Lecture Supplements

Plant Bacteriology

Bacterial Diagnosis-Part 3

Compiled by N. Hassanzadeh

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Website Address:
http://www.phytobacteriology.com

Contact address

- Department of Plant Protection, Faculty of Agricultural Sciences and Food Industries, Science & Research Branch, Islamic Azad University, Tehran-Iran.
- P.O. Box: 14155/775, Postal Code: 1477893855
- Branch website: www.srbiau.ac.ir
- e-mail addresses:
- hasanzadehr@srbiau.ac.ir
- hasanzadehr@yahoo.com

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Characteristics of the major groups of phytopathogenic bacteria

Gram-Positive Bacteria

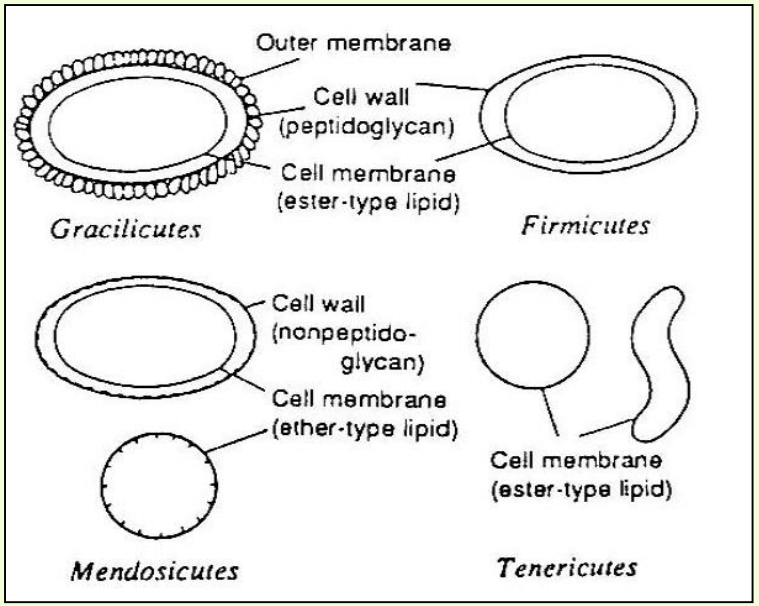
Kingdom Monera
Classification of Bacteria

- Within the prokaryote kingdom, four divisions are recognized, distinguished largely on the basis of cell wall characteristics (Murray, 1984). These are:
- Division I Gracilicutes: Gram-negative type cell wall (contains Gram-negative bacteria)
- Division II Firmicutes: Gram-positive type cell wall (contains Gram-positive bacteria and actinomycetes)
- Division III Tenericutes: No cell wall (contains class Mollicutes)
- Division IV Mendosicutes: Give evidence of earlier phylogenetic origin (contains class Archaeobacteria).
- Plant pathogenic prokaryotes are present in divisions I to III, and can thus be divided into organisms with a cell wall (true bacteria plus actinomycetes) and those without.

Sigee,1993 and 2005

Kingdom Monera
Classification of Bacteria

- **Phylum Firmicutes**
  - **Class Firmibacteria (Mostly single celled bacteria e.g. *Bacillus* & *Clostridium*).**
  - **Class Thallobacteria (Branching bacteria e.g. *Clavibacter*, *Rhodococcus*, *Streptomyces*, *Curtobacterium* and *Leifsonia*).**
    - **Order Actinomycetales**
- **Phylum Gracilicutes**
  - Class Scotobacteria
    - Order Spirochaetales
    - Order Pseudomonadales
    - Order Rickettsiales
    - Order Chlamydiales
  - Class Anoxyphotobacteria
  - Class Oxyphotobacteria
- **Phylum Tenericutes**
  - Class Mollicutes
- **Phylum Mendosicutes**
  - Class Archaebacteria



Zeuter Development Corporation, CANADA,1996;Goto,1992

Cavalier-Smith Bacterial megaclassification

Negibacteria as a root of the universal tree

- Prokaryotes constitute a single kingdom, Bacteria.
- Bacteria is divided into two new subkingdoms:

1. Negibacteria, with a cell envelope of two distinct genetic membranes;
2. Unibacteria, comprising the new phyla Archaebacteria and **Posibacteria**.

- Archaea and Eucarya are relatively recent (850 Mya) emergents from a line that also gave rise to the modern Gram-positive bacteria and actinobacteria.

Gram-Positive Bacteria

Plant pathogenic genus

- **Posibacteria**
- Subdivision 1. Endobacteria
- Class 1. Togobacteria
- Class 2. Teichobacteria → order: Bacilllales, Family: Bacilleaceae:
  Genera: *Bacillus* and *Clostridium*
- Class 3. Mollicutes → Genera: *Spiroplasma* & phytoplasma
- Subdivision 2. Actinobacteria
- Class 1. Arthrobacteria
- Class 2. Arabobacteria
- Class 3. Streptomycetes → order Actinomycetales, Families:
  Microbacterieacea : coryneforms
  Streptomycetaceae: *Streptomyces*

Cavalier-Smith,2002

Gram-Positive Bacteria

Plant pathogenic genus

Division rank is equivalent to phyla

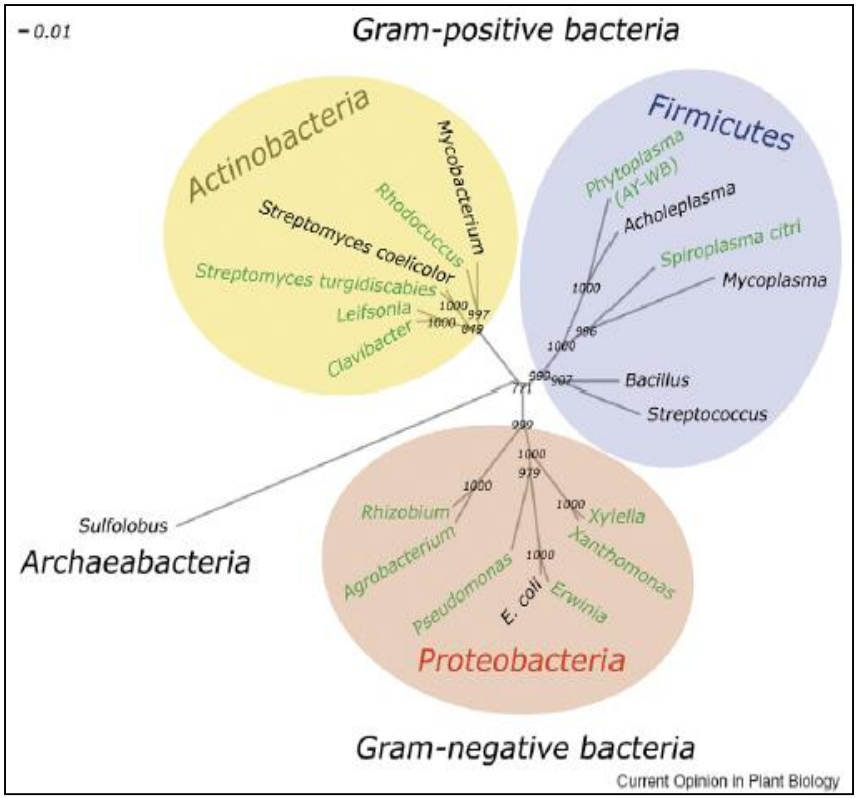
- **Division Posibacteria**
- **Subdivision Endobacteria [low G+C] (below 50% GC)**
- **Class 1. Togobacteria**
- **Class 2. Teichobacteria [Firmicutes]**
- *Bacillus anthracis*
- *Bacillus cereus*
- *Bacillus subtilis*
- *Bacillus halodurans*
- *Bacillus megaterium*
- *Clostridium acetobutylicum*
- *Enterococcus faecium*
- **Class 3. Mollicutes**
- **Subdivision Actinobacteria [high G+C] (greater than 50% GC)**
- **Class 3. Streptomycetes**
- coryneforms
- *Streptomyces*

Cavalier-Smith,2002

Gram-Positive Bacteria

Plant pathogenic genus

- The two clades that compose the Gram-positive bacteria:
  1. The Actinobacteria (high G+C) and
  2. The Firmicutes (low G+C), diverged very early in evolutionary history.



Hogenhout and Loria,2008

Gram-Positive Bacteria
Plant pathogenic genus

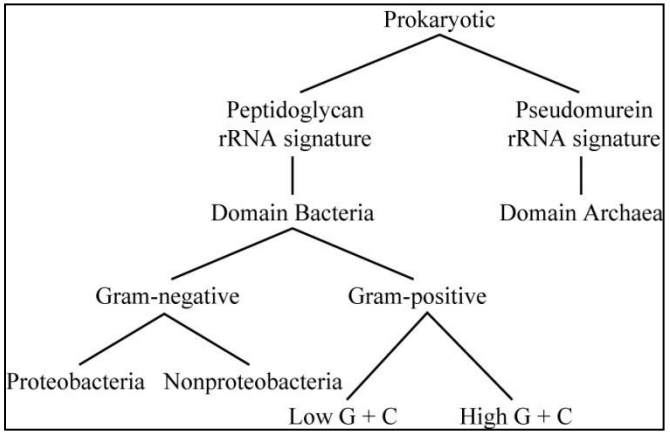
- **Firmicutes with High GC Content of DNA:**

1. **Actinomycetales:**

- *Mycobacterium*, *Streptomyces*, *Corynebacterium*, *Arthrobacter*, coryneforms

- **Firmicutes with Low GC Content of DNA:**

1. **Bacillales:** *Bacillus*, *Staphylococcus*, *Paenibacillus*
2. **Clostridiales:** *Clostridium*
3. **Mollicutes:** *Mycoplasma*, phytoplasma, *Spiroplasma*



The Prokaryotes,2006

Firmicutes

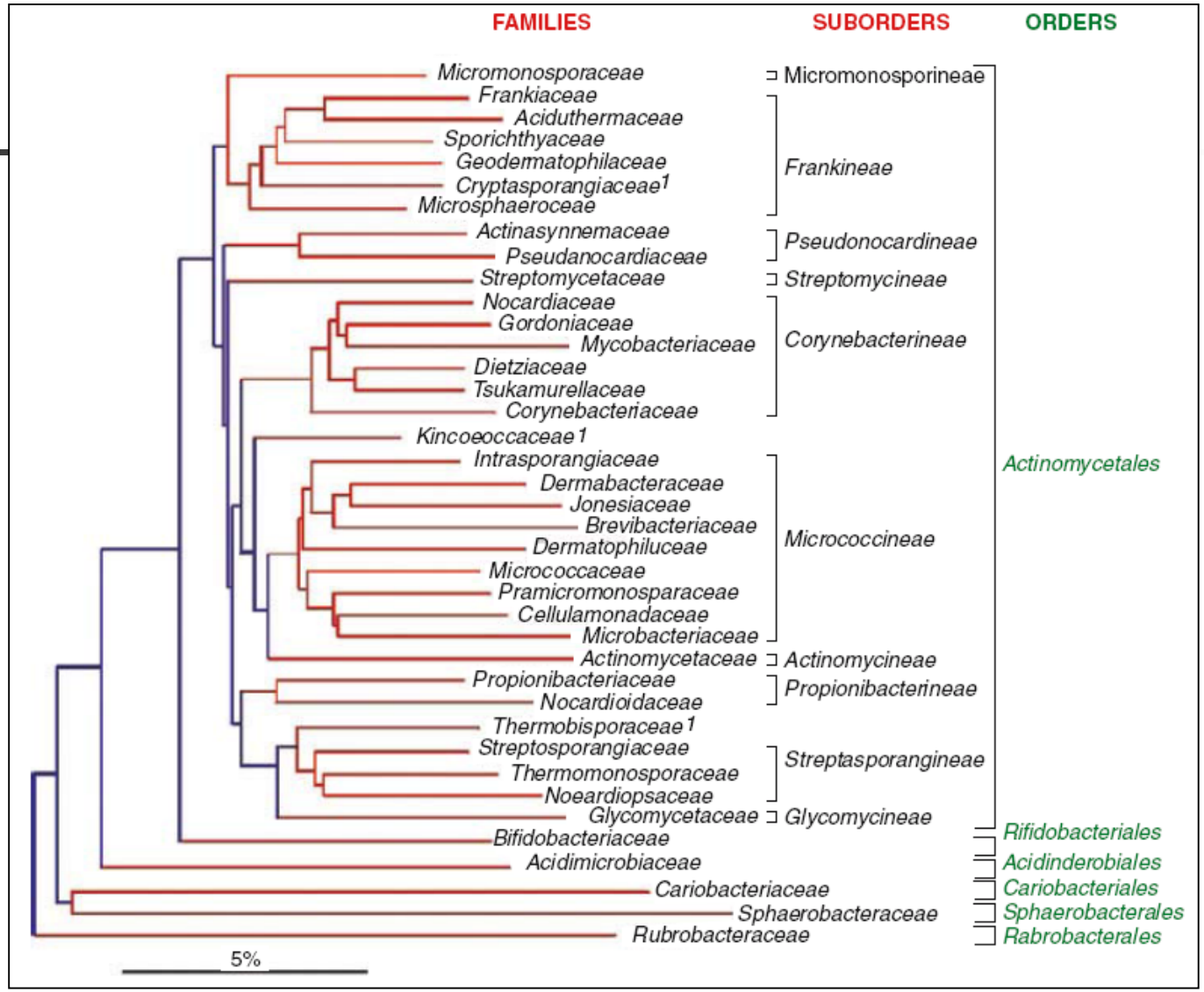
Plant pathogenic bacteria of the class Actinobacteria

Family	Genus
Order: Actinomycetales	
Microbacteriaceae (Most coryneforms)	*Clavibacter*, *Rathayibacter*, *Leifsonia*, *Curtobacterium*
Nocardiaceae	*Rhodococcus*
Streptomycetaceae	*Streptomyces*

The genera *Streptomyces* and *Rhodococcus* have very wide host ranges. Whereas, the genera *Clavibacter* and *Leifsonia* are host-specific at the species or subspecies level.

The Prokaryotes,2006; Hogenhout and Loria,2008

Intraclass relatedness of Actinobacteria, based upon 16S rDNA/rRNA sequence comparison



The Prokaryotes (chapter 1.1.1),2006

Two prokaryotic domain
Gram positive plant pathogenic bacteria

- There are two prokaryotic domains (or empires):

1. Bacteria (or *Eubacteria*)
2. Archaea (or *Archaeobacteria*), are subdivided into 29 phyla (or divisions):

- The domain Bacteria comprises of 27 phyla (or divisions).
- The Phylla related to Gram positive plant pathogenic bacteria:

1. Actinobacteria
2. Firmicutes
3. Tenericutes

J.P. Euzéby,2013

Gram-Positive Bacteria

Plant pathogenic genera

Screenshot of part of the classification of the domain bacteria/phylum Actinobacteria

- **Domain: Bacteria**
  Phylum: "Actinobacteria"
    Class: Actinobacteria
      Subclass: Actinobacteridae
        Order: *Actinomycetales*
          Suborder: Corynebacterineae
            Family: Nocardiaceae (*Rhodococcus*)
          Suborder: Micrococcineae
            Family: Microbacteriaceae (coryneforms)
          Suborder: Streptomycineae
            Family: Streptomycetaceae (*Streptomyces*)

J.P. Euzéby,2014

Gram-Positive Bacteria

Plant pathogenic genera

Firmicutes

- **Domain Bacteria**

  Phylum: "Firmicutes"

  Class: "Bacilli"

  Order: Bacillales

  Family: Bacillaceae (*Bacillus*)

  Family: Paenibacillaceae (*Paenibacillus*)

  Class: "Clostridia"

  Order: Clostridiales

  Family: Clostridiaceae (*Clostridium*)

J.P. Euzéby,2014

Gram-Positive Bacteria

Plant pathogenic genera

Tenericutes

- **Domain Bacteria**

  Phylum: "Tenericutes"

  Class: Mollicutes

  Order: Entomoplasmatales

  Family: Spiroplasmataceae

  Genus: *Spiroplasma*

J.P. Euzéby,2014

Identification of the bacterial pathogens

Coryneforms (coryneform plant pathogenic bacteria)

Disease diagnosis and pathogen diagnostics

The family Microbacteriaceae
Plant pathogenic coryneform bacteria

- The term "coryneform" is applied to any non-sporing Gram-positive rod of irregular outline.
- Some genera including *Clavibacter*, *Curtobacterium*, *Rathayibacter* and *Rhodococcus* are phytopathogenic species within the coryneform group of bacteria.
- However, most coryneform bacteria are saprophytes that live in soil, water and organic matter.
- The saprophytic coryneform bacteria are widely distributed in nature.

B. K. Schroeder;..

The Family Microbacteriaceae
Plant pathogenic coryneform bacteria

- The family Microbacteriaceae embraces a large group of predominantly aerobic Gram-positive bacteria of high G+C content that are distinguished from other actinobacteria by a combination of their:

1. Unusual B group cell wall peptidoglycan and
2. Unsaturated respiratory menaquinones.

- The family is a member of the order Actinomycetales, class Actinobacteria and currently harbors 15 genera including the plant pathogens:

1. *Clavibacter*,
2. *Curtobacterium*,
3. *Rathayibacter*,
4. *Leifsonia*,

- Only *Rhodococcus* belongs to the family Nocardiaceae.

The Prokaryotes (chapter 1.1.28),2006

Classification of Actinobacteria

Based on analyses of 16S ribosomal RNA and the distribution of 16S rDNA signature nucleotides

- Actinomycetes comprises of the genera such as *Corynebacterium*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Skermania*, *Tsukamurella* which form a monophyletic group in the 16S rRNA gene tree.
- The following pattern of 16S rDNA signature nucleotides was proposed as characteristic of the family: nucleotides at positions 45-396 (U-A), 144-178 (C-G), 258-268 (A-U), 497 (A), 615-625 (A-U), 694 (G), 771-808 (G-C), 839-847 (G-U), 1256 (G), 1310-1327 (A-U), and 1414-1486 (U-A).
- Table presents Phytopathogenic families and genera of the class Actinobacteria.

