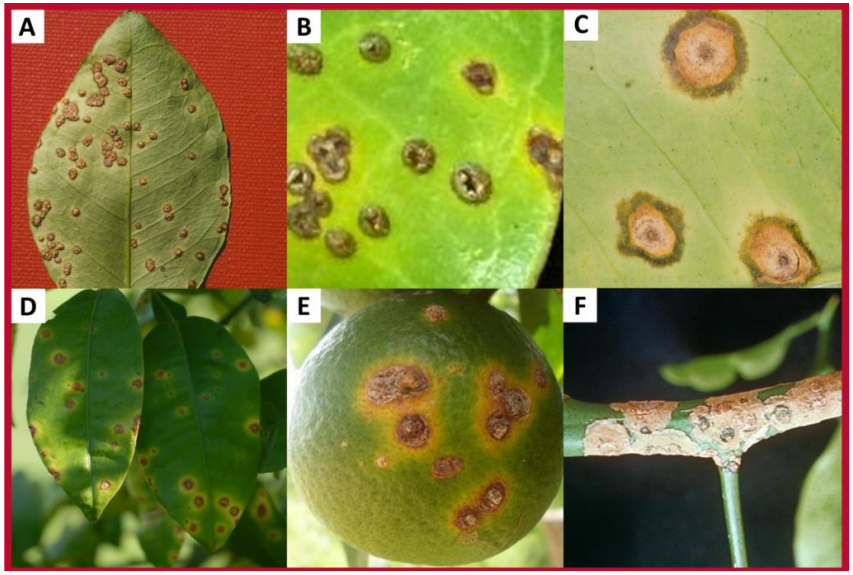


Janse,2001

Groups of citrus canker-causing bacteria *Xanthomonas citri* pv. *citri* (*Xcc*), and *X. citri* pv. *aurantifolii*

- Citrus canker lesions:

A. Raised canker lesions on lower leaf surface.
B. Corky lesions with crater in center.
C. Water-soaked margin around necrotic area.
D. Yellow halos around leaf lesions.
E. Canker lesions on fruit.
F. Canker lesions on stem.



Xanthomonas citri pv. *citri* (*Xcc*), and *Xanthomonas citri* pv. *aurantifolii* (*Xca*) are causal agents of Citrus Bacterial Canker (CBC). These two pathovars cause the so-called Asiatic and South American citrus bacterial canker, respectively.

Licciardello *et al.*,2022;The University of Georgia,2023

Diversity of xanthomonads associated with pepper and tomato pathogens

⇒*X. axonopodis* pv. *vesicatoria* (A group)
Proposal to rename as *X. euvesicatoria*.
⇒*X. vesicatoria* (B group)
⇒*X. perforans* (C group)
⇒*X. gardenri* (D group)

Jones *et al.*,2004

Diversity of xanthomonads associated with pepper and tomato pathogens
Groups, Host ranges and Races

A. Groups:

- *Xanthomonas campestris* pv. *vesicatoria* was found to be composed of two genetically and phenotypically distinct groups :

1. *X. axonopodis* pv. *vesicatoria (*A), and
2. *X. vesicatoria* (B).

- Two more bacterial spot-causing xanthomonads pathogenic on tomato and pepper plants, assigned to groups C and D.
- Based on <70% DNA relatedness, A, C, and D groups were renamed as:

1. *X. euvesicatoria* (A),
2. *X. perforans* (C), and
3. *X. gardneri* (D).

- B group was also retained in *X. vesicatoria.*

B. Host ranges:

1. *X. euvesicatoria* and *X. vesicatoria* cause significant losses on both tomato and pepper,
2. *X. gardneri* affects mainly tomato, and
3. *X. perforans* affects only tomato.

C. Races:

- On tomato plants three races, designated tomato races 1, 2, and 3 (T1, T2, and T3), were originally differentiated based on their reactions on three tomato genotypes.

Kizheva *et al.*,2011

Pathogenicity tests and pathothype determination

- The pathogenicity of the all isolates was confirmed and the pathotype was determined.

1. Pepper-tomato (pt) pathotype:

- 40 strains caused typical necrotic spot on tomato and pepper.

2. Tomato (t) pathotype:

- 14 isolates infected only tomato plants and belonged to the tomato (t) pathotype.

3. Pepper (p) pathotype:

- The rest 17 strains possessed characteristics of pepper (p) pathotype infecting only pepper plants and causing a common hypersensitive reaction on the leaves of tomato plants.

Kizheva *et al.*,2011

Race determination of tomato pathotype strains

- Races of the tomato pathotype strains were determined by the use of differentiator lines hawaii7998 and Hawaii7981 according to the scheme described by Jones *et al.*,1995 at the 4-5 leaf stage by the vacuum infiltration method.
- In the (pt) pathotype population (40 strains) the distribution of the races was as follows:
- 5 strains – raceT1,
- 26 strains –race T2,
- 9 strains – raceT3.
- In (T) pathotype population (14 strains):
- 8 strains – raceT1,
- 3 strains – raceT2,
- 3 strains – raceT3.

Kizheva *et al.*,2011

Pathogenicity tests, pathotype, and race determination

Designation of the *Xanthomonas* strains

Isolate/ strain	Host	Part of plants	Year	Pathogenicity	Pathotype	Race
3B	pepper	fruit	2006	Tomato/Pepper	PT	T2
14B	pepper	foliage	2006	Tomato/Pepper	PT	T1
18B	pepper	foliage	2006	Tomato/Pepper	PT	T3
20B	pepper	foliage	2006	Tomato/Pepper	PT	T2
22B	pepper	foliage	2006	Tomato/Pepper	PT	T2
25B	pepper	foliage	2006	Tomato/Pepper	PT	T2
31B	pepper	foliage	2006	Tomato/Pepper	PT	T3
36B	pepper	foliage	2006	Tomato/Pepper	PT	T3
37B	pepper	foliage	2006	Tomato/Pepper	PT	T3
43B	pepper	stem	2006	Tomato/Pepper	PT	T1
49B	pepper	foliage	2006	Tomato/Pepper	PT	T1
50B	pepper	foliage	2006	Tomato/Pepper	PT	T2
52B	pepper	foliage	2006	Tomato/HR	P	-
55B	pepper	stem	2006	Tomato/Pepper	PT	T2
62B	pepper	foliage	2006	Tomato/Pepper	PT	T2
70B	pepper	foliage	1999	Tomato/Pepper	PT	T1
73B	pepper	foliage	1999	Tomato/Pepper	PT	T3
75B	pepper	foliage	1999	Tomato/Pepper	PT	T2
83B	pepper	foliage	2002	Tomato/Pepper	PT	T3
89B	pepper	stem	2002	Tomato/Pepper	PT	T2
93B	pepper	seedlings	2002	Tomato/Pepper	PT	T1
94B	pepper	foliage	2003	Tomato/Pepper	PT	T2
97B	pepper	foliage	2003	Tomato/Pepper	PT	T2

Kizheva *et al.*,2011

Classification

Based on a polyphasic approach

- The core genome of *Xanthomonas* species much more closely predicts host association.
- *Xanthomonas* now comprises >20 species, many with pathovars.

1. Genus determination is largely based on 16S rDNA sequencing but it can also be achieved by fatty acid analysis.
2. The species determination within each can be differentiated by traditional tests.
3. Species determination is best achieved by gene sequencing e.g. *gyrase B* gene, which show good correlation with DNA homology. GyrB sequencing indicates c 7/8 new species.
4. Pathovar determination does not always rely on prior determination of species and traditional methods for identification and diagnosis are still very useful.

Plant Bacteriology Manual,2008; David Stead

Classification

Based on phytopathogenicity

- **A classification based on phytopathogenicity**
- In the past, the classification of the members of this genus has been based primarily on the criterion of host specificity.
- This practice, denounced as the 'new host – new species' concept led to a complex genus, finally containing more than 100 species.
- Extensive taxonomic examination of Xanthomonads shown that the phytopathogenic specialization of the bacteria is not correlated with the actual relationships within the genus.
- At present, non-pathogenic xanthomonads are frequently isolated from plant material.
- This indicates an actual continuous nature of biodiversity in the genus *Xanthomonas*.

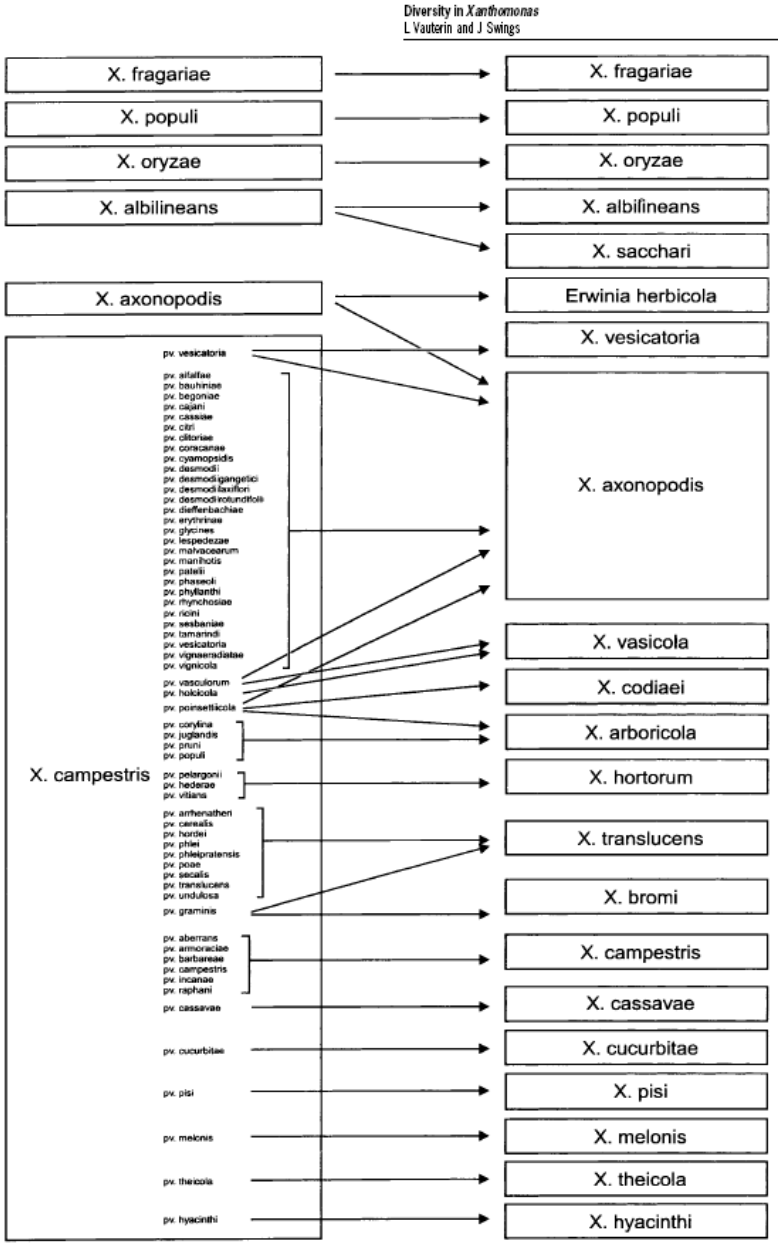
Classification
Based on DNA homology

- **A classification based on DNA hybridization matrices**
- Among DNA-based methods for bacterial characterization, DNA-DNA reassociation is considered to be a major determinant for definition of bacterial species.
- A hybridization value of 70 % or greater has been used to group related pathovars into species of *Xanthomonas*.
- Based upon total genomic DNA homology, the genus has been reclassified into 20 species.

Classification

Based on DNA homology

- Schematic representation of the rearrangements proposed within the genus *Xanthomonas*, resulting from a global taxonomic study of more than 1000 strains and DNA hybridization experiments between 183 selected strains.



Vauterin and Swings,1997

Classification

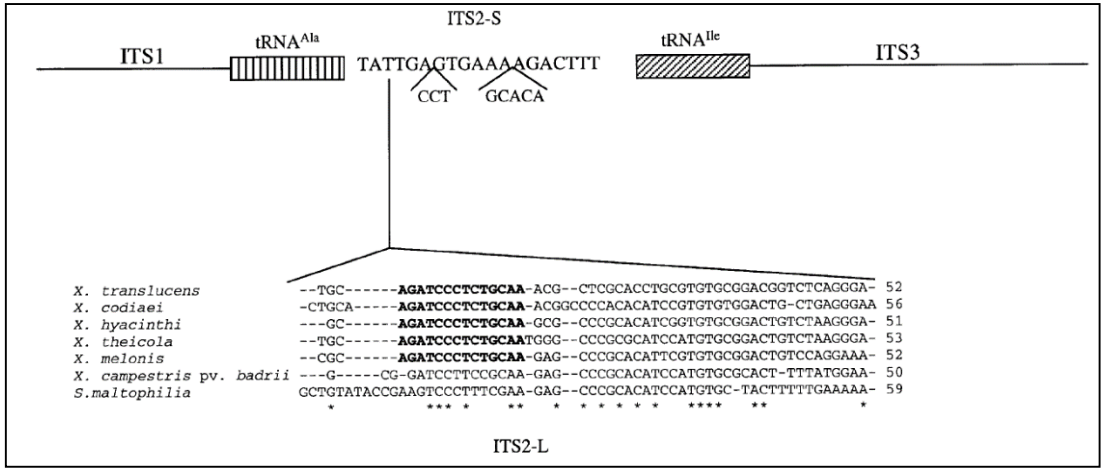
Based on 16S-23S rDNA ITS

- Nevertheless, several other criteria have also been applied for differentiation at the species, pathovar or strain levels.
- Comparative sequencing of rDNA subunits has been used to study the phylogeny of *Xanthomonas* and *Pseudomonas*.
- However, highly restricted variability has been shown in 16S rDNA within the genus *Xanthomonas*:
- All strains that showed 70% or more DNA–DNA homology exhibited 100% 16S rDNA sequence similarity.
- Better discrimination was obtained by analysis of the 16S-23S rDNA intergenic spacer (ITS) sequence, which showed approximately nine-fold higher diversity than 16S rDNA.

Classification

Based on 16S-23S rDNA ITS

- In bacteria and archaea, ITS is located between the 16S and 23S rRNA genes.
- In Xanthomonads, the tRNAs were highly conserved and divided the ITS sequence into three regions (ITS1, ITS2 and ITS3). Major differences in size were found in the ITS2 region.
- Most *Xanthomonas* species had a shorter sequence nt (named ITS2-S), but five species exhibited a longer sequence, of 78-85 bases (ITS2-L).



Gonçalves and Rosato,2002;..

Classification

Based on Length and GC content of ITS sequences from *Xanthomonas* species

Strain	Spacer length (bp)				G + C content (mol %)
	ITS1	ITS2	ITS3	Total	
X. albilineans ICMP 196	106	14	223	497	53·50
X. arboricola pv. *juglandis* ICMP 35^T	109	19	226	507	51·08
X. axonopodis pv. *axonopodis* LMG 538^T	113	18	232	519	51·63
X. axonopodis pv. *passiflorae* ICMP 3151^P	113	18	229	507	53·06
X. bromi LMG 947^T	112	19	228	510	51·96
X. campestris pv. *campestris* LMG 568^T	112	19	225	509	50·69
X. cassavae LMG 673^T	113	19	228	513	51·66
X. codiaei LMG 8678^T	113	79	228	578	54·30
X. cucurbitae LMG 690^T	110	19	219	501	52·49
X. fragariae LMG 708^T	114	19	226	512	51·40
X. hortorum pv. *hederae* LMG 733^T	113	19	226	510	51·17
X. hyacinthi LMG 739^T	103	75	166	493	55·57
X. melonis LMG 8670^T	112	75	230	569	53·08
X. pisi LMG 847^T	110	19	227	510	50·39
X. sacchari LMG 471^T	106	15	218	492	53·05
X. theicola LMG 8684^T	105	77	208	541	57·11
X. translucens pv. *translucens* LMG 876^T	103	76	208	540	55·37
X. vasicola pv. *holcicola* LMG 736^T	113	19	227	510	50·98

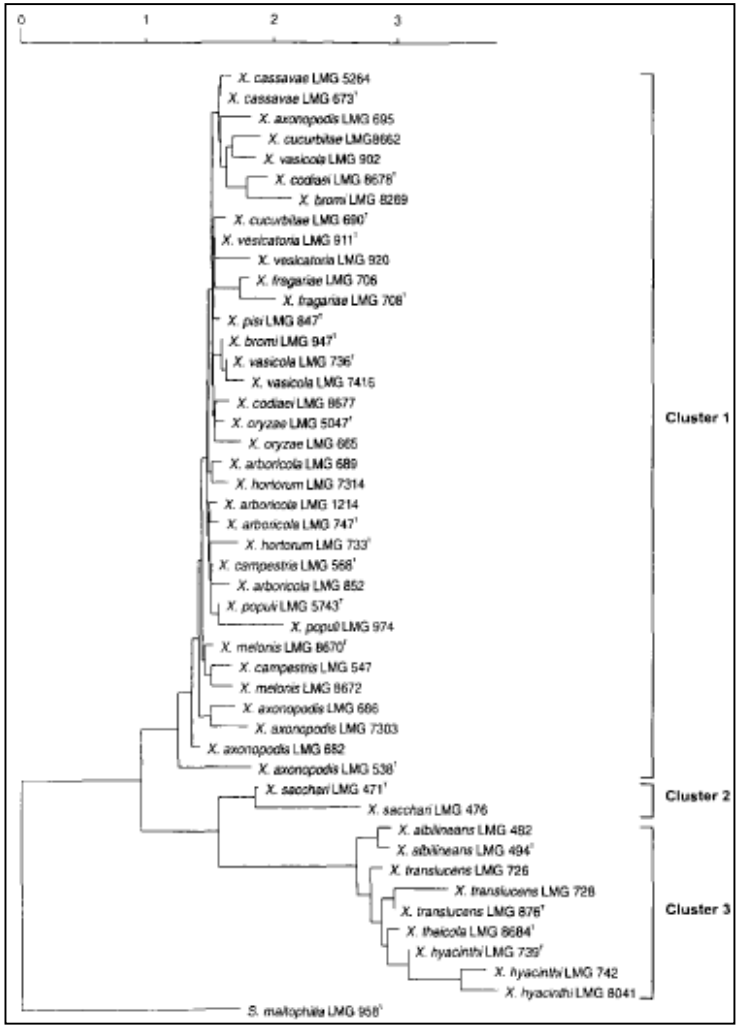
Gonçalves and Rosato,2002

Phylogeny

Based on of *Xanthomonas* species based upon 16S rDNA sequences

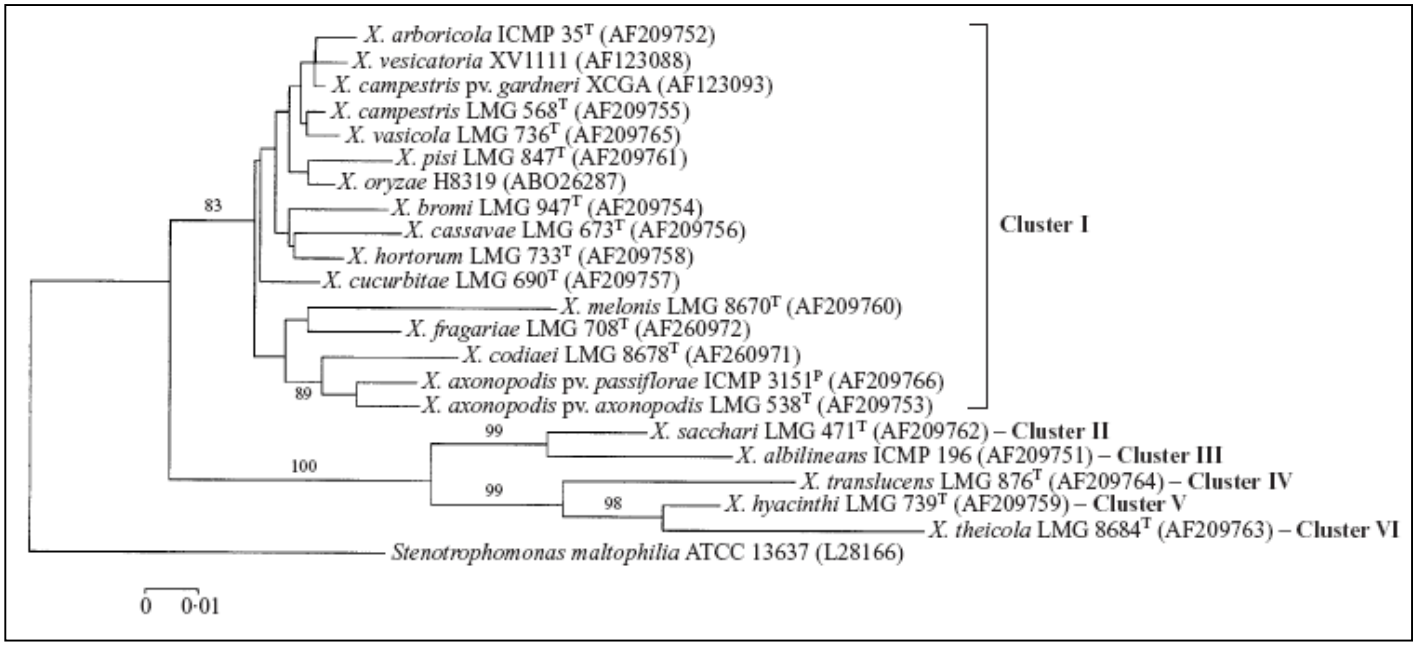
- Phylogenetic relationships based on pairwise comparisons of partial 16s rDNA sequences of all of the *Xanthomonas* strains examined, constructed by neighbor-joining.
- The sequences were BLASTed against NCBI's 16S rRNA GenBank.
- Clusters 1 and 2 represent the *X. campestris* and *X. albilineans* cores, respectively.

Hauben *et al.*,1997



Phylogeny

Based on of *Xanthomonas* species based upon 16S-23S rDNA ITS sequences



Six major clusters with bootstrap values higher than 98% were formed.

Classification

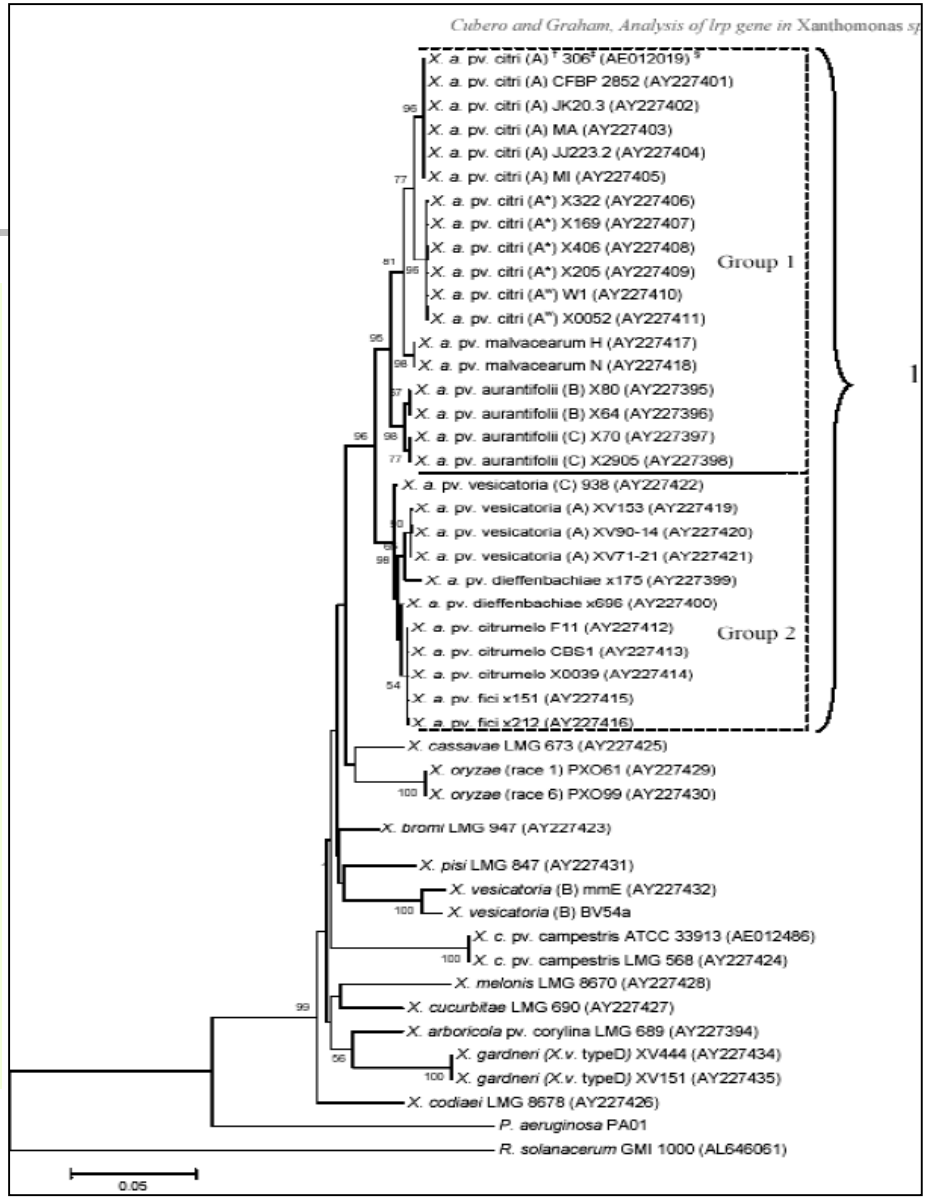
Based on *lrp* gene

Leucine-responsive regulatory protein

- Because of universal presence of *lrp* gene in genus *Xanthomonas*, as well as in other Gram-negative bacteria, and the fact that horizontal transmission is improbable, analysis of the *lrp* gene may be a widely adaptable tool for polyphasic taxonomic studies of bacteria.
- This method should be particularly valuable as a complement to current rDNA- and rep-PCR-based approaches, due to its capability for high resolution at the specific and infraspecific levels.

Phylogeny based on Leucine responsive regulatory protein (*LRP*) analysis

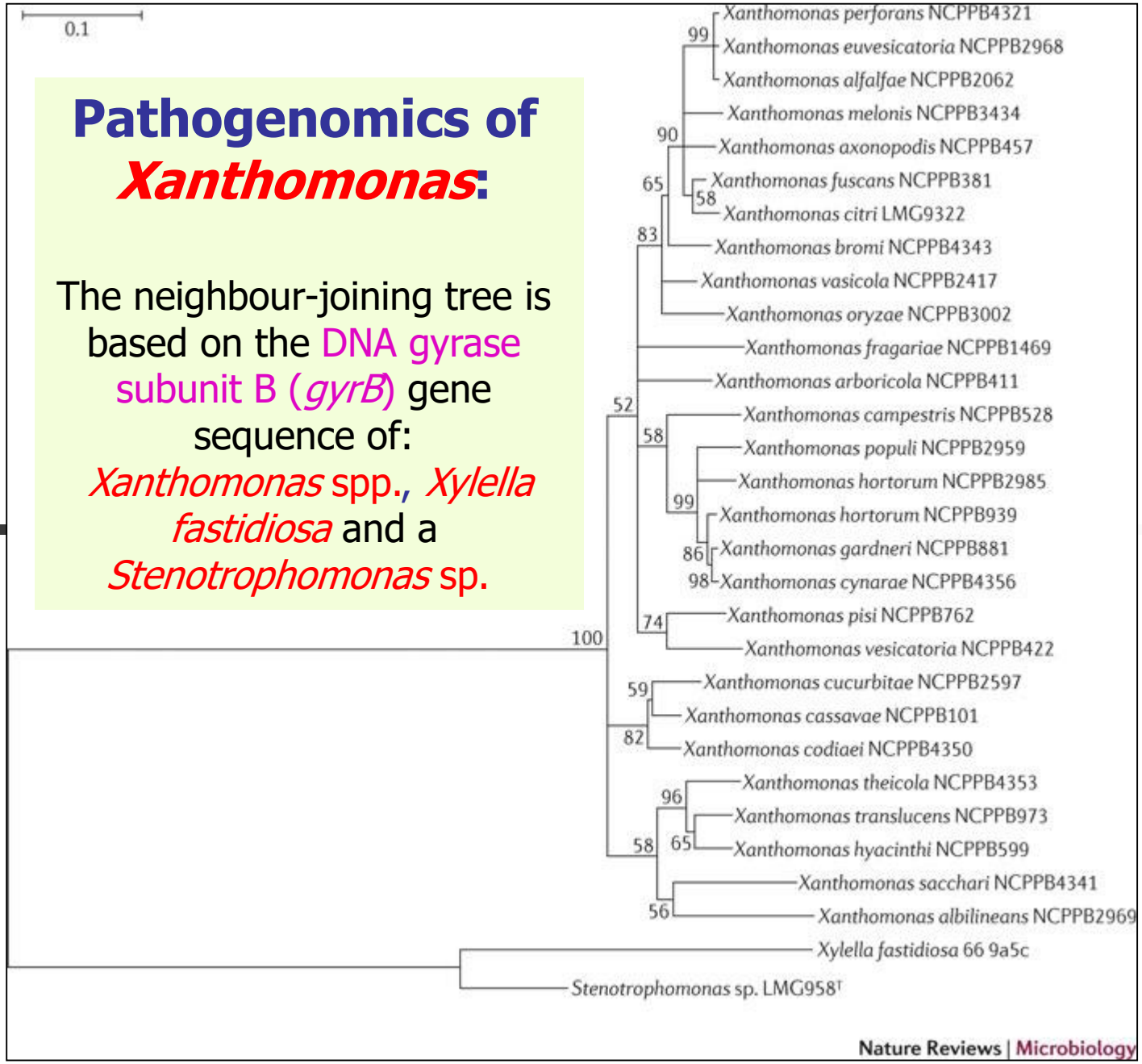
- A dendrogram based on pairwise comparison of all *lrp* sequences showed that *Xanthomonas* species grouped together, with *P. aeruginosa* and *R. solanacearum* as outgroups.
- The largest cluster within *Xanthomonas* was cluster 1 which encompassed all strains that are presently proposed as pathovars of *X. axonopodis* (Vauterin *et al.*,2000).



Cubero & Graham,2003

Pathogenomics of *Xanthomonas*:

The neighbour-joining tree is based on the DNA gyrase subunit B (*gyrB*) gene sequence of: *Xanthomonas* spp., *Xylella fastidiosa* and a *Stenotrophomonas* sp.



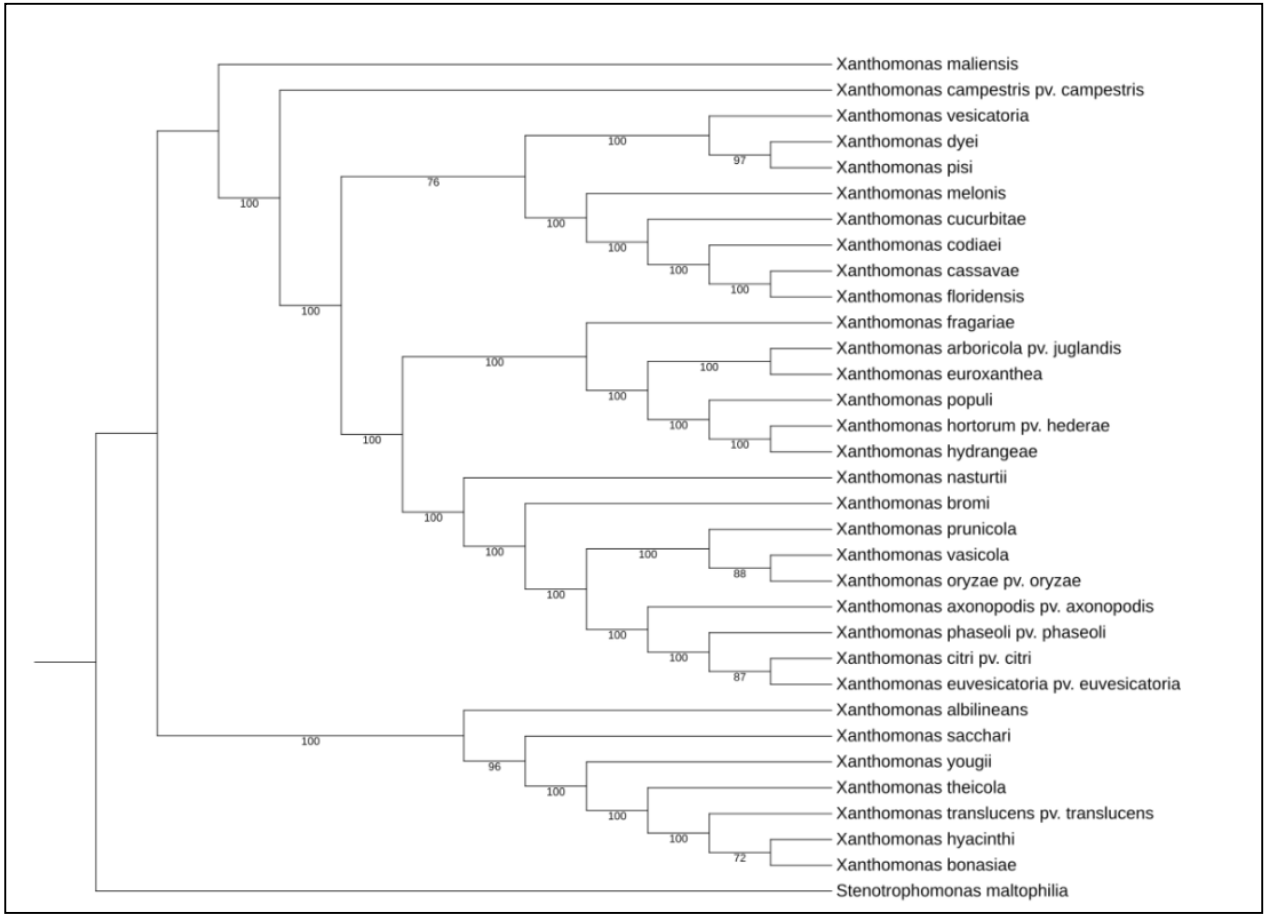
Ryan *et al.*,2011

Celebrating the 20th anniversary of the first *Xanthomonas* genome sequences

- Phylogenetic tree of the 32 valid species of *Xanthomonas* provided after TYGS analysis (Meier-Kolthoff *et al.*,2022).
- Tree inferred with FastME 2.1.6.1 (Lefort *et al.*, 2015) from GBDP distances calculated from genome sequences retrieved from Genbank.
- The branch lengths are scaled in terms of GBDP distance formula d5.
- The numbers on branches are GBDP pseudo-bootstrap support values > 70% from 100 replications, with an average branch support of 97.2% (Farris, 1972).
- The Newick file was edited in iTOL (https://itol.embl.de/) and rooted on the outgroup *Stenotrophomonas maltophilia*.
- The complete list of genomes and GenBank Assembly accession numbers are available in Supplementary Table S2.

Koebnik and Cesbron *et al.*,2024

Celebrating the 20th anniversary of the first *Xanthomonas* genome sequences



Koebnik and Cesbron *et al.*,2024

Proposed names

Based on16S-23S ribosomal intergeneric spacer sequences (Schaad *et al.*,2006)

Proposed name
Xanthomonas citri
X. citris subsp. *citri*
X. citris subsp. *malvacearum*
Xanthomonas alfalfae
Xanthomonas alfalfae subsp. *alfalfae*
Xanthomonas alfalfae subsp. *citrumelonis*

Note: *Xanthomonas fuscans* proposed by Schaad *et al.*,2006 is considered to be a synonym of *X. citri* (Euzeby,2021)

Isolation and Preservation
YPSA medium

- A loopful of the suspension was streaked on YPSA plates (Yeast extract, 5 g; Peptone, 10 g; Sucrose, 20 g; Agar, 12-15g in 1 liter distilled water with pH 7.4 and autoclaved at 121^{o}C for 15 minutes).
- The plates were incubated at 28^{o}C for 48-72 hours according to Schaad and Stall,1988.
- Bacterial colonies from each plate were further sub cultured and pure bacterial colonies were transferred to YPSA slants incubated at 28^{o}C for 48-72 hours and preserved at 4^{o} C for further work.

Differential Medium
Asparagine medium

- All Gram-negative isolates were allowed to grow on Asparagine medium (Asparagine, 0.5 g; KH_2PO_4, 0.1 g; $MgSO_4.7H_2O$, 0.2 g; KNO_3, 0.5 g; $CaCl_2$, 0.1 g; NaCl, 0.1 g and agar, 12-15 g in 1 liter distilled water with pH 7 and autoclaved at 121°C for 15 minutes) at 28°C for 48-72 hours without any other carbon and nitrogen sources (Dye 1962 cited in Bradbury,1984).
- This is used as a diagnostic test for *Xanthomonas* because they are not able to grow on it while others like yellow Enterobacteriaceae and many Pseudomonads can grow on it.
- In all cases, uninoculated medium was taken as negative control.
- Isolates that grew on Asparagine but which formerly showed yellowish mucoid growth and found grown alone on YPSA medium initially were included in further tests.

Bobosha,2003

Differential Medium
Levan medium

- **Growth on Nutrient agar with 5% Glucose**
- Each isolate was streaked on nutrient agar with 5% glucose (Nutrient agar, 23g; 5% Glucose in 1 liter distilled water with pH 7 and autoclaved at 121˚C for 15 minutes) and incubated at 28°C for 48-72 hours.
- Mucoid and yellow colony growth on this medium is one of the characteristics that differentiate *Xanthomonas campestris* from other *Xanthomonas* species (Bradbury,1984).

Bobosha,2003

Differential Medium

Xan-D medium

Adapted from Tween medium and milk-Tween medium

- The basal medium consisted of 10 g of Bacto-Soytone, 10 ml of Tween 80, 10 g of potassium bromide, and 15 g of Bacto agar in 500 ml of water.
- Skim milk solution was prepared by adding 10 g of skim milk to 500 ml of distilled water.
- The basal medium and skim milk solution were autoclaved separately and mixed when still hot to a final volume of 1 liter. After being cooled to 50 to 60°C, the above medium was supplemented with 24 mg of bromothymol blue (1.5 ml of 1.6% [wt/vol] bromothymol blue in ethanol) per liter under sterile conditions and poured into petri dishes.
- The resulting Xan-D medium had a pH of 6.5 and a light orange-yellow color.
- Bromothymol blue is a pH indicator which changes color from yellow to green to blue when pH transitions from 6.0 to 7.6. Cycloheximide (75 μg/ml), cephalexin (cefalexin) (65 μg/ml), and 5-fluorouracil (12 μg/ml) were added as necessary to prevent the overgrowth of contaminating fungi and bacteria in plant materials.
- The Xan-D medium amended with the three antibiotics was called Xan-D(CCF) hereafter.

Lee *et al.*,2018

Differential Medium
Xan-D medium

- To test the color development of *Xanthomonas* spp. and other bacteria on the Xan-D medium, four to six bacterial cultures were streaked per plate and incubated for 3 to 4 days at 28°C.
- For observation of colony morphology, 100 µl of bacterial suspension (10^3 CFU/ml) was pipetted onto the surface of the Xan-D medium and spread evenly with an L-shaped rod. The plate was incubated for 3 to 4 days at 28°C prior to observation.

1. The colonies of xanthomonads tested on the Xan-D medium were also yellow-green, mucoid, circular, convex, and surrounded with two zones, a bigger clear zone of skim milk hydrolysis and a smaller milky zone of Tween 80 lipolysis.
2. Most yellow nonxanthomonads isolated from plant seeds, such as *Arthrobacter* sp., *Pseudomonas* sp., and *Pantoea* sp., were flat without a milky zone, remained yellow, and did not turn green on the medium.

Lee *et al.*,2018

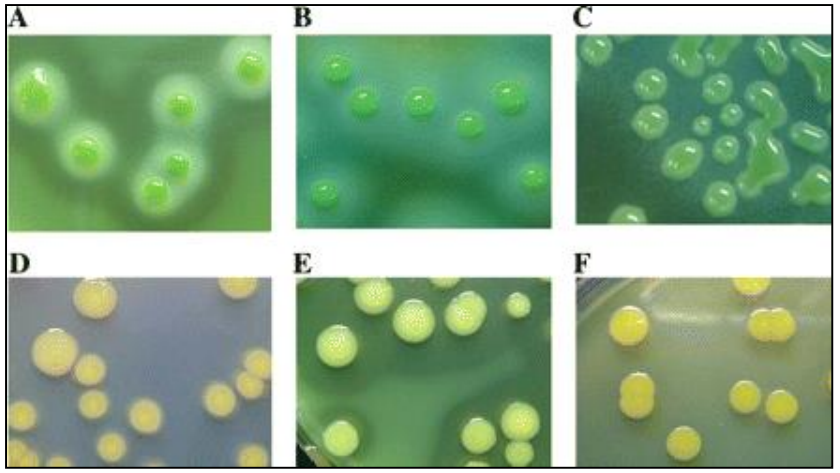
Differential Medium

Xan-D medium

Adapted from Tween medium and milk-Tween medium

- Colony color and morphology of xanthomonads and yellow nonxanthomonads on the Xan-D medium incubated at 28°C for 4 days.

A. *X. campestris* pv. *campestris* XCC1-1;
B. *X. campestris* pv. *coriandri* NPCCB 1457;
C. *X. citri* pv. *citri* XW134;
D. *Pseudomonas* sp. strain LS1-1;
E. *Arthrobacter* sp. strain Y6;
F. *Pantoea* sp. strain YBB12.



The colonies of xanthomonads were yellow-green, mucoid, circular, convex, and surrounded with two zones, a bigger clear zone of skim milk hydrolysis and a smaller milky zone of Tween 80 lipolysis.

Lee *et al.*,2018;..

Diagnostic tests

Characters used to differentiate *Xanthomonas* from other yellow pigmented bacteria

- *X. fragariae* is distinguishable from other phytopathogenic xanthomonads by at least seven characteristics:

1. No growth at 33°C
2. No hydrolysis of aesculin
3. No acid from arabinose, galactose, trehalose or cellobiose
4. 0.5-1.0% maximum NaCl tolerance (D. Noble).

Character[a]	*Xanthomonas*	*Pseudomonas*	*Flavobacterium*	*Pantoea*
Flagellation	1, polar	>1, polar	None	Peritrichous
Xanthomonadin	Yes	No	No	No
Fluorescence	No	Yes	No	No
Litmus milk	Alkaline[b]	Alkaline	Unchanged	Not Determined
Growth at 40°C	Yes[c]	No	Yes	Yes
Levan from sucrose	Yes	Yes	No	No
H_2S from cysteine	Yes	No	No	No
Oxidase	Negative	Negative[d]	Positive	Negative
Fermentative	No	No	No	Yes
Growth on:				
0.1% TTC	No	Yes	Yes	Yes

[a] For flagellation, fluorescence, levan, H_2S, oxidase, fermentation, and TTC, see pp. 10, 97, 93, 47, 10, 9, 154, respectively.
[b] *X. fragariae* is unchanged
[c] *X. fragariae* will not grow at 40°C
[d] P. *cichorii* is positive

Schaad *et al.*,2001

Diagnostic tests

Diagnostic tests for differentiation of several *Xanthomonas* spp.

Tests	*campestris*[a]	*fragariae*	*albilineans*	*cassavae*	*hyacinthi*	*oryzae*	*pisi*	*transculens*
Mucoid growth on YDC	+	+	-	+	+	+	+	+
Growth at 35°C	+	-	+	+	+	+	+	+
Growth on SX	+	-	-	-	-	-	+	-
Starch hydrolysis	+	+	-	+	+	-	+	+
Esculin hydrolysis	+	-	+	+	+	+	+	+
Protein digestion	+	-	-	+	+	+	+	+
Litmus milk	Alk	Alk	NC	Alk	NC	NC	Alk	Alk
Ice nucleation	-	+	-	-	-	-	-	+
Acid from:								
Arabinose	+	-	-	-	-	-	-	-
Utilization of:								
Glycerol	+[D]	-	+	-	-	-	+	-
Melibiose	V	-	-	-	-	-	+[D]	-

[a] These results are typical for pv. *campestris*; many other pathovars were not tested.

Abbreviations: +, 80% or more strains positive; -, 80% or more strains negative; V, between 21-79% of strains positive; +[D], delayed or weak positive; ND, not determined; Alk, alkaline; NC, no change. Data taken from Vauterin et al. (52) and original data (NWS).

Schaad *et al.*,2001

Arabinose, cellobiose and fructose were selected for this test because utilization of these sugars can be used to differentiate *Xanthomonas campestris* from other pathovars (Bradbury,1984).

Diagnostic tests

Characteristics of some *Xanthomonas* species that can be determined by classical methods[a,b]

Characteristic	*X. albilineans*	*X. fragariae*	*X. oryzae*	*X. populi*
Mucoid growth on nutrient agar + 5% glucose	–	+	+	+
Hydrolysis of gelatin	D	+	–[c]	–
H_2S from peptone	–	–	+	–
Maximum growth temperature, °C	37	33	32	27.5
Maximum salt tolerance (%, w/v)	0.5	0.5–1.0	0.5–2.0	0.4–0.6
Acid production from:[d]				
Arabinose	–	–	d[e]	–
Glucose, sucrose	+	+	+	+
Mannose	+	+	+	+
Galactose	d	–	+	+
Trehalose	–	–	+	+
Cellobiose	–	–	+	–
Fructose	–	+	+	+
Lactose	–	–	–	–
Maltose	–	–	–	–
Xylose	+	–	–	
Utilization of:				
Acetate, citrate, malate			+	
Propionate		–	+	
Succinate		+	+	
DL-Tartrate	–	–		
Benzoate	–	–		
Growth on Oxoid NA:				
None	+			
Good			+	
Poor to very poor		+		+

[a]for symbols see standard definitions.

[b]Data from Dye (1962, 1966b); Ridé and Ridé (1978, 1992; Swings et al. (1990); Vera Cruz et al. (1984).

[c]Reaction is positive for pathovar *oryzicola*.

[d]In the medium of Hayward (1964).

[e]In the medium of Starr (1946).

Bergey's Manual of Systematic Bacteriology,2005

Diagnostic tests

Discriminating bacteriological tests for preliminary identification and differentiation of pathovars of *Xanthomonas oryzae*

	X. oryzae pv. *oryzae*	*X. oryzae* pv. *oryzicola*
Gram staining	-	-
Oxidase test	-[a]	-[a]
2-ketoglucontate production	-	-
Fluorescence on King's B medium	-	-
Nitrate reduction	-	-
Acetoin production	-	+
Oxidation-fermentation of glucose	O/-	O/-
Gelatin hydrolysis	-/v	-/v
Starch hydrolysis	-	+
Sensitive to 0.001% cupric nitrate (w/v)	+	-
Utilization of L-alanine as carbon source	-	+/v
Growth on 0.2% vitamin-free casamino acids	-	+
Strong peptonization of litmus milk	-	+
Phenylalanine deaminase	-	+[b]

+ = positive; - = negative; O = oxidative; v = variable

[a] Weak positive reactions can be observed.

[b] Positive response in 50% of strains.

Sullivan,2011

Diagnostic tests

Morphological and biochemical characteristics that differentiate *Xanthomonas oryzae* pv. *oryzae and Xanthomonas oryzae* pv. *oryzicola*

	X. oryzae pv. *oryzae*	*X. oryzae* pv. *oryzicola*
Size	0.5-0.8 X 1.3-2.2 µm	0.4-0.6 X 1.0-2.5 µm
Oxidase test	–	–
Oxidation-fermentation (O-F) test	0	0
Nitrate reduction	–	–
2-ketogluconate production	–	–
Starch hydrolysis[a]	±	+
Carbon source utilization		
Trehalose	+	+
Inositol	–	–
Alanine	–	+
0.001% $CuNO_3$	+	–

[a] Bradbury (1986) registered Xoo as – and Xcola as +. Swings et al (1990) registered Xoo as +, + = positive, – = negative, 0 = oxidative.

Mew and Misra,1994

Diagnostic tests

Conventional and BIOLOG tests which differentiate the artichoke strains of *Xanthomonas* spp.

Test	*Xanthomonas* species																			
	1	2	3	4	5	6	7	8	9	10	11	2	13	14	15	16	17	18	19	20
Conventional tests																				
Gelatin hydrolysis	+	+	+	+	+	+	+	−	−	−	+	+	+	+	−	−	+	−	+	−
Starch hydrolysis	−	−	+	+	+	+	−	−	+	−	−	+	+	+	+	−	−	−	+	−
Milk proteolysis	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	−
Tween esterase	+	−	+	+	−	+	−	−	+	−	+	+	+	−	−	+	−	−	+	+
Acidification of:																				
Arabinose	−	−	−	−	−	−	−	+	−	−	+	+	+	+	−	+	−	−	−	−
Cellobiose	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Glucose	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Mannose	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
BIOLOG tests																				
D-Raffinose	+	−	+	−	−	+	−	−	+	−	−	+	+	−	+	−	−	−	+	−
Formic acid	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−
α-Ketobutyric acid	+	−	−	+	+	+	−	−	−	−	−	+	+	+	+	−	−	−	+	−
Propionic acid	−	−	−	+	−	+	−	−	+	−	−	+	+	−	+	−	−	−	+	−
D-Saccharic acid	−	−	−	−	−	+	−	+	−	−	−	+	+	−	+	−	−	−	+	−
L-Asparagine	+	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−
L-Aspartic acid	+	+	−	+	−	+	+	+	−	−	−	+	−	−	−	+	+	−	+	−
L-Ornithine	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
L-Threonine	+	−	+	+	+	−	−	−	−	−	−	+	+	−	+	−	−	−	+	−
Urocanic acid	+	−	−	+	−	−	+	+	−	+	−	+	−	+	+	−	−	−	+	−
Uridine	+	−	−	+	+	−	−	−	−	−	−	+	+	−	−	−	−	−	+	−
D,L-α-Glycerophosphate	+	−	+	+	−	−	−	−	+	−	−	+	−	−	+	+	+	−	+	−

+, 90-100% of the strains are positive ; -, 0-10% of the strains are positive. Species (from CFBP): 1, *X. cynarae*; 2, *X. fragariae*; 3, *X. hortorum* pv. *pelargonii*; 4, *X. arboricola* pv. *juglandis*; 5, *X. cassavae*; 6, *X. codiaei*; 7, *X. bromi*; 8, *X. cucurbitae*; 9, *X. phaseoli*; 10, *X. oryzae* pv. *oryzae*; 11, *X. vasicola* pv. *holcicola*; 12, *X. pisi*; 13, *X. melonis*; 14, *X. vesicatoria*; 15, *X. campestris* pv. *campestris*; 16, *X. translucens* pv. *translucens*; 17, *X. hyacinthi* ; 18, *X. theicola*; 19, *X. sacchari*; 20, *X. albilineans*.

Trébaol *et al.*,2000

Diagnostic tests

Comparison of biochemical and physiological characteristics of bacterial leaf blight pathogen of strawberry(*X. arboricola* pv. *fragariae*) and angular leaf spot of strawberry(*X. fragariae*)

	Bacterial leaf blight pathogen (= *X. arboricola* pv. *fragariae*)	*X. fragariae*
Gram stain	–	–
Growth on YDC	YMFa	YMS
Growth on GYCA	YMFa	YMS
Growth on BSCAA	+	–
Metabolism of glucose	O + F –	O + F –
Oxidase	– or slow	–
Tobacco hypersensitivity	+	–
Assimilation of D-galactose, glycerol, Tween 80, maltose, cellobiose, L-glutamic acid	+	–
Arginine dihydrolase	–	–
Catalase	+	+
Nitrate reduction	–	–
Esculin hydrolysis	+	–
Starch hydrolysis	+	–
Urease	–	–
Gelatin hydrolysis	+	+
Tween 80 hydrolysis	+	–
Digestion of milk proteins	+	–
Max. NaCl tolerance (%)	2·5	0·5
Growth at 4°C	+	–
Max. growth temperature	39°C	32°C
Cellulase activity	+	–
Presence of pectic/pectinolytic enzymes	+	–
Soft rot of potato/carrot slices	+	–
Ice-nucleation activity	–	–

Y, yellow; M, mucoid; Fa, fast growth; S, slow growth,

Janse,2001

Diagnostic tests

Comparison of biochemical and physiological characteristics of bacterial leaf blight pathogen of strawberry(*X. arboricola* pv. *fragariae*) and angular leaf spot of strawberry(*X. fragariae*)

Utilization of:	*X. euvesicatoria*	*X. vesicatoria*	*X. perforans*	*X. gardneri*
Dextrin	+	+	+	–
Glycogen	+	V	V	–
N-acetyl-D-glucosamine	+	V	+	–
D-galactose	+	V–	+	–
Gentibiose	+	V	+	–
α-D-lactose lactulose	V	V–	+	–
Acetic acid	V	–	+	–
Cis-aconitic acid	+	–	V	–
Malonic acid	+	V	+	–
Propionic acid	V–	V	+	–
D-alanine	V	V	+	–
Glycyl-L-aspartic acid	–	V–	+	–
L-threonine	V	V–	+	–

+ = positive reaction by all strains; V, 50% or more of strains utilized compound; V–, <50% of strains utilized compound; –, none of strains utilized compound.

Jonse,2000

Xanthomonadins

A useful chemotaxonomic markers for *Xanthomonas*

- All yellow *Xanthomonas* spp. produce xanthomonadins.
- Xanthomonadins are membrane bound, brominated aryl polyene esters pigments.
- These brominated, aryl-polyene, yellow, water-insoluble pigments that are associated exclusively with the outer membrane of the bacterial cell wall.
- In general, xanthomonadin is a unique pigments for *Xanthomonas* which can be easily distinguished from other yellow pigmented bacteria.

Xanthomonadins

A useful chemotaxonomic markers for *Xanthomonas*

- The xanthomonadins were initially thought to be carotenoids, but later Xanthomonadins were considered as a unique class of carotenoid-like pigments produced by members of the phytopathogenic genus *Xanthomonas*.
- Xanthomonadins and carotenoids are chemically different, but both having polyene chains.

Polyenes are poly-unsaturated organic compounds that contain one or more sequences of alternating double and single carbon–carbon bonds.
Aryl is any functional group or substituent derived from an aromatic ring, usually an aromatic hydrocarbon, such as phenyl and naphthyl.

Carotenoid pigments
In yellow-pigmented bacteria

- Carotenoids are yellow-orange, water soluble pigments that occur widely in plants, algae and photosynthetic bacteria.
- Zeaxanthin (3,3'-dihydroxy-β-carotene) is a natural, fat-soluble, yellowish carotenoid that is found in photosynthetic bacteria such as *Sphingomonas* spp. and *P. agglomernas*.
- Canthaxanthin (4,4’-diketo-β-carotene) is produced by Photosynthetic *Bradyrhizobium* strains.
- Carotenoids function as:
- A blue light-harvesting pigment (antenna or accessory pigment) for photosynthesis,
- Protectant against the toxic effects of UV radiation and oxygen radicals.

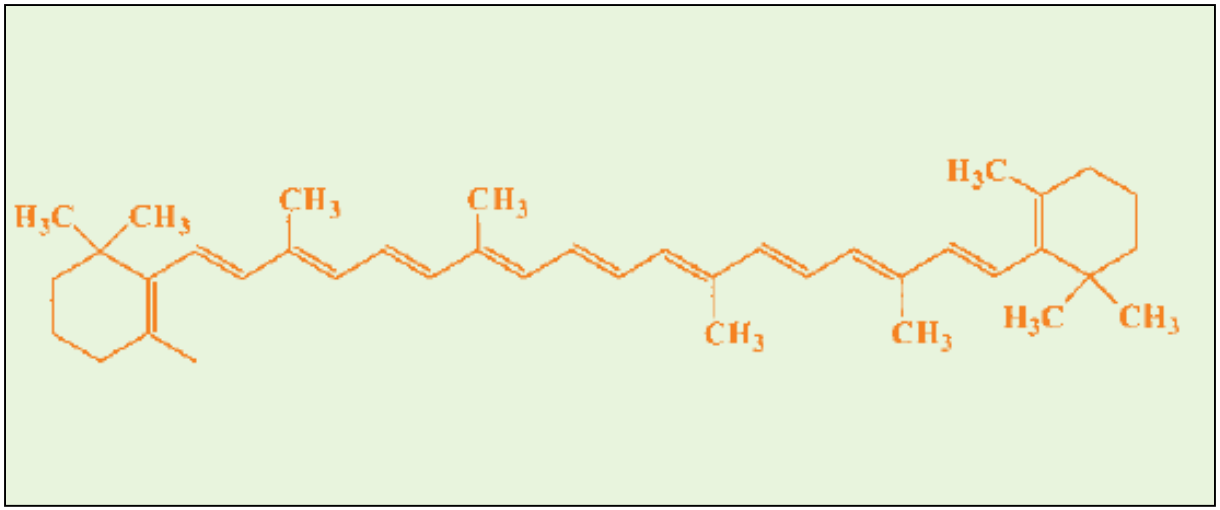
Absorption spectra
Carotenoids vs xanthomonadins

- *Xanthomonas* spp. show a peak at 440 nm and shoulders at 420 and 460 nm characteristic of xanthomonadin.
- Whereas, in carotenoid pigments, different pigments absorb different wavelengths of light. E.g.
- The carotenoid extracts of photosynthetic *Bradyrhizobium* strains gave three-peaked spectrum (460, 490, and 525 nm).
- In *Sphingomonas* and *Rhizomonas*, absorption spectrum of is characterized by maxima at 452 and 480 nm.

See also *Curtobacterium* spp.

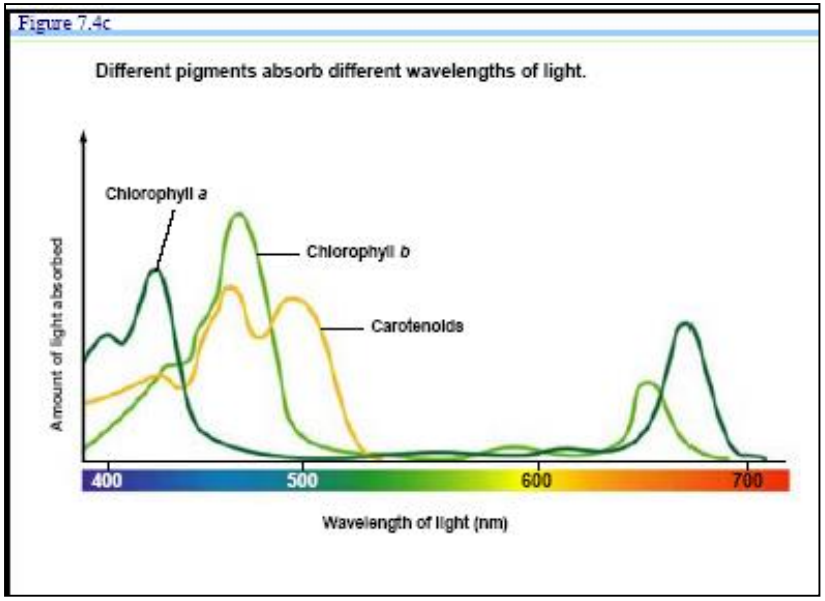
Carotenoids
β-carotene

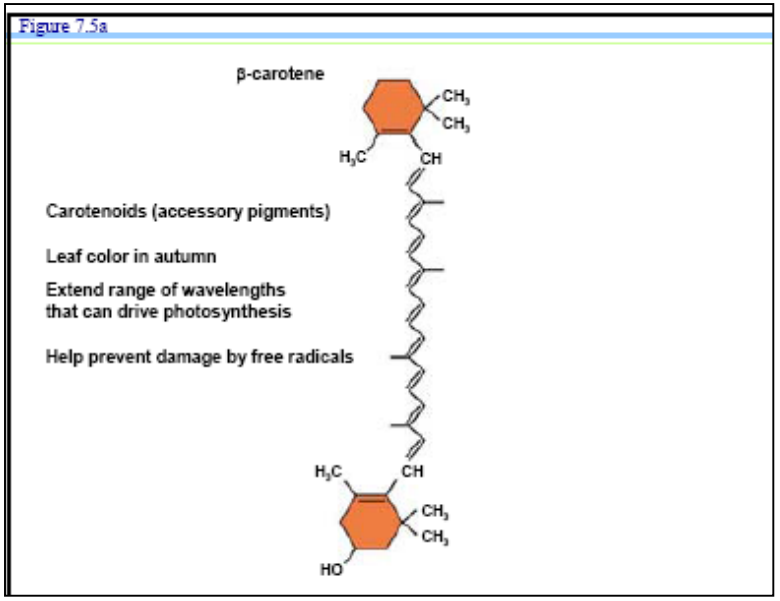
- There are several hundred different carotenoids known but they all have an extended system of conjugated double bonds such as that of β-carotene.



Moss,2002

Carotenoids
β-carotene





See pathogenesis file for terpene functions

See also *Curtobacterium* spp.

Xanthomonadins
Structure

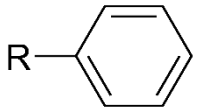
- Xanthomonadins from different xanthomonads differed in bromination and methylation.
- These different bromination and methylation patterns of xanthomonadins were useful for identification of members within the genus.
- Xanthomonadin pigments are differentiated into 15 groups according to:

1. The number of bromine atoms,
2. The absorption maxima and mass spectra value, and
3. Methylation.

Chun,2005

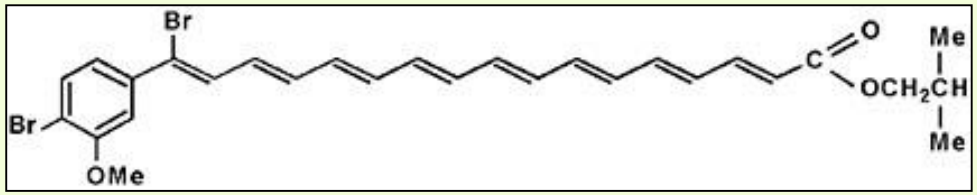
Xanthomonadins

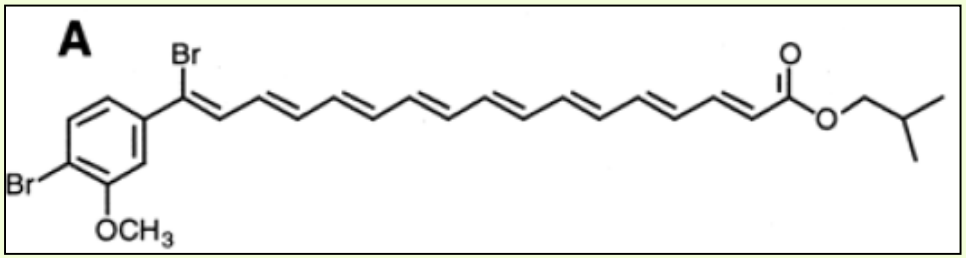
Structure of xanthomonadin I



Aryl

- Structure of xanthomonadin I produced by *Xanthomonas juglandis* (Xaj).