Family	Genus
Microbacteriaceae	***Clavibacter*** ***Curtobacterium*** ***Rathayibacter*** ***Leifsonia***
Nocardiaceae	***Rhodococcus***
Streptomycetaceae	***Streptomyces***

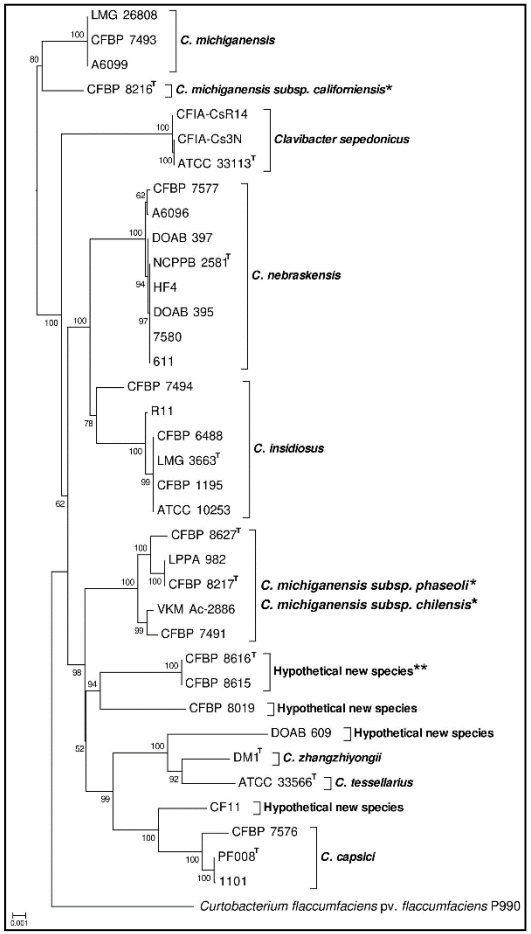
The Prokaryotes (chapter 1.1.1),2006

Mycobacterium vaccae is a natural soil bacterium make you smarter.

Classification

Whole-genome sequence-based phylogenetic analysis of plant-pathogenic members of *Clavibacter*

- Whole-genome sequence-based phylogenetic analysis of plant-pathogenic members of *Clavibacter*.
- The neighbour-joining tree was generated based on the whole-genome sequences of 49 Clavibacter strains using the galaxy Europe online service (https://galaxyproject.eu/) and 1000 bootstrap replications. *Clavibacter nebraskensis* strains were clustered in a monophyletic clade phylogenetically related to the alfalfa pathogen *C. insidiosus*.
- *Needs taxonomic re-evaluation.
- **Taxonomic description is in preparation.



Osdaghi *et al.*,2023

Thallobacteria

Clavibacter

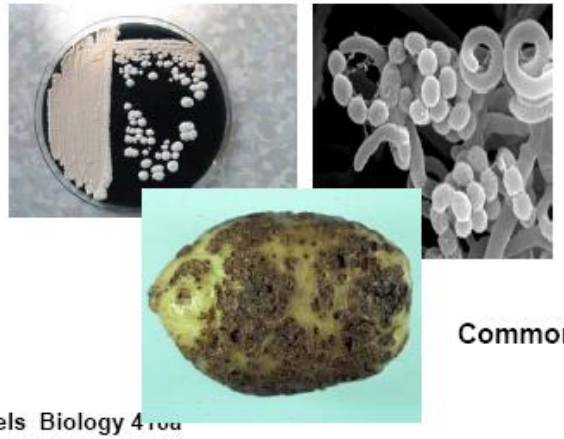
- pleomorphic, mostly rod-like
- diaminobutyric acid in cell wall
- usually non-motile

C. michiganensis subsp. sepedonicum
Ring rot of potato

C. michiganensis subsp. michiganensis
Bacterial canker of tomato
www.umassvegetable.org

Streptomyces scabies

- spore-forming
- branched vegetative hyphae
- soil inhabitants
- attacks potato and other root vegetables



Common scab of potato

Rhodococcus fascians

Fasciation of sweet pea

Cuppels Biology 4

Cuppels,2007

Diseases caused by coryneform plant pathogens *Clavibacter* spp.

Clavibacter capsici	Bacterial canker disease in pepper
C. insidiosus	Bacterial wilt of alfalfa
C. michiganensis	Bacterial canker of tomato
C. nebraskensis	Bacterial wilt and blight of corn (Goss's wilt)
C. phaesoli	Bacterial leaf yellowing on bean
C. sepedonicus	Bacterial ring rot of potato
C. tessellarius	Mosaic-like syndrome on wheat
C. zhangzhiyongii	Leaf brown spot and decline of barley

Euzeby,2024

Diseases caused by coryneform plant pathogens *Curtobacterium* spp.

- Plant pathogenic members of *C. flaccumfaciens* are divided into five pathovars based on their host of isolation, pathogenicity and host range (Collins and Jones,1983), i.e.
- *C. flaccumfaciens* pv. *flaccumfaciens* causing bacterial wilt of dry beans (Hedges, 1922),
- *C. flaccumfaciens* pv. *poinsettiae* causing bacterial canker of poinsettia (Pirone and Bender, 1941),
- *C. flaccumfaciens* pv. *betae* the cause of silvering disease of red beet (Keyworth *et al*., 1956), *C. flaccumfaciens* pv. *ilicis* causing bacterial blight of American holly (Mandel *et al*., 1961, Young *et al*., 2004), and
- *C. flaccumfaciens* pv. *oortii* the agent of bacterial wilt and leaf spot of tulip (Saaltink and Maas Geesteranus, 1969).

Osdaghi *et al*.,2024

Diseases caused by coryneform plant pathogens *Curtobacterium* spp.

- More recently, two additional pathovars were described, namely *C. flaccumfaciens* pv. *basellae* the causal agent of bacterial leaf spot of malabar spinach (Basella rubra [B. alba]) (Chen *et al.*, 2000) and *C. flaccumfaciens* pv. *beticola* the causal agent of bacterial leaf spot of sugar beet (Chen *et al.*, 2007).
- However, none of the latter new pathovars has so far been included in the list of plant pathogenic bacteria provided by the International Society of Plant Pathology Committee on the Taxonomy of Plant Pathogenic Bacteria (ISPP-CTPPB; Bull *et al.*, 2010, Bull *et al.*, 2012, Bull *et al.*, 2014).
- A recently published study described *Curtobacterium allii* causing onion bulb rot as the only plant pathogenic species of Curtobacterium outside *C. flaccumfaciens* (Khanal *et al.*, 2022).

Osdaghi *et al.*,2024

Diseases caused by coryneform plant pathogens *Curtobacterium*, *Leifsonia* and *Rathayibacter* spp.

Curtobacterium flaccumfaciens pv. *betae*	Vascular wilt and leaf spot of red beet (*Beta vulgaris*)
C. f. pv. *flaccumfaciens* (multicolored bacterium)	Bacterial wilt bean (*Phaseolus*, *Vigna*)
C. f. pv. *ilicis* (syn. *Arthrobacter ilicis*)	Blight of American holly (*Ilex opaca*)
C. f. pv. *oortii*	Yellow pustule of tulip (bulb symptoms) or hell fire (leaf symptoms)
C. f. pv. *poinsettiae*	Leaf spot and stem canker of Poinsettia (*Euphorbia pulcherrima*)
Leifsonia xyli subsp. *xyli*	Ratoon stunt in sugarcane
Leifsonia xyli subsp. *cynodontis*	Bermuda grass stunting disease
Rathayibacter iranicus	Gumming (tundu) disease or yellow slime of grains (wheat)

Diseases caused by coryneform plant pathogens

R. rathayi	**Gumming disease or yellow slime of grains (*Dactylis glomerata*)**
R. toxicus	**Gumming disease or yellow slime of grains (rye grass, *Lolium rigidum*)**
R. tritici	**Gumming disease or yellow slime of grains (wheat)**
Rhodococcus fascians	**Bacterial fasciation, leafy gall, cauliflower-like galls on *Gladiolus* corms, Abnormal sprout formation on phlox.**

Rhodococcus has very wide host ranges, whereas the two other Actinobacteria genera i.e. *Clavibacter* and *Leifsonia* are host-specific at the species or subspecies level.

Note: *Arthrobacter ilicis* is excluded from ISPP list of plant pathogenic bacteria and *Curtobacterium flaccumfaciens* pv. *ilicis* has been proposed for the pathogen of American holly.

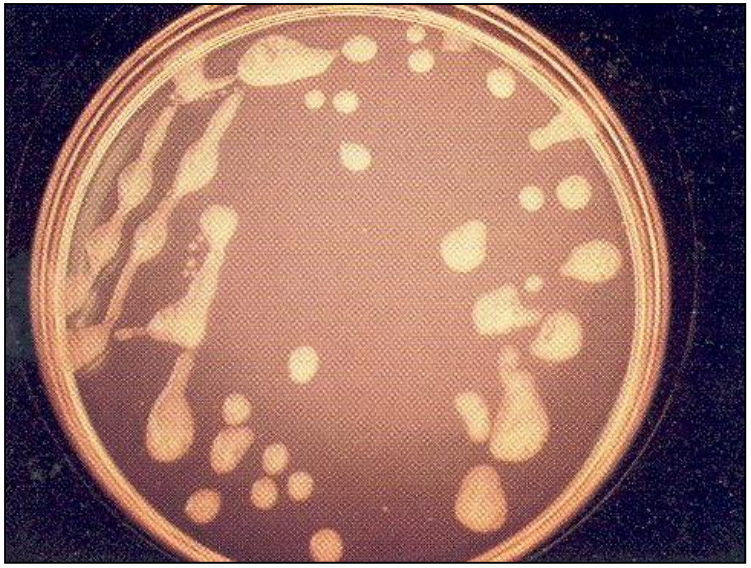
Characteristics of coryneform plant pathogens

Phenotypic criteria

- These are obligately aerobic to facultatively anaerobic;
- Non-spore forming organisms;
- With varying cell morphology, ranging from:

1. Coccoid(*Rhodococcus*), small irregular rods to
2. Branched fragmenting hyphae (*Leifsonia*).

- Mycolic acids and arabinogalactan are not present.

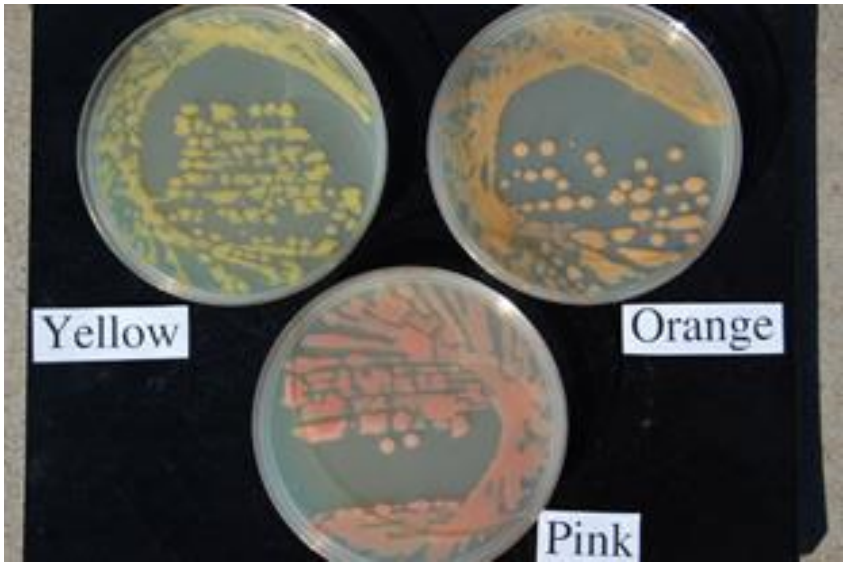


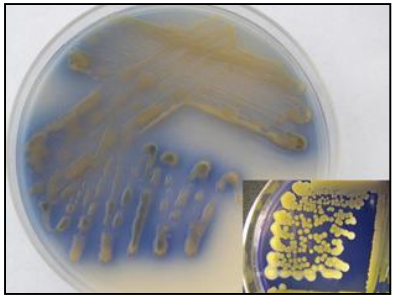
Colonies of *Clavibacter m.* subsp. *michiganensis* on SCM:
Colonies are small, light to dark gray, glistening, fludial and often irregularly shaped.

Characteristics of coryneform plant pathogens

Phenotypic criteria

- Bacterial wilt of dry beans caused by multicolored bacterium *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*.
- Young wilt pathogen culture exhibiting purple color variant on nutrient broth yeast extract medium (NBY).
- Note purple-blue pigments diffusing into media.
- Aged culture showing remnants of purple pigments.





Harveson *et al.*,2011

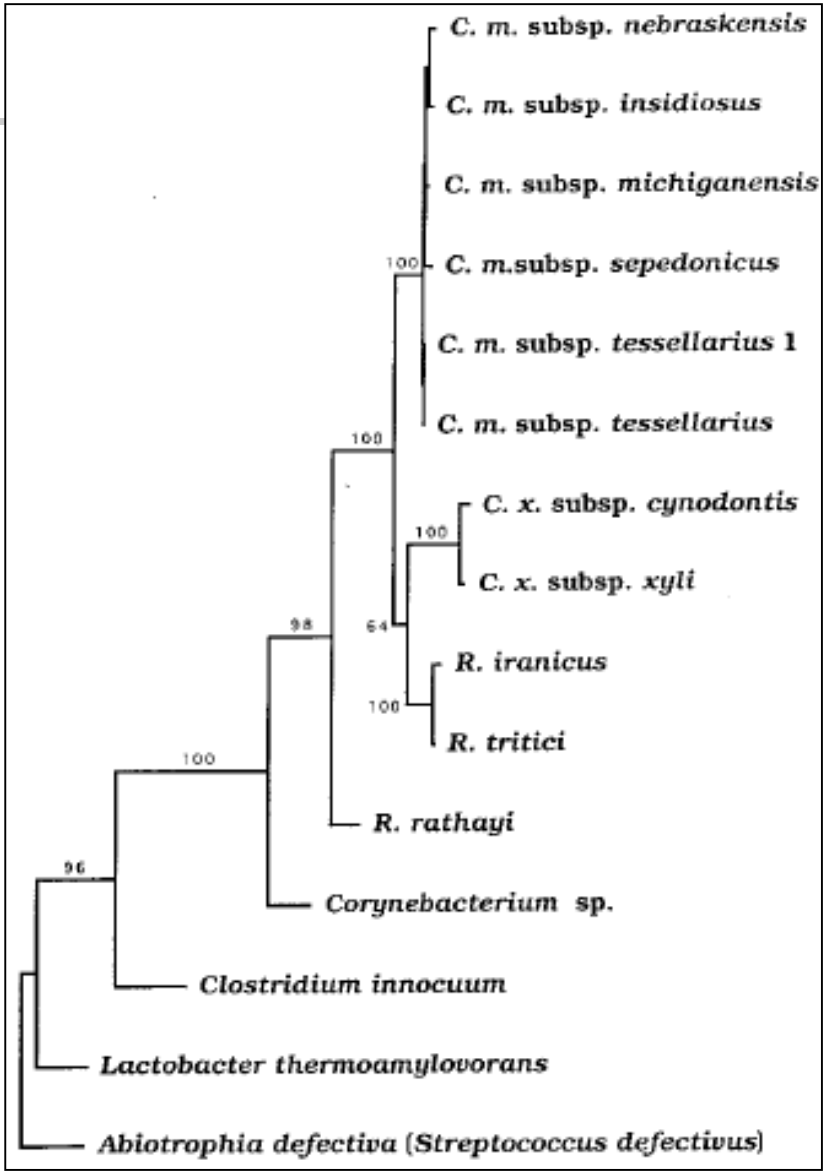
Characteristics of coryneform plant pathogens

Some other phenotypic criteria

1. **Based on common phenotypic criteria such as:**
   - Cell wall components,
   - Pigmentation of cell culture, and
   - Selected physiological features have been widely applied to coryneform genera such as of *Clavibacter* and *Rathayibacter* can be differentiate.
2. **On the basis of cell wall composition:**
   - The genus *Clavibacter* comprises five species and several subspecies of coryneform phytopathogenic bacteria which contain 2,4-diaminobutyric acid.
   - Mycolic acids and arabinogalactan are not present.
3. **Based on additional phenotypic markers**, including menaquinone-type markers and others, these phytopathogenic coryneform bacteria have been reclassified into two genera, *Clavibacter* and *Rathayibacter*.

Phylogenetic tree
Based on 16S rDNA analysis

- Phylogenetic tree constructed by parsimony analysis of 16S rRNA gene sequences from 11 members of the genera *Clavibacter* and *Rathayibacter* and 4 other related gram-positive bacteria, with *Abiotrophia defectiva* as the outgroup.



Lee *et al.*,1997

Differential characteristics of phytopathogenic coryneform bacteria

			Growth		Acid Production[a]			Utilization[b]		Hydrolysis[c]	
Pathogen	Motility	Pigment[d]	CNS	TTC	Ribose	Sorbitol	Inulin	Acetate	Formate	Casein	Esculin
Arthrobacter ilicis	+	Y	+	+	+	-	-	+	+	+	+
Clavibacter iranicum	-	Y	-	ND	-	-	+	-	-	-	+
Clavibacter michiganensis											
subsp. *insidiosus*	-	Y/B	-	+	-	-	-	-	-	-	+
subsp. *michiganensis*	-	Y/V_1	+	+	-	-	-	-	-	-	+
subsp. *nebraskensis*	-	O/V_2	+	-	-	+	-	+	-	-	+
subsp. *sepedonicus*	-	W	-	-	-	+	-	+	-	-	+
subsp. *tessellarius*	-	O	+	+	-	+	-	-	-	-	+
Clavibacter rathayi	-	Y	V	ND	-	-	-	-	-	-	+
Clavibacter tritici	-	Y	+	ND	-	-	+	+	-	-	+
Clavibacter toxicus	-	Y	ND	ND	ND	ND	-	-	ND	ND	ND
Clavibacter xyli											
subsp. *cynodontis*	-	Y	ND	ND	-	-	-	-	-	-	-
subsp. *xyli*	-	W	ND	ND	-	-	-	-	-	-	-
Curtobacterium flaccumfaciens											
pv. *betae*	V	Y	V	+	+	+	-	+	-	-	0
pv. *flaccumfaciens*	V	Y/O/P	V	+	$+^D$	-	-	+	-	+	+
pv. *oortii*	+	Y	V	+	$+^D$	-	-	-	-	+	+
pv. *poinsettiae*	V	O	V	+	$+^D$	+	-	+	-	+	+
Rhodococcus fascians	-	O	-	+	$+^D$	+	-	+	+	-	-

Abbreviations: +, 80% or greater positive; -, 80% or greater negative; V, variable, depending on the strain; $+^D$, delayed positive; ND, not determined.