Absorption maxima of xanthomonadins of Xaj are 437 and 463 nm on petroleum ether extracts; 454 and 481 nm on benzene ether extracts; and 441 nm on methanol ether extracts (Starr *et al.*, 1964).

Andrewes *et al.*,1976

Biological Role of Xanthomonadins/Xanthan

- They confer protection against damage by visible light in the presence of oxygen (photodynamic damage) during the bacterium's epiphytic phase (Biological role of Xanthomonadins).
- When both xanthomonadin and EPS production (Xanthan) are deficient, populations of *X. c.* pv. *campestris* are as much as 1000-fold lower *in planta*.
- This resulted in significantly fewer lesions (from 8.7 to 1.7 lesions per leaf) on spray-inoculated crucifer leaves.
- *Xanthomonas campestris* pv. *campestris* also requires a functional *pig B* for epiphytic survival and host infection (Poplawsky and Chun,1998).

Xanthomonadins

Genetics of xanthomonadin production

pig genes and diffusible factor(DF)

- Xanthomonadin encoding region is called as *pig* region.
- Xanthomonadin production in *Xanthomonas* is regulated by a diffusible factor (DF).
- Subsequent investigations showed that the DFs produced by *Xcc* and *Xoo* are:

1. 3-hydroxybenzoate (3-HBA), and
2. 4-hydroxybenzoate (4-HBA)

Wang *et al.*,2015

Xanthomonadins

Genetics of xanthomonadin production

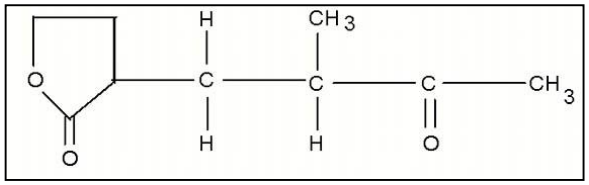
Diffusible factor (DF)

- DF production is essential for both:

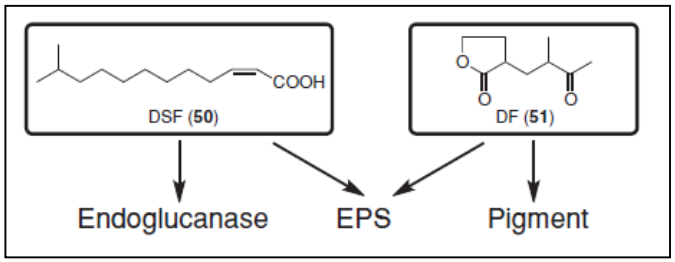
1. Xanthomonadin, and
2. EPS production.

- The function of DF is very similar to that of DSF(a small diffusible signal factor).
- DSF is produced by *rpf* genes, regarding the

1. Quorum sensing mechanism(cell-to-cell signaling);
2. Production of enzymes such as protease, endoglucanase and polygalacturonate lyase.



Chemical structure of the DF in *Xcc*.

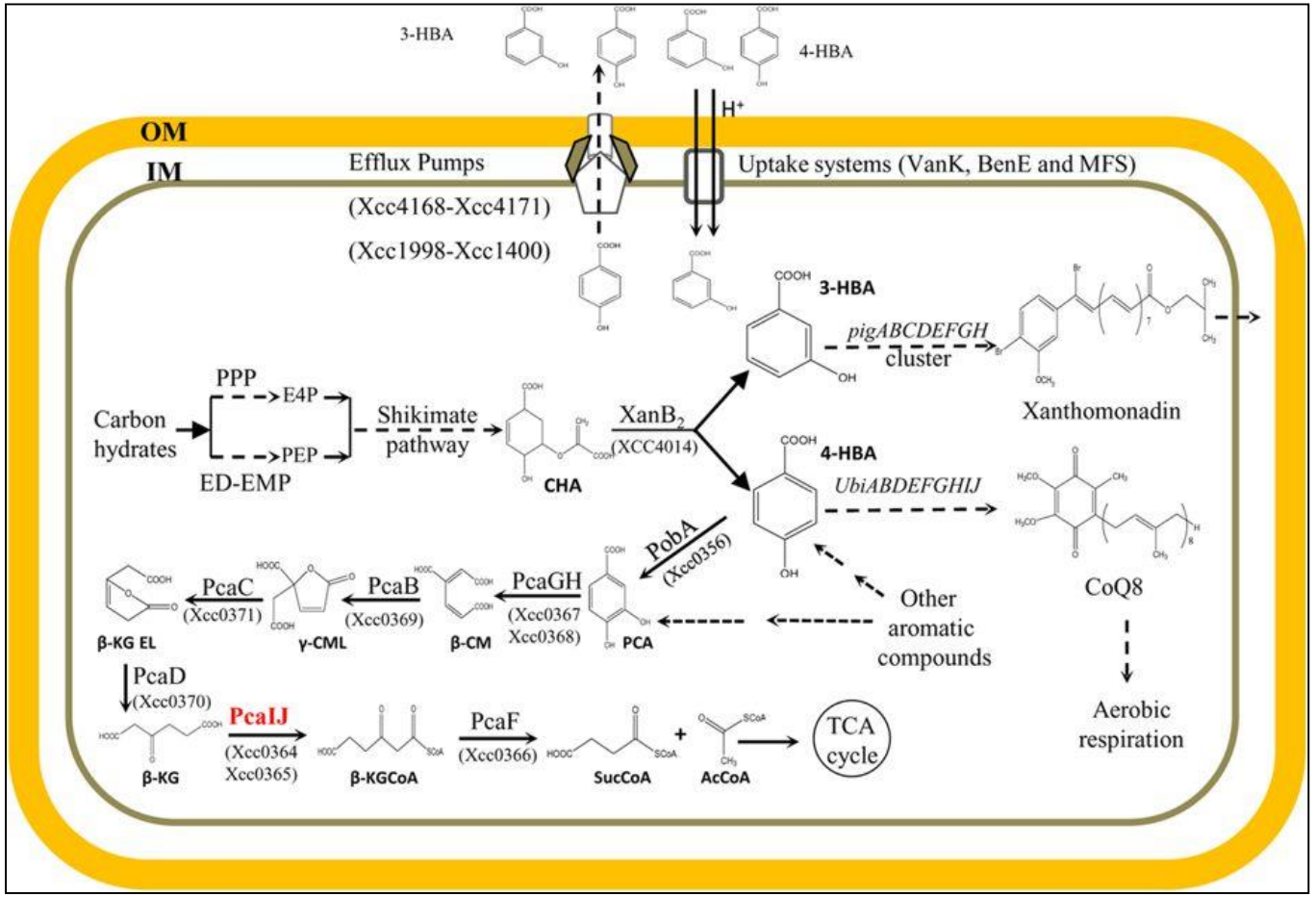


DF and DSF autoregulartory systems in *Xcc*.

Xanthomonadins

Genetics of xanthomonadin production

pig genes and diffusible factor(DF)



Wang *et al.*,2015

Xanthomonadins

Genetics of xanthomonadin production

pig gene cluster

- Xanthomonadin encoding region is called as *pig* region.
- An 18.6-kb genomic region from *X . campestris* pv. *campestris* that contains seven transcriptional units (*pig A-G*) required for xanthomonadin production.
- One of its transcriptional units is known as *pig B*.
- *pigB* is required for production of:

1. Extracellular polysaccharide (EPS),
2. Xanthomonadin pigments, and the
3. Diffusible signal molecule(diffusible factor, DF).

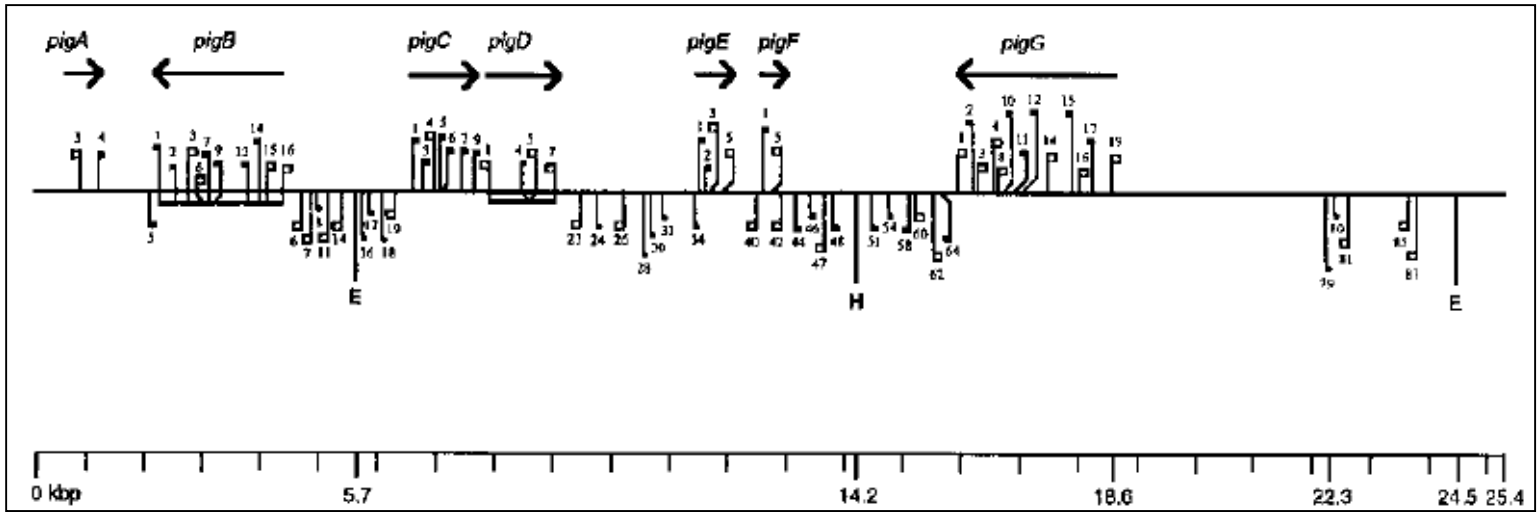
DF production is essential for xanthomonadin, EPS production, as well as for epiphytic survival and host infection.

Chun,2005;..

Xanthomonadin

pig region from *Xanthomonas campestris* pv. *campestris*

- Seven *pig* transcriptional units (*pigA* through *pigG*) were identified by transposon saturation mutagenesis within an 18.6-kbp portion of 25.4-kbp *pig* region from *Xanthomonas campestris* pv. *campestris*.

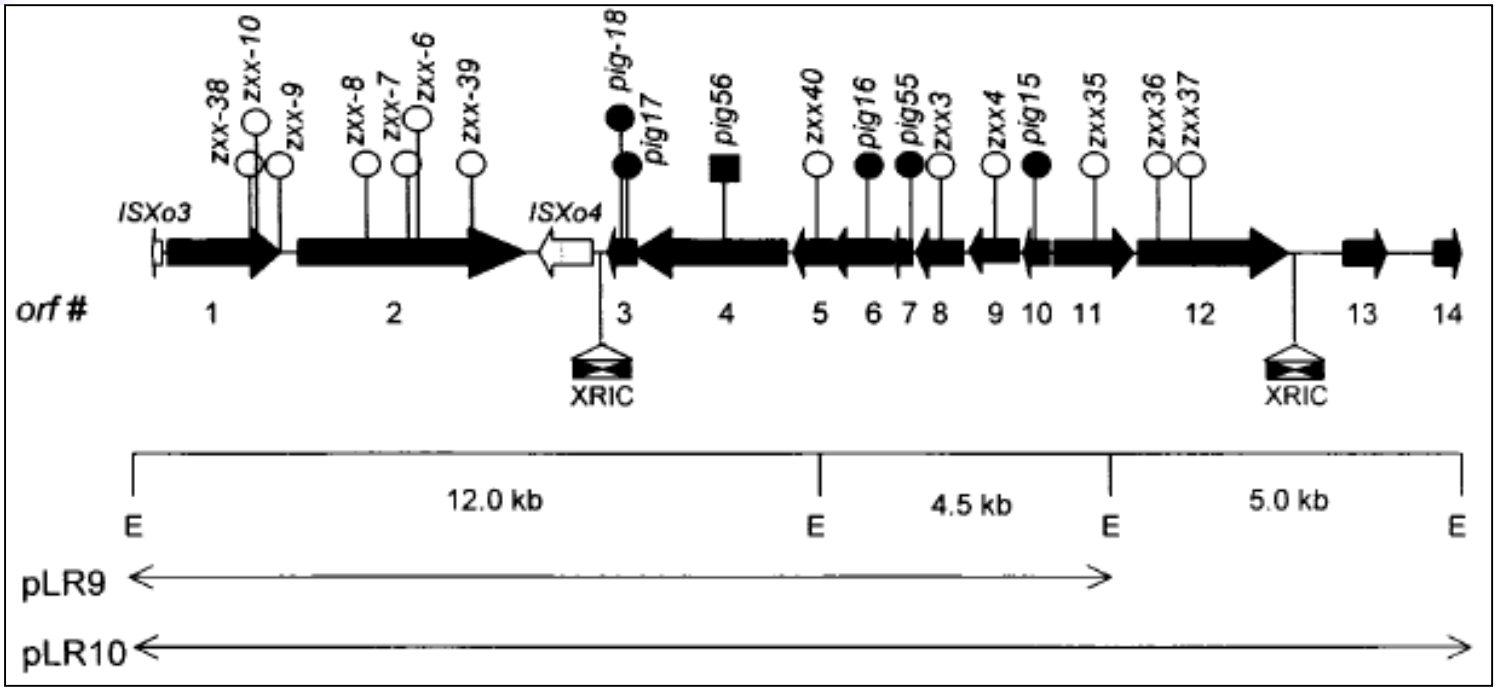


Xanthomonadins
xcc vs. *xoo*

- In *X. campestris* pv.*campestris*, the xanthomonadin gene cluster (*pig*), consists of seven transcriptional units (pig ABCDEFG) scattered within an 18.6-Kbp region in the main chromosome.
- Recently, a 20-Kbp region was partially sequenced and characterized in *Xanthomonas oryzae* pv.*oryzae* (XOO) as a xanthomonadin gene cluster.
- There are 14 genes in this region, of which genes 3, 6, 7, and 10 are required for xanthomonadin production.

Xanthomonadin

pig region from *Xanthomonas oryzae* pv. *oryzae*



Genetic organization of the *pig* locus of *X. oryzae* pv. *oryzae*.

Goel *et al.*,2002

Xanthomonadins

Genetics of xanthomonadin production

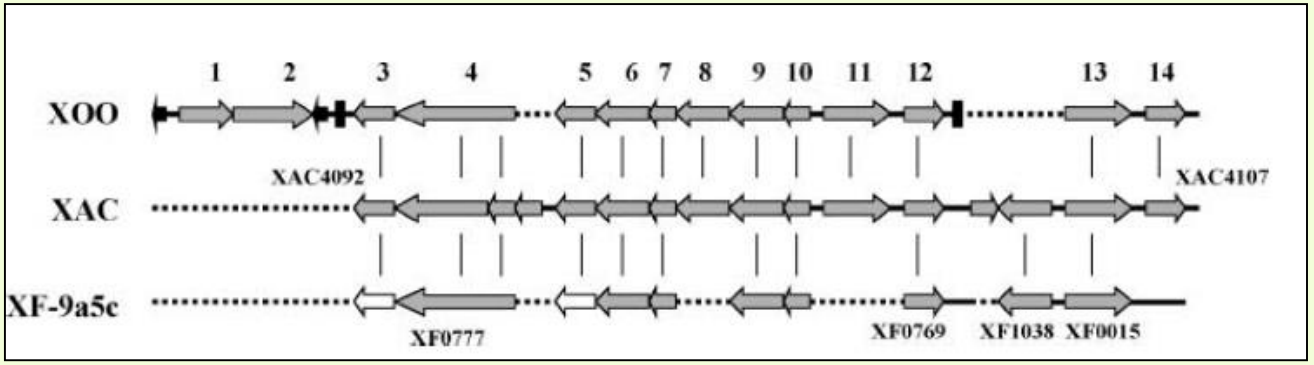
pig gene cluster

- All genes within the cluster appear to be related to the production and modification of the xanthomonadin.
- But, single site *pigB* (*xanB2*) mutants are typically white and produce less EPS.

Chun,2005

Xanthomonadins
XCC, XOO and *X. fastidiosa*

- Schematic alignments of the cluster of xanthomonadins in the genomes of *Xanthomonas oryzae* pv. *oryzae*, *X. axonopodis* pv. *citri* (now XCC), and *X. fastidiosa* (XF-9a5c).
- Gray arrows represent putative genes.
- White arrows represent intergenic regions of the *Xylella* genome identified by blastX to have amino acid similarity to protein sequence of the corresponding genes in *Xoo*.



Presence of Xanthomonadin pigment

Xanthomonadin pigment production

1. Spectrophotometry

- Each isolate was streaked on nutrient agar and incubated at 28^{o} C for 48 hours.
- After 48 h growth, scrape the bacteria from the surface and add to 3 ml of spectrophotometry grade methanol in a test tube with a screw cap.
- Enough bacteria including a known xanthomonad as a control should be added to the methanol to give a turbidity equivalent to near 10^{10} CFU/ml, (approximately 0.5 OD at 600 nm).

Schaad and Stall,1988; Chun,2002

Presence of Xanthomonadin pigment
Spectrophotometry

- Place the capped tube in a boiling water bath until the pigment has been removed from the bacteria (solution becomes yellow).
- The suspension was then centrifuged at 13,000 rpm for 15 minutes to remove cell debris.
- The supernatant was decanted and the methanol was allowed to evaporate to half of the original volume by flash evaporation by keeping the methanol extract in 50-60°C water bath until the optical density of the pigment extract reaches 0.4 at 443 nm.
- The absorption spectrum of the extract was determined using a scanning spectrophotometer.

Schaad and Stall,1988; Chun,2002

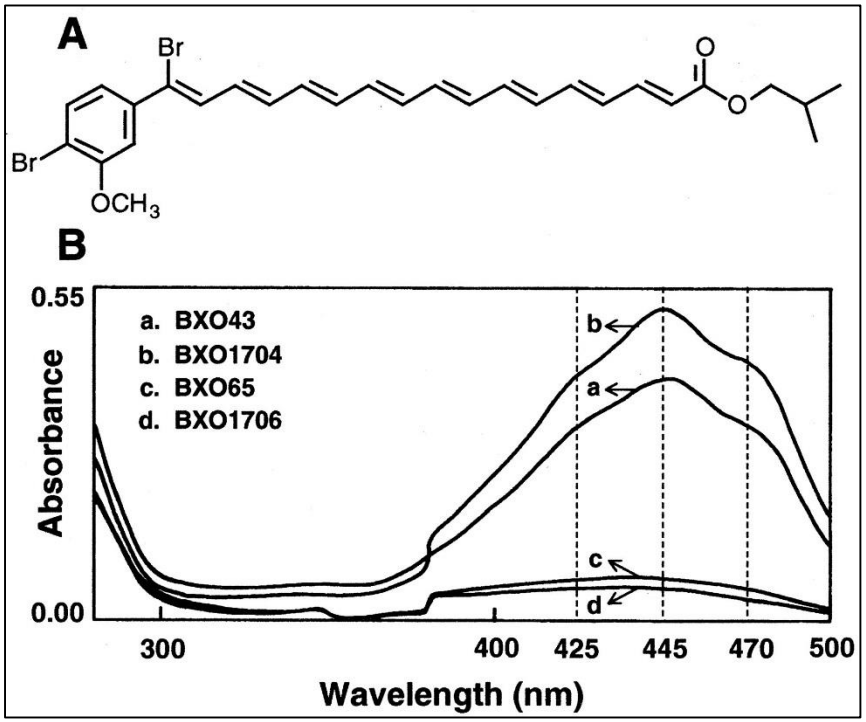
Spectral absorption property

Absorption spectra of xanthomonadin in *X. a.* pv. *oryzae*

Spectrophotometry

- Both wild-type strains showed a peak at 440 nm and shoulders at 420 and 460 nm characteristic of xanthomonadin while the pigment-deficient mutants showed neither the peak nor the shoulders(Rajagopal *et al.*,1997).

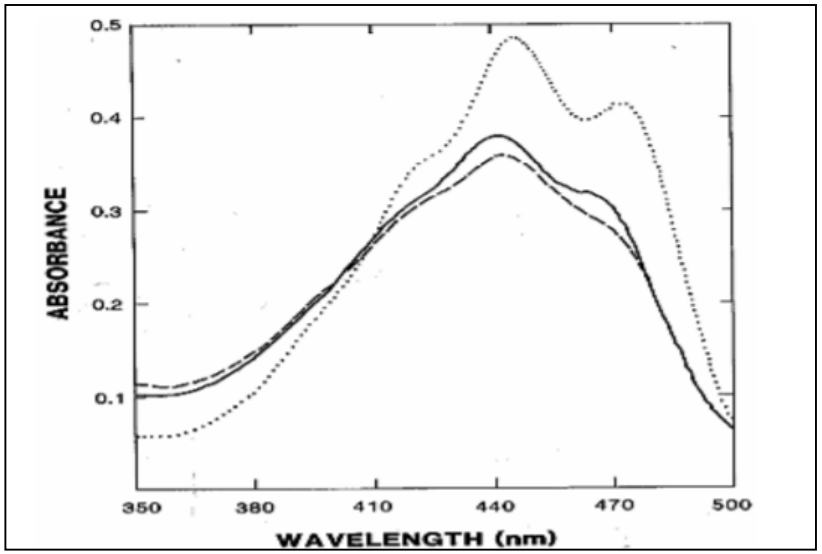
Structure of xanthomonadin I and absorption spectra of crude pigment extracts from *X. oryzae* pv. *oryzae* cultures.



Goel *et al.*,2001

Presence of Xanthomonadin pigment
Spectrophotometry and TLC

1. The absorption maxima of crude pigment extracts from bacterial isolates was 443 nm.
2. Thin-layer chromatography(TLC) showed migration of the pigments at retardation factor (R_f) values of 0.43-0.48 (mean 0.46).



Absorption spectra of crude pigment extracts from *Xanthomonas oryzae* pv. *oryzae*, isolate XOO 1 (solid line), strain of *Pseudomonas paucimobilis* (dotted line), and strain of *Xanthomonas fragariae* (dashed line)

Tolba *et al.*,2011

Presence of Xanthomonadin pigment
2. TLC method

- Spot five 5 µl aliquots on a precoated, thin-layer chromatography sheet of silica gel 60 of 0.2 mm thickness.
- Apply a total of 25 µl per spot, allowing each 5 µl amount to dry before applying the next.
- Place plate in developing apparatus with anhydrous spectrophotometry grade methanol as the solvent.
- Allow the solvent front to move approximately 10 cm.
- Outline the yellow spots with a pencil when the silica gel is still wet.
- A yellow spot with an average Rf value of 0.45 (range of 0.42 to 0.49) is positive for xanthomonadins.

Chun, 2002

Chromatographic property

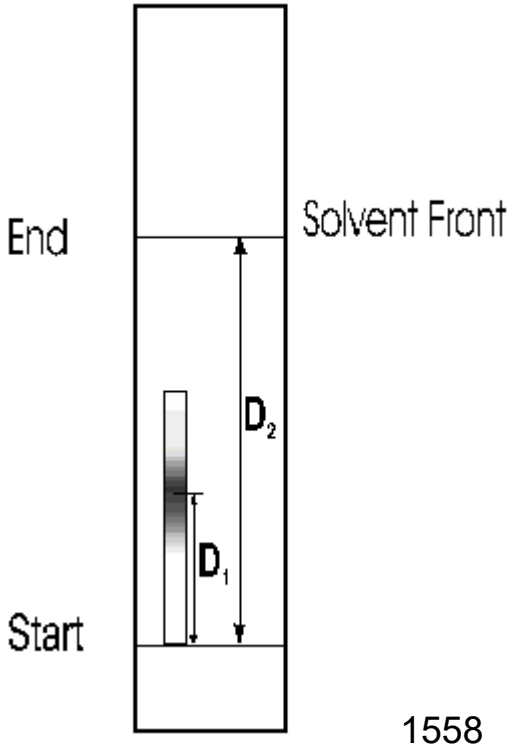
Identification of yellow Xanthomonadin pigment of *Xanthomonas*

TLC method

- Spot five 5 µl aliquots (a total of 25 µl per spot) on TLC silica gel and allow the solvent front to move ca 10 cm.
- A yellow spot with a range Rf (retention factor) value of 0.45 (range of 0.42-0.49) is positive for xanthomonadins.
- The retention factor, Rf, is defined as distance the solute (D1) moves divided by the distance traveled by the solvent front (D2):

  Rf = D1 / D2

- Where:
- D1= distance that color traveled, measured from center of the band of color to the point where the food color was applied
- D2= total distance that solvent traveled.



Xanthan gum

A heteropolymer

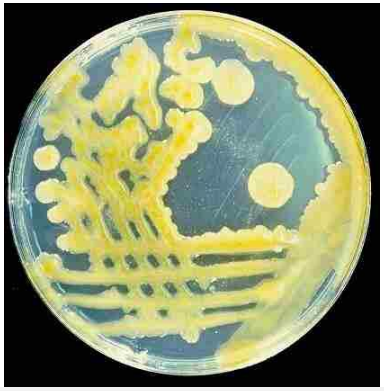
Chemical Structure

- Xanthan or xanthan gum is a complex polysaccharide composed of more than one type of sugar (a heteropolymer).
- Xanthan Gum consists of:

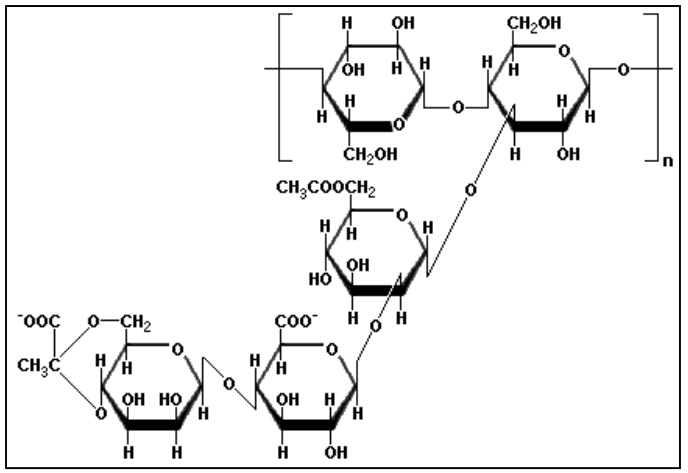
1. repeated pentassaccharide units to form cellulosic backbone through the 1, 4B-D glucosidic linkage and
2. a side chain.

- The side chain consists of

1. two mannose and
2. one glucuronic acid, so the chain consists of repeating modules of five sugar units.
3. About half of the terminal mannose units have a pyruvic group.



The gums help the bacteria stick to the leaves of host plants.



Xanthan gum

Functions in survival & pathogenicity

Functions of xanthan in survival

1. Long term survival of bacterial cells,
2. Increased thermal death point,
3. Protection against UV.

Functions of xanthan in pathogenicity

1. May be involved in wilting associated with bacterial blight of rice (*X. oryzae*),
2. Induction of water-soaking,
3. Gel-like slime may be associated with bacterial colonization.

Xanthan gum
Stability and Viscosity

- Xanthan Gum is stable in applications with a wide range of pH values (2-12).
- It has a tolerance to enzymes, salt, and heat.
- For instance, Xanthan Gum in a 1.1% citric acid/citrate solution at a pH of 3.4 at 90°C for 24 hours showed excellent thermal stability.
- Xanthan Gum also exhibits excellent freeze-thaw stability.
- Viscosity values are generally not affected by changes in pH, addition of salt and thermal changes for extended periods of time.

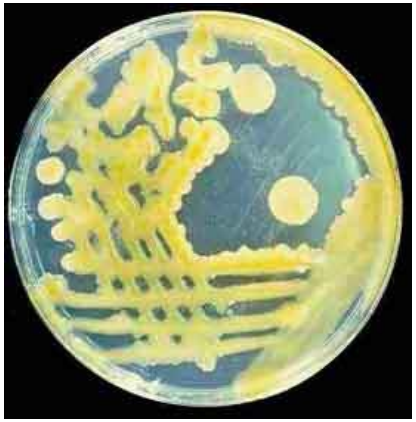
Colony Gums,2011

Xanthan gum
The applications

- The applications for Xanthan Gum seem to be endless.
- Food grade xanthan gum can be widely used as salt/acid resistant thickener, high efficient suspension agent and emulsifier, high viscosity filling agent in various food and beverage.
- It can not only enhance the performance of water-keeping and shape-keeping, but also improve the freeze/thaw stability and mouth-feeling of food and beverage product.

Xanthan gum
Physical property

- Xanthan Gum is a white to pale white powder and mainly is an 80-100 mesh.
- Particle size can vary depending on the customer specifications and can consist of an agglomerated product to fine 200 mesh powder.



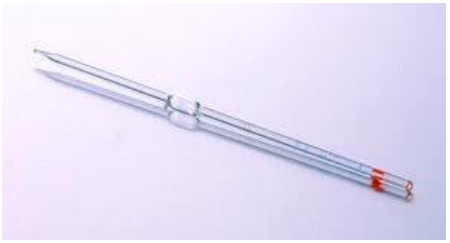
Xanthan gum
An commercial bioproduct

- About 20,000 tones of xanthan are produced industrially from *X. campestris* each year.
- The gum itself is colourless.
- Xanthan gums are extracellular polysaccharides produced by the aerobic fermentation of *Xanthomonas* bacteria.
- The gums are commercially harvested from fermentation tanks and used for a variety of purposes such as the food thickeners commonly found in dipping sauces, salad dressings, and chewing gum.

Viscosity Test

Xanthan gum

For preliminary identification of strains of *Xanthomonas campestris*



- A diagnostic test based on the viscosity of suspensions prepared from 6- to 7-day-old cultures of bacteria on SPA medium in order to differentiate 16 of 17 pathovars of *Xanthomonas campestris* from miscellaneous yellow bacteria.
- The viscous suspensions of each xanthomonas spp. (viscosity presumed to be caused by xanthan gums) was pipetted and delivered.
- ≥26 sec for 0.9 ml required to flow through a 1-ml Pyrex pipet,
- It is 5.6 sec or less for other yellow bacteria.

Pierce *et al.*,1990

Viscosity Test of bacterial suspension

Viscosity of suspensions of known pathovars and species *Xanthomonas*

Culture no.[y]	Name	Viscosity[z] (sec)	Suspension[z] (cfu/ml)
XA 123	*Xanthomonas albilineans*	5.4	5×10^{10}
XA 144	*X. albilineans*	1.2	2×10^{11}
XA 131	*X. axonopodis*	0.9	4×10^{10}
UC 855	*X. campestris* pv. *campestris*	>30.0	1×10^{12}
XC 145	*X. c.* pv. *celebensis*	>30.0	3×10^{10}
UC 867	*X. c.* pv. *incanae*	26.4	1×10^{10}
UC 873	*X. c.* pv. *juglandis*	5.6	2×10^{11}
UC 1060	*X. c.* pv. *juglandis*	2.2	1×10^{11}
UC 874	*X. c.* pv. *malvacearum*	>30.0	2×10^{10}
UC 876	*X. c.* pv. *malvacearum*	>30.0	2×10^{11}
XO 111	*X. c.* pv. *oryzicola*	>30.0	4×10^{9}
UC 131	*X. c.* pv. *pelargonii*	>30.0	9×10^{12}
UC 878	*X. c.* pv. *pelargonii*	>30.0	4×10^{10}
UC 879	*X. c.* pv. *pelargonii*	>30.0	2×10^{11}
UC 880	*X. c.* pv. *phaseoli*	>30.0	1×10^{11}
UC 673	*X. c.* pv. *pruni*	27.0	2×10^{9}
XT 129	*X. c.* pv. *secalis*	>30.0	1×10^{10}
UC 886	*X. c.* pv. *vesicatoria*	>30.0	1×10^{12}
UC 895	*X. c.* pv. *vitians*	>30.0	1×10^{11}
UC 861	*X. fragariae*	0.8	4×10^{10}

[y] From the collection of Plant Pathogenic Bacteria, University of California, Berkeley.
[z] Viscosity and cfu/ml readings represent the mean of four trials; concentration of suspension was determined for one replicate.

Pierce *et al.*,1990

Viscosity Test

Viscosity and other characteristics of initially unknown yellow Gram-negative bacteria

Culture no.	Plant source	Viscosity[y] (sec)	Suspension[y] (cfu/ml)	Physiological characteristics[x]				
				OX	HL	Growth on Tween A	HR	Use of asparagine
875-3	Alder	0.9	2×10^{9}	−	+	−	−	
119-7	Alder[z]	>30.0	6×10^{11}	−	−	+	+	−
948-3	Cactus	1.7	2×10^{7}	−	+	−	−	
638-5	Geranium	0.9	2×10^{9}	−	−	−	−	
723	Hebe	0.9	4×10^{9}	−	−	−	−	
632-1	Hyacinth[z]	>30.0	5×10^{11}	−	−	+	−	−
597-2	Ivy	0.8	2×10^{11}	−	−	+	−	
446-3	Ivy[z]	>30.0	3×10^{12}	−	−	+	+	−
138	*Malus sylvestris*[z]	0.8	6×10^{10}	−	+	−	−	
351-1	Manzanita[z]	>30.0	3×10^{12}	−	−	+	+	−
417	Oleander	0.8	4×10^{10}	−	+	+	−	
695	Passiflora	0.6	1×10^{11}	−	−	−	−	
91-2	Geranium	0.8	4×10^{10}	−	−	+	−	
604-4	Petunia	0.6	7×10^{10}	−	−	−	−	
626-1	Stock[z]	>30.0	2×10^{10}	−	−	+	+	−
668-5A	Strawberry	1.0	2×10^{10}	−	−	+	−	
668-5B	Strawberry	1.1	2×10^{11}	−	−	+	−	
ML5	Sugar Beet[z]	0.9	1×10^{11}	+	−	−	−	
447-2	Tomato	0.7	3×10^{11}	−	+	+	−	
58	Zinnia[z]	>30.0	1×10^{12}	−	−	+	+	−

[x]OX = Oxidase test; HL = Hugh-Leifson medium, covered with mineral oil; HR = hypersensitive reaction of *Nicotiana glutinosa* at 32 C; use of asparagine as sole carbon and nitrogen source.
[y]Viscosity readings represent the mean of four replicates. Concentration of suspension was determined for one of the replicates.
[z]Bacteria from these sources subsequently were identified as: alder = *Xanthomonas campestris* pv. unnamed; hyacinth = *X. c.* pv. *hyacinthi*; ivy = *X. c.* pv. *hederae*; *Malus sylvestris* = *Erwinia herbicola*; manzanita = *X. c.* pv. unnamed; stock = *X. c.* pv. *incanae*; sugar beet = *Pseudomonas fluorescens*; zinnia = *X. c.* pv. *zinniae*.

Pierce *et al.*,1990

Fatty acid analysis

- Lipids account for 20% of the weight of bacteria.
- *Xanthomonas* is rich in fatty acids.
- Certain fatty acids are characteristic for genus and not detected in other plant pathogenic bacteria. e.g.
- 11:0 iso,
- 11:0 iso 3OH,
- 13:0 iso 3OH.

Percentage concentration of fatty acid methyl esters useful for species differentiation within the genus *Xanthomonas*[a,b]

Fatty Acid	1. *X. campestris*	2. *X. albilineans*	3. *X. arboricola*	4. *X. axonopodis*	5. *X. bromi*	6. *X. cassavae*	7. *X. codiaei*	8. *X. cucurbitae*	9. *X. fragariae*	10. *X. hortorum*	11. *X. hyacinthi*	12. *X. melonis*	13. *X. oryzae*	14. *X. pisi*	15. *X. populi*	16. *X. sacchari*	17. *X. theicola*	18. *X. translucens*	19. *X. vasicola*	20. *X. vesicatoria*
Saturated acids:																				
$C_{16:0}$	3.6	13.4	4	4.1	4.9	1.4	6.3	6.7	3.7	2	3.8	4.3	18.5	3.4	7.4	4.2	8.8	4.3	15.8	3.3
Unsaturated acids:																				
$C_{15:1\ \omega8c}$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**2.9**	0	0	0	0	0
$C_{16:1\ \omega9c}$	0.9	0	0.9	0.7	0.7	0.5	1.5	0.1	0	0.1	0	1.6	2.3	1	**16.9**	1.1	0.2	1	0.7	0.8
$C_{16:1\ \omega7c}$	12.7	13.2	15	16.2	8.9	10.3	15.1	16	11.7	18.6	12.1	10.4	24.9	13.8	0	15	12.9	21.1	25	15.6
Branched saturated acids:																				
$C_{14:0\ iso}$	0.7	0	0.8	0.5	0	0	0.6	1.2	0.2	0.7	3.7	0.2	0	0.7	0	**14**	9.8	2.6	0	0.9
$C_{15:0\ iso}$	26.5	**2.4**	31.1	28.2	21.3	31.8	26	31.5	36.8	33.2	11.1	28.5	6.3	31.8	23.4	17.3	12.9	29.4	15.5	24.6
$C_{15:0\ anteiso}$	13.9	**0.1**	13.7	9.3	2.6	9.5	7.4	10.2	10.5	15.7	26.8	6.6	0.7	9.4	1.6	5.6	15.9	5	3	15.5
$C_{16:0\ iso}$	3.2	4.7	2	2.6	1.7	1.1	3.6	3.2	0.9	1.7	6.6	1.9	0.2	1	0	**18.8**	9.9	3.6	1.3	4.1
$C_{17:0\ iso}$	6.8	**19.3**	4.5	6.7	10.9	9.5	7.4	3.9	3.7	2.7	2.1	10.2	14.6	5.3	2.5	2.7	2.4	4.8	7.3	6.8
$C_{17:0\ anteiso}$	0.8	**2.9**	0.3	0.4	0.1	1	0.5	0.2	0.2	0.1	1.1	0.2	0.3	0.5	0	0.2	1.2	0	1.1	1.1
$C_{19:0\ iso}$	0	**1.6**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Branched unsaturated acids:																				
$C_{15:1\ iso\ G}$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1.6**	0	0	0	0	0
$C_{17:1\ iso\ \omega9c}$	8.5	23.3	3.8	6.5	25.7	17.5	7.5	6.1	3.5	3.9	1.7	10.4	9.1	6.2	0	2.2	2.4	7.4	11.2	8.3
Others:																				
$C_{16:0\ alc}$	0	0.3	0	0	0	0	0	0	0	0	**1.6**	0	0	0	0	0	0	0	0	0
$C_{17:0\ cyclo}$	0	0	0	0	0	0	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0

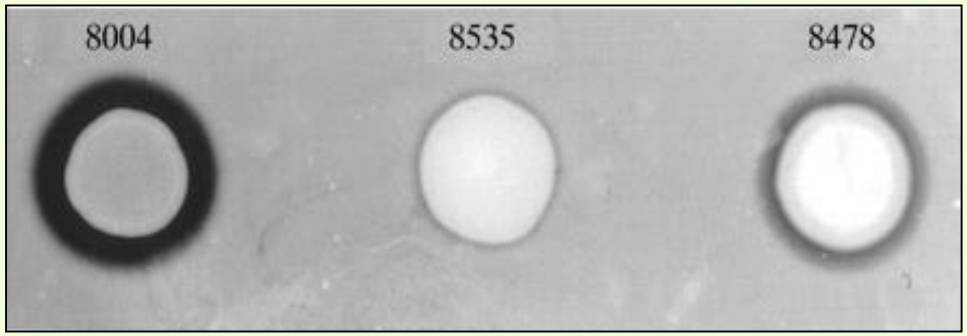
[a]The most distinctive components are highlighted in bold.

[b]Adapted from Vauterin et al. (1996b).

Bergey's Manual of Systematic Bacteriology,2005

Enzyme assay
Xcc

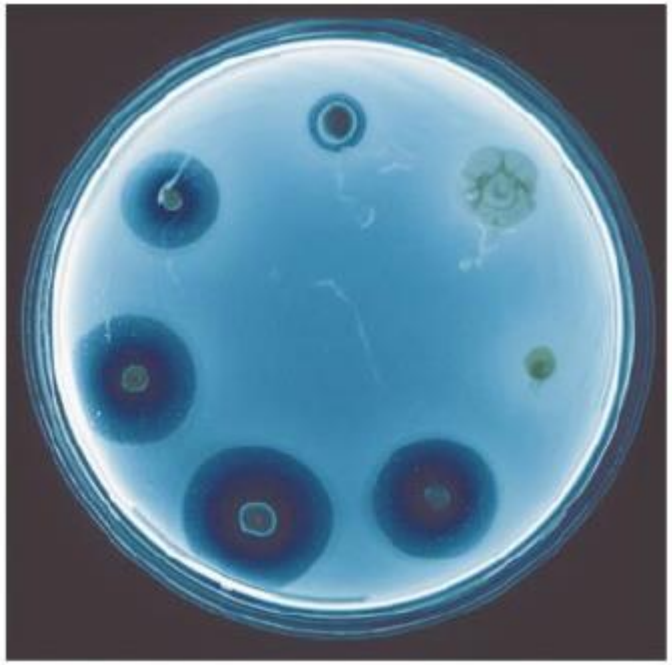
- Protease production by strains of *Xcc* as revealed by zones of clearing around colonies on skimmed milk indicator plates.
- Indicator plates for protease production contained NYGB supplemented with 1% (w/v) skimmed milk and solidified with 2% (w/v) agar (Tang *et al.*,1987).
- The strains were 8004 (wild-type) and two mutants (8478 and 8535).



Albicidins assay

Xanthomonas albilineans

- *X. albilineans* produces a family of antibiotics and phytotoxins(albicidins), which are not only implicated in chlorosis induction but also play a larger role in pathogenesis.
- Inhibition of *E. coli* lawn growth around colonies of *X. albilineans* due to albicidin antibiotics.
- Other tested *Xanthomonas* spp. (top right) are not inhibitory.

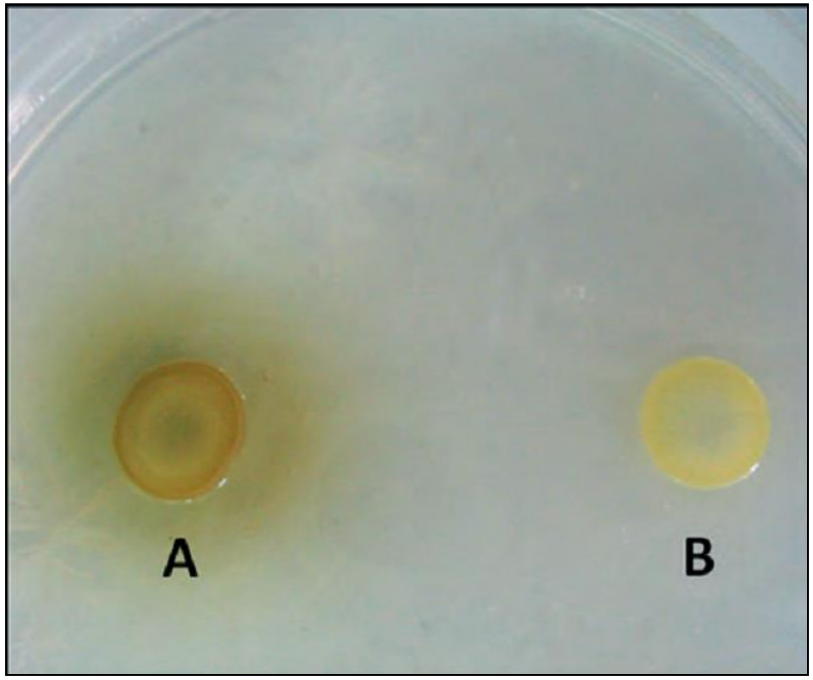


Chlorosis-inducing isolates of *X. albilineans* produce a family of antibiotics called albicidins in culture.

Birch,2001

Quinate metabolism test
Xanthomonas arboricola species

- *X. arboricola* species may be differentiated from the other *Xanthomonas* spp. by the ability to degrade quinate in succinate-quinate (SQ) medium (Lee *et al.*, 1992) where *X. arboricola* colonies produce a deep green colour, diffusing around the bacterial streak, which is recorded after 5-6 days of growth on the medium.
- Other *Xanthomonas* spp. will grow on this medium, but no colour change is produced.



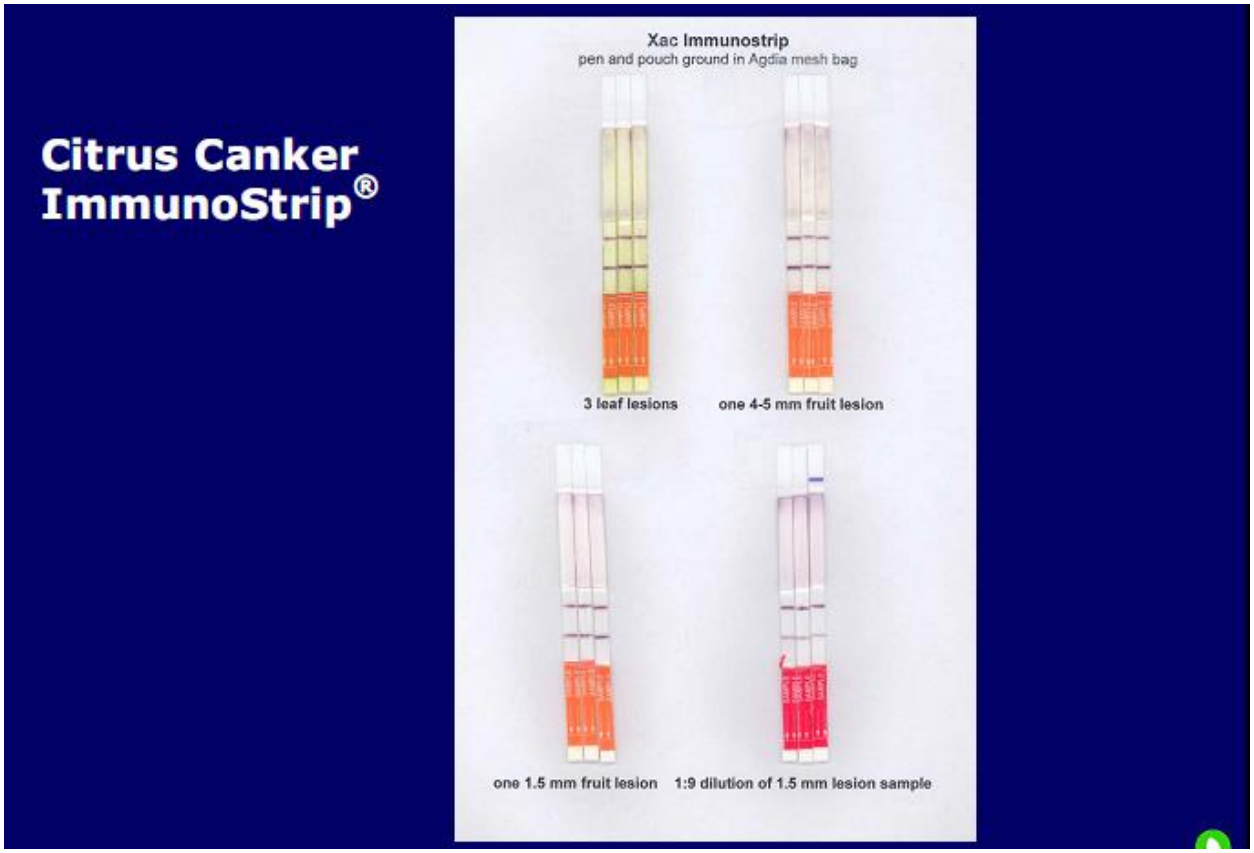
Quinate metabolism test:
A - positive reaction: production of a deep green discoloration around colony growth,
B - negative reaction.

Prokic *et al.*,2012

Serological test

Immunoassay

ImmunoStrips for *Xanthomonas citri*



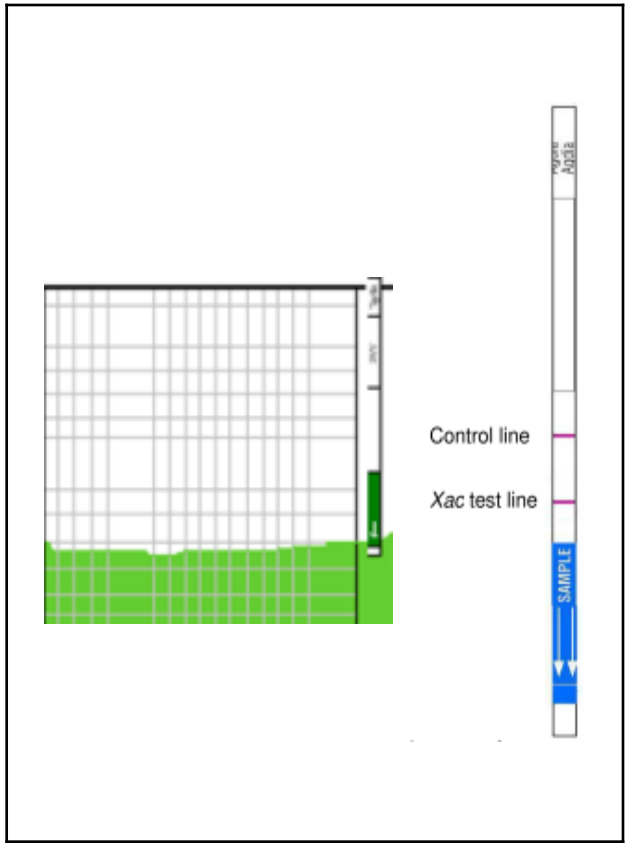
Positive samples produce two colored bands;
Negative samples produce a single control band.

Sutula,2008

Xac ImmunoStrip®Test

For detection of *Xanthomonas citri*

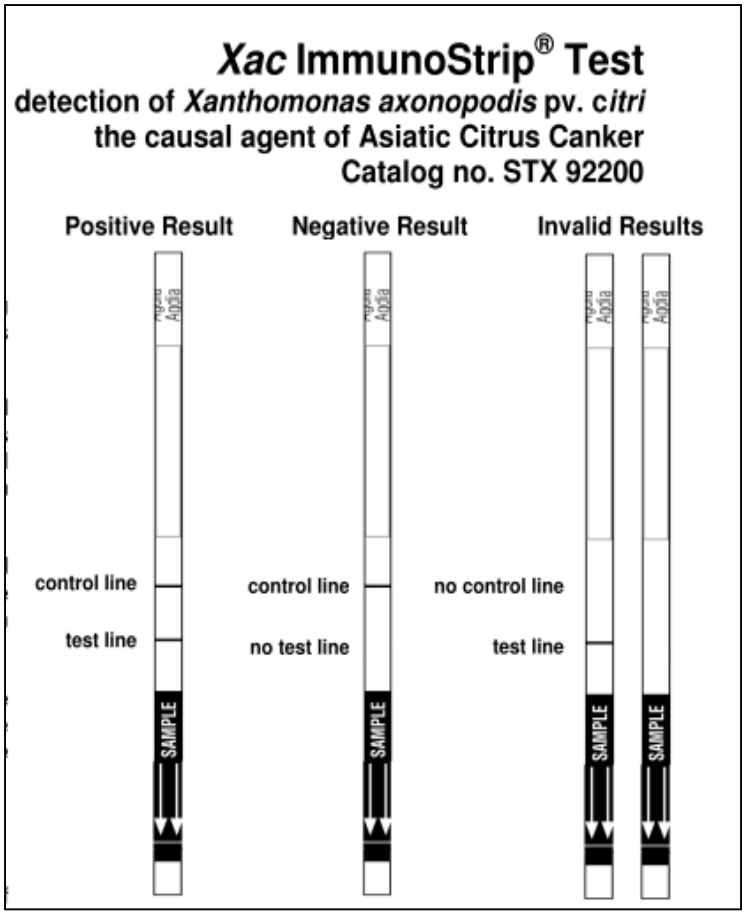
- ImmunoStrip® test for detection of *Xanthomonas citri* the causal agent of Asiatic Citrus Canker.
- Insert the end of the ImmunoStrip marked "sample" into the pouch, as illustrated.
- Allow the strip to run for a 30 minutes or until both the control line and test line appear.
- Note: Do not allow much more than 0.5 cm or ¼ inch of the end of the strip to be submerged in the extract.
- The end of the strip should remain in the extract during test.



Xac ImmunoStrip®Test

For detection of *Xanthomonas citri*

- The control line assures that the test is working properly.
- If the control line does not appear, the test is invalid.
- If the sample is positive (+) for Xac, the test line will appear.
- The test line will be red to purple in color just as the control line.
- A positive result can appear and be interpreted in less than 30 minutes.
- If the sample is negative (-) for Xac, the test line will not appear.
- Allow the strip to run the full 30 minutes before ruling the sample negative.
- Use the images to the right as a guide to determine results.

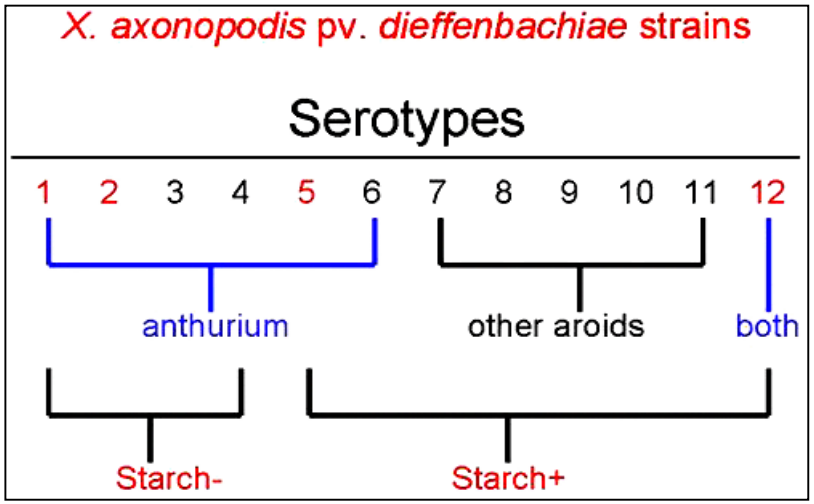


Agdia,Inc

Serotyping

Xanthomonas axonopodis pv. *dieffenbachiae*

- The strains were serotyped (assigned to serogroups) based on their reactions to a panel of monoclonal antibodies.
- A comparison of 323 strains resulted in a clear separation of anthurium strains from those isolated from the other aroids.
- Relationship between serotypes and capacity to hydrolyze starch among strains of *X. axonopodis* pv. *dieffenbachiae*.
- Strains from anthurium were in serotypes 1-6; strains from other aroids were mostly in serotypes 7-11.
- Nonpathogenic strains were often serotype 12, reacting only with a *Xanthomonas*-specific monoclonal antibody.



Alvarez *et al.*,2006

PCR primers for Xanthomonads

Specificity	Primer Designation	Sequence	Size (bp)
Nonspecific, many xanthomonads	RST2 RST3	(5'AGGCCCTGGAAGGTGCCCTGGA3') (5'ATCGCACTGCGTACCGCGCGCG3')	840
	RS21 RS22	(5'GCACGCTCCAGATCAGCATCGAGG3') (5'GGCATCTGCATGCGTGCTCTCCGA3')	1075
Nonspecific, certain xanthomonads	RST9 RST10	(5'GGCACTATGCAATGACTG3') (5'AATACGCTGGAACTGCTG3')	355
X. albilineans	Ala4 L1	(5'CCCGACTGGCTCCACCACTG3') (5'CAAGGCATCCACCGT3')	360
	XAF1 XAR1	(5'CCTGGTGATGACG TGGGTT-3') (5'CGATCAGCGATGCACGCAGT-3')	600
	XaAlb2-f3 XaAlb2-3	(5'CACACACACAATACAGCATTGCGG3') (5'CCCAACTTACTTGAGGCTATGG3')	440
	Nested primers		
	XaAlb2-f4 XaAlb2-r4	(5'CTTCTGCAGCTTGCTCGTC3') (5'GCTCAGTTACGCTCAGCTAATC3')	308
X. campestris pv. *citri*	3 2	(5'TGGTGTCGTCGCTTGTAT3') (5'CACGGGTGCAAAAAATC3')	222
	4 7	(5'TGTCGTCGCTTGTAT3') (5'GGGTGCGACCGTCAGGA3')	467
	Nested primers		
	94-3 94-4	(5'CTCGATCACGATGTCCTTCTCC3') (5'GTGGATGGCATGAGCATGAAG3')	315
X. campestris pv. *manihotis*	XV XK	(5'TTCGGCAACGGCAGTGACCACC3') (5'TCAATCGGAGATTACCTGAGCG3')	898
X. campestris pv. *phaseoli*	X4c X4c	(5'-CGCCCGGAAGCACGATCCTCGAAG3) (5'GGCAACACCCGATCCCTAAACAGG3)	730
X. fragariae	XF9 XF11	(5'TGGGCCATGCCGGTGGAACTGTGTGG3') (5'TACCCAGCCGTCGCAGACGACCGG3')	537
	XF9 XF11	(5'TGGGCCATGCCGGTGGAACTGTGTGG3') (5'TCCCAGCAACCCAGATCCG3')	458
	241A 241B	(5'GCCCGACGCGAGTTGAATC3') (5'GCCCGACGCGCTACAGACTC3')	550
	245A 245B	(5'CGCGTGCCAGTGGAGATCC3') (5'CGCGTGCCAGAACTAGCAG3')	300
	2956A 295B	(5'CGTTCCTGGCCGATTAATAG3') (5'CGCGTTCCTGCGTTTTTTCG3')	615
X. translucens[a]	T1 T2	(5'CCGCCATAGGGCGGAGCACCCCGAT3') (5'GCAGGTGCGACGTTTGCAGAGGGATCTTCTGCAAA3')	139

[a] Also includes *X. campestris* pvs. *arrhenatheri, phlei, poae, graminis*

Schaad *et al.*,2001

PCR identification of the genus
Based on 16S rRNA analysis

- Bacteria belonging to the genus *Xanthomonas* are found world-wide.
- The genus consists mainly of plant-associated bacteria.
- Usually, identification of the phytopathogenic yellow-pigmented xanthomonads is possible when present in their expected habitat.
- However, new bacteria and bacterial associations are found routinely.
- In addition to the plant pathogens, the group of saprophytic and epiphytic *Xanthomonas* bacteria is expanding.
- Also the position within the *Xanthomonas* genus of bacteria isolated from totally different sources such as soil, animal and clinical samples, has been discussed and questions the status of *Xanthomonas* as a phytobacterial genus.

Maes,1993

PCR identification of the genus
Based on 16S rRNA analysis

- To define the genus *Xanthomonas*, based on a specific nucleic acid sequence recognition, deduced and synthesized a 17-mer with the following sequence:
- 5'-AAGGATCGGGTATTAAC-3', homologous(corresponding) to nucleotide positions 461/477 of the *Xanthomonas* 16S rRNA.
- This oligonucleotide was tested for its specificity in PCR.
- The 17-mer was used as reverse primer, in combination with a universal 16S rDNA primer (5'- AGAGTTTGATCITGGCTCAG-3',position 8/27, *E. coli* position numbering).

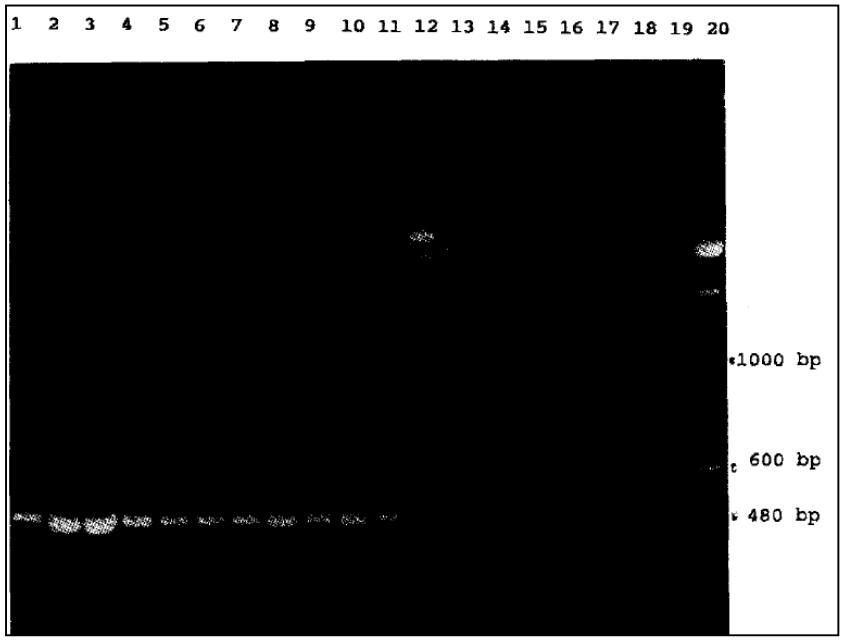
'I' indicates inosine, which can pair with base T, A, or C.

Maes,1993

16S	16S rRNA/8/27 – GAG TTT GAT CNT GGC TCA G	480	Maes *et al.*, 1996
	16S rRNA/461/477- AAG GAT CGG GTA TTA AC		Trindade,2007

PCR identification of the genus
Based on 16S rRNA analysis

- Among the 52 *Xanthomonas* species and *X. campestris* pathovars tested, 49 produced a single intense 480-bp fragment.
- Of 4 saprophytic isolates, 2 were recognized as xanthomonads.
- But the other two(strains A4070 and A4093) have not be classified within the *Xanthomonas* genus since they produced other fragments in addition to a 480-bp band.



Maes,1993

PCR identification of the genus
Based on 16S rRNA analysis

Genus *Xanthomonas*

Genus *Xanthomonas*					
Species/pathovars	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
Xanthomonas (genus)	**8/27** **461/477** 16S rRNA gene	Conventional	Bacteria (boiled) or seed extract	Maes, 1993	

Palacio-Bielsa *et al.*,2009

Oligonucleotide primers used for 16S rDNA amplification and sequencing of *Xanthomonas* spp. e.g. the universal primers of 27F/907R

- The two primers used for PCR amplification are listed below:

Primer[a]	Sequence	Target positions[b]	Application
16F27	5′ AGAGTTTGATCMTGGCTCAG 3′	8-27	Amplification
16R343	5′ ACTGCTGCCTCCCGTA 3′	358-343	Sequencing
16F355	5′ ACTCCTACGGGAGGCAGC 3′	337-355	Sequencing
16R519	5′ GTATTACCGCGGCTGCTG 3′	536-519	Sequencing
16F530	5′ TTCGTGCCAGCAGCCGCGG 3′	512-530	Sequencing
16R685	5′ TCTACGCATTTCACCGCTAC 3′	704-685	Sequencing
16F704	5′ GTGTAGCGGTGAAATGCGTAGA 3′	685-704	Sequencing
16F946	5′ CCCGCACAAGCGGTGGA 3′	930-946	Sequencing
16R1087	5′ CTCGTTGCGGGACTTAACCC 3′	1206-1087	Sequencing
16F1195	5′ AGGAAGGTGGGGATGACGTC 3′	1195-1214	Sequencing
16R1389	5′ ACGGGCGGTGTGTACAAG 3′	1389-1372	Sequencing
16R1525	5′ TTCTGCAGTCTAGAAGGAGGTGWTCCAGCC 3′	1525-1496	Amplification

[a] F, forward primer; R, reverse primer.
[b] The numbering of target positions is based on the numbering of the *E. coli* 16S rRNA sequence (6).