[a]RSD broth (see d, p. 225) with yeast extract reduced to 0.1 g/L, and the test compound (0.5% w/v) replacing glucose.
[b]RSD broth as above, except the test compound at 0.1% w/v and bovine serum albumin omitted from the medium when testing *Arthrobacter* and *Corynebacterium* species.
[c]SC medium (see b, p. 225) supplemented with 1% (w/v) casein or 0.1% (w/v) esculin and 0.05% (w/v) ferric citrate. Clearing of the medium with casein or the development of a brown color in the medium with esculin indicates hydrolysis.
[d]NBY agar (see c, p. 4) used for all pathogens, except SC agar used for *C. xyli* subspecies. Y, yellow; B, blue; O, orange; W, white or colorless; P, purple; V_1, various pigments (occasional variants are pink, red, orange, and white or colorless); V_2, occasional variants are yellow; ND, not done. Colors refer to those of non-diffusable pigments, except as noted. The blue pigment is intracellular indigoidine granules sometimes produced in addition to yellow pigment. The purple is extracellular and occasionally found.

Schaad *et al.*,2001

Differential characteristics of phytopathogenic coryneform bacteria

Bacterial species	Organic acids		Enzymes		Selective medium			Acid from carbohydrate using: Purple Broth plus			Medium C plus				Colony characteristics		
	Fm	Na	T80	Gel	CNS	TTC	Mot	Man	Rha	Suc	Lac	Tre	Man	Rha	Ind	Ep	Colour†
A. ilicis	v	+	−	+	+	+	v	+	+	−	v	+	+	+	−	−	Y;W/Be
Cl. michigenense subsp. *insidiosum*	−	−	−	−	−	−	−	+	−	−	v	v	+	−	+	+	DY
Cl. m. subsp. *michiganense*	+	+	−	−	+	+	−	−	−	v	v	+	+	−	−	−	Y;DY
Cl. m. subsp. *nebraskense*	+	v	−	−	+	−	−	−	−	−	v	+	v	−	−	−	O
Cl. rathayi	+	−	−	v	−	−	−	+	−	−	−	+	+	−	−	−	BrY
Cl. m. subsp. *sepedonicum*	v	v	−	−	−	−	−	+	−	+	−	−	+	−	−	−	W/Be
Cl. m. subsp. *tessellarius*	+	+	+	−	+	+	−	−	−	v	+	+	+	−	−	−	O
Cl. tritici	+	v	+	−	+	−	−	+	−	+	−	v	+	−	−	−	BrY
Cur. flaccumfaciens pv. *betae*	−	+	+	v	+	+	+	−	+	v	+	+	+	+	−	−	Y;DY
Cur. fl. pv. *flaccumfaciens*	+	v	+	v	+	+	v	−	v	v	v	+	+	+	−	v	Y;V
Cur. fl. pv. *oortii*	−	+	+	+	+	+	+	−	+	v	+	+	+	+	−	−	Y;V
Cur. fl. pv. *poinsettiae*	−	+	+	v	+	+	v	−	−	v	+	+	+	−	−	−	O;Y/O
R. fascians	+	+	+	−	−	+	−	−	−	−	−	+	+	−	−	−	Y/O;BrY

+, 80–100% of organisms positive; −, 80–100% of organisms negative; v, organisms were variable either between strains or when test was repeated.

* Abbreviations: Fm, fumaric acid; Na, sodium acetate; T80, lipase for Tween 80; Gel, gelatinase; CNS, growth on CNS; TTC, growth on TTC; Mot, motility; Man, mannitol; Rha, rhamnose; Suc, sucrose; Lac, lactose; Tre, trehalose; Ind, presence of indigoidine; Ep, presence of extracellular pigments.

† Colony colour on NBY: DY, dull yellow; Y, yellow; O, orange; BrY, bright yellow; Y/O, yellow-orange; W/Be, white/beige; V, variable.

Henningson and Gudmestad,1991

Physiological and morphological characteristics of the 16S rRNA restriction types in comparison to reference strains of the genera *Curtobacterium, Clavibacter* and *Rathayibacter*

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Number of strains	2	1	1	2	2	2	5	5	1	1	1	1	1	1	1	1	1	5	5	5	1	1	1	1	5
Colony colour	y	o	y	y	o	y	o	o	y	y	ly	i	o	o	y	y/b	w	o	y/yo	y	y	y	y	y	y
Motility	+	−	+	d	d	d	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Hydrolysis of:																									
Gelatin	+	w+	+	−	d	+	d	d/+*	−	−	+	+	w+	−	−	−	−	d	d	−	−	−	−	−	−
Aesculin	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	w	−	+
Casein	+	+	+	−	d	+	d	d/+*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Starch	−	−	−	d	d	d	d	−	−	−	−	−	−	−	−	−	−	−	d	d/+†	−	−	−	−	−
Tween 80	−	w+	−	d	+	d	+	+	+	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	+
Tween 60	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	d	−	+	+	+	+	−	+
DNase	w+	w+	w+	−	−	−	−	−	−	−	−	−	+	+	+	+	−	w+	+	+	−	−	−	−	−
Levan	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
Oxidative acid production from:																									
Adonitol	+	+	+	+	+	+	+	−	+	−	+	−	−	−	−	−	−	−	d	−	−	−	−	−	−
Inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	d	+	−	−	−	−	−	d/−‡
Inulin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	d/−†	+	+	−	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−
D-Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	+
D-Melezitose	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	+	−	−	−	−	−
Melibiose	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+	+	d/−†	−	−	−	−	w+
D-Sorbitol	+	+	+	+	+	+	+	+	−	−	−	−	+	w+	−	−	−	+	+	d/−†	w+	w+	w+	w+	−
L-Sorbose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	d/−†	−	−	−	−	−
Raffinose	+	+	+	+	+	+	+	+	−	−	+	+	−	−	−	−	−	+	+	+	−	−	−	−	+
L-Rhamnose	+	+	+	+	−	+	d	d/−*	+	+	+	+	−	−	−	−	−	d	d	+	−	−	−	−	d/+‡
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	−	−	−	−	+

Behrendt *et al.*,2002

Physiological and morphological characteristics of the 16S rRNA restriction types in comparison to reference strains of the genera *Curtobacterium, Clavibacter* and *Rathayibacter* Continued

- Abbreviations: +, positive reaction; w, weakly positive ; -, negative; d, reaction differs among strains ; y, yellow; i, ivory; ly, light yellow; o, orange; y/b, blue pigment of the intracellular indigoidine granules sometimes produced in addition to the yellowpigment; yo, yellow-orange; w, white.
- Species/genotype:
- 1, *Curtobacterium accumfaciens* pv. *accumfaciens*; 2, *Curtobacterium*
- *accumfaciens* pv. *accumfaciens* ; 3, *Curtobacterium accumfaciens* pv. *accumfaciens* (*Corynebacterium accumfaciens* subsp. *violaceum*); 4, *Curtobacterium accumfaciens* pv. *betae*; 5, *Curtobacterium accumfaciens* pv. *poinsettiae*; 6, *Curtobacterium accumfaciens* pv. *oortii* ; 7, genotype A; 8, genotype D; 9, *Curtobacterium luteum*; 10, *Curtobacterium citreum*; 11, *Curtobacterium pusillum*; 12, *Curtobacterium albidum*; 13, *Clavibacter tessellarius* ; 14, *Clavibacter. nebraskensis*; 15, *Clavibacter michiganensis*; 16, *Clavibacter michiganensis* subsp. *insidiosus* ; 17, *Clavibacter sepedonicus*; 18, genotype B1; 19, genotype B2; 20, genotype

Behrendt *et al.*,2002

Characteristics the genus *Rathayibacter*

- Four species:

1. *R. rathayi*
2. *R. tritici*
3. *R. toxicus*
4. *R. iranicus*

- All nematode transmitted, in grasses;
- Produce toxins important in grazing;
- Not easy to differentiate other than by genetic fingerprinting.

Characteristics of the Rathayibacteria

- *Rathayibacter* spp. cause gumming diseases, usually characterized by yellow bacterial slime on seed heads, stems and leaves of the plant host.
- Rathayibacters use as vectors the highly specialized plant-pathogenic nematodes of the genus *Anguina*.
- It should also be noted that *Anguina tritici* was demonstrated to transmit *R. rathayi* into *Triticum* spp. and initiate a disease similar to that caused by *R. tritici*.
- *Rathayibacter toxicus* annual raygrass toxicity bacterium can produce a neurotoxin that can be fatal to grazing animals.

Two novel *Rathayibacter* species

- Two novel species, *Rathayibacter caricis* sp. nov. and *Rathayibacter festucae* sp. nov. are proposed for two coryneform actinomycetes:
- *Rathayibacter caricis* found in phyllosphere of *Carex* sp.
- *Rathayibacter festucae* found in leaf gall induced by the plant-parasitic nematode *Anguina graminis* on *Festuca rubra* L.

Isolation methods

Two new *Rathayibacter* species

- ***Rathayibacter caricis***
- The gall surface was sterilized with 75% (v/v) ethanol for 1 min and dried; the galls were cut into pieces, added to 2 ml 0±85% (w/v) NaCl solution and milled.
- The plants of *Carex* sp. (without any visible symptoms of disease) were collected for pathogenicity tests.
- ***Rathayibacter festucae***
- An overhead part of a fresh plant was placed into a flask with saline solution (0.85% NaCl) and shaken for 1 h on a rotary shaker.
- The above plant gall and phyllosphere suspensions were plated by adding 1 drop into corynebacterium agar (CB agar) and incubated for 1 month at room temperature (18-24°C).

Characteristics of species in the genus *Rathayibacter*

Characteristic	1	2	3	4	5	6
Colony colour	Yellow	Rose-orange	Yellow	Yellow	Yellow	Yellow
Observation of visible growth (days)	2	2	2	2	2	4
Cell-wall sugar:*						
Glucose	1·0	1·0	+	+	+	+
Galactose	–	–	+	+	–	–
Mannose	4·5	1·8	+	+	+	+
Rhamnose	7·0	1·3	+	+	+	+
Xylose	0·7	0·3	+	+	–	–
Fucose	1·8	–	–	–	–	–
Oxidase	w	+	w	w	w	w
Methyl red test	+	+	–	–	–	–
Voges–Proskauer	+	+	–	–	–	–
Utilization of:						
Dulcitol	+	+	–	–	–	–
meso-Inositol	+	+	–	–	–	–
Inulin	+	+	–	+	+	–
Melibiose	+	+	–	–	–	–
meso-Erythritol	–	+	–	–	–	–
Salicin	+	+	–	+	+	–
Sorbitol	+	+	–	+	–	–
Hydrolysis of:						
Tween 60	–	+	+	+	+	–
Tween 80	–	+	–	+	+	–
Tolerance to:						
5% NaCl	+	–	–	+	–	–
0·03% Tellurium acetate	–	–	+	+	+	–
Source plant†	*Carex* sp.	*Festuca rubra* L.	*Dactylis glomerata* L.	*Triticum aestivum* L.	*Triticum aestivum* L.	*Lolium rigidum* Gaud.
Nematode associated‡	No data	*Anguina graminis*	*Anguina* sp.§	*Anguina tritici*	*Anguina tritici*	*Anguina funesta*‖

Abbreviations: +, positive ; -, negative; w, weak or negative, depending on growth phase. Strains: 1, *Rathayibacter caricis* sp.; 2, *Rathayibacter festucae* sp.; 3, *R. rathayi*; 4, *R. tritici*; 5, *R. iranicus* VKM Ac-1602T; 6, *R. toxicus*.

Dorofeeva *et al.*,2002

Characteristics the genus *Clavibacter*

- Vascular diseases caused by *Clavibacteria* and disease symptoms *Clavibacter michiganensis* causes a vascular disease with very high titers in a variety of agriculturally important plants and, in fact, may be considered the most important bacterial pathogen.
- *Clavibacter sepedonicus* is pleomorphic, slightly club-shaped short rod and this rod size is about 0.5-1.0 µm.
- Latent infection is observed in contaminated seeds.
- Pathogenicity Factors originally, extracellular polysaccharides (EPS) were proposed to be the main pathogenicity factor inducing wilt symptoms.

The Prokaryotes (chapter 1.1.28),2006. Euzeby, 2024

Isolation Media

Clavibacteria spp.

1. Nutrient agar supplemented with 1% glucose or 5% sucrose.
2. Glucose-yeast-calcium (GYCA) medium.
3. NBY medium.
4. Doppel's medium.
5. CB agar or any other media based on peptone, yeast extract, and glucose may be used as nonselective media for isolation of plant pathogenic *clavibacteria*.

Doppel's Medium (Lelliott and Stead, 1987)

Glucose	10 g
Yeast extract	8 g
Casein hydrolysate (not vitamin free)	8 g
K_2HPO_4	2 g
$MgSO_4 \cdot 7H_2O$	0.3 g
Agar	12 g
Water	1 liter
pH 7.2	

NBY Medium (Gross and Vidaver, 1979a; Vidaver and Davis, 1988)

Nutrient broth	8 g
Yeast extract	2 g
K_2HPO_4	2 g
KH_2PO_4	0.5 g
Agar	15 g
Water	1 liter
pH 7.2	

Fifty ml of 50% glucose and 1.0 ml of 1M $MgSO_4 \cdot 7H_2O$ are both added after separate filter sterilization.

The Prokaryotes (chapter 1.1.28),2006

Isolation and pigment development
Semi-selective media
Clavibacter michiganensis

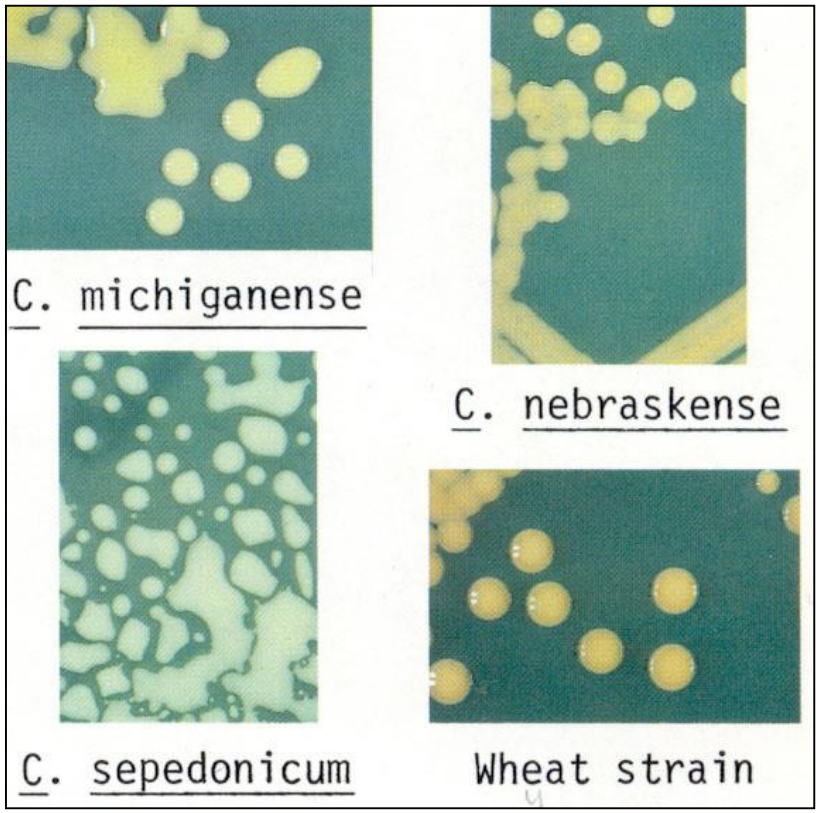
- Six representative semi-selective media for the detection of *C. michiganensis* subsp. *michiganensis*, namely CMM1T, SCM, SCMF, BCT, and SMSMM:
- The composition of each medium per 1 L were as follows:
- CMM1T (10 g sucrose, 3.32 g Tris base, 11.44 g Tris-HCl, 0.25 g $MgSO_4.7H_2O$, 5 g LiCl, 2 g yeast extract, 1 g NH_4Cl, 4 g casamino acids, 15 g agar, 10 mg polymyxin B sulfate, 28 mg nalidixic acid, 100 mg nystatin).
- SCM (10 g sucrose, 0.1 g yeast extract, 1.5 g H_3BO_3, 0.25 g MgSO4.$7H_2O$, 2 g K_2HPO_4, 0.5 g KH_2PO_4, 18 g agar, 30 mg nalidixic acid, 50 mg nicotinic acid, 100 mg nystatin, 10 mg potassium tellurite),
- SCMF (10 g sucrose, 2 g yeast extract, 1.5 g H_3BO3, 0.25 g $MgSO_4.7H_2O$, 2 g K_2HPO_4, 0.5 g KH_2PO_4, 18 g agar, 20 mg nalidixic acid, 50 mg nicotinic acid, 100 mg nystatin, 10 mg potassium tellurite, 80 mg trimethoprim),
- BCT (2.5 g mannitol, 2 g yeast extract, 1 g K_2HPO_4, 0.1 g KH_2PO_4, 0.05 g NaCl, 0.1 g $MgSO_4$. $7H_2O$, 0.015 g MnSO4. H_2O, 0.015 g $FeSO_4$. $7H_2O$, 0.6 g H_3BO_3, 15 g agar, 20 mg nalidixic acid, 100 mg trimethoprim, 20 mg polymyxin B, 4.2 mg epoxiconazole, 12.5 mg fenpropimorph), and
- SMCMM (20 g glycerol, 5 g peptone, 3 g yeast extract, 2 g K_2HPO_4, 0.5 g KH_2PO_4, 0.25 g $MgSO_4$. 7H2O, 5 g LiCl, 15 g agar, 20 mg nalidixic acid, 40 mg cycloheximide, 80 mg $K2Cr_3O_7$, 2 mg NaN_3, 2.1 mg 2,4,5,6-tetrachloroisophthalonitrile).
- All tested semi-selective media contained nystatin or cycloheximide. Both antibiotics inhibit the growth of fungi. Moreover, it is considered that other antimicrobial agents influence selectivity for *C. michiganensis* subsp. *michiganensis* in bacteria.
- R2A (per 1 L: 0.5 g glucose, 0.5 g soluble starch, 0.5 g yeast extract, 0.5 g proteose peptone no. 3, 0.5 g casamino acid, 0.3 g K_2HPO_4, 0.05 g MgSO4. 7H2O, 0.3 g sodium pyruvate, 15 g agar) medium containing nystatin at 100 μg mL^{-1} was used to detect total cultural bacteria in seed samples(SOMEYA *et al.*,2020).