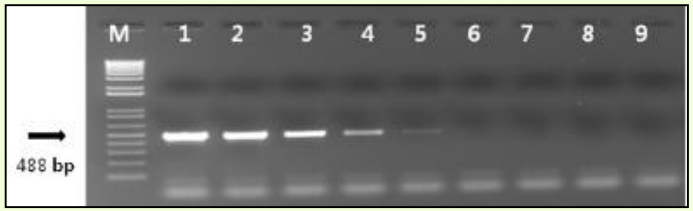
- Partial sequences were determined by using the following five sequencing primers: 16R343,16R519,16F530,16F946,and 16R1389.

Hauben *et al.*,1997

PCR assay

Targeting a membrane fusion protein gene *Xanthomonas oryzae* pv. *oryzicola*

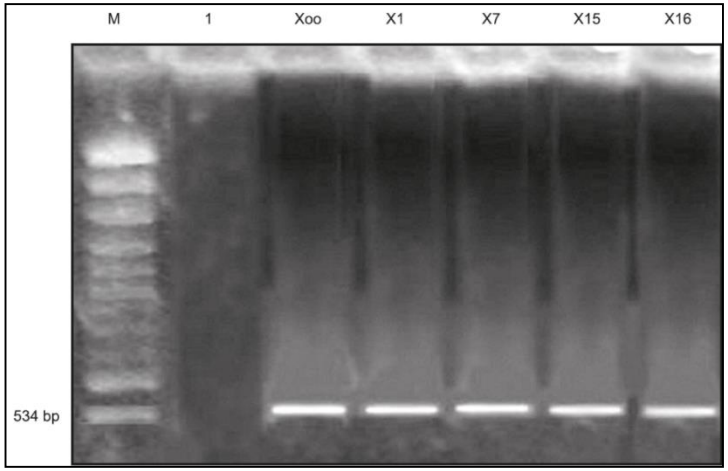
- It has been known that many kinds of membrane proteins in the bacterial cell play various and important roles.
- It was reported that membrane protein was developed for preliminary screening and comparing large numbers of isolates in taxonomic and epidemiological studies.
- Based on differences in a membrane fusion protein gene of *Xanthomonas oryzae* pv. *oryzicola* and other microorganisms, one pair of pathovar-specific primers, XOCMF/XOCMR, was synthesized.
- The XOCMF and XOCMR primers were tested against *Xanthomonas oryzae* pv. *oryzicola*.
- As expected, a 488-bp DNA fragment was amplified.
- None of the other *Xanthomonas* strains and reference microorganisms reacted with the primers.



Kang *et al.*,2008

PCR assay
Xanthomonas oryzae pv. *oryzae*

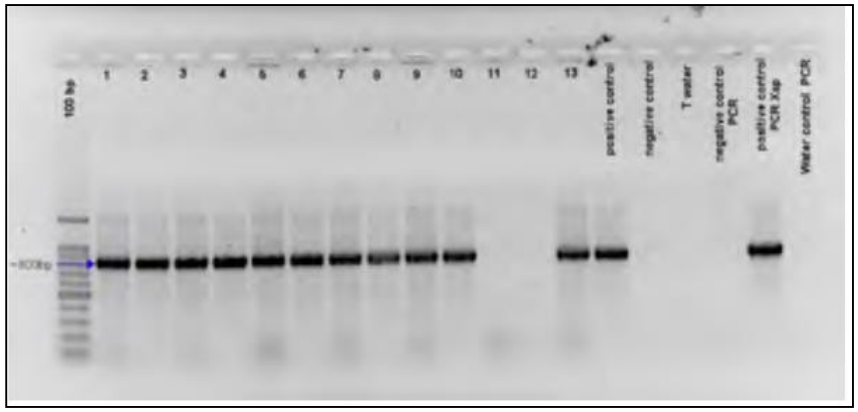
- The 20 oligonucleotide
- XOF, 5'-ATGCCGATCACCATGCCGAT, and
- XOR 5'-TGGCCTTGTCGTACGAGCTC-3' were designed and tested for *X. oryzae* pv.*oryzae* (Lee *et al.*,2004).
- lane 1 is negative control (distilled water);
- lane Xoo is positive control (CFBP.2532) showing the amplification approximately 534 bp;
- X1, X7, X15 and X16 isolates from rice fields in Guilan province.



Ghasemie *et al.*,2008

PCR assay
Xanthomonas phaseoli

- With the specific pair of primers p7X4c and p7X4e from Audy *et al.*,1994, all *X. axonopodis* pv. *phaseoli* will give a product of 800bp.
- The sequence of the prime pair are:
- p7X4c: 5′ ggcaacacccgatccctaaacagg 3′
- p7X4e: 5′ cgccggaagcacgat cctcgaag 3′



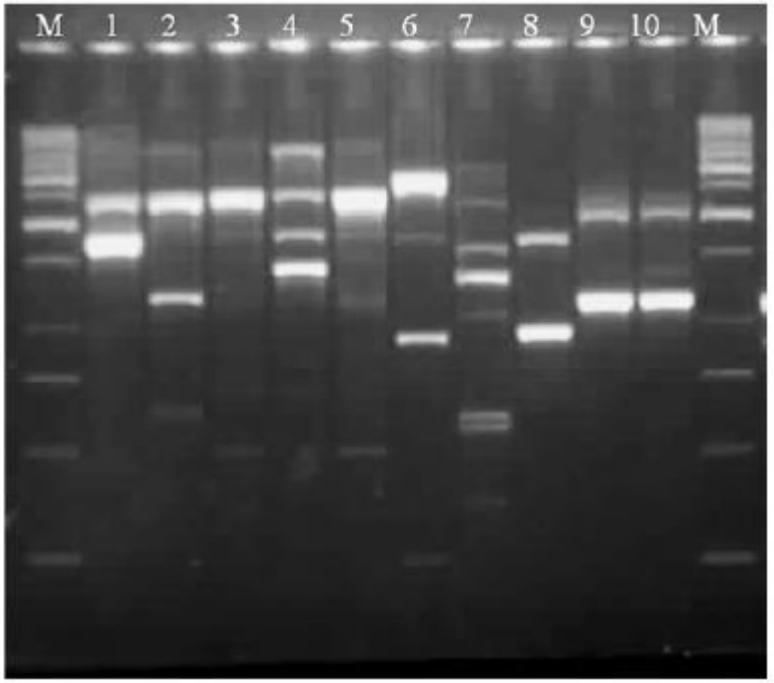
Agarose gel showing Xap specific band at 800 bp (Audy *et al.*,1994 primers).

Grimault *et al.*,2014; Corzo-López *et al.*,201

RAPD analysis

Xanthomonas malvacearum

- A gel showing RAPD amplification patterns generated using primer OP-R20 (CACAGCTGCC) on 9 *Xam* races and isolate Ms.
- M: marker;
- Lane 1, isolate Ms; lane 2, race 1; lane 3, race 3; lane 4, race 4; lane 5, race 8; lane 6, race 11; lane 7, race 21; lane 8, race 26; lane 9, race 28; lane 10, race 32.



Abdo-Hasan *et al.*,2008

RAPD analysis

Xanthomonas phaseoli

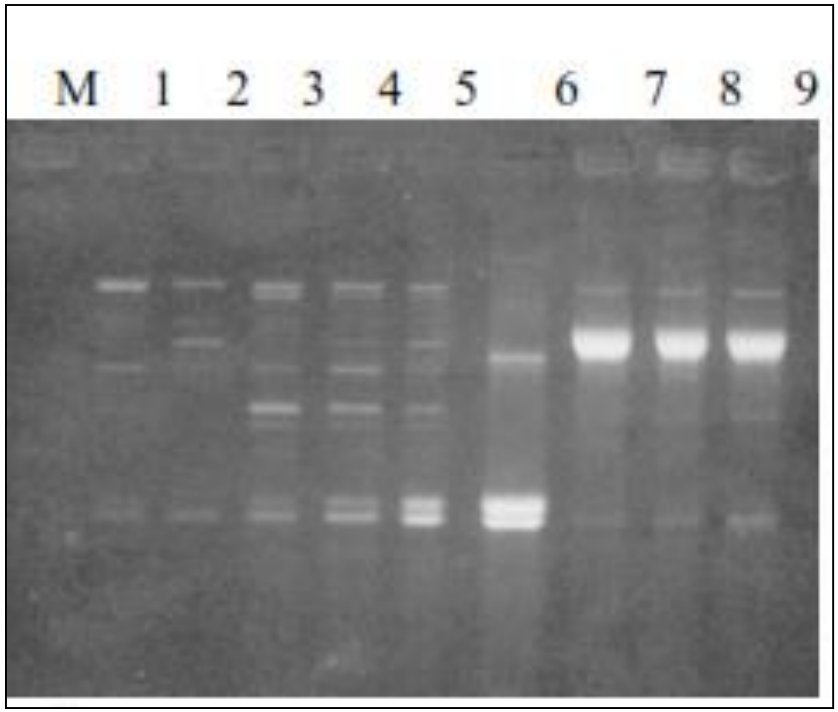
- PCR reaction was performed in 20 µl volume containing RAPD buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 2.5 mM MgCl2, 0.01% gelatin), 0.2 mM of each dNTP (Boehringer Mannheim), 15 ng of ten mer primer, 17.5 ng of DNA, 1.5 units Taq Polymerase (Gibco).
- Nine primers were used for RAPD analysis: OPG-03, OPG-06, OPH-01, OPH-04, OPH-06, OPH-07, OPH-08, OPH-09 and OPH-12.
- The amplifications were performed in a MJ Research thermocycler, programmed for 35 cycles of 1 min at 94°C, followed by 1 min at 35°C, 1.5 min at 72°C and a final extension at 72°C for 5 min.
- Aliquots of the final amplified products were visualized in 1.4% agarose gels containing 0.5 µg/ml of ethidium bromide.

Mário de Carvalho Nunes *et al.*,2008

RAPD analysis

Xanthomonas phaseoli

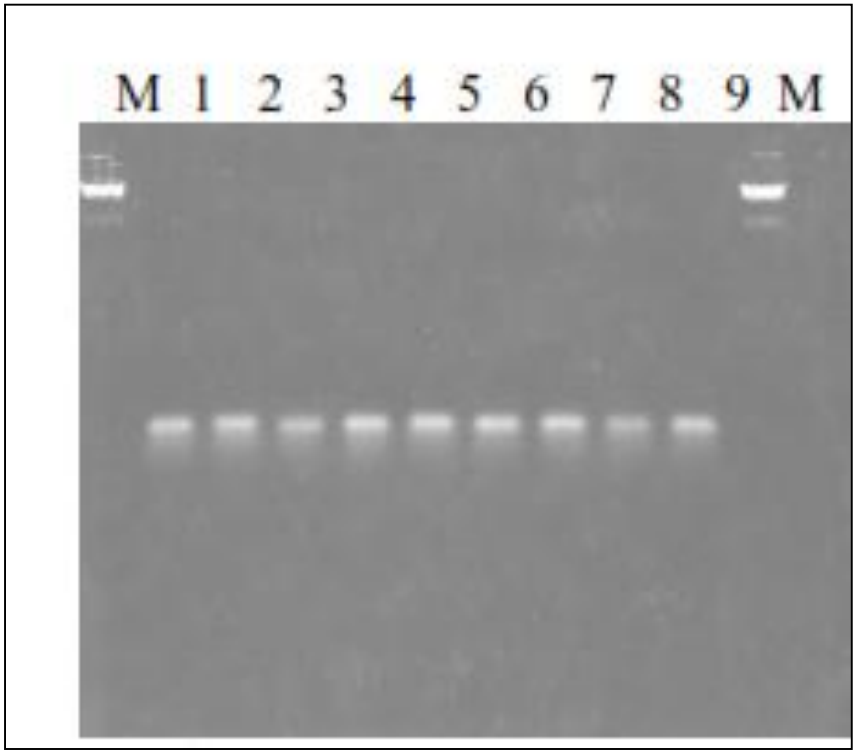
- RAPD polymorphism of nine isolates of *Xanthomonas axonopodis* pv. *phaseoli*, with primer OPG-06.
- Lane M: molecular size marker (1 Kb ladder Gibco BRL);
- lane 1: Davis isolate; lane 2: W33 isolate; lane 3: W43 isolale; lane 4: W44 isolate; lane 5: W45 isolate; lane 6: W63 isolate; lane 7: W67 isolate; lane 8: W68 isolate; lane 9: W69 isolate.



Mário de Carvalho Nunes *et al.*,2008

RAPD analysis
Xanthomonas phaseoli

- Amplified PCR product of *Xanthomonas axonopodis* pv. *phaseoli* isolates, with primer RST21 and RST22.
- Lane M: molecular size marker (1 Kb ladder Gibco BRL);
- lane 1: Davis isolate; lane 2: W33 isolate; lane 3: W43 isolale; lane 4: W44 isolate; lane 5: W45 isolate; lane 6: W63 isolate; lane 7: W67 isolate; lane 8: W68 isolate; lane 9: W69 isolate.



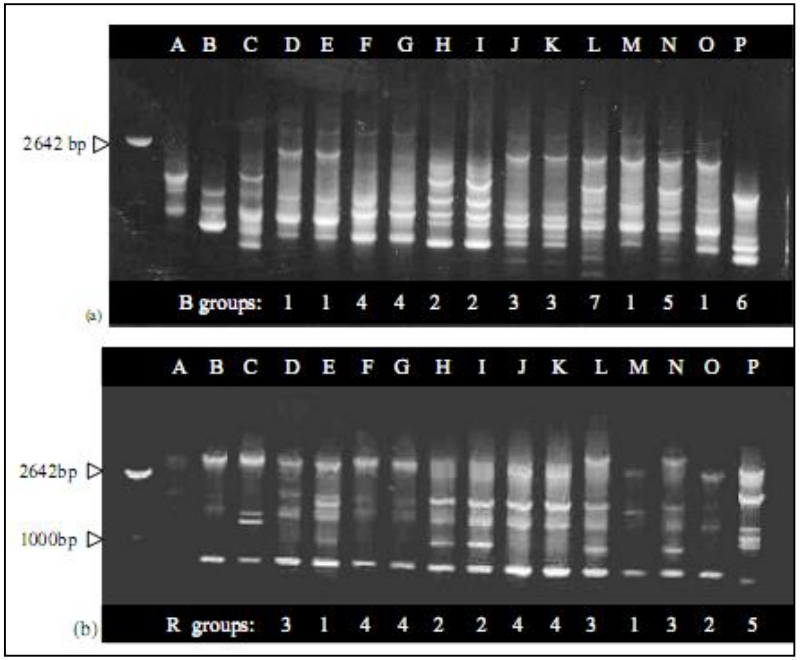
Mário de Carvalho Nunes *et al.*,2008

Genomic fingerprinting

Rep-PCR and Box-PCR analysis

Xanthomonas campestris pv. *campestris*

- Two rep-PCR protocols, namely BOX-PCR and REP-PCR, were used.
- BOX AIR primer (5-CTACggCAAggCgACgCTgACg-3).
- REP-PCR, using REP1R (5-IIIICgICgICATCIggC3) and REP2I (5-ICgICTTATCIggCCTAC-3) primer set (Versalovic *et al.*,1991; 1994).
- Both protocols gave reproducible genomic PCR profiles consisting of approximately 400–3000bp.
- With BOX-PCR, Xcc strains generated more than seven fingerprint patterns.



Massomo *et al.*,2003

Genomic fingerprinting

Rep-PCR

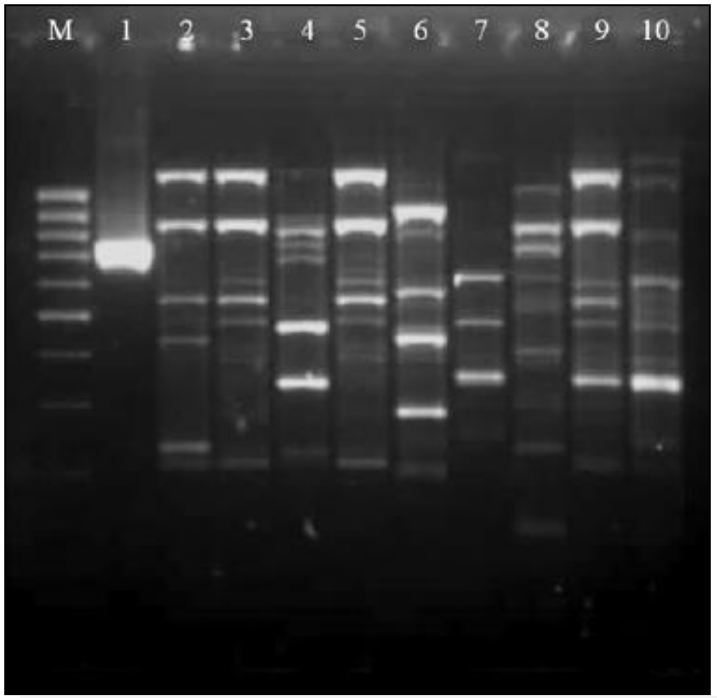
Xanthomonas fragariae

- The two sets of primers are:
- REP1R-I: 5′-IIIICGICGICATCIGGC-3′
- REP2-I: 5′-ICGICTTATCIGGCCTAC-3′
- ERIC1R: 5′-ATGTAAGCTCCTGGGGATTCAC-3′
- ERIC2: 5′-AAGTAAGTGACTGGGGTGAGC G-3′
- The reaction buffer contains 16.6 mM $(NH4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 6.7 µM EDTA, 30 mM 2-mercaptoethanol, 0.17 mg BSA/ml, 10% (v/v) dimethyl sulfoxide, 1.2 mM of each dNTP, 62 pmol each primer and 2 U Taq DNA polymerase.
- Bacteria from a representative colony of the test strain are transferred, using a sterile 10 µl pipette tip (or other suitable implement), to a PCR tube containing 25 µl of the reaction mixture.
- The cycling parameters are 95 °C for 6 min followed by 35 cycles at 94°C for 1 min, 44 °C (REP primers) or 52°C (ERIC primers) for 1 min and 65°C for 8 min. The amplification cycles are followed by a final extension step of 68°C for 16 min.

ISSR analysis

X. anopodis pv. *malvacearum*

- ISSR (Inter Simple Sequence Repeat) is a general term for a genome region between microsatellite loci.
- Twenty four primers used for Inter Simple Sequence Repeats (ISSRs) analysis.
- The amplification patterns generated using primer A-31 (AGCAGCAGCAGCR) are shown in this figure.
- Lane 1, isolate Ms; lane 2, race 1; lane 3, race 3; lane 4, race 4; lane 5, race 8; lane 6, race 11; lane 7, race 21; lane 8, race 26; lane 9, race 28; lane 10, race 32.



Microsatellite loci which are widely distributed throughout the genome can be used to classify individuals by relatedness.

Abdo-Hasan *et al.*,2008

Nested PCR
X. arboricola pv. *fragariae*

- Incubate leaf, petiole and crown tissue (30-70 g) in 10-20 ml of 0.01 M sodium phosphate buffer(pH 7.2) per gram of tissue at room temperature overnight.
- Extract DNA and analyse by single and nested PCR as described by Zimmerman *et al.* (2004).
- The primers are:
- 245A: 5′-CGCGTGCCAGTGGAGATCC-3′
- 245B: 5′-CGCGTGCCAGAACTAGCAG-3′
- 245.5: 5′-GGTCCAGTGGAGATCCTGTG-3′
- 245.267: 5′-GTTTTCGTTACGCTGAGTACTG-3′

IPCC,2016

Nested PCR

X. arboricola pv. *fragariae*

Continued

- PCR is carried out in 25 µl reaction mixtures containing PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P-40, 2.5 mM $MgCl_2$), 0.2 mM each dNTP, 0.2 µM each primer and 0.5 µl Taq DNA polymerase.
- The cycling parameters are an initial denaturation step of 94°C for 4 min; 35 cycles of 94°C for 1 min, 68°C for 1 min and 72°C for 1 min; and a final extension step of 72°C for 7 min.
- For nested PCR, after amplification of DNA with the first round primers (245A and 245B), 1 µl of the first PCR product is used as template in a second PCR with the internal primers 245.5 and 245.267.
- The same cycling parameters are used except the annealing temperature is 62°C for the internal primers 245.5 and 245.267.
- PCR products are analysed by 1.2% agarose gel electrophoresis in 0.5× TAE buffer.

IPCC,2016

Nested PCR

X. arboricola pv. *fragariae*

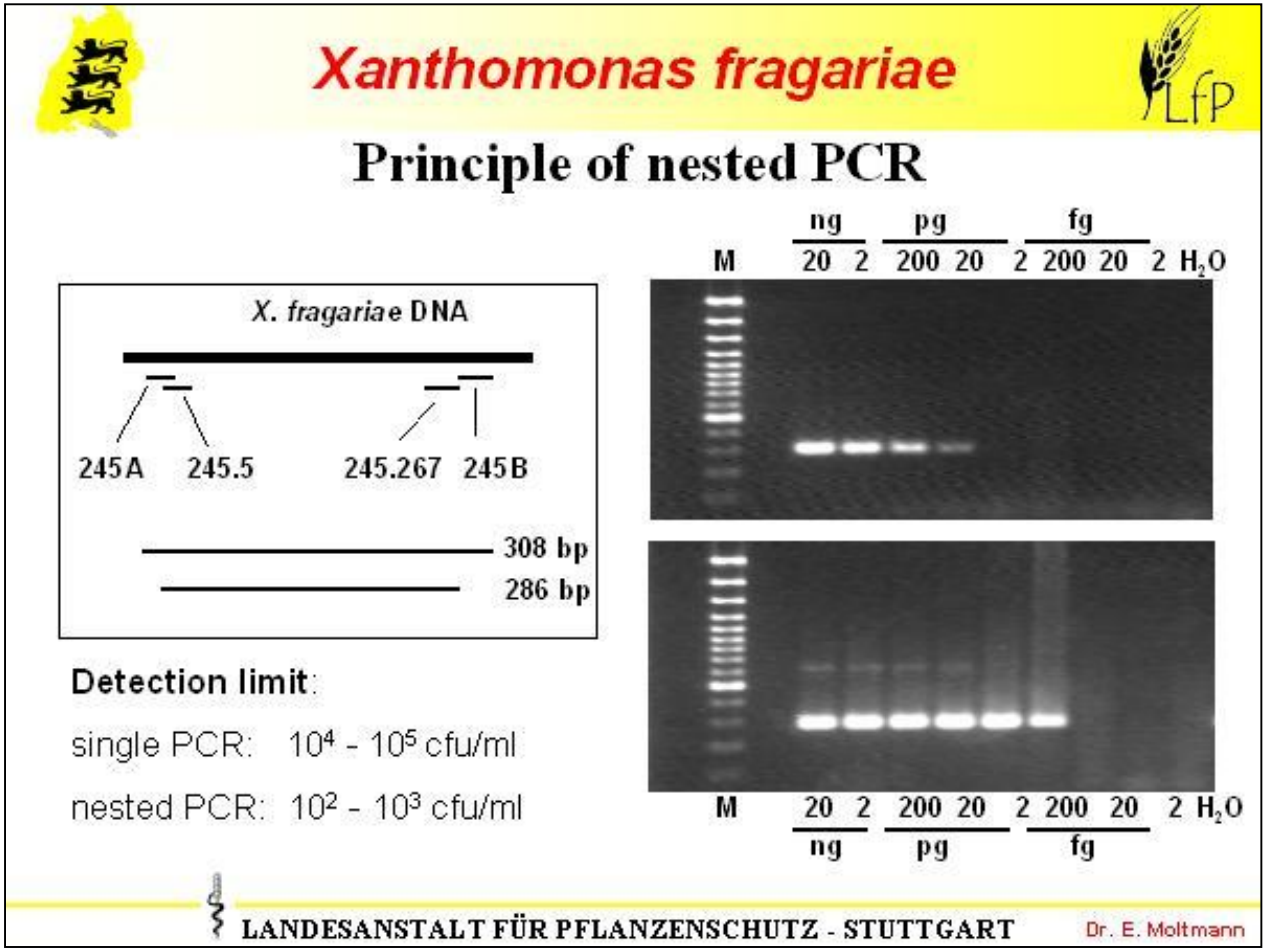
Continued

- Specific PCR amplicons for *X. fragariae* are 300 bp in the first round PCR using the 245A and 245B primers, and 286 bp in the nested PCR using the internal primers 245.5 and 245.267.
- With high template concentrations, a second fragment of approximately 650 bp is sometimes amplified.

IPCC,2016

Nested PCR

X. arboricola pv. *fragariae*



Moltmann & Zimmermann,2004

PCR

Amplification of *hrp* gene region

Detection and identification of phytopathogenic bacteria

- The *hrp* gene clusters that determine hypersensitivity and pathogenicity may be appropriate for selection of probes for detection and identification of phytopathogenic bacteria.
- The *hrp* gene cluster is required by bacterial plant pathogens to produce symptoms on susceptible hosts or nohosts.

Leite *et al.*,1994

PCR

Amplification of *hrp* gene region

Xanthomonas phaseoli

- The primers RST21 (5'GCACGCTCCAGATCAGCATCGAGG 3') and RST22 (5'GGCATCTGCATGCGTGCTCTCCGA 3') delineated a 1,075-bp fragment.
- DNA was amplified in a total volume of 25 µl. The reaction mixture contained 2.5 µl of 10X buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin), 0.2 mM of each dNTP, 12.5 pmol of each primer, 50 ng of DNA, and 1.5 units *Taq* Polymerase.
- PCR amplifications were programmed for 30 cycles of 30s at 95ºC (denaturation), 40s at 61°C (annealing), 45s 72ºC (extension), and a final extension at 72ºC for 5 min.
- Aliquots of the final amplified products were analyzed and visualized in 1.0% agarose gels containing 0.5 µg/ml of ethidium bromide.

Nunes *et al.*,2003

PCR

Amplification of *hrp* gene region

Xanthomonas phaseoli

- The set of primers used for *hrp* region was that described by Leite Jr. *et al.*,1994.
- The primers RST21 (5'GCACGCTCCAGATCAGCATCGAGG 3') and RST22 (5'GGCATCTGCATGCGTGCTCTCCGA 3') delineated a 1,075-bp fragment. DNA was amplified in a total volume of 25 µl. The reaction mixture contained 2.5 µl of 10X buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin), 0.2 mM of each dNTP (Boehringer Mannheim), 12.5 pmol of each primer, 50 ng of DNA, and 1.5 units *Taq* Polymerase.
- PCR amplifications were performed in a MJ Research thermocycler programmed for 30 cycles of 30s at 95ºC (denaturation), 40s at 61ºC (annealing), 45s 72ºC (extension), and a final extension at 72ºC for 5 min. Aliquots of the final amplified products were analyzed and visualized in 1.0% agarose gels containing 0.5 µg/ml of ethidium bromide.

Mário de Carvalho Nunes *et al.*,2008

PCR

Amplification of *hrp* gene region

Xanthomonas campestris pv. *vesicatoria*

- Three sets of oligonucleotide primers were selected from the nucleotide sequence of the hrp region of *X. campestris* pv. *vesicatoria*.

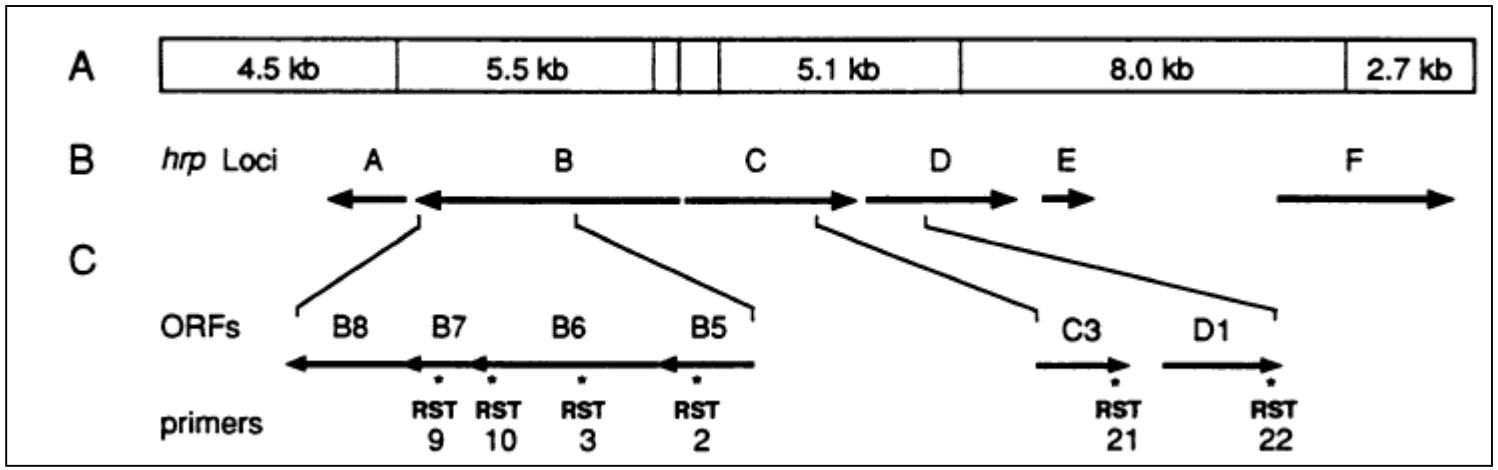
1. Primers RST2 (5'AGGCCCTGGAAGGTGCCCTGGA3') and RST3 (5'ATCGCACTGCGTACCGCGCGCGA3') delineated an 840-bp fragment.
2. Primers RST9 (5'GGCACTATGCAATGACTG3') and RST10 (5'AATACGCTGGAACTGCTG3') delineated a 355-bp fragment, and
3. Primers RST21 (5'GCACGCTCCAGATCAGCATCGAGG3') and RST22 (5'GGCATCTGCATGCGTGCTCTCCGAY) delineated a 1,075-bp fragment.

Leite *et al.*,1994

PCR

Structural organization of the *hrp* region in *Xanthomonas campestris* pv. *vesicatoria*

- Below is the primers map to the complementation groups *hrpB*, *hrpC*, and *hrpD* of *X. campestris* pv. *vesicatoria*.



(A) EcoRI fragments of the hrp region. (B) Position and orientation of the *hrp* loci, designated *hrpA* to *hrpF*. (C) Position and orientation of the open reading frames (ORFs). The sizes of the loci are based on a combination of genetic and sequence analysis from which possible open reading frames are predicted. Only the open reading frames relevant for this study, *hrpB5* to *hrpB8*, *hrpC3*, and *hrpD*, are shown here. For each RST oligonucleotide primer used for DNA amplification, its position in the DNA sequence is indicated by an asterisk (*).

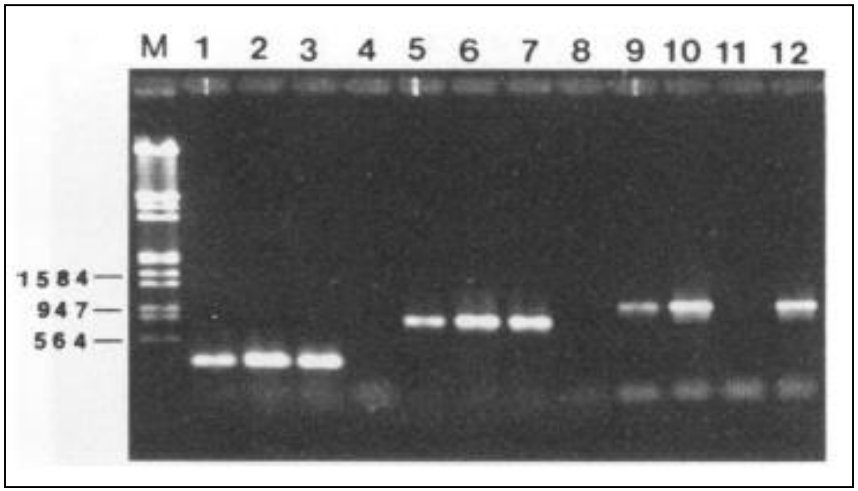
Leite *et al.*,1994

PCR

Amplification of *hrp* gene region

Xanthomonas campestris pv. *vesicatoria*

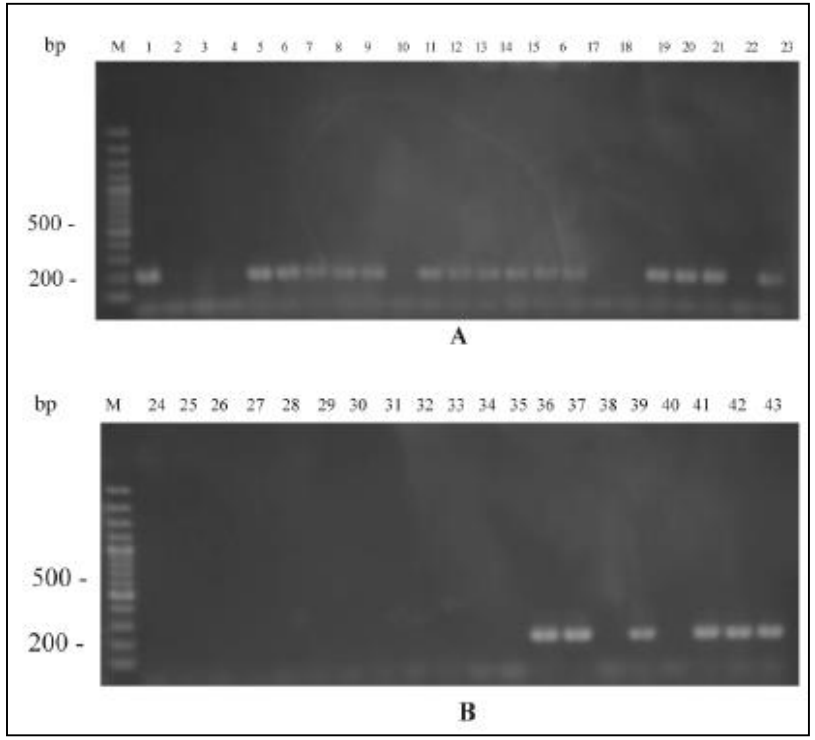
- Amplification of fragments of the *hrp* gene cluster from *X. campestris* pv. *vesicatoria* 75-3.
- Lanes:
- M, phage λ restricted with EcoRI and HindIII;
- 1 to 4, amplification with primers RST9 and RST10;
- 5 to 8, amplification with primers RST2 and RST3;
- 9 to 12, amplification with primers RST21 and RST22.
- Molecular sizes are given in base pairs.



Leite *et al.*,1994

PCR amplification with specific primers *Xanthomonas euvesicatori*

- PCR was performed using the following specific primers for the species *X. euvesicatoria*:
- Primer Xeu 2.4 (ctG GGA AAc tcA ttc GcA Gt), and
- Xeu 2.5 (ttG tGG cGc tct tAt ttc ct) (Moretti,2009).
- All previously proposed *X. euvesicatoria* strains (except two) gave positive results with amplification of the typical 208-bp product in confirmation of their identification.

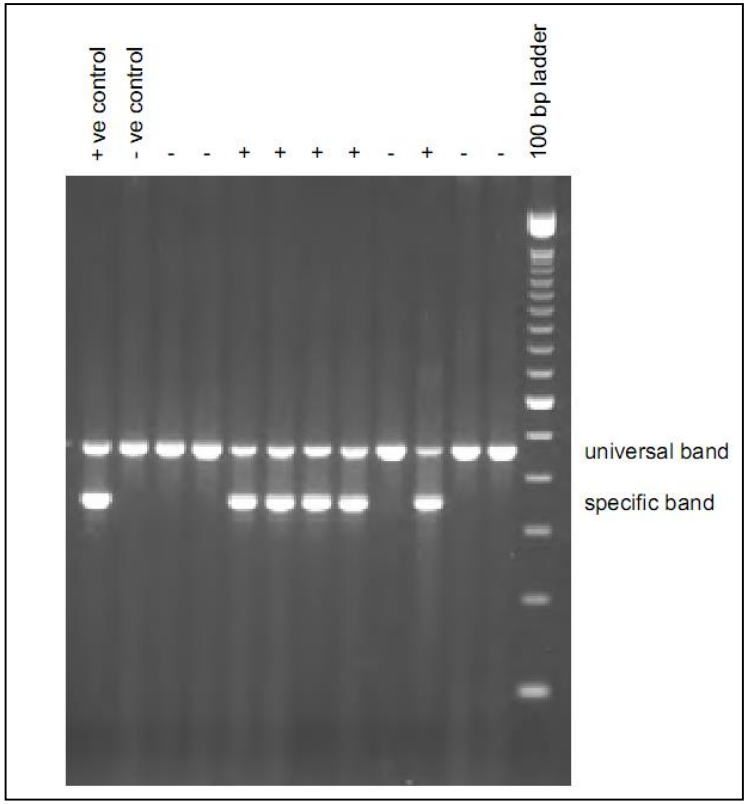


Kizheva *et al.*,2011

PCR amplification with specific and universal primers

Xanthomonas hortorum pv. *carotae*

- Agarose gel showing *Xanthomonas hortorum* pv. *carotae* specific products of 355 bp with primer pair 3Sforw/3Srev and universal bacterial products of 441 bp with primers 1052F/BacR.
- Two bands (specific and universal) = positive identification; one band (universal) = negative identification.



1052F	5' gCA.Tgg.TTg.TCg.TCA.gCT.CgT 3'
Bac R	5' TAC.ggC.TAC.CTT.gTT.ACg.ACT.T 3'

3Sforw	5' CAT.TCC.AAg.AAg.CAg.CCA 3'
3Srev	5' TCg.CTC.TTA.ACA.CCg.TCA 3'

International Rules for Seed Testing,2014

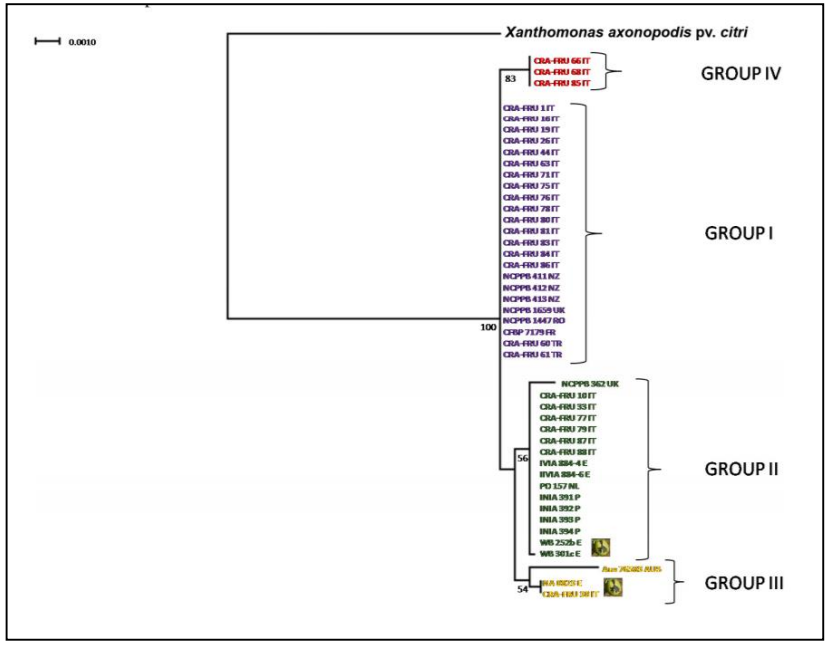
PCR
Multilocus Sequence Typing (MLST)
Xanthomonas arboricola pv. *juglandis* (*XAJ*)

- Gene fragments of *acnB* (second aconitase), *gapA* (glyceraldehyde-3-phosphate dehydrogenase), *gyrB* (subunit B of DNA gyrase) and *rpoD* (sigma subunit of RNA polymerase) were amplified from genomic DNA of the 45 *Xaj* strains.
- The DNA gene fragments were extracted using the alkaline lysis method. Briefly, a loopful of pure colonies was suspended into Eppendorf tubes containing sterile saline (0.85% of NaCl in distilled water) and mixed with a Vortex. Subsequently, the tubes were centrifuged for 2 min at 10,000 g. Then, the pellet was resuspended in 100 µL of 0.05 M NaOH and heated at 95°C for 15 min. After centrifugation for 2 min at 10,000 g, the supernatant was used as a DNA template or stored at −20°C.
- Gene fragments were amplified and sequenced with primers described by Parkinson *et al.*,2007 (i.e., *acnB*, *gapA* and *rpoD*) and Young *et al.*,2008 (i.e., *gyrB*).
- PCR was carried out in a total volume of 25 µL containing 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, pH 9); 50 pmol of each primer; 1.25 U GoTaq® DNA polymerase (Promega); 0.2 mM each dNTP (Promega); 2 mM $MgCl_2$ and 1 µL of 50 ng DNA. All PCR reactions were performed in a BioRad MJ Mini thermal cycler using the following conditions: denaturation at 95°C for 5 min; 30s of annealing at 58°C for *acnB* and *gapA* and at 60°C for *gyrB* and *rpoD* and extension at 72°C for 1 min for 35 cycles; and 5 min at 72°C for the final extension. The PCR reactions were then sent to Primm (Milano, Italy) for sequencing.

Marcelletti *et al.*,2010

PCR
Multilocus Sequence Typing (MLST)
Xanthomonas arboricola pv. *juglandis* (*XAJ*)

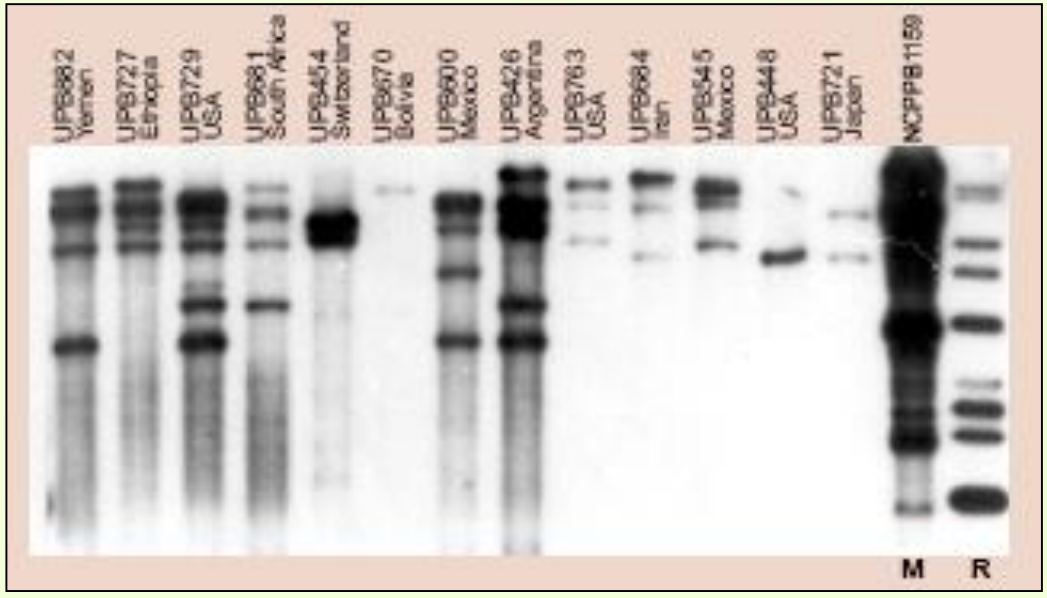
- Dendrogram of relationships among *X. a.* pv. *juglandis* strains obtained by using the amino acid sequences of *acnB*, *gapA*, *gyrB* and *rpoD*.
- Bootstrap values are reported at the main nodes.
- The *X. a.* pv. *juglandis* strains isolated from apical necrosis of walnut fruits are pointed out.



Marcelletti *et al.*,2010

RFLP analysis
Xanthomonas translucens

- Southern hybridization of *Eco*RI digested genomic DNA of 13 *Xanthomonas translucens* strains from several countries pathogenic to small grains using plasmid probe pBSF2 from *X. c.* pv. *manihotis*.
- M = *X. a.* pv. *manihotis* ; R = molecular mass standard Raoul I.



RFLP technique

Multiplex PCR

Xanthomonas oryzae pv. *oryzae* (Xoo) and *Xanthomonas oryzae* pv. *oryzicola* (Xoc)

- In this study, 51 non-pathogenic, yellow-colony forming, *Xoo* and *Xoc* look-alike bacteria isolated from rice seeds, were used to test the specificity of a multiplex PCR for the detection and differentiation of *Xoo* and *Xoc*.
- Four primer pairs used in this multiplex PCR specific for 2 pathovars of *Xanthomonas oryzae* (*Xo*)which are *Xoo* and *Xoc* were used.
- All 51 isolates did not amplify any band, indicating that they are not Xo, Xoo or Xoc.
- These results imply that the multiplex PCR used in this study is robust in detecting and differentiating Xoo and Xoc from non-pathogenic, rice seed-associated, yellow colony-forming bacteria.

Lee and Vera Cruz,2014

Multiplex PCR

Xanthomonas oryzae pv. *oryzae* (Xoo) and *Xanthomonas oryzae* pv. *oryzicola* (Xoc)

- Primers used in the multiplex polymerase chain reaction (PCR) analysis for detection of *X. oryzae*, *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* (Lang *et al*.,2010).

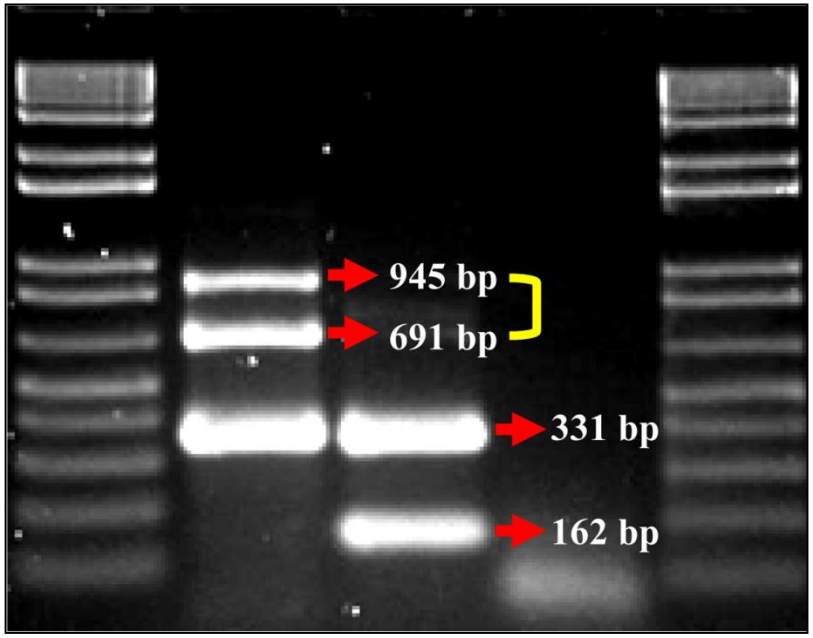
Target	Primer	Sequence	Product Size (bp)
X. oryzae	Xo3756F	CATCGTTAGGACTGCCAGAAG	331
	Xo3756R	GTGAGAACCACCGCCATCT	
X. oryzae pv. *oryzae*	Xoo80F	GCCGCTAGGAATGAGCAAT	162
	Xoo80R	GCGTCCTCGTCTAAGCGATA	
X. oryzae pv. *oryzicola*	Xoc3866F	ATCTCCCAGCATGTTGATCG	691
	Xoc3866R	GCGTTCAATCTCCTCCATGT	
	Xoc3864F	GTGCGTGAAAATGTCGGTTA	945
	Xoc3864R	GGGATGGATGAATACGGATG	

Lee and Vera Cruz,2014

Multiplex PCR

Xanthomonas oryzae pv. *oryzae* (Xoo) and *Xanthomonas oryzae* pv. *oryzicola* (Xoc)

- Band amplification of multiplex PCR products on a 1.4% agarose gel resolved at 100-110V for two hours and stained with GelRed.
- Amplification of no DNA control was also used in the multiplex PCR (Lanes 1&5- 1kb ladder; lane 2- BLS256; lane 3-PXO99, and lane 4- sterile distilled water).



Two *Xoc*-specific amplicons, 691 bp and 945 bp bands, were generated for BLS 297(*Xoc*), (lane 2), while a 162-bp fragment specific for *Xoo* was resolved for PXO 99(*Xoo*) (lane 3). In addition, a 331 bp-fragment was amplified for both *Xoo* and *Xoc*, confirming that these belong to *X. oryzae*.

Lee and Vera Cruz,2014

Multiplex PCR
X. arboricola pv. *fragariae*

- This multiplex PCR enabled detection to 10^3 cfu/ml in plant tissue (Pooler *et al.*, 1996; Hartung and Pooler 1997).
- The three sets of primers described by Pooler *et al.* (1996) are:
- 241A: 5′-GCCCGACGCGAGTTGAATC-3′
- 241B: 5′-GCCCGACGCGCTACAGAC TC-3′
- 245A: 5′-CGCGTGCCAGTGGAGATCC-3′
- 245B: 5′-CGCGTGCCAGAACTAGCAG-3′
- 295A: 5′-CGT TCC TGGCCGATT AATAG-3′
- 295B: 5′-CGCGTTCCT GCG TTTTTT CG-3′
- The cycling parameters are an initial activation step of 95°C for 15 min; 35 cycles of 95°C for 1 min, 57°C for 1 min and 72°C for 1 min; and a final extension step of 72°C for 7 min.

Multiplex PCR

Molecular markers selected for the assays *Xanthomonas* that infect citrus

- The oligonucleotides and amplicons generated by PCR are protected by the patent application registration under process number BR 10 2018 067332 7.

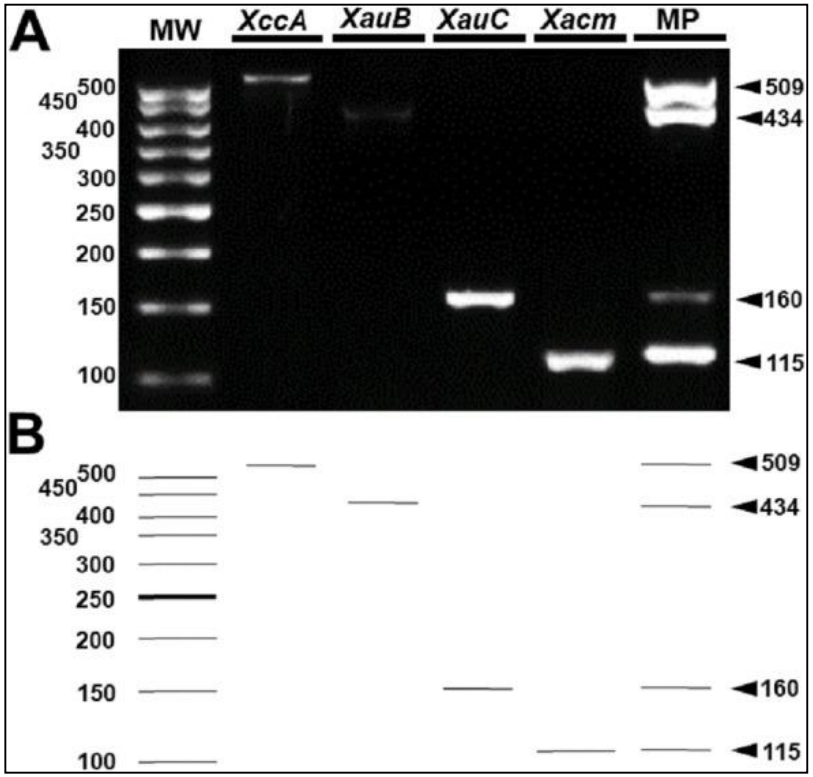
Pathotypes	Markers	Forward	Reverse	Amplicon size (bp)	Tm (°C)
XccA	XccAm	ATGCTGAGCAAGCCTTCGAT	AGCTGGGAACGATGATGGTG	509	59
XauB	XauBm	TCGATCGCACGGACTACTTG	AAAATGCGGCTCTCCCTCTC	434	59
XauC	XauCm	CACTGGAGGCAGGAGTCGAG	CCACCCTCAAGTTCAGCAACA	160	59
Xacm	Xacmm	ACCAACACCTTGTGGTCGTA	TGTTCGTCAAACCGGCCA	115	59

Fonseca *et al.*,2019

Multiplex PCR

Xanthomonas that infect citrus

- (A) Multiplex PCR electrophoresis containing the combination of the DNA of one species with all pairs of specific primers generating only their amplicon (lines 2–5).
- MP-Combination of DNAs of all pathotypes with all primers (line 6).
- MW, molecular weight (ladder 100 bp) and
- (B) Theoretical electrophoresis of multiplex PCR.

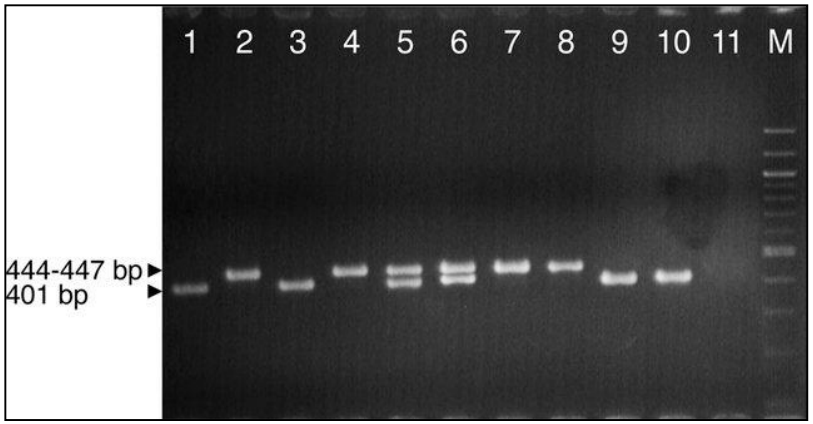


Fonseca *et al.*,2019

Multiplex nested PCR

Multiplex nested PCR detection of *X. axonopodis* pv. *allii* strains.

- Lanes 1 to 10, strains CFBP 6384, CFBP 6385, CFBP 6380, CFBP 6379, CFBP 6369, CFBP 6107, JY 276, CFBP 6387, CFBP 6368, and CFBP 6382, respectively;
- lane 11 negative PCR control;
- lane M, 100-bp ladder (Invitrogen, Merelbeke, Belgium).



The internal primers used in the multiplex PCR assay directed amplification for all 86 *X. axonopodis* pv. *allii* strains tested, resulting in a 401-bp amplicon, a 444- to 447-bp amplicon, or both amplicons, depending on the strain.

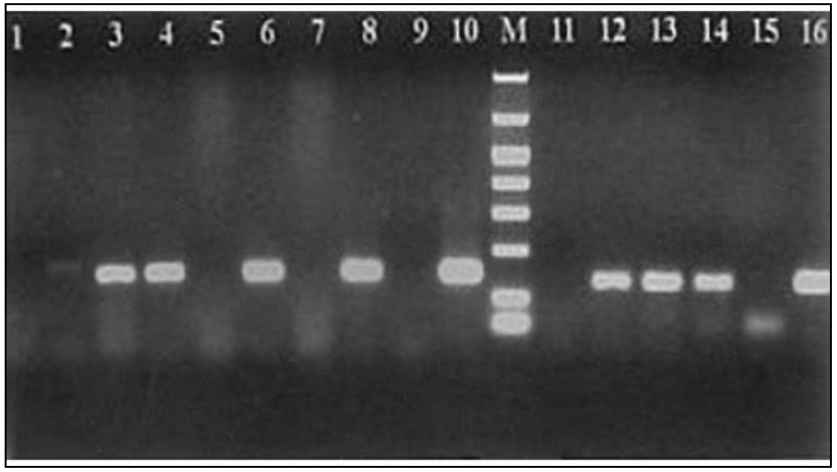
Robene *et al.*,2010

Real-time PCR
X. arboricola pv. *fragariae*

- The primers, based on sequences of the *gyrB* gene, and TaqMan probe, covalently labelled at the 5′ end with the reporter dye JOE and at the 3′ end with the quencher dye TAMRA, are:
- Xf gyrB-F: 5'-CCG CAG CGA CGC TGA TC -3'
- Xf gyrB-R: 5'-ACG CCC ATT GGC AAC ACT TGA-3'
- Xf gyrB-P: 5'-TCC GCA GGC ACA TGG GCG AAG AAT TC-3‘
- PCR is carried out by adding 4 µl template DNA to a reaction mixture containing 1×TaqMan Buffer A (Applied Biosystems1), 5.5 mM $MgCl_2$, 200 µM dNTPs (Promega1), 300 nM each primer, 100 nM probe and 0.63 U AmpliTaq Gold DNA polymerase (Applied Biosystems1).
- The cycling parameters are an initial activation step of 2 min at 50°C then 15 min at 95°C followed by 40 cycles of 10 s at 95°C and 1 min at 60°C.

Real-time PCR
Xanthomonas citri

- Real-time polymerase chain reaction (PCR) products of dried herbarium citrus leaf samples infected by *Xanthomonas citri* pv. *citri* using direct tissue extracts or DNA purified using Idaho Technology (IT) Kit from extracts, electrophoresed in 2% agarose gels and stained with ethidium bromide.
- Lanes 1 to 8: VM3 and 4 primer pair PCR products.
- Lane 15-water control;
- Lane M-Marker DNA Lane.



Narayanasamy,2006

PCR detection and identification of xanthomonads

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X. albilineans	**Ala4/IIe2** Inter tRNA region **16S+IIe1 or Ala1+23S** Region between 16S rRNA gene and $tRNA^{ala}$ or $tRNA^{ile}$ and 23S rRNA	Conventional Nested	Bacteria (boiled) or leaf (DNA extraction)	Honeycut *et al.*, 1995	
X. albilineans	**Ala4/L1** Inter tRNA region	Conventional	Bacteria, sap, leaf (untreated)	Pan *et al.*, 1997	
X. albilineans	**XAF1/XAR1** Genomic DNA (unknown)	Conventional BIO	Bacteria, sap, leaf (boiled)	Wang *et al.*, 1999	
X. arboricola pv. *pruni*	**Y17CoF/Y17CoR** RAPD fragment	Conventional	Bacteria, plant (untreated)	Pagani, 2004	
X. axonopodis pv. *dieffenbachiae*	**$KJM11^f$/$KJM12^r$** + **$KJM34^f$/$KJM36^r$** + **$KJM74^f$/$KJM73^r$** RAPD fragment	Conventional Multiplex Previous immunocapture	Bacteria, plant (DNA extraction or immunocapture)	Khoodoo *et al.*, 2005	
X. axonopodis pv. *dieffenbachiae*	**PXadU/PXadL** (external) **NXadU/NXadL** (internal) RAPD fragment	Nested	Bacteria (boiled), plant (PP buffer with 5% PVP)	Robène-Soustrade *et al.*, 2006	
X. axonopodis pv. *manihotis*	Plasmid fragment (unknown)	Conventional	Plant extracts (without DNA extraction)	Verdier *et al.*, 1998	
X. axonopodis pv. *phaseoli* *X. campestris phaseoli* var. *fuscans*	**X4c/X4e** Plasmid DNA	Conventional	Bacteria, leaf (DNA extraction)	Audy *et al.*, 1994	*Xanthomonas campestris* pv. *phaseoli* *X. campestris phaseoli* var. *fuscans* is not a valid name according to the ISPP list.
X. axonopodis pv. *phaseoli* *X. campestris phaseoli* var. *fuscans*	**OP-G11** Random primer	RAPD	Bacteria (DNA extraction)	Birch *et al.*, 1997	*X. campestris* pv. *phaseoli*
X. axonopodis pv. *phaseoli* *X. campestris phaseoli* var. *fuscans*	**Xf1/Xf2** RAPD fragment + **X4c/X4e** Plasmid DNA	Conventional Multiplex	Bacteria, plant (DNA extraction)	Toth *et al.*, 1998	*X. campestris* pv. *phaseoli* Xf1/Xf2 specific for *Xanthomonas campestris* pv. *phaseoli* var. *fuscans*. X4c/X4e amplify both *X. arboricola* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans*.
X. axonopodis pv. *phaseoli*	**X4c/X4e** **(Xanthomonas)** Plasmid DNA	Conventional	Seeds (alkaline treatment)	Audy *et al.*, 1996	*X. campestris* pv. *phaseoli* *Pseudomonas savastanoi* pv. *phaseolicola* (*P. syringae* pv. *phaseolicola*) also amplified.