Isolation and pigment development *C. michiganensis* subsp. *insidiosus*

- Modified Burkholder's agar (Straley *et al.*,1974), containing 250 ppm actidione (cycloheximide), is recommended for primary isolation and development of pigment (Close & Mulcock,1972).
- Isolations should be incubated at 20°C and take 5-7 days to develop.

Cultural morphologies of different species of *Clavibacter*

- *Clavibacter michiganense* subsp. *insidiosum* (*Cmi*),
- *C. michiganense* subsp. *michiganense* (*Cmm*),
- *C. michiganense* subsp. *nebraskensus* (*Cmn*).



Bacteriocins
Clavibacter subspecies

- Inhibition of closely related strains by other *Clavibacter* subspecies was reported early.
- Gross and Vidaver (1979a) showed, that 85% of the tested strains of the subspecies *Cmi*, *Cmm*, and *Cmn* produced one or more bacteriocins.
- The size of inhibition zones varied with strains, incubation temperature, and media used.
- In most cases all the other *Clavibacter* subspecies and additionally some *Rathayibacter* and *Curtobacterium* strains were affected by the bacteriocin.

Eichenlaub *et al.*,2006

Test for production of bacteriocin by *Clavibacter*

- Typing of bacterial strains by the pattern of sensitivity to bacteriocins or phages is a useful aid for identifying plant pathogenic bacteria.
- Bacteriocins are toxic, narrow-spectrum protein metabolites of bacteria that inhibit/kill related bacteria.
- Inhibition of growth of (most of the) related bacteria tested, visible as a clear halo (no growth) around the colonies of different *Clavibacter* species.

Janse,2006;..

Phages of *Clavibacter*

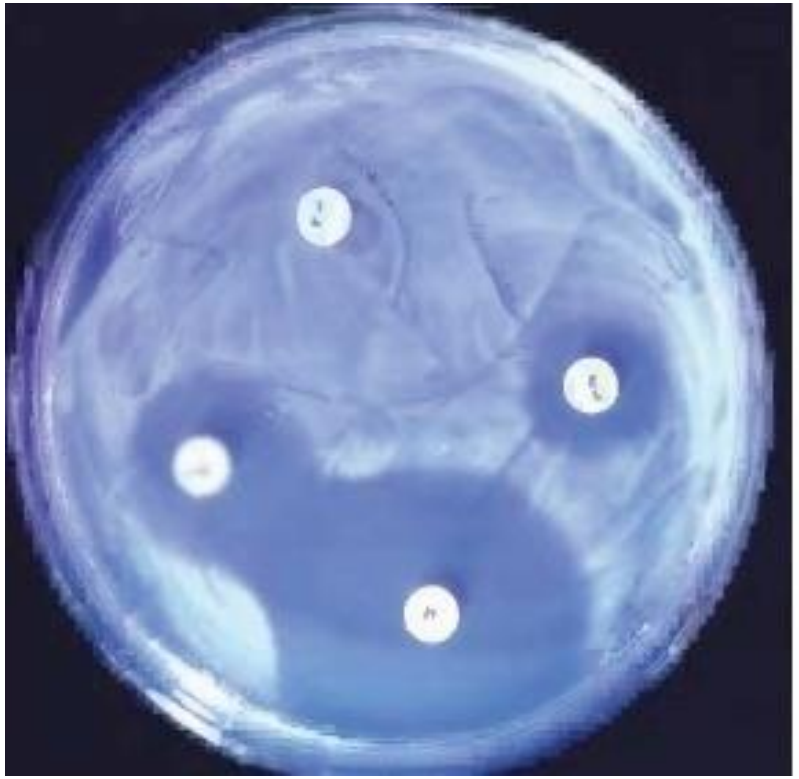
- A few phages have been isolated for
1. *Clavibacter insidiosum* (*Ci*),
2. *C. michiganense* (*Cm*),
3. *Clavibacter nebraskensis* (*Cn*).
- All are virulent and subspecies-specific.
- They have isometric heads and long non-contractile tails.
- CMP1 from *Cm* was shown to be specific for virulent *Cm* strains (Echandi and Sun,1973).
- It is a DNA phage with a size of about 60 kb.
- No lysogenic phage was reported up to now.

Eichenlaub *et al.*,2006

Sensitivity test for antibiotics

Clavibacter michiganensis subsp. *michiganensis*

- The bacterium *C.michiganensis* subsp. *michiganensis* is:
- Sensitive to tetracycline (TE).
- Moderately sensitive to streptomycin (S),and erythromycin (E).
- Insensitive to penicillin (P).



Selected differentiating characteristics of the *C. michiganensis* subspecies

Subspecies	Pigmentation	Gelatin liquefaction	Utilization of acetate	Reducing sugar production	Levan production
Cmm	yellow	+	+	variable	-
Cms	white	-	+	+	-
Cmi	blue-gray (yellow)	-	-	-	-
Cmn	orange	-	+	+	+
Cmt	orange		-		

Eichenlaub *et al.*,2006

On NBY medium certain species exhibited very characteristic colony colours that could be used tentatively to identify the organisms. For example, *Clavibacter michiganense* subsp. *insidiosus* produced indigoidine (a deep blue and indigo mix) on the colony edges.

Table 4. Characteristics that differentiate the *Clavibacter michiganensis* subspecies.

Characteristic	*Cl. m.* subsp. *michiganensis*	*Cl. m.* subsp. *insidiosus*	*Cl. m.* subsp. *nebraskensis*	*Cl. m.* subsp. *sepedonicus*	*Cl. m.* subsp. *tesselarius*
Pigment, NBY agar	Y	Y, BL	O (rarely Y)	W	O
Pigment, CB agar	Y	W, YW, BL	O	YW	RO
Cell wall sugars					
Glucose	d	+	+	+	+
Fucose	+	+	+	–	+
Acid from					
Lactose	+	+	+	–	+
Mannose	+	+	+	d	+
L-Ribose	+	+	+	–	+
Inositol	+	–	+[a]	–	+[a]
Sorbitol	–	–	+[b]	–[c]	+
Growth on C-source					
Dulcitol	d	–	+	–	+
D-Rhamnose	+	–	–	–	–
Utilization					
Acetate	+[d]	–	+	+	–
Succinate	+	–	+	+	+
Hydrolysis					
Starch	+	–	+	+	+
Tween 40	+	–	d	+	+
DNA	+	+	+	–	+
H_2S from peptone	+	+[b]	+	–	+
Levan production	–[c]	–[c]	+[e]	–	+[e]
Growth on CNS[f]	+	–	+	–	+
Growth on TTC[f]	+	+	–	–	–
5% NaCl	+	–	+	–	d
0.03% K-Tellurite	d	–	+	–	+
Voges-Proskauer	+[e]	+[e]	–	–	+[e]
G+C (mol%)[f]	73	73	73.5	72	74
Plant host[g]	Tomato and pepper	Lucerne	Corn	Potato	Wheat
Predominant symptoms[g]	Wilt and fruit spot	Wilt and stunting	Wilt and leaf blight	Wilt and tuber rot	Leaf spot

Symbols and abbreviations: +, positive; –, negative; d, different between strains; Y, yellow; O, orange; RO, rose-orange; W, white; YW, yellowish white; and BL, blue to black somewhat diffusible pigment (indigoidine).

[a,b,c,d,e]The opposite results were reported by Zgurskaya (1993a), Dye and Kemp (1977), Davis et al. (1984b), Vidaver and Davis (1988), and Behrendt et al. (2002), respectively, using different methods.

[f]Data from Carlson and Vidaver (1982). Somewhat different values of the G+C content of DNA were reported by other authors.

[g]Data from Vidaver (1982).

Adapted from Dye and Kemp (1977), Carlson and Vidaver (1982), Davis et al. (1984b), Vidaver and Davis (1988), Zgurskaya (1993a), and Behrendt et al. (2002).

The Prokaryotes (chapter 1.1.28),2006

Serological test

Immunoassay

BID Assay for *Clavibacter michiganensis* subsp. *michiganensis*

- 96-well ELISA kit, self-contained
- Cmm1 monoclonal antibody
- 10 minute boiling step for sample extraction
  - Replaced by new buffer - no boiling
- Results read by eye or spectrophotometer

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The BID ELISA test available in 2002

Miller,2006

ImmunoStrip test system
Detection method

- Add approximately 3 ml of sample extract buffer to the grinding bag.
- Take a sample of plant tissue that shows symptoms.
- The plant tissue should be diluted approximately 1:20 in extract buffer.
- Rub the bag with a pen or blunt object to completely crush sample.
- Remove one strip from the packaging. When handling the strip, always grasp the top of the strip marked with the test name. Do not remove protective covering.
- Keeping the strip in a vertical position, insert the end of the strip marked "sample" into the extract.
- Do not allow much more than 0.5 cm or 1/4 inch of the end of the strip to be submerged in the extract.
- Be sure the strip remains in the extract during the test.

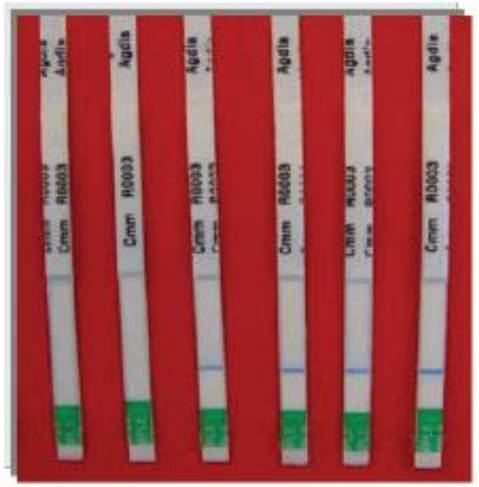
Laboratory 9. Pathogen detection methods,2008

ImmunoStrip test system
Detection method

- **Results:**
- The control line will usually appear in 3 to 5 minutes.
- Maximum reaction occurs in 20 minutes, at which time the ImmunoStrip should be removed from the buffer.
- 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, the test line will also appear.
- If the sample is negative, the test line will not appear.
- The test line will be red to purple in color just as the control line.
- If you wish to keep the strips as permanent records, cut off the sample pads (green ends marked "sample") and discard.

Serological test
Immunostrips for Cmm

- Immunostrip format
- Cmm1 monoclonal antibody
- Test complete in <5 minutes



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Miller,2006

Cmm Immunostrip Results

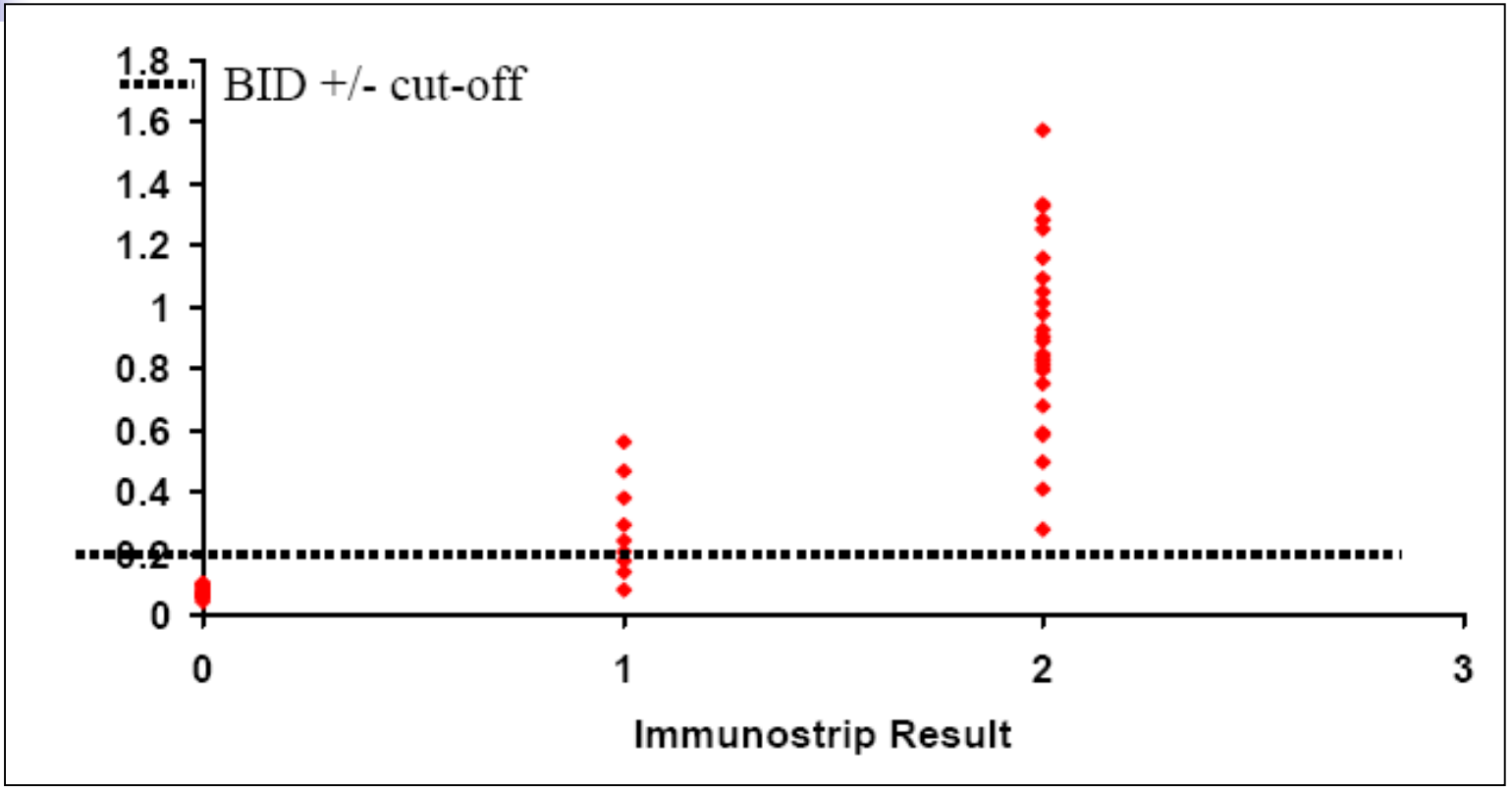
- Positive (+)
- Weak positive (+/-)
- Negative

Miller,2006

Accuracy of Cmm Immunostrip Assay

Immunostrip	Culture	Number of Samples
+	+	11
-	-	12
weak +	+	3
+	-	1
weak +	-	4

Comparison of BID-Cmm and Immunostrips for Detection of Cmm



Miller,2006

Comparison result
BID-Cmm/Cmm Immunostrip

- BID-Cmm is relatively sensitive
  - Detection limit ~ 10^6 CFU/ml *in planta*
  - Fast, easy to use

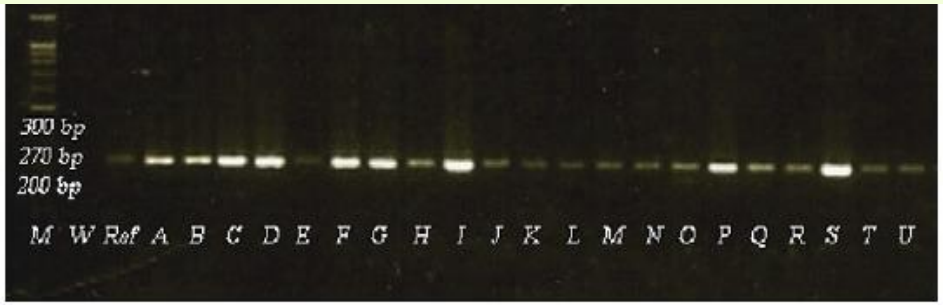
- Cmm Immunostrip
  - As sensitive as BID
  - Very fast, very easy to use: suitable in field
  - No false negatives observed in field sample evaluations
  - A few false positives - experimental error?

Miller,2006

Conventional PCR

Amplification of16S-23S rDNA region space of *Clavibacter michiganensis* subsp. *michiganensis*

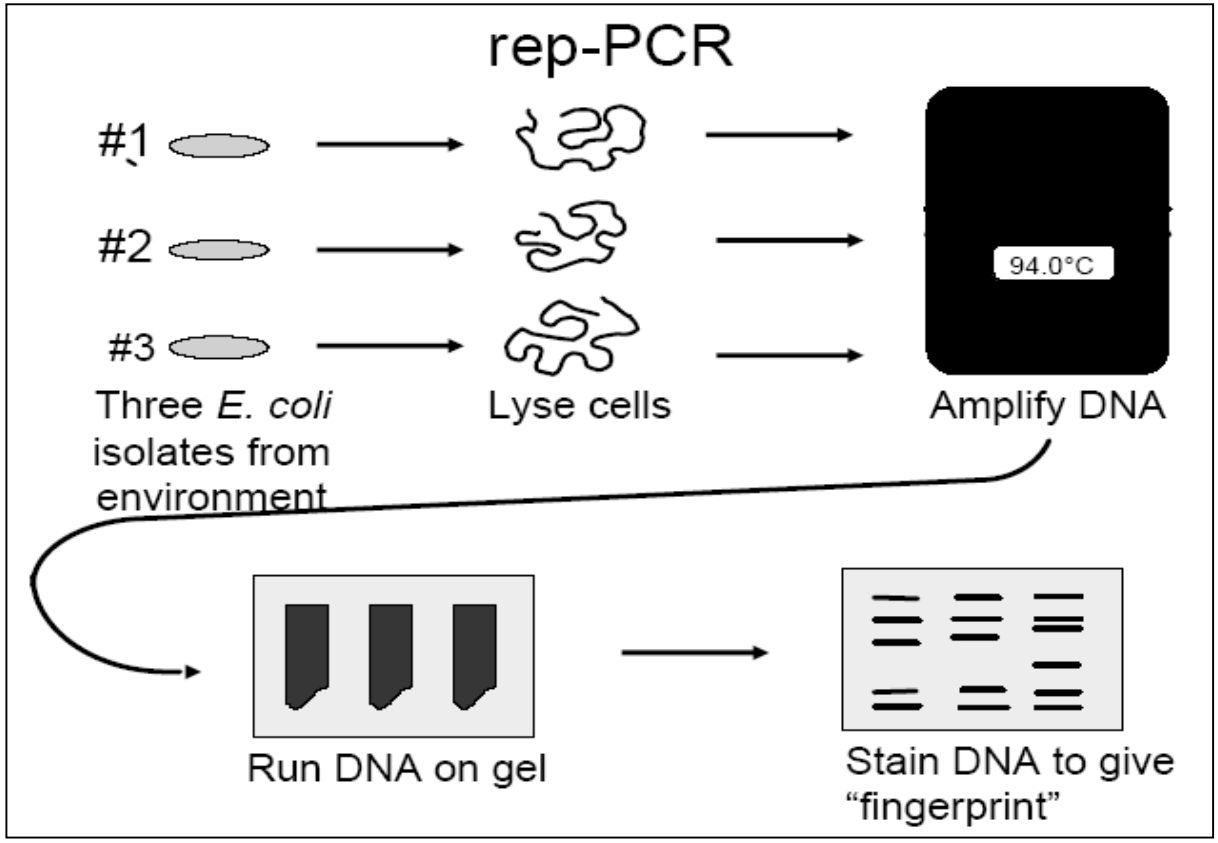
- Pathogen confirmation by specific PCR with primers PSA-4/PSA-R.
- Forward primer PSA-4 (5′-TCA TTG GTC AAT TCT GTCTCC C-3′) and reverse primer PSA-R (5′-TAC TGA GAT GTT TCA CTT CCC C-3′).
- The expected band size is 270 bp.
- M: Gen Ruler Fermantas 100-1000 bp.
- W: Water is negative control.



Baysal *et al.*,2011

Repetitive-PCR

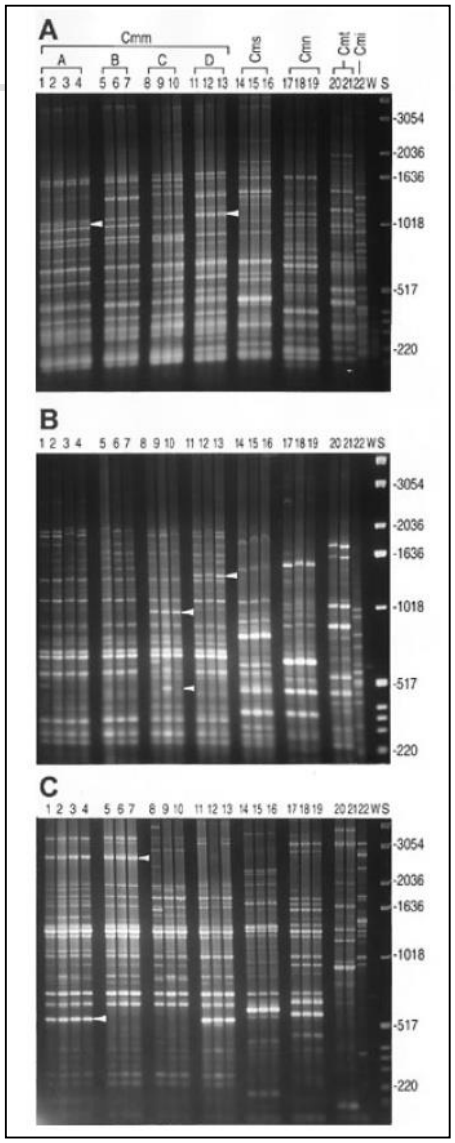
Rep-PCR



rep-PCR

A rapid and effective method to identify *Clavibacter michiganensis*

- Three rep-PCR primers (BOX, ERIC, and REP) were used for genomic fingerprinting.
- Agarose gel electrophoresis of polymerase chain reaction (PCR) fingerprint patterns obtained from strains representing five subspecies of *Clavibacter michiganensis* using primers corresponding to repetitive extragenic palindromic sequences (rep-PCR).
- **A,** BOX-PCR pattern,
- **B,** ERIC-PCR pattern,
- **C,** REP-PCR pattern.

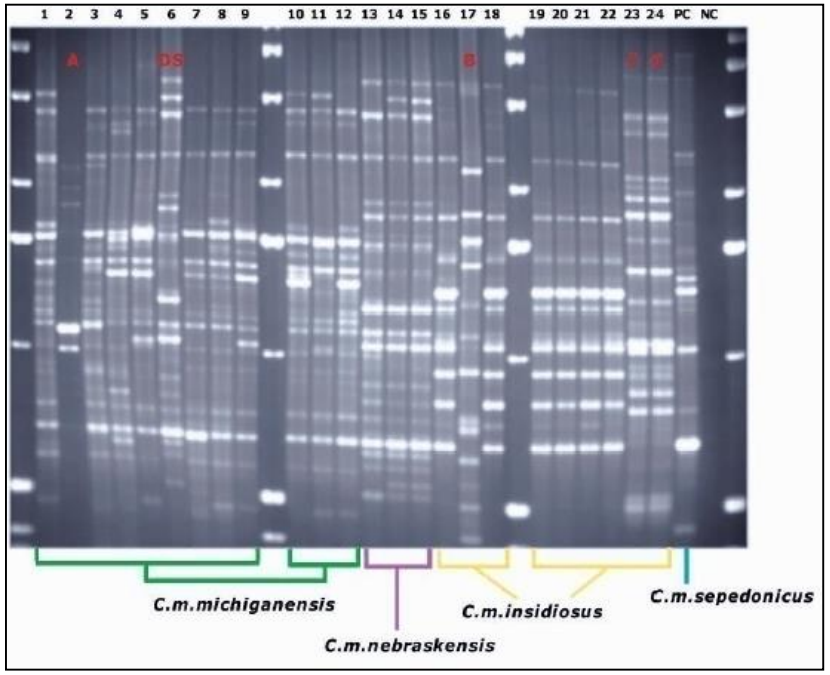


Louws *et al.*,1998

rep-PCR analysis

Used to discriminate several ex. subsp. of *Clavibacter michiganensis*

- BOX-PCR results for several ex. subspecies of *Clavibacter michiganensis*.
- Non-numbered lanes 1 kb DNA ladder;
- lanes 1-9 and 10-12 *C. m.* subsp. *michiganensis*;
- lanes 13-15 *C. m.* subsp. *nebraskensis*;
- lanes 16-18 and 19-24 *C. m.* subsp. *insidiosus*;
- lane 25 positive control *C. m.* subsp. *sepedonicus*;
- lane 29 negative control, ultra pure water.
- A, B and C = misidentified strains, strain C tested twice; DS = deviating strain of *C. m.* subsp. *michiganensis*, probably a mislabelled strain of *C. nebraskensis* (compare profiles!).

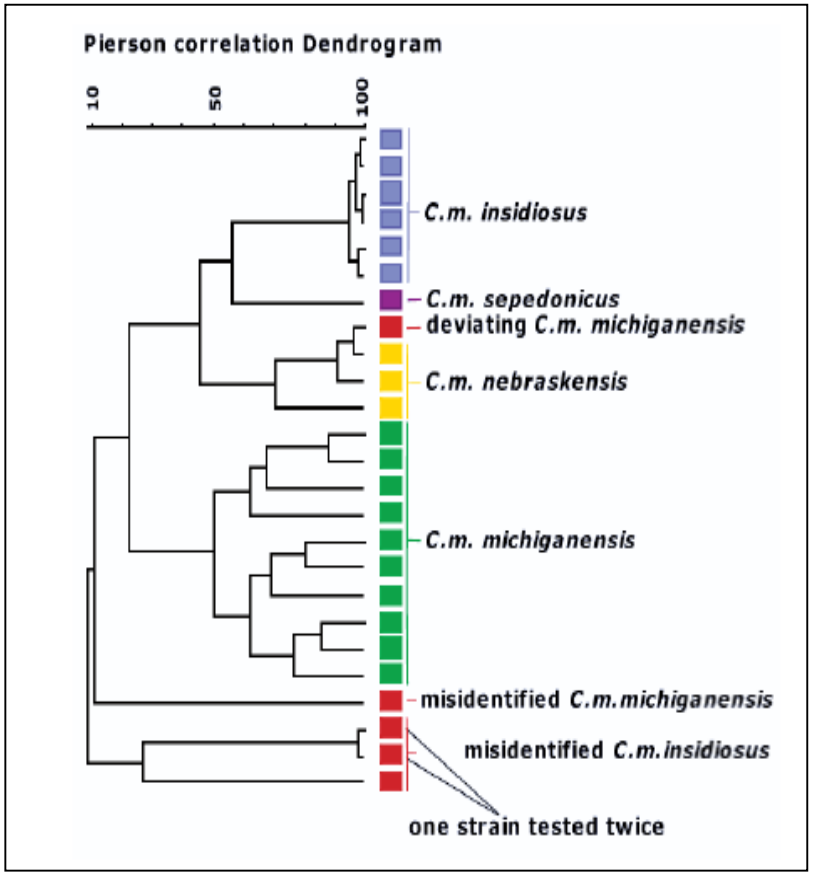


Janse,2006

rep-PCR analysis

Used to discriminate several ex. subsp. of *Clavibacter michiganensis*

- Dendrogram from data shown in previous fig., using Bionumerics software (AppliedMaths, Kortrijk, Belgium).
- There is a clear separation between the subspecies of *Clavibacter michiganensis*.

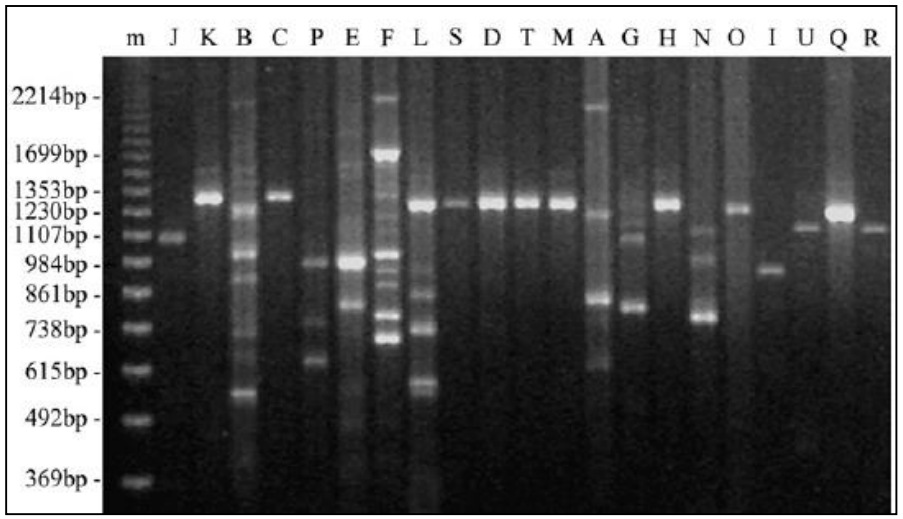


Janse,2006

ISSR analysis

C. michiganensis subsp.*michiganensis*

- ISSR (Inter Simple Sequence Repeat) is a general term for a genome region between microsatellite loci
- A total of eight ISSR primers were used to amplify the DNA:
- (ACC)6CC, CCA(TGA)5TG, CCA(TG)7T, GCA(AC)7, (CA)8RG, (AG)8YT, GGG(AC)7 and (GA)8GG.
- Of the screened primers used to analyze isolates, the most polymorphic loci amplified were obtained from the ISSR primers (CA)8RG (This Figure).



Microsatellite loci which are widely distributed throughout the genome can be used to classify individuals by relatedness.

Baysal *et al.*,2011

The *Clavibacter* subspecies
Infection mode

- The *Clavibacter* subspecies are probably mainly wound pathogens (Strider,1969).
- Infection of *Cm* through stomata and through hydathodes was described.
- Another important mode of transmission is through contaminated seeds (Strider,1969).
- Infection through animal vectors was never described for the *Clavibacter* subspecies (contrasting with the infection mode of *Rathayibacter* which is associated with different nematode species like *Anguina*) although the severity of the disease can be influenced by the presence of nematodes (Hunt *et al.*,1971).

Eichenlaub *et al.*,2006

Characteristics of the genus *Curtobacterium*

- The genus comprises of six spp., of which only *curtobacterium flaccumfaciens* is considered as plant pathogenic.

1. *Curtobacterium citreum*
2. *Curtobacterium albidum*
3. *Curtobacterium luteum*
4. *Curtobacterium pusillum*
5. *Curtobacterium herbarium*
6. *Curtobacterium flaccumfaciens*

- The five recognized pathovars of *Curtobacterium flaccumfaciens* are:

1. pv. *betae*
2. pv. *flaccumfaciens*
3. pv. *ilicis*
4. pv. *oortii*
5. pv. *poinsettiae*.

Schaad *et al.*,2001; Dunleavy,1989

Characteristics of the genus *Curtobacterium*

- Colonies of both pathogenic and non-pathogenic curtobacteria on nutrient agar are yellow, orange or ivory, shiny, slightly convex and round, with entire margins.
- The exception is with *C. albidum* and *C. pusillurn* which produce white colonies.
- All yellow pigmented curtobacteria produced β-carotene on tryptic soy agar.
- Colonies grown on nutrient dextrose agar ranged in diameter from 2 mm (*C. luteurn*) to 12 mm (*C. albidum*).
- *C. albidum* and *C. luteurn* are known antagonists against plant pathogenic fungi.

Dunleavy,1989;..

Test for carotenoid pigments in *Curtobacterium* spp.

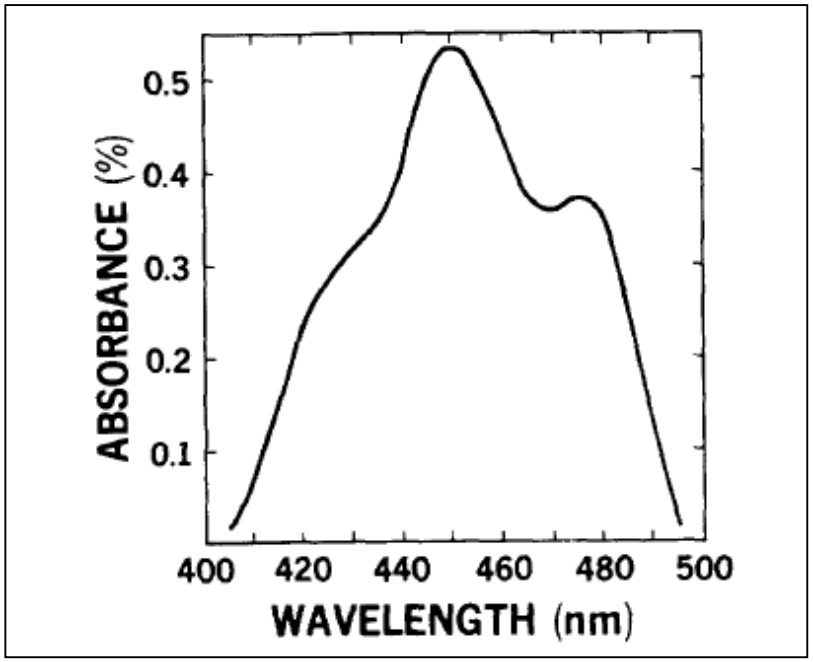
β-carotene

- The test bacteria were grown for 5 days on tryptic soy agar.
- Cells were washed from the plate with methanol and suspended in 10 ml of methanol for 30 min.
- Cells were removed by centrifugation, and the supernatant was added to a cuvette for spectrophotometric analysis.
- A β-carotene (Sigma Chemical Co., St. Louis, Mo.) standard was used for comparison with the bacterial product and consisted of a few crystals of β-carotene dissolved in 10 ml of methanol.
- β-Carotene in methanol gives an absorbance maximum at 447 nm (Goodwin, 1980).

Dunleavy,1989

Test for carotenoid pigments
β-carotene

- Absorption spectrum of β-carotene in methanol.
- The β-carotene was produced by yellow-pigmented *Curtobacterium* spp.
- This spectrum was identical to that of purified β-carotene.