Palacio-Bielsa *et al.*,2009

PCR detection and identification of xanthomonads

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	HB 14F/HB 14R (***Pseudomonas***) Phaseolotoxin gene cluster Simultaneous detection **HB 14F+HB 14R+ X4c+X4e**	Concurrent detection			
X. campestris pv. *campestris*	**HrcCF2/HrcCR2** *hrcC* gene (pathogenicity-associated)	Conventional	Bacteria, plant and seeds (DNA extraction)	Zaccardelli *et al.*, 2007	
X. citri subsp. *citri*	**2/3** Pathotype A strains **4/5; 6/7; 1/5** Pathotype A strains (variable for pathotypes B and C) Plasmid DNA	Conventional	Bacteria, plant (DNA extraction)	Hartung *et al.*, 1993	*X. campestris* pv. *citri*
X.citri subsp. *citri*	(first round) + **94-3 bio/94-4 lac** (second round) Plasmid DNA	Nested	Plant (immunocapture)	Hartung *et al.*, 1996	*X. axonopodis* pv. *citri*
X.citri subsp. *citri*	**CiH2/CiH3** Contains 5´termini for a plasmid DNA of *X. axonopodis* pv. *citri* and 3´termini homologous to Figwort mosaic virus (FMV)	Competitive (Internal standard)	Plant (DNA extraction)	Cubero *et al.*, 2001	*X.axonopodis* pv. *citri*
X. citri subsp. *citri*	**Xac01/Xac02** *rpf* gene cluster	Conventional	Bacteria, plant (DNA extraction)	Coletta-Filho *et al.*, 2006	*X. axonopodis* pv. *citri*
X. citri subsp. *citri*	**A5, C5, A2, D2, A3, D7, A9, A10** Genomic and plasmid DNA (unknown)	Conventional	Plant (DNA extraction)	Li *et al.*, 2006a	*X. axonopodis* pv. *citri*
X. citri subsp. *citri* (Pathotypes A, B and C)	**J-pth1/J-pth2** Pathotypes A, B and C strains *pthA* gene (involved in virulence) **J-RXg/J-RXc2** Pathotype A strains ITS region	Conventional	Bacteria (DNA extraction)	Cubero and Graham, 2002	*X. axonopodis* pv. *citri*

Palacio-Bielsa *et al.*,2009

PCR detection and identification of xanthomonads

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X. citri subsp. *citri* (Pathotypes A, B and C)	**2/3** Pathotype A strains Plasmid DNA **J-pth1/J-pth2** Pathotype A, B and C strains *pthA* gene (involved in virulence) **J-RXg/J-RXc2** Pathotype A strains ITS region	Conventional	Plant (DNA extraction)	Hartung *et al.*, 1993, 1996; Cubero *et al.*, 2001; Cubero and Graham, 2002; Anon., 2005b	*X. axonopodis* pv. *citri* Recommended in the EPPO protocol.
X .citri subsp. *citri* *X. citri* pv. *aurantifolii*	**VM1/VM2** **VM3/VM4** **VM5/VM6** *pthA* gene family **Kingsley forward/reverse** *X. citri* pv. *citri* A chromosome	Real-time (SBYR® Green Master Mix)	Bacteria, plant (DNA extraction)	Mavrodieva *et al.*, 2004	*X. citri* pv. *citri* *X. citri* pv. *aurantifolii* is not included in the ISPP List.
X. citri subsp. *citri* *X. axonopodis* pv. *citrumelo*	**J-RT pth3/J-RT pth4** *pth* gene, citrus bacterial canker strains **J-RTRib 16Sup/J-RTRib downXC2** Ribosomal sequence, *X. axonopodis* pv. *citrumelo* **J-AdlrpU1J-AdlrpU2** *lrp* gene, *Xanthomonas* spp. **J-Taqpth2** *pth* gene, citrus bacterial canker strains **J-Taq16S-1** Ribosomal sequence, *X. axonopodis* pv. *citrumelo* **J-Alrpallelic1** *lrp* gene, *X. citri* pv. *citri* wide host range strains **J-Awlrpallelic1** *lrp* gene, *X. citri* pv. *citri* restricted host range strains	Real-time (TaqMan)	Bacteria, plant (DNA extraction)	Cubero and Graham, 2005	*X. axonopodis* pv. *citri* *X. axonopodis* pv. *citrumelo* is not included in the ISPP list. Allelic discrimination of citrus *Xanthomonas* strains allowed and a single nucleotide difference detected.

Palacio-Bielsa *et al.*,2009

PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for plant pathogenic bacteria according to ISPP nomenclature. When the nomenclature reported in articles differs, the originally cited names of side of the table bacteria are indicated on the right

X. fragariae	**REP1R-I, REP2-I, ERIC1R, ERIC2**	rep	Bacteria (untreated)	Opgenorth *et al.*, 1996	Bacterial identification.
X. fragariae	**241A/241B** **245A/245B** **295A/295B** **Multiplex** (different primer pairs combinations) **241+245, 241+295, 245+295, 241+245+295** RAPD fragment	Conventional Multiplex	Bacteria (DNA extraction)	Pooler *et al.*, 1996	
X. fragariae	**XF9/XF11** (first round) + **XF9/XF12** (second round) *hrp* gene	Nested	Bacteria, plant (DNA extraction)	Roberts *et al.*, 1996; Mahuku and Goodwin, 1997	
X. fragariae	**JJ9/JJ12** *hrp* gene	Conventional	Bacteria, plant (DNA extraction)	Zhang and Goodwing, 1997	
X. fragariae	**XF10/XF12** *hrp* gene	Conventional	Plant (DNA extraction)	Stöger and Ruppitsch, 2004	
X. fragariae	**245A/245B** (first round) RAPD fragment **245.5/245.267** (second round) 245A-245B fragment	Conventional Nested	Bacteria, plant (DNA extraction)	Pooler *et al.*, 1996; Zimmermann *et al.*, 2004	Both pairs of primers can be used in conventional or nested PCR.
X. fragariae	**245A/245B** (first round) RAPD fragment **245.5/245.267** (second round) 245A-245B fragment **XF9/XF11** (first round) + **XF9/XF12** (second round) *hrp* gene	Conventional Nested	Plant (DNA extraction)	Roberts *et al.*, 1996; Zimmermann *et al.*, 2004; Moltmann and Zimmermann, 2005	Primers pair 245A/245B and 245.5/245.267 can be used in both conventional and nested PCR.
X. fragariae	**REP1R-I, REP2-I, ERIC1R, ERIC2** **241A, 241B, 245A, 245B, 29 A, 295B** RAPD fragments	rep Multiplex	Plant (with or without DNA extraction) (enrichment)	Opgenorth *et al.*, 1996; Anon., 2006d Pooler *et al.*, 1996; Stöger and Ruppitsch, 2004; Anon., 2006d	Recommended in the EPPO protocol.

Palacio-Bielsa *et al.*,2009

PCR detection and identification of xanthomonads

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	XF9/XF11 *hrp* gene	Conventional		Roberts *et al.*, 1996; Anon., 2006d	
X. hortorum pv. *carotae*	**3SF/3SR** RAPD fragment	Conventional	Bacteria (boiled), plant, seeds (DNA extraction)	Meng *et al.*, 2004	*X.campestris* pv. *carotae*
X. hortorum pv. *pelargonii*	RAPD fragment	Conventional	Bacteria (DNA extraction)	Manulis *et al.*, 1994	*X. campestris* pv. *pelargonii*
X. hortorum pv. *pelargonii*	**XcpM1/XcpM2** *X. c.* pv. *pelargonii* DNA (ERIC)	Conventional	Bacteria, plant (DNA extraction)	Sulzinski *et al.*, 1996, 1997, 1998	*X. campestris* pv. *pelargonii*
X. hortorum pv. *pelargonii*	RAPD fragment	Conventional	Bacteria, plant (DNA extraction)	Chittaranjan and De Boer, 1997; Manulis *et al.*, 1994	*X. campestris* pv. *pelargonii*
X. hyacinthi	**JAAN/JARA** *fimA* gene (type IV structural fimbrial-subunit)	Conventional	Bacteria, plant (untreated)	van Doorn *et al.*, 2001	
X. oryzae pv. *oryzae*	**XOR-F/XOR-R2** ITS region	Conventional	Bacteria, plant (boiled)	Adachi and Oku, 2000	A fragment of the same size also obtained from *X. campestris* pathovars *citri, incanae* and *zinniae*.
X. oryzae pv. *oryzae*	**TXT/TXT4R** *IS1113* insertion element	Conventional BIO	Pure cultures, and plant tissue (DNA extraction) or BIO-PCR from seeds (without DNA extraction)	Sakthivel *et al.*, 2001	
X. oryzae pv. *oryzae*	**PF/PR** Putative siderophore receptor gene *cds*	Real-time (TaqMan)	Rice seeds washes (untreated)	Zhao *et al.*, 2007	At an annealing of 60°C both pv. *oryzae* and pv. *oryzicola* and *oryzicola* are amplified, whereas at 68°C only *X. oryzae* pv. *oryzae* results in a fluorescent signal.
X. oryzae pv. *oryzae* *X. oryzae* pv. *oryzicola*	**XOR-F/XOR-R2** ITS region **TXT/TXT4R** *IS1113* insertion element Differentiation of pathovars *oryzae* and *oryzicola* **R16-1/R23-2R** ITS region	Conventional BIO	Bacteria, plant (DNA extraction or BIO-PCR from seeds without DNA extraction)	Kim and Song, 1996; Adachi and Oku, 2000; Sakthivel *et al.*, 2001; Anon., 2007	
X. translucens	**PAf/PBf/PABr** ITS region	Multiplex	Bacteria (DNA extraction) or plant (PVP addition)	Marefat *et al.*, 2006	Groups A and B of *X. translucens* from pistachio differentiated. *X. translucens* pv. *cerealis* also amplified.

Palacio-Bielsa *et al.*,2009

PCR detection and identification of xanthomonads

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X. translucens pv. *poae*	**XAN1/XAN2** **XAN3/XAN4** **XAN5/XAN7** Encompassing 16S rRNA, ITS, 23S rRNA	Conventional	Bacteria (DNA extraction)	Mitkowski *et al.*, 2005	
X. vesicatoria	**RST2/RST3** **RST9/RST10** *hrpB* (hypersensitive reaction and pathogenicity gen cluster)	Conventional	Seed washes (DNA extraction) (sodium ascorbate and PVPP)	Leite *et al.*, 1995	*X. campestris* pv. *vesicatoria*
X. campestris pathovars: *aberrans, armoriaceae, barbarae, campestris, incanae, raphani*	**DLH120/DLH125** *hrpF* gene (Specific for *X. campestris*) + **DLH138/DLH139** ITS region from *Brassica* spp. (host internal control) **DHL153/DHL154** *hrpF* gene (Specific for *X. campestris*) + **DHL155/DHL156** ITS region and 5.8S rRNA gene from *Brassica* spp.	Multiplex-Conventional Multiplex-Real-time (SBYR® Green Master Mix) (fluorescently labeled probes)	Bacteria, seed-washes (DNA extraction)	Berg *et al.*, 2005, 2006	
Xanthomonas Numerous pathovars (not *translucens* group)	**RST2/RST3** **RST9/RST10** *hrpB* (hypersensitive reaction and pathogenicity gen cluster) **RST21RST22** *hrpC, hrpD* groups	Conventional and RFLP	Bacteria (DNA extraction)	Leite *et al.*, 1994	
X, campestris hordei *X. translucens* pathovars: *arrhenatheri, cerealis, graminis, phlei, phleipratensis, poae secalis, translucens* and *undulosa*	**T1/T2** ITS region	Conventional	Bacteria, seeds (boiled)	Maes *et al.*, 1996b	*X. campestris* pathovars: *cerealis, secalis, translucens, undulosa, arrhenatheri, graminis, phlei, phleipratensis, poae* No distinction of the five cereal leaf streak pathovars from the other five pathovars.

Palacio-Bielsa *et al.*,2009

PCR detection and identification of xanthomonads

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X. fragariae *X. arboricola* pv. *fragariae*	*X. fragariae* **Xf gyrB-F/ Xf gyrB-R** (primers) **Xf gyrB-P** (probe) *GyraseB* gene *X. arboricola* pv. *fragariae* **Xaf pep-F/ Xaf pep-R** (primers) **Xaf pep-P** (probe) *pep* propyl endopeptidase gene	Real-time (TaqMan)	Bacteria (boiled) and plant (DNA extraction)	Weller *et al.*, 2007	*Xanthomonas arboricola* pv. *fragariae* not included in the ISPP List. Primers *Xf gyrB* specific for *X. fragariae*. *Xaf pep* primers detect other *X. arboricola* pathovars assayed also, but not *X. fragariae*.
X. hortorum pv. *pelargonii*	**XcpM1/XcpM2** DNA (ERIC) **RS3/Rs4** *R. solanacearum pehB* gene **DG1/DG2** 18S rRNA gene (host internal control)	Multiplex	Bacteria (DNA extraction), plant (GeneReleaser)	Glick *et al.*, 2002	*X. campestris* pv. *pelargonii* *Ralstonia solanacearum* also amplified.
X. oryzae (pathovars *oryzae* and *oryzicola*)	**R16-1/R23-2R** 16S-23S rDNA spacer region	Conventional	Bacteria (DNA extraction)	Kim and Song, 1996	Differentiation between *X. oryzae* pathovars *oryzae* and *oryzicola*. *Acidovorax avenae* (*Pseudomonas avenae*), *Burkholderia glumae* (*Pseudomonas glumae*), *Pantoea agglomerans* (*Erwinia herbicola*), *Pseudomonas fuscovaginae* and *Pseudomonas syringae* pv. *syringae* also amplified and differentiated by primary and secondary fragments.
X. vesicatoria *Pseudomonas syringae* pv. *tomato*	**BSX1/BSX2 (Xanthomonas)** Genomic DNA (unknown) **COR1/COR2 (Pseudomonas)** Coronafacic acid *cfa7* gene	Conventional	Bacteria, plant (freeze-boiled method DNA extraction)	Cuppels *et al.*, 2006	*X. axonopodis* pv. *vesicatoria* and *X. gardneri* are not valid names according to the ISPP List. BSX primers amplify *X. vesicatoria*. Other coronatine-producing *P. sryringae* pathovars also amplified with COR primers.

Palacio-Bielsa *et al.*,2009

Pathogenicity tests
Xanthomonas spp.

- All strains of *X. malvacearum*, *X. phaseoli*, *X. campestris* pv. *campestris*, *X. a.* pv. *alfalfae* caused disease when inoculated into their respective hosts:
- Cotton (angular water soaked lesions),
- Bean (water soaked spots),
- Cabbage (black veins and chlorosis), and
- Alfalfa (yellow water-soaked spots).
- None of these strains caused disease in citrus and none of the 26 citrus strains caused disease in cotton, alfalfa or beans.
- Both strains of *X. axonopodis* pv. *axonopodis* produced narrow (0.1-0.2 mm wide), linear (2-3 cm long) and chlorotic lesions in sugarcane leaves.

Pathogenicity tests

Pathovar system of taxonomy

X. malvacearum, X. campestris, X. vesicatoria

Xanthomonas campestris* pv. *campestris – crucifer black rot

***X. c.* pv. malvacearum** – cotton angular leaf spot

X. c.* pv. *vesicatoria – tomato & pepper bacterial spot

host→ pathovar↓	cabbage	cotton	pepper
campestris	disease	HR	HR
malvacearum	HR	disease	HR
vesicatoria	HR	HR	disease

Pathogenicity tests

Mid vein injection

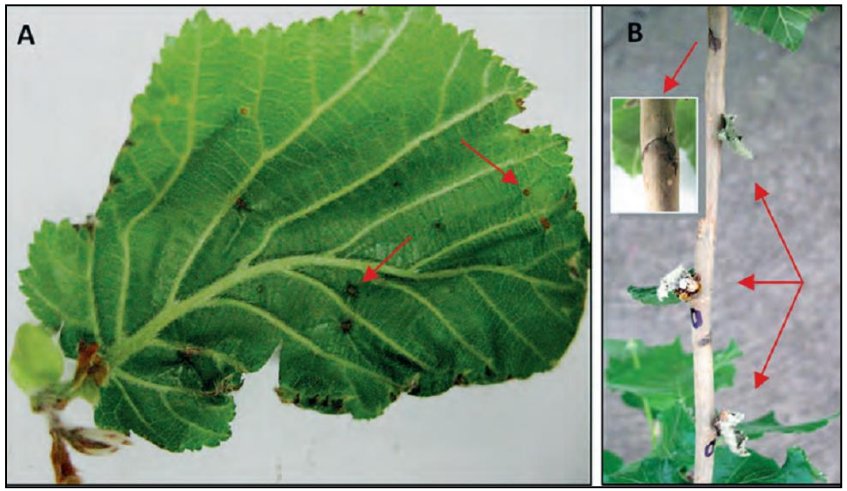
Xanthomonas spp.

- Cells were grown in liquid NBY and the resulting log phase suspensions adjusted in 0.85% saline to contain 10^5 cfu/ml.
- For citrus strains (*X. citri*): Newly unfolded leaves of Mexican lime, Mandarin orange, Duncan grapefruit, or lemon seedlings and first true leaves of cotton seedlings were infiltrated with an inoculum using the blunt end of a 1.0 ml syringe.
- For cabbage (*X. campestris* pv. *campestris*) and sugarcane (*X. axonopodis* pv. *axonopodis*): The leaf mid vein and stem (growing point), respectively, of 2-3 leaf-stage plants were injected with a suspension of 10^5 cfu/ml using a 26-gauge needle and syringe.
- For Bean, alfalfa, and cotton leaves were atomized with similar prepared inocula.
- Control inoculations were made with 0.85% saline.
- After 10-14 days at 30°C in a lighted (14 h) dew chamber, results were recorded.

Pathogenicity assay

Xanthomonas arboricola pv. *corylina*

- Prepare the inoculum to an approximate concentration of 10^8 (OD_{600}CFU ml^{1-}= 0.3).
- Water-soaked angular spots and lesions on leaves 30 days after inoculation.
- A. bud necrosis and dieback and necrotic lesions on the stem 6 months after inoculation.



Prokic´ *et al.*,2012

Pathogenicity assay
Xanthomonas arboricola pv. *fragariae*

- Pathogenicity was tested in winter in a glasshouse on pot-cultivated strawberry cv. Chandler.
- Leaves were inoculated with *X. arboricola* pv.*fragariae* (bacterial leaf blight of strawberry) by puncturing major veins, the blade, edge of blade, midrib and peduncle.
- Also strawberry cv. Chandler leaves were inoculated with other xanthomonads at 1-2 x 10^7 and 1-2 x 10^6 cfu mL^{-1}.
- Control leaves were inoculated with sterile 8.5 g L^{-1} NaCl solution.
- Symptom development was assessed up to 60 days after inoculation.
- Re-isolations were carried out after the appearance of symptoms.

Janse,2001

Pathogenicity assay
Xanthomonas arboricola pv. *fragariae*

- For expression of the disease, plants require specific conditions of temperature and humidity and need to be observed for a period of four weeks after inoculation.
- The isolates were tested for pathogenicity on strawberry plants in the greenhouse.
- It takes time and is laborious.
- The detached leaf assay may be used as an alternative to whole plant pathogenicity testing.

D. Noble, Moltmann *et al.*,2005;..

Pathogenicity assay

X. arboricola pv. *fragariae* vs. *X. fragariae*

- In both cases, symptoms only appeared ≈30 days after inoculation.
- The midrib showed reddish discoloration 8-10 mm above and below the inoculation site.

1. *X. arboricola* pv.*fragariae* (bacterial leaf blight of strawberry):

- Only on some cases, induce water-soaked areas along the midribs.
- The presence of bacterial exudate was never observed.

2. *X. fragariae* strains(angular leaf spot of strawberry):

- All induce water-soaked symptoms.
- Samples with young lesions should be examined for the presence of bacterial ooze as this is the best indictor that the cause is bacterial.

Janse,2001;D. Noble

Pathogenicity assay

Xanthomonas fragariae

Symptoms

Ooze formation at high humidity

Baden-Württemberg
LANDESANSTALT FÜR PFLANZENSCHUTZ

Moltmann *et al.*,2005

Pathogenicity test
Inoculation of whole plants
Xanthomonas arboricola pv. *juglandis*

- 1 to 2-year-old potted plants of a cultivar susceptible to walnut blight should be used. Bacterial infections require young leaves, which are more susceptible than the mature and old ones. Inoculum suspensions are prepared from 4 to 5-day-old *Xaj* cultures grown on NA (nutrient agar) medium at 27ºC.
- Suspensions on SDW are adjusted to 1-5 × 10^8 CFU ml^{-1} with a spectrometer (A_{600nm} =0.2) and serial dilutions are plated on YDC medium and counted for confirmation of inoculum concentration.
- Inoculum suspensions must be prepared on the same day of inoculation and maintained at 4ºC until use. To facilitate infection diatomaceous earth (0.2 g l^{-1}) can be added to the inoculum.
- Bacterial suspensions are sprayed under pressure (1-2 bar) on both faces of walnut leaves until runoff. Inoculated plants are immediately covered with plastic bags which are internally sprayed with sterile water and incubated for 24-48 h at 25ºC in a climatic chamber. Then, plastic bags are removed and plants are re-introduced into the climatic chamber at 25ºC, 70-80% RH, 16 h photoperiod for 7-10 days for symptom development.
- Pathogenic bacterial strains produce brown spots often surrounded by a yellowish halo.

Moragrega,2012

Pathogenicity test

Inoculation of whole plants

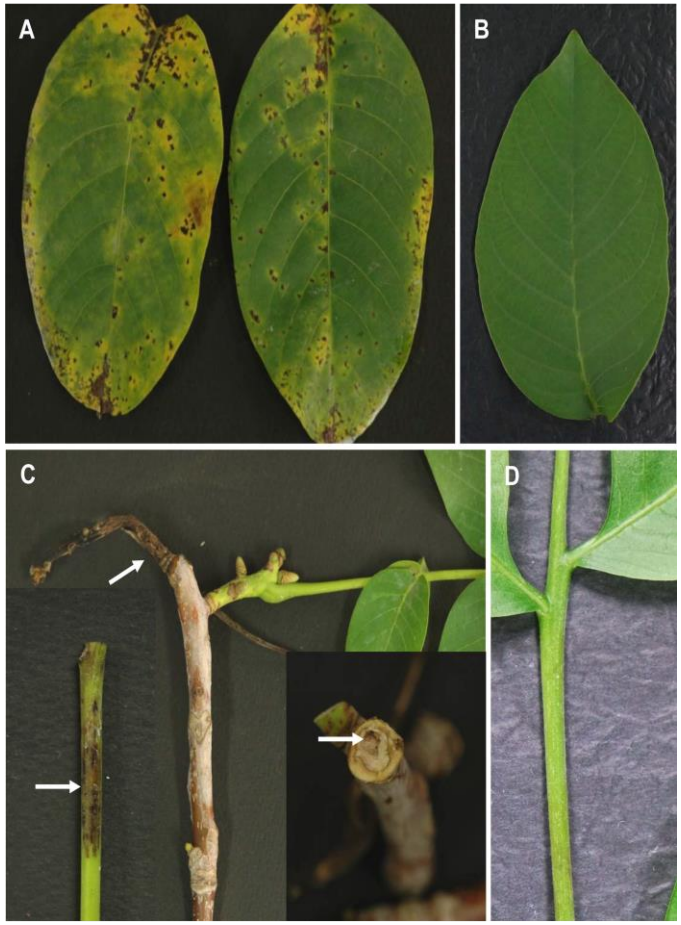
Xanthomonas arboricola pv. *juglandis*

1. Pathogenicity test can be carried out on detached walnut leaves and stem by artificial inoculation:

A. on wounded leaves, 2 weeks after inoculation with yellow halos around the black spots.

C. on inoculated stem 1 week after inoculation (the sites of inoculation noted by arrows).

- A cross-section of the stem shown at the corner represents the disease spread inside the stem tissue.
- B and C, control



Sup Kim *et al.*,2021

Pathogenicity test

Xanthomonas arboricola pv. *juglandis*

2 **Pathogenicity test**

Field Inoculation (Feb. 2006)

Inoculum :

- Bacterial suspensions (1 x 10^9 ufc/ml iin water)

Plant material :

- 2 varieties : Franquette (RA 311) and Fernor (RA 1156)

Protocole :

- Inoculation on 15 trees/strain/varieties
- On each plant, 3 inoculation points

Photos D. Meyer, INRA-Angers

ngers 17-19 April 2007 *Action COST 873*

Meyer *et al.*,2007

Pathogenicity test
Xanthomonas arboricola pv. *juglandis*

- Koch postulate is OK.

Inoculation of one *Xanthomonas* strain isolated from black juice collected on a oozing canker on a trunk.

Photos M. Ménard, INRA-Angers

Meyer *et al.*,2007

Pathogenicity assay

Immature healthy fruits

Xanthomonas arboricola pv. *juglandis*

- To evaluate walnut susceptibility or resistance to bacterial blight a detached immature fruit inoculation test has been developed and widely used in:

1. cultivar susceptibility evaluation assays,
2. assessment of strain virulence, or
3. determination of the efficacy of chemicals in walnut blight control.

- The method consists of artificial inoculation of bacterial suspensions on immature fruits (Gf+30) and incubation under controlled environment conditions.

Moragrega,2012

Pathogenicity assay

Immature healthy fruits

Xanthomonas arboricola pv. *juglandis*

- Swab immature healthy fruits with alcohol and wash in sterile water.
- Place a drop of inoculum (10^6 cfu per ml) on the fruit surface and puncture the fruit by pricking (making pinholes) through the drop with a sterile needle.
- Incubate at 28°C in closed boxes lined with damp blotting paper.
- Use a known reference culture of as a positive control.
- Use sterile water as a negative control.

Plant Bacteriology Manual,2008

Pathogenicity assay

Immature healthy fruits

Xanthomonas arboricola pv. *juglandis* (*Xaj*)

- Evaluation of walnut susceptibility or resistance to bacterial blight.

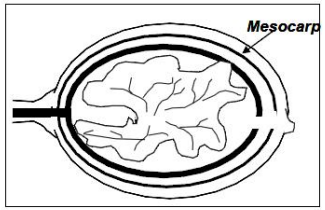


Image 2: Inoculation of bacteria at mesocarp level.

Inoculation of bacteria at mesocarp level.

Symptoms of *Xaj* infection on inoculated walnut fruit tissues. Infection expanded laterally in the mesocarp and internally to the endocarp and the seed, developing on cultivar susceptibility.

Aletà *et al.*,2001

Pathogenicity assay

Immature healthy fruits

Xanthomonas arboricola pv. *juglandis*

- Evaluation of walnut susceptibility or resistance to bacterial blight.
- Incubation of inoculated fruits in climatic chambers under high relative humidity and optimal conditions for disease development.

Aletà *et al.*,2001

Pathogenicity assay

Immature healthy fruits

Xanthomonas arboricola pv. *juglandis*

- Disease severity indexes established to evaluate disease severity in walnut fruits inoculated with *X. arboricola* pv. *juglandis*.

Disease severity is determined according to established Infection Severity Indexes (I) from 0 to 4: 0:no infection; 1: necrosis located to the inoculation point; 2: necrosis extending from the inoculation point through the green tissue; 3: necrosis extending through the mesocarp and reaching the endocarp and 4: necrosis affecting the seed (Image 4).

Disease severity per replicate (S) is calculated from disease severity indexes (I) as follows:

$$S = \sum_{n=1}^{N} \frac{I_n}{N \cdot 4} \cdot 100$$

Where: I_n is the disease severity index of an inoculation (n)
N is the amount of inoculations in a replicate

From disease severity (S) per replicate values mean severity per walnut cultivar or selection can be obtained and statistical analysis can be performed.

Aletà *et al.*,2001

Pathogenicity assay
Xanthomonas phaseoli

- Grow seedlings of a known susceptible *Phaseolus vulgaris* cultivar (e.g. Helda) under suitable conditions until first leaf is just visible (approx. 10 days after sowing).
- Water plants 1-3 hours before inoculation to provide better conditions for infection.
- Dip a sterile toothpick or needle in the bacterial culture on YDC medium.
- Inoculate the seedling by stabbing the toothpick or needle until it emerges from the opposite side of the node.
- Turn the toothpick or needle slightly while drawing to release bacteria.
- Use two seedlings per suspected isolate.

Pathogenicity assay
Xanthomonas phaseoli

- Place seedlings in an environment with high relative humidity and light adequate for plant growth for 7 days.
- Incubate inoculated plants at 20-30°C and record symptoms after 4-5 days, 8-10 and 14 days. Check symptoms on plants and compare them with the negative (no bacteria) and positive (reference strains) controls.
- Evaluate the inoculated seedlings for symptoms of *X. phaseoli*.
- Typical *X. phaseoli* symptoms include dark green water-soaked lesions at the point of entry of the toothpick or needle.
- Lesions can become red-brown, elongate extending into the stem causing slight to severe stem cracking.
- Wilting and flagging of the top foliage followed by necrosis can occur 14-18 days after inoculation.

Pathogenicity assay
Xanthomonas phaseoli

- *Phaseolus vulgaris* leaves 5-11 days after inoculation with typical Xap
- Watersoaked spots (a),
- necrosis (b,c) and
- dead tissues (d).

Grimault *et al.*,2014

Pathogenicity assay

Xanthomonas axonopodis pv. *poinsettiicola*

- To fulfil Koch's postulates, bacterial suspensions (10^8 CFU per ml) were injected into the leaves of four poinsettia plants.
- Inoculated plants were kept in a growth chamber at 28°C.
- Typical symptoms were observed in 6-10 days in all inoculated plants and were identical to those observed on the nurseries.
- Control plants, inoculated with sterile distilled water, showed no symptoms.
- The bacterium was readily re-isolated from diseased leaves.
- *Euphorbia milii* and *Codiacum variegatum* (both Euphorbiaceae) were also inoculated; symptoms appeared on *E. milii* but not on *C. variegatum*.

Lee *et al.*,2006

Plant tests

Xanthomonas campestris pv. *campestris*

- For growth assays, bacterial suspensions in water at 10^5 c.f.u. ml^{-1} were introduced into the leaf lamina of mature leaves of turnip using a syringe with no needle, and plants were maintained as described by Collinge *et al.*,1987.
- For pathogenicity tests, bacterial suspensions (10^6 c.f.u. ml^{-1}) were introduced into the vascular system of turnip through nicks at the vein endings and the plants were maintained in high humidity conditions as described by Dow *et al.*,1990.
- The ability of bacterial strains to induce the hypersensitive response was tested in pepper cv. ECW10R.

Plant tests

Xanthomonas campestris pv. *campestris*

- Pathogenicity was confirmed by inoculating susceptible cabbage by the cotyledon pricking method (Anonymous, 1985) and foliar spraying of 4-6-week-old plants with bacterial inoculum, grown on YDC agar for 48 h at 28 °C, harvested and adjusted to adjusted to 10^8 cfu/ml in 0.85% saline solution.
- Plants were incubated in polyethylene humid chambers for 24 h in growth rooms maintained at 28 °C with 14/10 h light regime.
- Thereafter, they were removed from the humid chambers and kept under the same light and temperature conditions.
- Types of symptoms induced by spray inoculation were recorded 14-21 days after inoculation (DAI).
- Small greyish spots around the stomata, followed by characteristic necrotic V-shaped lesions on the margins of leaves with chlorosis were observes after 14-21 DAI.

Massomo *et al.*,2003

Pathogenicity tests

Lesion progress (mm)on cabbage by using six different inoculation methods

Xanthomonas campestris pv.*campestris*

- Amongst different inoculation methods tested, 'Vein inoculation' method gave quicker symptom expression and highest lesion progression followed by 'Hydathode inoculation' and 'Clip inoculation' methods, respectively.

Disease Progress Towards Days After Inoculation	Average Lesion Progress on (mm)					
	Spraying the Inoculums with Hand Atomizer	Carborundum Abrasion Method	Spraying Inoculation After Multineedle Pricking	Injection Infiltration Method	Hydathode Inoculation Method	Scissor Clipping
4	1	2	1	1	2	1
6	3	6	3	2	6	2
8	6	10	7	3	9	5
10	11	14	12	5	16	9
12	16	18	17	5	21	12
14	21	24	21	5	28	15
16	26	32	25	6	32	19
18	29	43	28	7	39	23
20	33	52	31	7	48	27
22	34	61	38	8	54	31
24	38	66	44	12	59	34
26	42	74	51	13	61	35
28	44	80	56	18	68	38
30	48	89	61	19	72	40

Maji and Nath,2015

Races within *Xanthomonas campestris* pv.*campestris*

- Reaction of the differential Brassica spp. series to the nine races of *Xanthomonas campestris* pv. c*ampestris*.