Dunleavy,1989

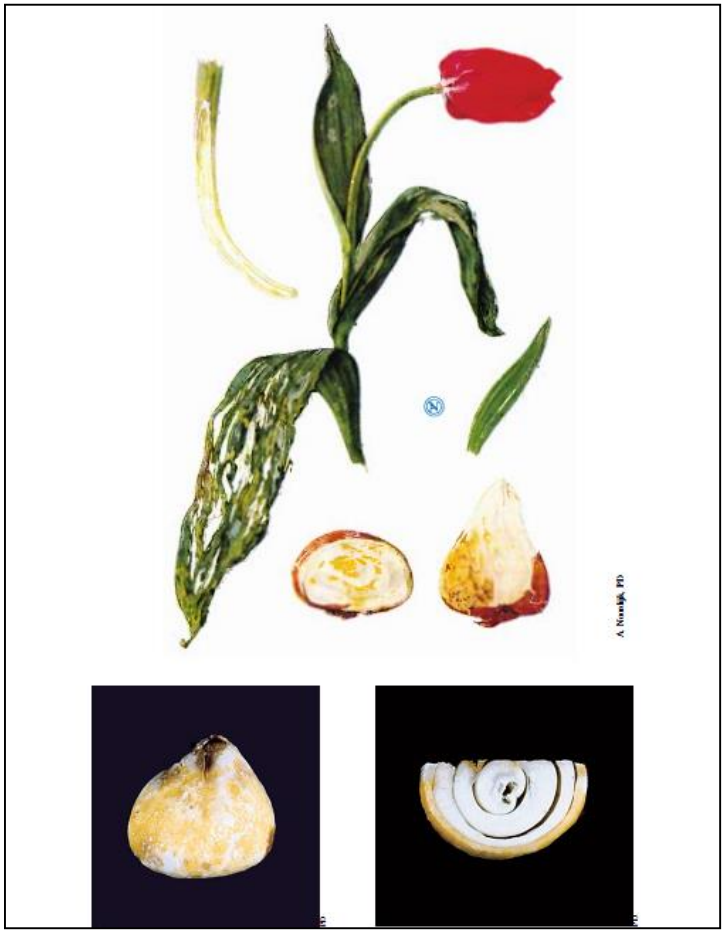
Characteristics typical of the genus *Curtobacterium*

- Gram positive, nonsporeforming cells usually move by means of lateral flagella.
- Cells are small, irregular, unbranched rods, tending to become shorter in the older culture.
- Obligately aerobic, catalase positive, chemoorganotrophic, mesophilic, and capable of growth on a nutrient agar and related media.
- Growth usually occurs at 5% NaCl but not at 10% NaCl.
- Acid is produced rather slowly and weakly from carbohydrates.
- The species differentiation is based on:
- Growth characteristics,
- Content of G+C of DNA,
- Fatty acid composition, and
- Some physiological properties.

Yellow pustule and hellfire

Curtobacterium flaccumfaciens pv. *oortii*

- Top: Leaf symptoms: silver grey stripes and cracks along the main vein (called hell fire) and yellow pustules on the outer white scales of the bulbs. Yellow discoloration of the vascular tissue in stems and bulbs. Colour drawing.
- Bottom: Natural tulip infection.
- Pustules on outer scale (left) and yellow discoloration of vascular and surrounding tissue of outer scale (right).



Janse,2006

Characteristics that differentiate species of the genus *Curtobacterium*

Table 5a. Characteristics that differentiate species of the genus *Curtobacterium*.[a]

Characteristics	*C. albidum*	*C. citreum*	*C. flaccum-faciens*[b]	*C. herbarum*	*C. luteum*	*C. pusillum*
Motility	–	+	+	+	+	+
Colony color	I	Y	Y/O	O	Y	LY
DNAse	–	–	w	–	–	–
Hydrolysis of						
Casein	+	–	d	+d[c]	–	–
Esculin	+	+	+	+	–	+
Gelatin	+	–	d	+d[c]	–	+
Starch	–	–	d	–	–	–
Tween 80	+	–	d	+	+	–
Acid from						
Adonitol	–	–	+	–	+	+
Raffinose	+	–	+	+	–	+
L-Rhamnose	+	+	d	–d[c]	+	+
D-Sorbitol	–	–	+	+	–	–
Cyclohexylundecanoic fatty acid[d]	–	–	–	–	–	+
G+C (mol%)[e]	70	70.5	68.3–68.5	71	69.8	69.3

Symbols and abbreviations: +, positive; –, negative; w, weakly positive; d, differs among strains; nd, not determined; colony color on nutrient agar II (SIFIN): I, ivory; Y, yellow; O, orange; and LY, light yellow.

[a]Table is based on the data reported by Behrendt et al. (2002). The type strain of *Curtobacterium plantarum* (Dunleavy, 1989) does not belong to the genus *Curtobacterium* (DSMZ, Catalogue of Strains), and this species is not included in the Table.

[b]Characteristics that differentiate pathovars of the species *C. flaccumfaciens* are given in Table 6.

[c]Reaction of the type strain is positive (+d) or negative (–d).

[d]Data from Suzuki and Komagata (1983).

[e]Data from Yamada and Komagata (1970), exept for the data for *C. herbarum* taken from Behrendt et al. (2002); somewhat higher values of G+C content were reported by Döpfer et al. (1982).

The Prokaryotes (chapter 1.1.28),2006

Table 6. Characteristics that differentiate pathovars[a] of the species *Curtobacterium flaccumfaciens*.

Characteristic	*Curt. flac.* pv. *betae*	*Curt. flac.* pv. *laccumfaciens*	*Curt. flac.* pv. *ootrii*	*Curt. Flac.* pv. *poinsettiae*
Colony color, NBY agar	Y	Y/O/P	Y	O
Cell wall sugar				
Galactose	–	+	–	+
Glucose	–	–	+	w
Fucose	+	–	+	–
Hydrolysis of				
Gelatin	–	+[b]	+[b]	–/d[c]
Casein	–	+	+	+/d[c]
DNAse	–	+	–	–
β-Galactosidase (pH 7)	+	d	d	–
Cystin arylamilase (pH 8.5)	d	d	–	–
Acid from L-rhamnose	+	+	+	–[b]
Acid from D-sorbitol	+	–[d]	–[d]	+
Assimilation of				
Acetate	+	+	–	+
Fumarate	–	+	–[e]	+[f]
Propionate	–	–	–	+[e]
KCN-tolerance, 0.0075% (w/v)	+[b]	+	+	–
Production of H_2S	+	+	+	–
Max. temperature for growth (°C)	37	37	37	34–35
G+C (mol%)[g]	73.7	72.2	72.2	72.5
Plant host[h]	Beet	Bean	Tulip	Poinsettia
Predominant symptoms[h]	Silvering of leaves; wilt	Wilt	Leaf, bulb spot; wilt	Leaf spot; wilt

Abbreviations: w, weak or questionable; and for other definitions, see footnote in Table 5a.

[a]According to Carlson and Vidaver (1982) and Vidaver and Davis (1988), the pathovars of *Curtobacterium flaccumfaciens* should be classified as separate subspecies (see the text).

[b]The opposite result was reported by Day and Kemp (1977) using different methods.

[c]Different results between strains (Behrendt et al. 2002).

[d,e,f]The opposite results were obtained by Behrendt et al. (2002), Davis et al. (1984b), and Carlson and Vidaver (1982), respectively, using different methods.

[g]Data from Döpfer et al. (1982).

[h]Data from Day and Kemp (1977) and Vidaver (1982).

Additional characteristics differentiate species of the genus *Curtobacterium* Continued

Table 5b. Additional characteristics that differentiate *Curtobacterium albidum*, *C. citreum*, *C. flaccumfaciens*, *C. luteum* and *C. pusillum*.

Characteristics	*C. albidum*[a]	*C. citreum*	*C. flaccum- faciens*[b]	*C. luteum*[a]	*C. pusillum*
Colony color	I	DuY	Y/O/P	DaY	PY/GW
Assimilation of					
Lactic acid	+	+	+	−	+
Malic acid	−	+	+	+	−
Fumaric acid	+	+	+	+	d
α-Ketoglutaric acid	−	+	−	−	−
Citric acid	+	d	+	−	−
Glyoxylic acid	+	+	−	−	−
Gluconic acid	+	+	+	+	−

Abbreviations: I, ivory; DuY, dull yellow; DaY, dark yellow; Y, yellow; O, orange; P, pink; PY, pale yellow; GW, grayish white (colony color on nutrient agar); for other definitions see footnote in Table 5a.

[a]Data for type strain only.

[b]Data for *C. flaccumfaciens* pv. *flaccumfaciens* and *C. flaccumfaciens* pv. *poinsettiae* only; characteristics that differentiate all pathovars of the species *C. flaccumfaciens* are given in Table 6.

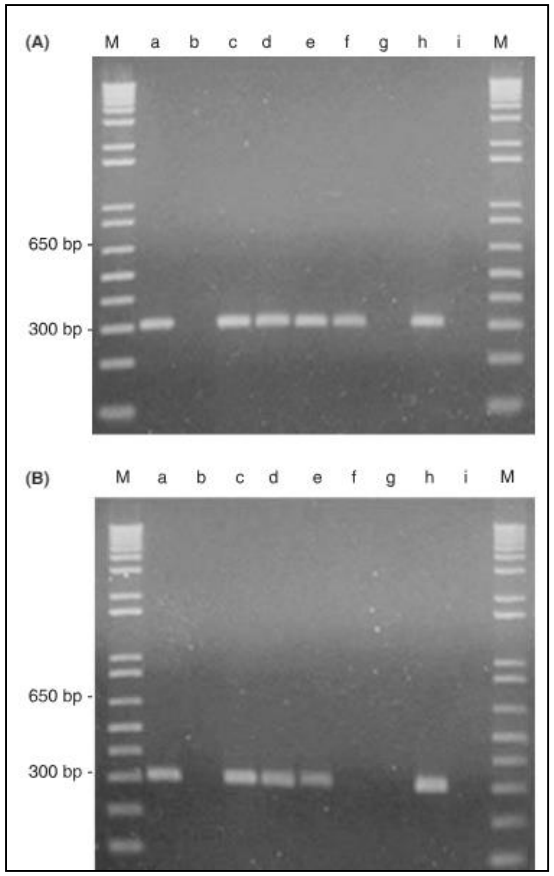
Data from Yamada and Komagata (1972a; 1972b) and Komagata and Suzuki (1986).

The Prokaryotes (chapter 1.1.28),2006

Specific PCR amplification

Curtobacterium flaccumfaciens pv. *flaccumfaciens*

- Gel electrophoresis of the PCR products obtained with the primers CffFOR2 and CffREV4, using as template DNA extracted with either (A) the Instagene Matrix (Biorad) or (B) the Puragene DNA Isolation Kit (Gentra) from:
- (Lane a) soakings of bean seeds naturally infected with *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Cff),
- (b) soakings of healthy bean seeds,
- (c-g) soakings of healthy bean seeds artificially contaminated with 10^5-10^1cells $ml^{-1}$of Cff strain ICMP 2584,
- (h) Cff strain ICMP 2584;
- (i) negative control with sterile distilled water.
- (M) 1 Kb plus DNA ladder



Telgi *et al.*,2002

Characteristics of the genus *Rhodococcus*

- Large genus with a single pathogenic sp. (*R. fascians*).
- Can be damaging to some horticultural crops.
- Pathogenicity associated with a plasmid.
- Other *Rhodococcus* spp. are often abundant on plant surfaces and in soil.
- Can be difficult to differentiate *R. fascians* from other spp.

David Stead

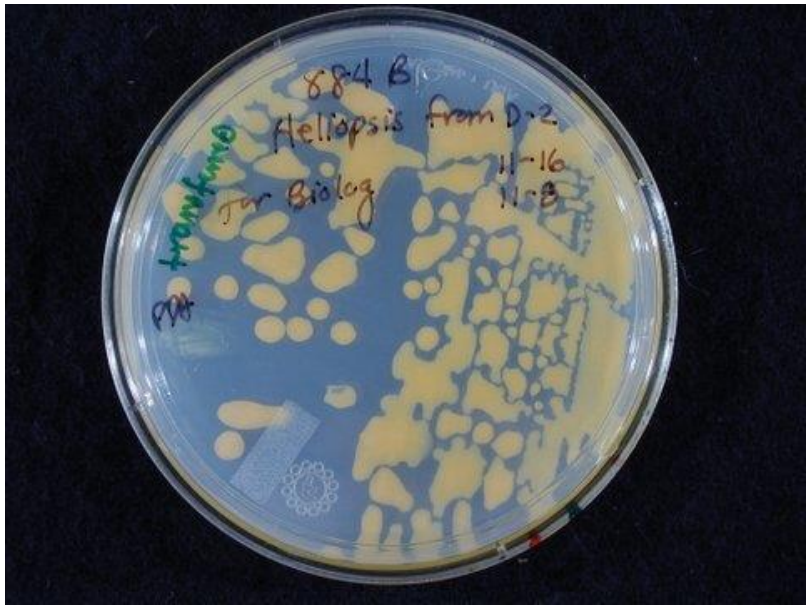
Disease symptoms caused by *Rhodococcus fascians*

	
Infected *Iberis* and negative control about 4 mo. post-inoculation.	Shoot proliferation on *Iberis*.
	
Leafy gall on *Oenethera*.	Bud proliferation on *Tiarella*.

Sandberg *et al.*,2011

Characteristics of *Rhodococcus fascians*

- Gram positive, aerobic bacterium.
- The only phytopathogen within its genus.
- Pleiomorphic
- Strains used in this experiment were extremely virulent: they produce dramatic symptoms on a wide range of hosts.



Sandberg *et al.*,2011

Characteristics of the genus *Leifsonia*

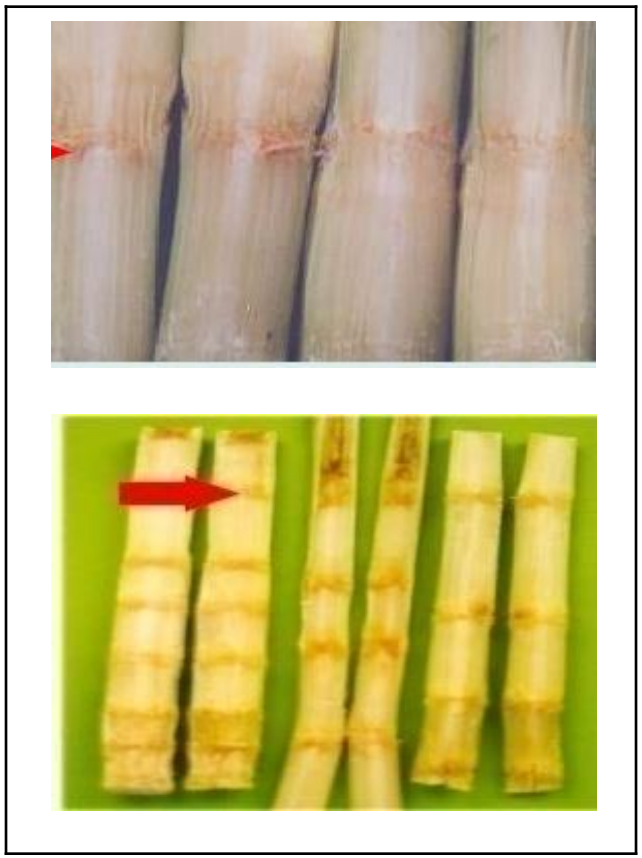
- The genus *Leifsonia* (Evtushenko *et al.*,2000) was proposed for:
- *Leifsonia poae*
- Isolated from *Poa annua* root galls induced by the grass root-gall nematode *Subanguina radicicola*.
- Two pathogenic *Leifsonia* (*Clavibacter*) spp. are:

1. *Leifsonia* (*Clavibacter*) *xyli* subsp.*xyli*
2. *Leifsonia* (*Clavibacter*) *xyli* subsp.*cynodontis*

Etymology: Leif.so'ni.a N.L. fem. n. *Leifsonia*, named after Einar Leifson, who isolated and described the first organism of this genus (Euzeby, 2020)

Internal nodal caused by RSD

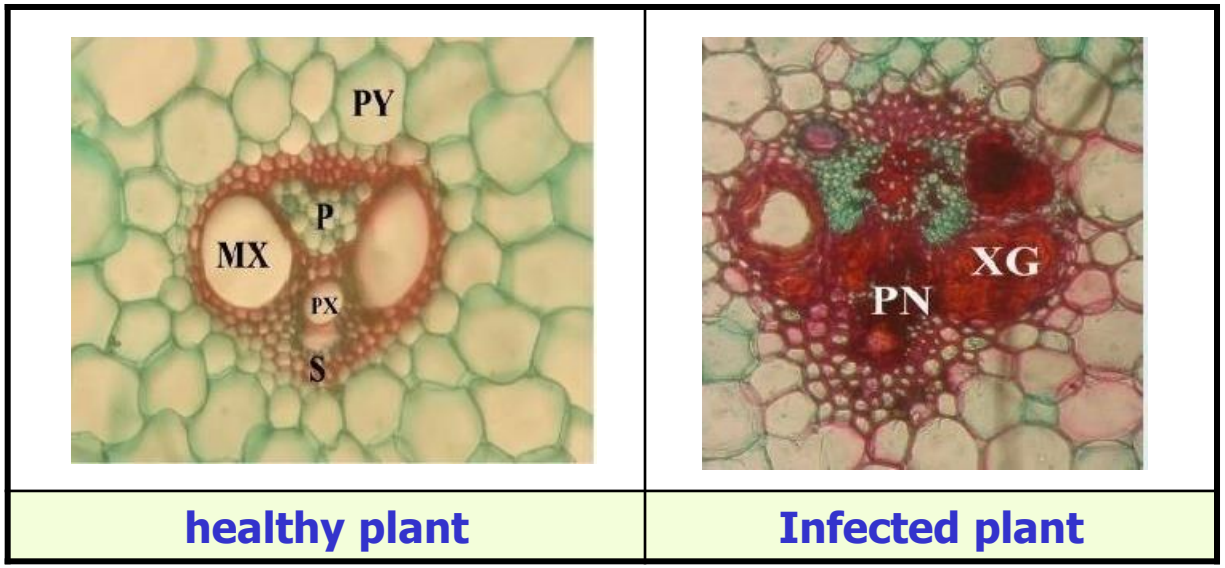
- RSD produces no visible symptoms other than stunting.
- The only other visible symptoms are red-orange dots or commas in the vascular traces in the nodal tissue (which can be seen when stalks are sliced with a sharp knife).



Ibrahim and coworkers

Cross section of vascular bundles RSD

- A: Histological changes in the vascular bundles of the stalks of sugar cane that infected with the isolated bacteria as shown in the cross sections of the lower part of the node.
- B: The cross section of the healthy plant.



Ibrahim and coworkers

Isolation
Culture media

- Different media based on glucose, peptone and yeast extract are appropriate for cultivation of the rapidly growing organisms of the genus *Leifsonia*.
- Successful isolation of these two pathogens requires surface sterilization of plant material and other precautions to avoid or reduce the growth of saprophytes.
- The xylem-inhabiting bacteria can be isolated from affected vascular bundles at the nodes, which are usually inhabited by bacteria in the infected plant.
- Under the microscope, affected bundles appear plugged with bacteria contained in a colored gummy substance.
- SC medium is used for growing of *Leifsonia xyli* subsp. *cynodontis* and *Leifsonia xyli* subsp. *xyli*.

Isolation and cultivation of *Leifsonia xyli*

SC medium

- The successful isolation of slowly growing plant pathogens of the genus *Leifsonia* from diseased plants require culture- amendment.
- Extracts of Bermuda grass and maize, as well as xylem fluid collected from maize plants, enhance the growth of *Leifsonia xyli* subsp. *Cynodontis*.
- The growth was enhanced even with 1% (v/v) maize xylem sap in a rich culture medium, giving both an increased growth rate and a higher stationary phase cell density.

- Cornmeal agar 17 g/L
- Papaic digest of soy meal (or phytone peptone or soytone) 8 g
- K_2HPO_4 1 g
- KH_2PO_4 1 g
- $MgSO_2.7H_2O$ 0.2 g
- Water 1 liter
- Bovine hemin chloride 15 mg
- Bovine serum albumin (20% solution),fraction V 2 g
- Glucose (50% solution) 0.5 g
- Cystcine (10% solution) 1 g
- The 15 mg of bovine hemin chloride is 15 ml of 0.1% bovine hemin chloride in 0.05N NaOH.
- The bovine serum albumin (20%), glucose (50%) and cysteine (10%) solutions should be filter sterilized and then 10, 1 and 10 ml, respectively, added to the sterilized molten medium at 50°C.
- The pH is then adjusted to 6.6.

The Prokaryotes (chapter 1.1.28),2006

Salient phenotypic characteristics of *Leifsonia*

- The species differentiation is based on:

1. Growth;
2. Morphological and physiological characteristics;
3. Cell wall sugar composition;
4. Menaquinone pattern.

Vitamin K_1 (phylloquinone) and Vitamin K_2 (menaquinone) is normally produced by bacteria.

Salient phenotypic characteristics of *Leifsonia*

- Salient phenotypic characteristics that differentiate *Leifsonia* gen. nov. from other genera containing DAB in their peptidoglycan.

Genus	Morphology	Motility	Major menaquinone	Peptidoglycan amino acid*	Polyamine pattern	Total polyamine (μmol g^{-1})	Growth at 18 °C
Leifsonia gen. nov.	R, F	+	MK-11, 10	DL-DAB	PUT	0·6	+
Agromyces	F, R	–	MK-12	L-DAB	PUT	0·2–0·3	+
Clavibacter	R	–	MK-9	DL-DAB	SPD, SPM	2·0–7·0	+
Rathayibacter	R	–	MK-10	L-DAB	SPD, SPM	4·8–18·0	+
Cryobacterium	R	–	MK-10	L-DAB	ND	ND	–
Leucobacter	R	–	MK-11	L-DAB, GABA	ND	ND	+
Agrococcus	C	–	MK-12, 11	L-DAB, Asp, Thr	SPM	0·9	+

* All organisms also contain alanine, glutamic acid and glycine.

The table is based on the data obtained in this study and extracted from relevant references (see text). R, Rods; F, Fllaments; C, cocci ; +, positive ; -, negative; DL-DAB, d and L isomers of diaminobutyric acid; GABA, *c*-aminobutyric acid; Asp, asparagine; Thr, threonine; PUT, putrescine; SPM, spermine; SPD, spermidine; nd, no data.

A **polyamine** is an organic compound having two or more primary amino groups-NH_2

Morphological and biochemical characteristics

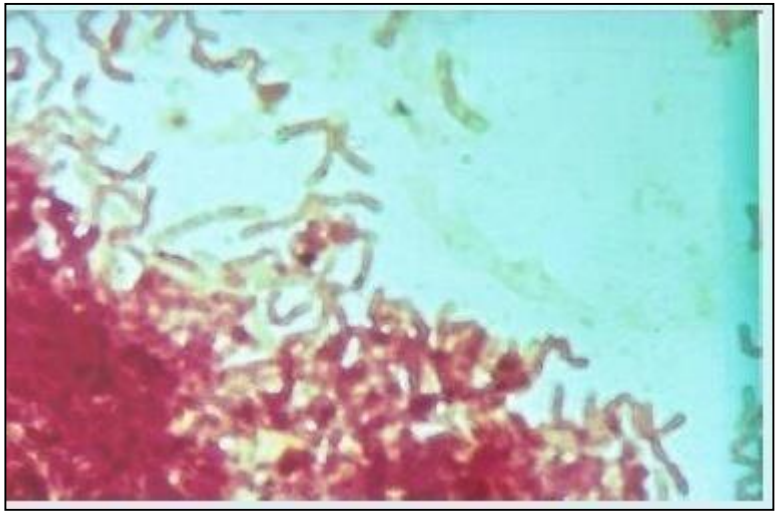
Leifsonia

- Organisms of the genus *Leifsonia* usually form yellow, yellowish-white, white or brownish-white colonies, which are circular, somewhat convex, glistening, opaque, and butyrous.
- Cells are nonspore forming, irregular rods, usually fragmenting into shorter rods or coccoid elements.
- Filaments and primary branching occur in young cultures of some species.
- Organisms are usually motile, mesophilic, obligately aerobic, and catalase positive.
- Oxidase test is variable among species.

Bacterial cells

Leifsonia xyli subsp. *xyli*

- The reisolated bacteria from the cultivated infected plant.



Ibrahim and coworkers

Characteristics that differentiate species of the genus *Leifsonia*

Table 8. Characterictics that differentiate species of the genus *Leifsonia*.

Characteristics	*L. aquatica*	*L. nagano-ensis*	*L. shinshu-ensis*	*L. poae*	*L. xyli* subsp. *xyli*	*L. xyli* subsp. *cynodontis*
Colony color	Y	W, PB	W, PB	PY, Y	W, PY	PY, Y
Growth on R agar	+	+	+	+	–	–
Visible colonies, day	2	2	2	2	7–10	5–7
Cell width (μm)	0.4–0.7	0.3–0.5	0.3–0.4	0.4–0.8	0.2	0.2–0.3
Cell length (μm)	1.2–2.5	1.8–3.0	2.5–3.0	1.5–8.0	5.0	3.0–6.0
Fucose in the cell wall	+	ND	ND	–	+	+
Utilization of						
Citrate	+	–	–	–	–	+
Gluconate	+	–	–	–	–	–
Propionate	+	–	+	+	–	–
Acid from						
D-Galactose	+	+	–	+	–	–
Salicin	+	+	–	+	–	–
D-Sucrose	w	+	–	+	–	–
Glucose	+	+	–	+	+	+
Mannose	+	+	–	+	w	+
Melibiose	–	+	–	–	–	–
Inulin	–	–	+	–	–	–
Hydrolysis of starch	+	+	+	–	–	+
Growth at NaCl, 5%	+	–	–	–	–	–
Major menaquinone, MK	11, 10	11, 10	11, 12	11, 10	ND	11, 12
G+C (mol%)	70	71	71	69	66	69
Source of isolation	Distilled water	Soil	Soil	Root gall on *Poa annua*	*Saccharum*, interspecific hybrid	*Cynodon dactylon*

Symbols and abbreviations: +, positive; –, negative; w, weakly positive; ND, not determined; W, white; Y, yellow; PB, pale brown; PY, pale yellow; for other definitions see footnote in Table 1.
Data from Davis et al. (1984b), Suzuki et al. (1999), and Evtushenko et al. (2000).

The Prokaryotes (chapter 1.1.28),2006

Phenotypic differences between strains VKM Ac-1401T, '*Corynebacterium aquaticum*' and *Leifsonia xyli* subspecies.

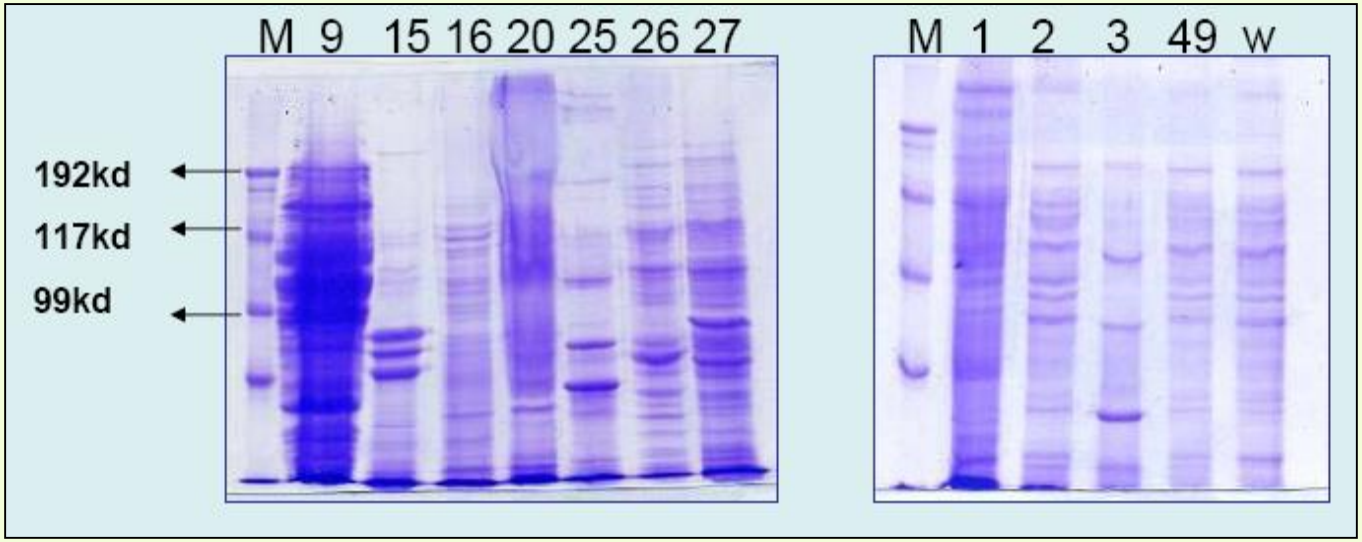
Character	VKM Ac-1401^T	'*Corynebacterium aquaticum*' VKM Ac-1400^T	*Clavibacter xyli* subsp. *xyli**	*Clavibacter xyli* subsp. *cynodontis**
Cell width (μm)	0·6–0·9	0·4–0·7	0·2	0·2–0·3
Cell length (μm)	8·0–15·0	1·2–2·5	5·0	3·0–6·0
Visible colonies (day)	2	2	7–10	5–7
Colony colour	Yellow	Yellow	White	Yellow
Growth on CB agar	+	+	−	−
Fucose in the cell wall	−	+	+	+
Oxidase	−	+	−	−
Production of H_2S	−	+	−	−
Voges–Proskauer test	+	−	−	+
Methyl red test	+	−	−	+
Utilization of:				
Citrate	−	+	−	+
Gluconate	−	+	−	−
Propionate	+	+	−	−
Hydrolysis of:				
Starch	−	+	−	+
Aesculin	+	−	−	−
Gelatin	+	−	−	−
Growth in 5% NaCl	−	+	−	−
Acid from:				
D-Arabinose	+	+	−	−
D-Galactose	+	+	−	−
Salicin	+	+	−	−
D-Sucrose	+	+	−	−
Used as C-source for growth:				
Adonitol	−	+	ND	ND
L-Arabinose	+	−	ND	ND
Inositol	−	+	ND	ND
Melezitose	−	+	ND	ND
Raffinose	+	−	ND	ND
L-Sorbose	−	+	ND	ND
Tolerance to antibiotics (μg ml^{-1}):				
Chloramphenicol (10)	−	+	ND	ND
Doxycycline (5)	−	+	ND	ND
Erycycline (10)	−	+	ND	ND
Gramicidin (50)	−	+	ND	ND
Rifampicin (30)	+	−	ND	ND
Major menaquinones	MK-11	MK-11, 10†	ND	MK-11, 12†
Source of isolation	Nematode gall on *P. annua* roots	Distilled water	*Saccharum*, interspecific hybrid	*Cynodon dactylon*

Evtushenko *et al.*,2001

Protein analysis

Leifsonia xyli subsp. *xyli*

- Protein profile of the bacterial isolates of LXX that were used in the injection of the mouths (the name of isolates is written above it and the molecular masses of the marker are written beside it.

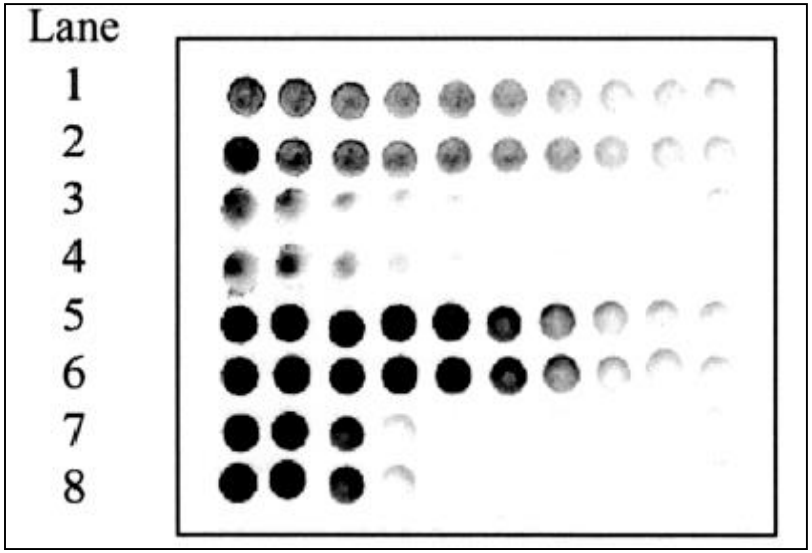


Ibrahim and coworkers

Dot blot assay

Leifsonia xyli subsp. *xyli*

- Lane 1 and 2, 2-fold dilution in sugarcane juice (CP72-1210),
- Lane 3 and 4, 10-fold dilution in reaction buffer,
- Lane 5 and 6, 2-fold dilution in sugarcane juice (CP87-3388);
- Lane 7 and 8, 10-fold dilution in sugarcane juice (CP87-3388).



Nitrocellulose membrane submitted to the standard colorimetric dot blot ELISA.

Westphal and Mirkov,2003

PCR detection

Leifsonia xyli subsp. *xyli*

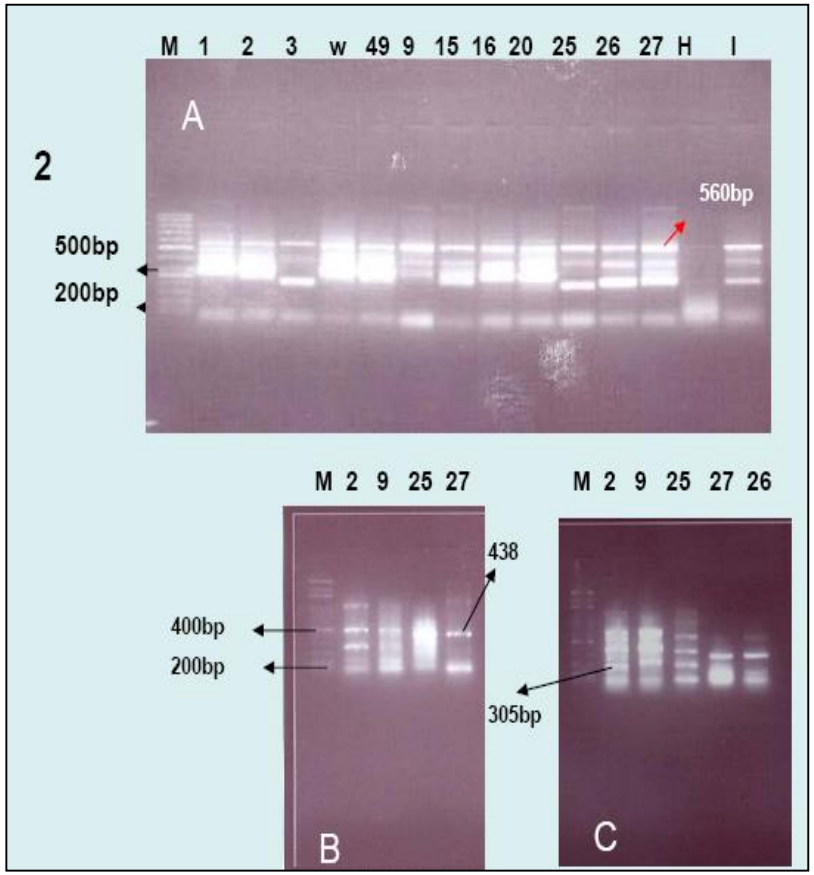
- The primers used were:

A. L1&G1,

B. Cxx1 and Cxx2 that give 438 bp PCR product.

C. ITSf#5 and ITSr#5 that give 305 bp PCR product.

- DNA marker used in A was 50bp and in both B&C was 100 bp ladder.



Ibrahim and coworkers

Characteristics of the genus

Endophytic bacteria

Plantibacter

- Phenotypically close to the *Rathayibacter.*
- Isolated from grass phyllosphere and surface litter after mulching the sward.
- The genus *Plantibacter* is characterized by aerobic, nonsporeforming, nonmotile irregular cells.
- Oxidase test varies between the species.
- *Plantibacter agrosticola* sp. nov., *Plantibacter elymi* sp. nov., and *Plantibacter cousiniae* sp. nov. isolated from plant galls induced by three different nematodes of the subfamily Anguininae.
- CNS medium or a modified CNS medium without lithium chloride was successfully used for isolation of endophytic bacteria, including *Plantibacter*.