Identification	Race	Host
HRI 3811	1	*Brassica oleracea*
HRI 3849A	2	*Brassica oleracea botrytis*
HRI 5212	3	*Brassica oleracea botrytis*
HRI 1279A	4	*Brassica oleracea capitata*
HRI 3880	5	*Brassica oleracea capitata*
HRI 6181	6	*Brassica rapa*
CFBP 4953	7	*Brassica oleracea botrytis* cv. cortes
CFBP 1124	8	*Brassica oleracea botrytis*
CFBP 6650	9	*Brassica oleracea*

Manuscript Number: EJPP2341R3

Pathogenicity test
Xanthomonas vasicola pv.*musacearum*

- Susceptible enset (root crop) clone 'Arkiya' from Areka experimental field was used for this test.
- Three months old suckers were planted in pots with sun-dried soil, sand and manure mixture with the ratio of 3:1:1 (Quimio,1992).
- The suckers were kept in Green house at 25-30˚C day and 15-18˚C night temperature.
- Isolates that were preserved at 4^{o}C for this purpose were grown on YPSA medium at 28˚C for 48 hours, harvested, suspended in sterilized water and adjusted to 0.3 O.D at 460 nm (10^{7}-10^{8} cfu/ml bacterial cell concentration) using spectrophotometer.
- An aliquot of 3 ml of the bacterial suspension were inoculated using a hypodermic sterile syringe to the second innermost leaf petiole with 3 replication.
- Sterilized distilled water was also inoculated as negative control.
- The reaction of the plants was observed in every week for 3 months.

Bobosha,2003;..

Pathogenicity test
Xanthomonas vasicola pv.*musacearum*

- Regarding the pathogenicity test, the inoculated leaves of the unset suckers showed light yellow to dark brown necrosis around the inoculated area of the leaves within 7 days and no necrosis was observed in control suckers inoculated with sterilized water.
- However, no complete wilting of the suckers was observed in 12 weeks observation period.

Bobosha,2003;..

Hypersensitivity test

Xanthomonas vasicola pv. *musacearum*

- Forty-eight hours old cultures of each isolate were suspended in sterilized distilled water and adjusted to 0.3 O.D at 460 nm (10^7-10^8 cfu/ml bacterial cell concentration) using spectrophotometer.
- An aliquot of 2 ml of each bacterial culture suspension was injected using a sterilized hypodermic syringe into the intercellular spaces of expanded leaves of a one-month tobacco plant (*Nicotiana tabacum* var. white burley).
- Injection of sterilized distilled water was used as negative control.
- All the tobacco plants were kept in Green house at 25-30^o C and 15-18^o C day and night temperature until symptom developed and a complete collapse of tissues occurred with yellow chlorosis to brown necrosis around the injection point was taken as positive for the test (Quimio,1992).

Bobosha,2003;..

Pathogenicity tests

Xanthomonas citri pv.*citri*

- **Attached leaf assay**
- With a syringe without the needle, citrus leaves are infiltrated with the bacterial suspension adjusted to 10^8 CFU ml^{-1} (OD_{660}= 0.06) by gently pressing the blunt end against the abaxial leaf surface until about 2 cm^2 of the leaf is water-congested.
- Six different strains may be inoculated into the same leaf, three on each side of the mid-vein.
- The leaves are then observed for development of tissue hyperplasia.
- Symptoms can generally be observed after 7–14 days after incubation in a glasshouse at about 25°C, as lesions with a raised margin surrounding a slightly chlorotic region.
- The raised margin then becomes pronounced, roughened and corky, the central region of the lesion becomes necrotic and collapsed; the necrotic lesions may split and the leaves abscise after several weeks.

Pathogenicity tests
Xanthomonas citri pv.*citri*

- **Detached leaf assay**
- Pathogenicity of *Xcc* strains was evaluated on detached immature leaflets of grapefruit, or other susceptible citrus plants.
- Surface of young leaves was disinfected with 70% ethanol, washed with sterile water and placed on the surface of 1% water agar with their abaxial surfaces facing up-wards (Vernière *et al.*,1991).
- Ten wounds per leaf were performed with a needle and droplets (10 µl) of bacterial suspensions of 1×10^{8} CFU ml^{-1} were placed on each wound.
- Leaves were incubated for 1-2 weeks at 28°C until symptoms appearance (development of tissue hyperplasia).
- Negative controls had leaves treated with sterile water.

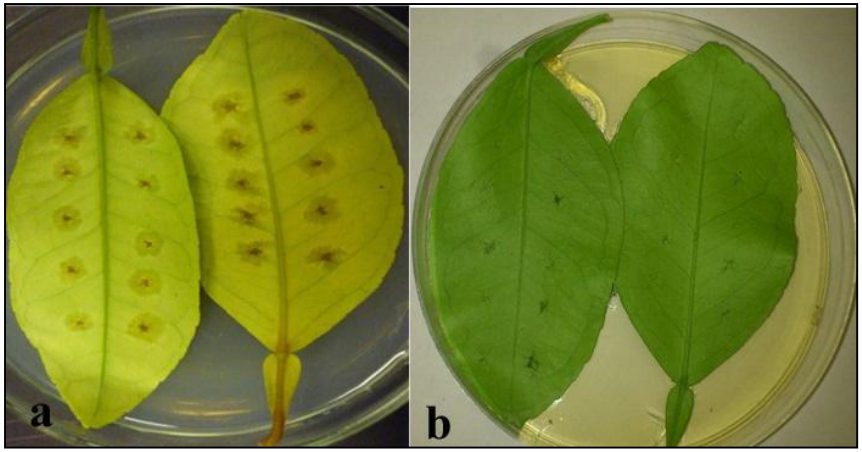
Pathogenicity tests
Xanthomonas citri pv. *citri*

- **Detached leaf assay:**
- Response on Duncan grapefruit in a detached leaf assay after one week post inoculation with:

a) Typical *Xcc*-A strains,

b) Atypical *Xcc*-A* strains.

- All *Xcc*-A strains produced erumpent callus-like tissue with water-soaked.
- In contrast, the *Xcc*-A* strains caused no symptoms after inoculation.



Al-Saleh *et al.*,2014

Pathogenicity test
Xanthomonas citri pv. *citri*

- **Inoculation of plants growing *in vitro***
- Use of *in vitro* plants for inoculation provides for greater security.
- Susceptible cultivars of grapefruit like 'Marsh', or of sweet orange like 'Parson Brown', can be used.
- The seedlings are grown under sterile conditions in Murashige and Skoog salt solutions by placing a drop of about 10^9 cfu mL^{-1} in wounded leaves.
- Incubation at 25-28°C for 7-14 days is required for symptom appearance (López & Navarro,1981).

EPPO Standards,2006;..

Tests of host/race specificity

X. citri subsp. *malvacearum* (ex. *X. axonopodis* pv. *malvacearum*)

- Cotton plants were grown in growth chambers with a 14-h light/10-h dark cycle.
- Maximum air temperature during the photoperiod was 30°C; minimum temperature during the dark period was 19°C.
- Plants were fertilized weekly with a mineral mixture.
- Inoculation was done 6 to 15 weeks after planting, using the youngest, fully expanded leaves.
- Transparent plastic bags were placed over the leaves 1 to 2 h before inoculation to encourage stomatal opening in response to the increased humidity.
- Inocula of *X. campestris* pv. *malvacearum* H (race 1) was prepared by diluting late logarithmic phase nutrient broth cultures in sterile, saturated $CaCO_3$ solution to $\approx 5 \times 10^6$ CFU ml^{-1}.
- Inoculation was accomplished with needleless syringes through open stomata on the abaxial leaf surface.
- A single leaf of each plant was inoculated in four places with each of the strains being tested.

Essenberg,2002

Tests of host/race specificity

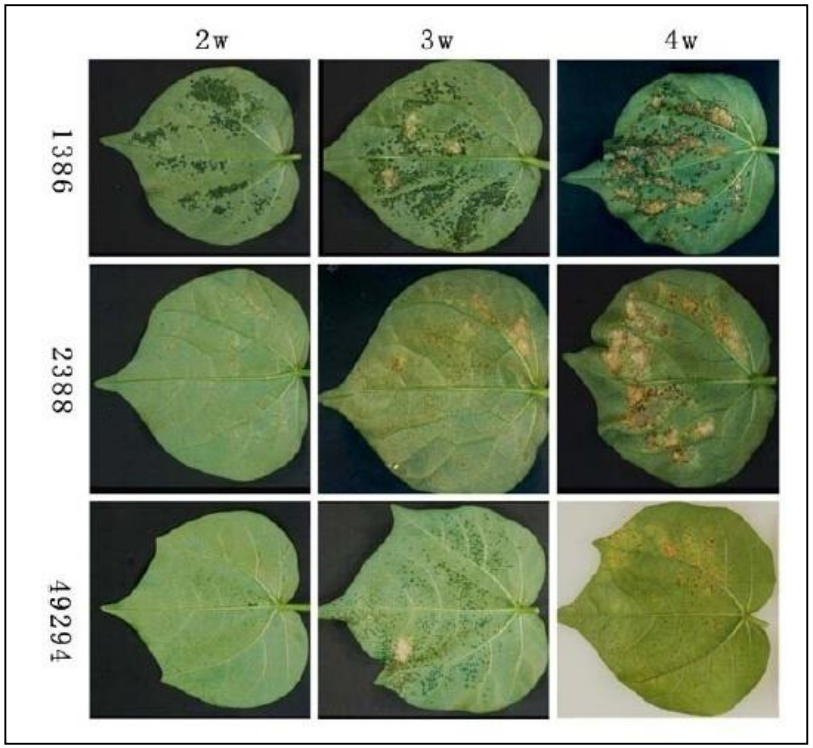
X. citri subsp. *malvacearum* (ex. *X. axonopodis* pv. *malvacearum*)

- The disease grade scale was based on the sizes of the macroscopically visible water-soaked areas:
- 0 = no water-soaking;
- 1 = pinpoint-sized dots;
- 2 = small, round speckles (≈ 0.3 mm);
- 3 = merged angular patches; and
- 4 = confluent areas.
- Intermediate grades between each of the established grades, e.g., 1.3, 2.7, and 3.3, were sometimes recorded.

Essenberg,2002

Identification of a highly virulent strain of *X. citri* subsp. *malvacearum* (ex. *X. axonopodis* pv. *malvacearum*)

- Comparison of the symptoms caused by race 18 (GSPB 1386),and race 20 (GSPB 2388, ATCC 49294) of *X. axonopodis* pv. *malvacearum* on cotton cv. 'Acala 44'.
- Bacterial strains were suspended in inoculation buffer (0.01 M $MgSO_4$) to a 10^5 cfu ml^{-1} concentration, and then evenly sprayed on the lower side of the first two not fully-grown young leaves (1/3 size of a mature leaf) following the cotyledons as described (Klement *et al.*,1990).



Differentiation of highly virulent strains from weakly virulent strains.

Huang *et al.*,2008

Pathogenicity test

X. citri subsp. *malvacearum* (ex. *X. axonopodis* pv. *malvacearum*)

- The tested lines/cultivars were infiltrated with a suspension of selected bacterial isolates prepared by suspending colonies in ddH_2O to a concentration of 10^7 CFU/ml and covered with polyethylene bags for 24 h.
- Plants were examined for the appearance of lesions from 3 to 15 days post infiltration.
- The next table indicates that race 1 was the most frequent of all with 21 pathogenic isolates but it was also the least virulent, showing symptoms on only one differential variety (Acalla 44).
- Races 3 and 4 were among the least virulent with symptoms on two differential varieties, Acalla 44 being one, and frequencies of 10 and 5% respectively.
- The most virulent races were in the descending order 32, 28 and 21, which showed symptoms on 9, 8 and 7 differential cultivars respectively.

Abdo-Hasan *et al.*,2008

Pathogenicity test

X. citri subsp. *malvacearum* (ex. *X. axonopodis* pv. *malvacearum*)

Frequency	No.of isolates	Variety	Origin	Reaction of differential varieties										Suggest race
				I	II	III	IV	V	VI	VII	VIII	IX	X	
52.50%	21	Aleppo 33,40,90	Aleppo Idlip El-Ghab	+	-	-	-	-	-	-	-	-	-	1
10%	4	Aleppo 33,40	Aleppo Idlip El-Ghab	+	-	+	-	-	-	-	-	-	-	3
5%	2	Aleppo 33,40	Aleppo Idlip	+	-	-	+	-	-	-	-	-	-	4
2.50%	1	Aleppo 90	Aleppo	+	+	-	+	-	-	-	+	+	-	8
5%	2	Aleppo 40	Idlip	+	-	+	+	-	-	-	-	-	-	11
7.50%	3	Aleppo 33, 40, 118	Aleppo Idlip El-Ghab	+	+	-	+	-	+	-	+	+	+	21
2.50%	1	Aleppo 40	Idlip	+	-	-	+	+	+	-	+	-	-	26
7.50%	3	Aleppo 33, 90,118	Aleppo Idlip	+	+	+	+	-	+	-	+	+	+	28
7.50%	3	Aleppo 90	Aleppo	+	+	+	+	+	+	-	+	+	+	32

I-Acala 44 , I I-Stoneville 2B-S9 , III -Stoneville 20 , IV-Mebane B-1, V-1-10B,VI-20-3 ,VII-101-102B, VIII-Gregg, EmpireB4, X-Dpxp4.
+Susceptible - Resistant

Abdo-Hasan *et al.*,2008

Pathogenicity test

Xanthomonas citri pv. *viticola* causes bacterial canker of grapevine

- Pathogenicity test on *Vitis vinifera* cultivar Sauvignon carried out by leaf and stem inoculations with *Xanthomonas* strains.

A. Symptoms 35 days after inoculation of: CFBP 7764 on leaf and stem (a, b); CFBP 7657(c), CFBP 7658(d), CFBP 7659 (e) and negative control at 21 days after infiltration (f).

B. Symptom development was recorded as (+) necrosis at the point of infiltration; (++) necrosis at the point of infiltration followed by multiple necrotic spots on the leaves and leaf veins, or development of canker-like lesions on the stems; strain CFBP 7694 was received as *X. campestris* pv. *viticola*, but is related to *X. hortorum* according to gyrB and rpoD sequencing.

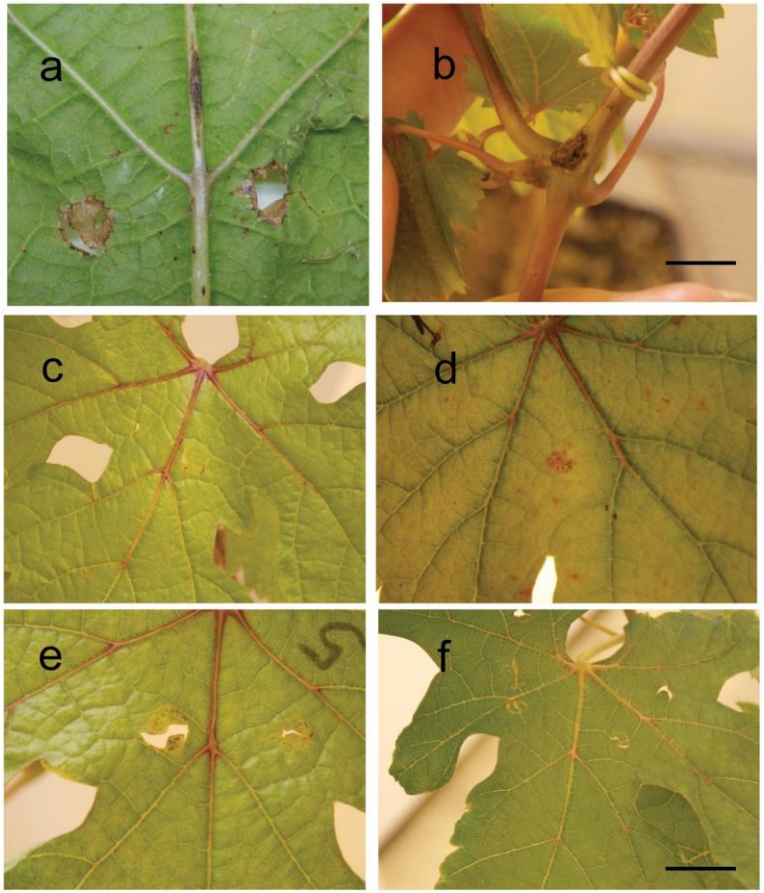
C. Scale bar=1.0 cm.

Ferreira *et al.*,2019

Pathogenicity test

***Xanthomonas citri* pv. *viticola* causes bacterial canker of grapevine.**

A



B

Strain	leaf		stem	
	symptom	isolation	symptom	isolation
CFBP 7660 *X. citri* pv. viticola	++	+	++	+
CFBP 7764 *X. citri* pv. viticola	++	+	++	+
CFBP 7658 *X. citri* pv. vitiscarnosae	+	+	+	+
CFBP 7659 *X. citri* pv. vitistrifoliae	-	+	+	+
CFBP 7657 *X. citri* pv. vitiswoodrowii	-	-	-	-
CFBP 7694 *X. hortorum*	-	+	-	-
Control (water)	-	-	-	-

Ferreira *et al.*,2019

Pathogenicity Reaction
Xanthomonas hortorum pv. *carotae*

1. Carrot seedlings at the 3-5 true leaf stage are sprayed to run off with bacterial suspension.
2. Inoculated seedlings are covered with clear plastic bags and are incubated in walk in incubator at 28°C for 48 hours.
3. Seedlings are transferred to the greenhouse and plastic bags are removed during the day but each night seedlings are sprayed with water and covered with plastic bags for up to 10 days.
4. Seedlings inoculated with water serve as negative checks; inoculate with in each set of seedlings a known strain of Xhcr.
5. Symptoms begin as small chlorotic areas on leaf margins that turn necrotic and brittle.
6. Re-isolate from seedlings displaying symptoms.

Kuan *et al.*,1985

Pathogenicity tests

Leaf clipping and spray inoculation methods

X. oryzae pvs. *oryzae* and *oryzicola*

- Isolates are tested on susceptible rice cultivars.
- Two main methods were suggested for routine pathogenicity tests as well as for breeding programmes:

1. The leaf clipping method (cutting and dipping leaf tips into the bacterial suspension) is widely used for the inoculation tests with *X. oryzae* pv. *oryzae* strains, and
2. Spray inoculations for *X. oryzae* pv. *oryzicola*.

- The leaf clipping method is widely used for the inoculation tests with *X. oryzae* pv.*oryzae* strains and spray inoculations for *X. oryzae* pv. *oryzicola*.

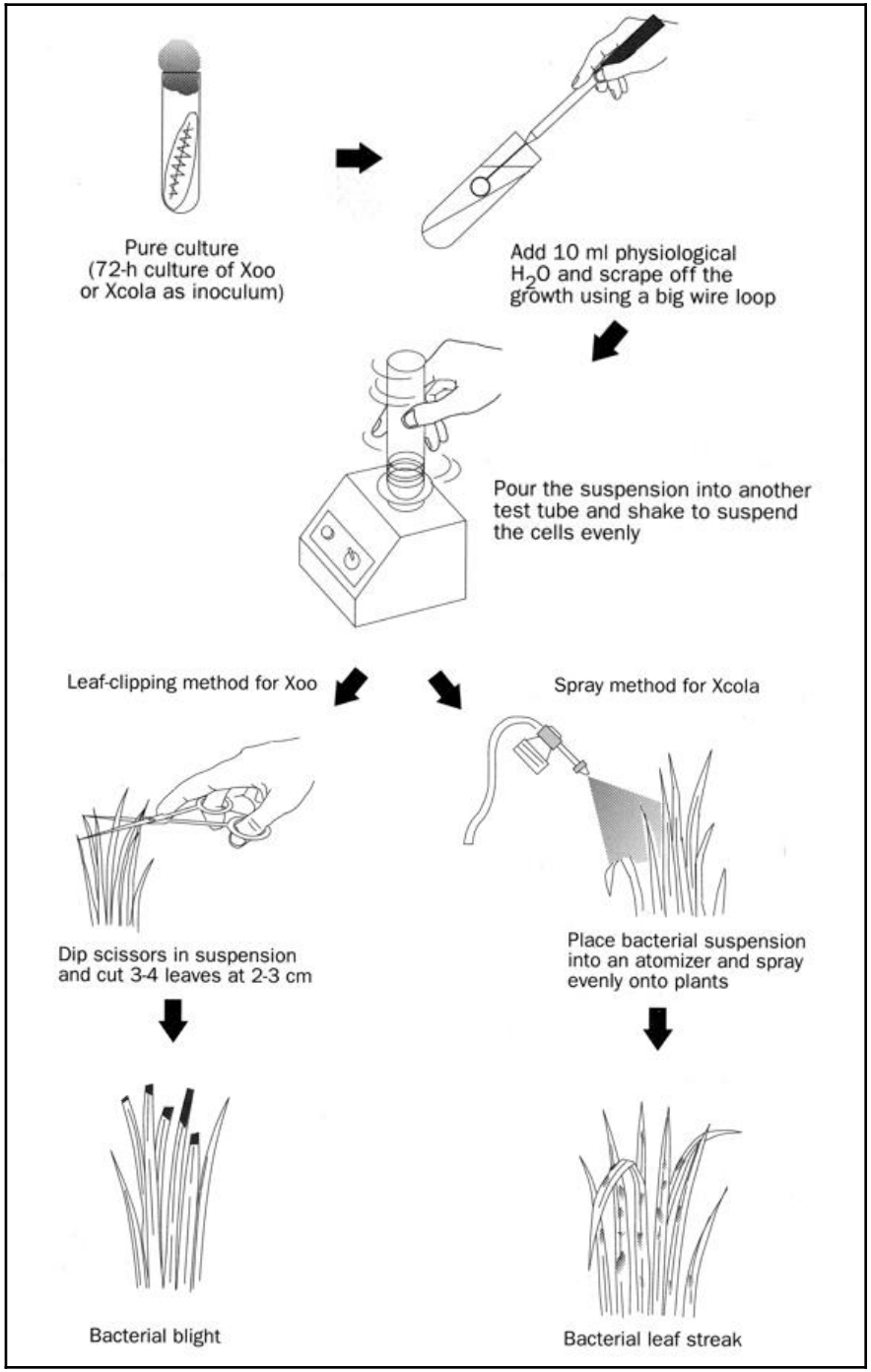
OEPP/EPPO Bulletin,2007

Pathogenicity tests

Leaf clipping and spray inoculation methods

- Pathogenicity test for Xoo and Xcola on mature plants in the greenhouse.
- What makes the method so successful is that the bacterial is directly expose to the xylem of the plant giving it a 90% success rating.

Mew and Misra,1994; Barker,2002



Pathogenicity tests

The clip-method

X. oryzae pvs. *oryzae* and *oryzicola*

- Cutting 2-3 cm of the tips of 30-40 leaves of rice plants with a pair of scissors while still immersed in the Xoo bacterial suspension (10^8 CFU mL^{-1}) prepared in sterile saline solution.
- Alternatively spray the clipped leaves with the bacterial suspension (particularly useful for *X. oryzae* pv. *oryzicola*).
- The inoculated plants are covered for 24 h with a polythene bag, and incubated at 30°C with 12 h light cycle, with care taken that the plants are not in direct contact with the bag.
- Plants are observed for symptoms after 48-72 h up to 14 days.
- Plants are checked for water-soaked areas in the inoculated leaves, usually beginning from the inoculated ends as water-soaked stripes, an indication of *X. oryzae* pv. *oryzae* symptoms.
- Lesions enlarge and may turn yellow within a few days.
- Milky drops of exudates can be observed. Make sure that the symptoms are not localized, but extend downwards.
- Appearance of reddish stripes from the point of inoculation of clipped leaves may be an indication of *X. oryzae* pv. *oryzicola*.

OEPP/EPPO Bulletin,2007

Pathogenicity tests

The clip-method

X. oryzae pvs. *oryzae* and *oryzicola*

- Cutting 2-3 cm of the tips of 30-40 leaves of rice plants with a pair of scissors while still immersed in the bacterial suspension.
- The rice plants were inoculated by clipping method (Kauffman *et al*.,1973) at boot leaf stage with a pair of scissors every time dipped into the bacterial suspension containing 10^8cfu/ml, prepared from 48 h old actively growing culture grown on nutrient agar medium.
- Plants were covered with polythene bags. The control plants were treated with distilled water.
- After 24 h, the bags were removed and the lesion length was measured in cm after 21 days.

Kumar and Balabaskar,2013

Pathogenicity tests

The clip-method

Xanthomonas oryzae pv. *oryzae*

- Rice leaves showing symptoms after inoculation with *Xanthomonas oryzae* pv. *oryzae* by the lead 'clipping' method.

OEPP/EPPO Bulletin,2007;Sullivan,2011

Pathogenicity tests

Leaf clipping and spray inoculation methods

X. oryzae pvs. *oryzae* and *oryzicola*

- Include a negative control (e.g. plants inoculated with sterile saline solution alone), and
- a positive control to monitor false negative reactions caused (e.g. by technical failure).
- Population levels of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in leaf tissue were estimated by serial dilutions and colony counts on plates of selective medium after 2 days of incubation at 28°C.

Wu *et al.*,2015; OEPP/EPPO Bulletin,2007

Pathogenicity tests

Detached leaf assay

X. oryzae pv. *oryzae*

- To determine the virulence and relative aggressiveness, the suspensions of different isolates of *Xanthomans oryzae* were applied on leaves of eight rice varieties.
- The detached leaves were placed on 3-folded blotting paper towel in petri plates and inoculated with bacterial suspension containing 10^8 cfu/ml through pin prick method.
- The inoculated leaves were incubated at 22°C, the lesion length measured in cm.

Measurement of BLB lesion length (cm) through detached leaf assay.

Jabeen *et al.*,2011

Pathogenicity tests

Spray inoculation

Xanthomonas oryzae pv. *oryzicola*

- Rice plants can be inoculated by spray inoculation with putative *X. oryzae* pv. *oryzicola* bacteria (Cottyn *et al.*,1994).
- The concentration of the suspension is adjusted to 10^8-10^9 CFU mL^{-1}.
- A drop of Tween 20 is added and the suspension is atomized evenly onto IR24 or IR50 plants (35-40 d-old), but taking care that the suspension does not run off.
- The plants are labelled and maintained at 28-30°C with 12 h daylight cycle under moist conditions in a growth room or greenhouse and examined for bacterial streak lesions 10 days after inoculation.
- The transparent streaks differentiate leaf streak lesions from those of the bacterial blight(Xoo), which are opaque against the light at earlier stages of infection.

OEPP/EPPO Bulletin,2007

Pathogenicity tests

Inoculation with a syringe

Xanthomonas oryzae pv. *oryzicola*

- For observation of water-soaking due to *X. oryzae* pv. *oryzicola*, a suspension (10^8 CFU/ml) of strain RS105 was infiltrated into the leaves of two-week old rice seedlings by needleless syringe.
- The disease symptoms were recorded after 15 days of incubation.

Wu *et al.*,2015

Pathogenicity tests
Vacuum infiltration method
X. oryzae pvs. *oryzae* and *oryzicola*

- Vacuum infiltration is another method that is have been successful, but hasn't been prove with rice.
- In theory using the rice plant natural's pore to inject the bacterial inside.
- The Vacuum filtration used a chamber; inside the chamber rice is placed in a bacterial suspension.
- The suspension is a mixture of the bacteria and a silwet coating solution.
- Using a vacuum compressor; all of the oxygen inside the chamber is removed, creating a vacuum.
- The process of taking the oxygen out of the chamber takes thirty seconds.
- After the chamber is completely empty a valve on top of the chamber is open and all of the oxygen rush back in.
- Using this pressure the bacterial is forced into the rice plant stomata and into its veins.

Barker,2002

Pathogenicity tests

Vacuum infiltration method

X. oryzae pvs. *oryzae* and *oryzicola* and *B. glumae*

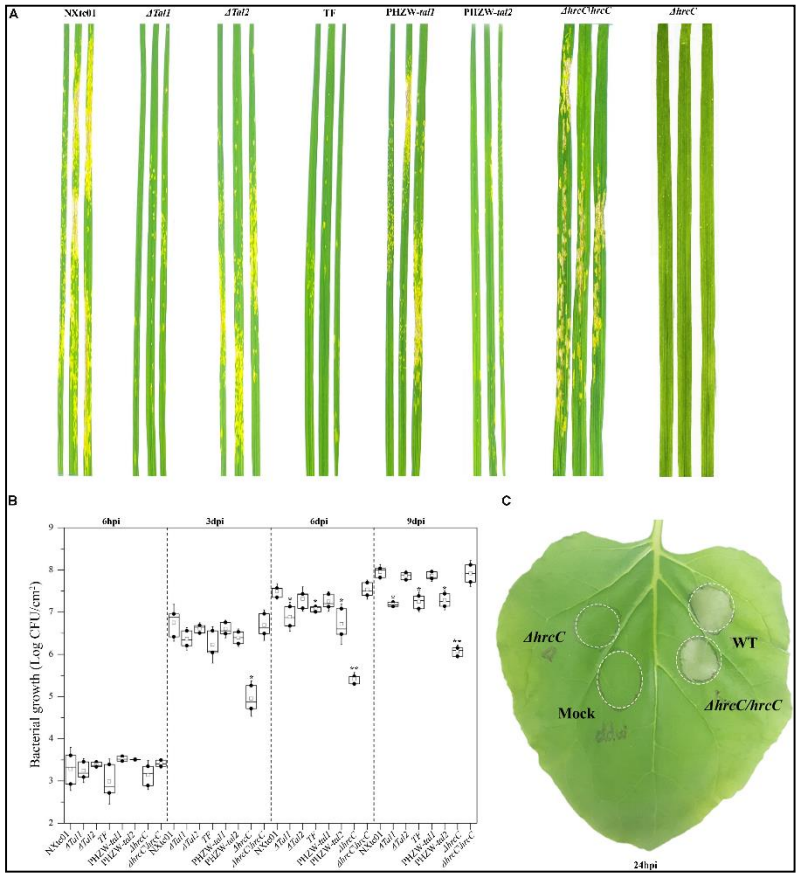
- Five grams (approximately 150 seeds) of rice cultivar Nipponbare were surface-disinfected in 75%ethanol for 10 min, incubated in approximately 0.5% chlorine solution for 30 min, and rinsed three times with sterilized distilled water.
- After disinfection, the seeds were transferred to Petri dishes containing sterilized filter paper and allowed to air-dry for 3 h in a laminar-flow chamber.
- The surface-disinfected seeds were inoculated with 5 mL g^{-1} of bacterial suspensions of *X. oryzae* pvs. *oryzae* and *oryzicola* or *B. glumae* or the mixture of the these three pathogens.
- OD_{600} values were measured using a Nanodrop (ND 100 spectrophotometer, NanoDrop).
- The inoculation was vacuum infiltrated for 60 min. After inoculation, the artificially infected seeds were allowed to air-dry in the laminar air flow chamber and stored at 4°C until use.

Lu *et al.*,2014

Pathogenicity tests

Xanthomonas translucens pv. *cerealis*

- Bacterial cells were grown for 12 h, harvested, washed and suspended in 10 mM $MgCl_2$ to OD_{600} = 0.2 (1.6 × 10^8CFU/ml).
- The inocula (OD_{600} = 0.2) of WT, ΔhrcC, ΔhrcC/hrcC, and mock (ddw) were infiltrated into three to four leaves of 20 days old *N. benthamiana* plants using a needless syringe, and infiltrated areas were marked.
- Symptoms were observed and compared at 24 hpi.



Ali Shah *et al.*,2019

Culture conservation
Xanthomonas spp.

- Yellow slime accumulates at the bottom of GYCA slants used to conserve *Xanthomonas translucens* strains.
- Tubes can be kept for several months at 4°C.