Characteristics that differentiate the genera *Plantibacter* and *Rathayibacter*

Table 13. Characteristics that differentiate the genera *Plantibacter* and *Rathayibacter*.[a]

Characteristic	Plantibacter	Rathayibacter
Colony color	Yellow	Yellow, pink-orange
Major menaquinone	MK-10 / MK-9, 10 / MK-10,11	MK-10
Cytochrome oxidase	aa_3	bb_3 / bb_3, and aa_3
Quinole oxidase	bo_3 / bb_3 / none	None
Polyamine pattern	Spermine, and 1,3-diaminopropane	Spermine, and spermidine
Polyamine (total amount, $\mu mol \cdot g^{-1}$)	1.3–1.43	4.8–18.0
Plant host/nematode vector[b]	*Agrostis sp.* / *Anguina agrostis*; *Elymus repens* / *Anguina agropyri*; *Cousinia onopordioides* / *Mesoanguina picridis*	*Festuca rubra* / *Anguina graminis*; *Dactylis glomerata* / *Anguina* sp.; *Triticum aestivum* / *Anguina tritici*; *Lolium rigidum* / *Anguina funesta*; *Agrostis avenacea* / *Anguina* sp.; *Polypogon monspeliensis* / *Anguina* sp.; *Carex* sp. / *Heteroanguina caricis* (?)
Other sources	Grass phyllosphere, surface litter after mulching the sward, agronomic crops, and rhizosphere of potato	No data on properly identified rathayibacteria

[a]Members of the genera also differ in FT-IR spectra (Behrendt et al., 2002).
[b]See Tables 14, 15 and the text for details.
Data from Sabet (1954), Gupta and Swarup (1972), Price et al. (1979), Riley (1987), Riley and McKay (1990), Riley and Ophel (1992), Zgurskaya et al. (1993b), Evtushenko et al. (1994; unpublished), Altenburger et al. (1997), McKay et al. (2001), Behrendt et al. (2002), Dorofeeva et al. (2002), Heuer et al. (2002), Zinniel et al. (2002), and Trutko et al. (2003).

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Characterictics that differentiate *Plantibacter* species

Table 14. Characterictics that differentiate *Plantibacter* species.

Characteristic	*P. flavus*	"*P. agrosticola*"	"*P. elymi*"	"*P. cousiniae*"
Fucose in cell wall	ND	–	–	+
Oxidase test	–	+	+	+
Cytochrome oxidase	ND	aa_3	aa_3	aa_3
Quinole oxidase	ND	bb_3	bo_3	–
Heme *O*	ND	–	+	–
Major menaquinone	MK-10,11	MK-9, 10	MK-9, 10	MK-10
Iso-16:0 Me (%)[a]	–	1,53	3,74	2,89
Hydrolysis				
Starch	d	d	+	+
Hypoxanthine	ND	–	d	+
Xanthine	ND	–	d	+
Tween 80	+	+	d	–
Methyl red test	ND	–	d	+
Growth at 6% NaCl	–	d	d	+
G+C (mol%)	68–70	67.2	66.5	67.8
Source	Grass phyllosphere, and surface litter after mulching the sward	*Agrostis* sp.[b]	*Elymus repens*	*Cousinia onopordioides*
Location of gall	ND	Seed	Stem	Leaf
Nematode vector	ND	*Anguina agrostis*	*Anguina agropyri*	*Mesoanguina picridis*

Symbols and abbreviations: +, positive reaction; –, negative; d, different among strains; ND, not determined.
[a]Inferred on the basis of retention time.
[b]Most probably *Agrostis capillaris* L. (S. A. Subbotin, personal communication).
Data from Evtushenko et al. (1994; unpublished), Behrendt et al. (2002), and Trutko et al. (2003).

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Fatty acid analysis

Gram positive plant pathogenic bacteria

	15:0 ISO	15:0 ANTEISO	15:1 ANTEISO A	17:0 ANTEISO	10 Methyl 18:0	18:1 ù9cis
Bacillus	+	+				
Clavibacter	+	+	+	+		
Curtobacterium	+	+		+		(+)
Rathayibacter	+	+	(+)	+		
Rhodococcus					+	

*Many Gram positive plant associated bacteria such as *Bacillus* spp., *Arthrobacter* spp. and *Curtobacterium* spp. have very similar fatty acids in their profiles.
+ = thought to occur in all strains
(+) = occurs in some taxa/strains

Prediction of Gram positive genera of plant pathogenic bacteria based on fatty acids.

15:1 anteiso A is a chemotaxonomic marker for *Clavibacter*

Schaad *et al.*,2001

PCR Primers for coryneforms

Specificity	Primer Designation	Sequence	Size (bp)
Species *michiganensis*	CMR16F1	(5'GTGATGTCAGAGCTTGCTCTGGCGGAT3')	1,500[a]
	CMR16R1	(5'GTACGGCTACCTTGTTACGACTTAGT3')	
	CMR16F2	(5'CCCCGACTCTGGGATAACTGCTA3')	1,300[a]
	CMR16R2	(5'CGGTTAGGCCACTGGCTTCGGGTGTTACCGA3')	
subsp. *michiginensis*	CMM5	(5'GCGAATAAGCCCATATCAA3')	614[b]
	CMM6	(5'CGTCAGGAGGTCGCTAATA3')	
sepedonicus	Sp1f	(5'CCTTGTGGGGTGGGAAAA3')	215
	Sp5r	(5'TGTGATCCACCGGGTAAA3')	
sepedonicus	CMS6	(5'CGCTCTCCCTCACCAGACTC3')	258
	CMS7	(5'TCCCGTGCTTGCCTGCGTTG3')	
sepedonicus	Cms50F	(5'GAGCGCGATAGAAGAGGAACTC3')	224
	Cms50R	(5'CCTGAGCAACGACAAGAAAAATAG3')	
	Cms72F	(5'AGTTCGAGTTGATAGCAATCCGC3')	247
	Cms72R	(5'GTGTCTCGGATTCACGATCACC3')	
	Cms85F	(5'AAGATCAGAAGCGACCCGCC3')	232
	Cms85R	(5'TCGCACAGCCAAATCCAGC3')	
sepedonicus	A47A	(5'CACCCCTCGACTGCGAGAAACG3')	670
	A47B	(5'TCCTCCGAGACTTTCGGGACGC3')	
sepedonicus	CSRSC	(5'GGCCATGACGTTGGTGACAC3')	1054[c]
sepedonicus	Cms50-2F	(5'GGCAGAGCATCGCTYCAGTACC3')	NA
	Cms133-R	(5'CGGAGCGCGATAGAAGAGGA3')	
	TaqMan Probe	(5'-FAM-AAGGAAGTCGTCGGATGAAGATGGG-TAMRA-3')	
subsp. *xyli*	*Cxc*ITSf#5	(5'TCAACGCAGAGATTGTCCA3')	305
	*Cxc*ITSr#5	(5'GTACGGGCGGTACCTTTTC3')	
cynodontis/xyli	*Cx*FOR	(5'AAGGAGCATCTGGCACCCT3')	446
	*Cxc*REV	(5'AGGATTCGGTTCTCATCTCA3')	
	*Cx*FOR	(5'AAGGAGCATCTGGCACCCT3')	278
	*Cxc*REV	(5'GAATCGATCGGCGTCTCCTC3')	
subsp. *xyli*	Cxx1	(5'CCGAAGTGAGCAGATTGACC3')	438
	Cxx2	(5'ACCCTGTGTTGTTTTCAACG3')	
xyli	RSD33	(5'CTGGCACCCTGTGTTGTTTTC3')	265
	RSD292	(5'TTCGGTTCTCATCTCAGCGT3')	
	RST60	(5'TCAACGCAGAGATTGTCCAG3')	229
	RST59	(5'CGTCTTGAAGACACAGCGATGAG3')	
R. fasciens	JRERIGHT	(5'CGGGATCCATATCGAACCGCCCTC3')	225
	JRELEFT	(5'GGGAATTCCGACCGTATCCAGTGT3')	

[a] Estimated value.
[b] Avirulent strains are negative.
[c] Gives same 1054 bp product with *C. michiganensis* subsp. *insidious*.
NA, not applicable

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 *Clavibacter*

Genus *Clavibacter*					
Species/subspecies	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
Clavibacter and *Rathayibacter* (genus specific)	**R16FO/CBR16R1** **+** **CBR16F2/CBR16R2** 16S rDNA	Nested	Bacteria (DNA extraction)	Lee *et al.*, 1997a	Restriction enzyme analysis required for differentiation species and subspecies inside both genera.
C. michiganensis subsp. *insidiosus*	**CIRS-1/CIRS2** Insertion element	Conventional	Plant tissue and seeds (DNA extraction)	Samac *et al.*, 1998	
C. michiganensis subsp. *michiganensis*	**CMM-5/CMM-6** *Pat-1* gene plasmid DNA	Conventional	Plant tissue and seeds (DNA extraction) bacteria (boiled)	Dreier *et al.*, 1995	
C. michiganensis subsp. *michiganensis*	**CM_3/CM_4** DNA fragment from a cloned pathogenic isolate	Conventional	Bacteria, seeds (alkaline lysis and boiled)	Santos *et al.*, 1997	
C. michiganensis subsp. *michiganensis*	**CMM5/CMM6** *Pat-1* gene plasmid DNA **PSA-4/PSA-R** 16S-23S rDNA spacer region	Conventional	Bacteria (boiled)	Anon., 2005a; Milijasevic *et al.*, 2006	Recommended in the EPPO protocol.
C. michiganensis subsp. *michiganensis*	**CMM-5/CMM-6** *Pat-1* gene plasmid DNA	Conventional	Bacteria (DNA extraction)	Hadas *et al.*, 2005	

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

	CM_3/CM_4 DNA fragment from a cloned pathogenic isolate	BIO			
C. michiganensis subsp. *sepedonicus*	**A47A/A47B** CS1 plasmid sequence fragment	Conventional	Bacteria (untreated)	Firrao and Locci, 1994	
C. michiganensis subsp. *sepedonicus*	**CMS-6/CMS-7** CS1 plasmid sequence fragment	Conventional Competitive (*Arabidopsis* genomic DNA as internal standard)	Plant tissue (DNA extraction)	Schneider *et al.*, 1993 Hu *et al.*, 1995	Both authors used the same primers but the second protocol can be quantitative.
C. michiganensis subsp. *sepedonicus*	**Spif/Sp5r** 16S-23S rDNA spacer region	Conventional	Bacteria, potato tubers (DNA extraction)	Li and De Boer, 1995	
C. michiganensis subsp. *sepedonicus*	**CSRS-C** Inverted repeat plasmid CS1	Conventional	Bacteria, plant tissue (alkaline treatment)	Slack *et al.*, 1996	
C. michiganensis subsp. *sepedonicus*	**Nested** **CMSIF1/CMSIR1** **+** **CMSIF2/CMSIR2** Insertion element	Nested	Bacteria, potato tubers (DNA extraction)	Lee *et al.*, 1997b	
C. michiganensis subsp. *sepedonicus*	**CMS50F/CMS50R** **CMS72F/CMS72R** **CMS85F/CMS85R** Three primer sets for single or multiplex PCR Chromosomal DNA (unknown)	Conventional Multiplex	Bacteria, potato tubers (DNA extraction)	Mills *et al.*, 1997	
C. michiganensis subsp. *sepedonicus*	**Primers** **Cms 50-2F/Cms 133R** Chromosomal DNA (unknown) **Probe** **Cms 50-53T**	Real-time (TaqMan) BIO+TaqMan	Bacteria (untreated)	Schaad *et al.*, 1999	BIO implies enrichment in solid medium.
C. michiganensis subsp. *sepedonicus*	**PSA-1/PSA-R** 16S-23S rDNA spacer region **NS-7-F/NS-8-R** DNA from potato, eggplant and tomato	Conventional Multiplex (Coamplification of host DNA as internal control)	Bacteria (untreated), potato tissue (DNA extraction)	Pastrik, 2000	

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

C. michiganensis subsp. *sepedonicus*	See: Schneider *et al.*, 1993; Firrao and Locci, 1994, Li and De Boer, 1995, Slack *et al.*, 1996, Mills *et al.*, 1997, Schaad *et al.*, 1999; Pastrik, 2000	Conventional Real-time (TaqMan)		Anon., 2006b	Recommended in the EPPO protocol
C. michiganensis subspecies: *insidiosus, michiganensis sepedonicus, nebraskensis, tessellarius*	**CMR16F1/CMR16R1** + **CMR16F2/CMR16R2** 16S rRNA gene **CMR16F1/CMR16R1**	Nested Conventional	Bacteria, potato tubers (DNA extraction)	Lee *et al.*, 1997b	Restriction analysis required for differentiation of *C. michiganensis* subsp. *sepedonicus*.
C. michiganensis subspecies: *insidiosus, michiganensis sepedonicus, nebraskensis, tessellarius*	**Universal all subspecies PAS-R/ Subspecies-specific PSA-1** (*C. m.* subsp. *sepedonicus*) **PSA-4** (*C. m.* subsp. *michiganensis*) **PSA-5** (*C. m.* subsp. *insidiosus*) **PSA-2** (*C. m.* subsp. *tesalarius*) **PSA-7** (*C. m.* subsp. *nebraskensis*) 16S-23S rDNA spacer region	Conventional	Bacteria (DNA extraction)	Pastrik and Rainey, 1999	*C. michiganensis* subsp. *insidiosus* and *nebraskensis* yield same band. RAPD-PCR for distinguishing subspecies.
C. michiganensis subspecies: *insidiosus, michiganensis sepedonicus, nebraskensis, tessellarius*	**Primers FP Cm/RP Cm** Common ITS in all subspecies **Subspecies specific probes Cms probe Cmm probe Cmn probe Cmi probe Cmt probe**	Real-time (TaqMan)	Bacteria (DNA extraction)	Bach *et al.*, 2003	

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 *Rhodococcus*

Genus *Rhodococcus*					
Species	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
R. fascians	**JRERIGHT/JRELEFT** *fas-1* gene (cytokinin biosynthesis)	Conventional	Plant (DNA extraction)	Stange *et al.*, 1996	

Genus *Curtobacterium*

Genus *Curtobacterium*					
Species/pathovars	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
C. flaccumfaciens pv. *flaccumfaciens*	**CF4/CF5** Chromosomal DNA (unknown)	Conventional	Bacteria (DNA extraction)	Guimaraes *et al.*, 2001	
C. flaccumfaciens pv. *flaccumfaciens*	**CffFOR2/CffREV4** Chromosomal DNA (unknown)	Conventional	Bacteria (DNA extraction) or seeds	Tegli *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

Genus *Leifsonia*

Genus *Leifsonia*					
Species/subspecies	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
L. xyli subsp. *xyli*	**CxxITSf # 5/CxxITSr # 5** ITS region **CxFOR/CxxREV/ CxcREV** ITS region	Conventional Multiplex	Bacteria (untreated), vascular fluid (PVP)	Fegan *et al.*, 1998	*Clavibacter xyli* subsp. *xyli* Multiplex assay allows differentiation between *C. xyli* subsp. *xyli* and *C. xyli* subsp. *cynodontis*.
L. xyli subsp. *xyli*	**Cxx1/Cxx2** ITS region	Conventional	Bacteria (untreated), vascular sap (PVP and Ficoll)	Pan *et al.*, 1998	*C. xyli* subsp. *xyli*
L. xyli subsp. *xyli*	**RSD 33/RSD 297 (primary) + RST60/RST59 (secondary)** ITS region	Nested	Not indicated	Falloon *et al.*, 2006	
L. xyli subsp. *xyli*	**Lxx82F/Lxx22R Lxx202F/Lxx331R** ITS region	Conventional Real-time (SBYR® Green Master Mix)	Plant (DNA extraction)	Grisham *et al.*, 2007	

Palacio-Bielsa *et al.*,2009

Pathogenicity tests

Seedling test for *C. michiganensis*

- Pathogenicity tests on tomato seedlings were performed by puncturing the stem four times with a needle that had been dipped in a suspension of bacteria containing 10^2-10^5 cells mL^{-1}.
- The inoculum was prepared from a culture grown overnight on NA and suspended in saline.
- The plants were maintained at 28°C and symptoms were recorded after 7-21 days.
- For each strain, five plants were inoculated, and the test was conducted three times.
- Using titers of 8×10^4 bacteria all plants were infected and show severe symptoms.
- All *Clavibacter* spp. are vascular plant pathogens causing systemic infections.

Hadas *et al.*,2006; Eichenlaub *et al.*,2006

Pathogenicity tests

Seedling test for *C. michiganensis* subsp. *michiganensis*

- The bacterium is re-isolated from wilting plants by removing a 1-cm stem section from 2 cm above the inoculation point and suspending in phosphate buffer and dilution plating on NGA or YPGA.
- Stripes on stems developed that split and exposed reddish brown cavities (stem cankers).

Pathogenicity and hypersensitive response (HR) assays
C. michiganensis subsp. *michiganensis*

- Cell suspensions were prepared and tomato seedlings (*Lycopersicon esculentum* Mill. cv. Bonny Best) and four o'clocks (*Mirabilis jalapa*)plants were tested for virulence on tomato and HR on four o'clock plants.
- Most *C. michiganensis* subsp. *michiganensis* strains were fully virulent (causing cankers and wilt in tomato), and a strong HR on four o'clock plants.
- Some strains showed intermediate virulence reactions (produced only cankers at the site of inoculation) and two strains of intermediate virulence were inconsistent (+/–) in HR response.

Louws *et al.*,1998

Pathogenicity and hypersensitive response (HR) assays

C. michiganensis subsp. *michiganensis*

Figure 1. Symptomatology of *Clavibacter michiganensis* subsp. *michiganensis* in different hosts: a) Bacterial canker on *Solanum cheesmanii*, b) Bacterial wilting and collapse of *Solanum cheesmanii* c) Hypersensitive response in *Nicotiana tabacum*, d) bacterial canker on naranjilla *Solanum quitoense* e) Diagnostic symptomatology of incurvature of leaves characteristic of bacterial wilt caused by *Clavibacter michiganensis* subsp. *michiganensis* in *Solanum quitoense* (compare to leaves in 1f plant to the extreme right) f) Experimental units (naranjilla plants) in the assay to evaluate the parasitic ability of *Clavibacter michiganensis* on *Solanum quitoense*: first to the left (T1: root damage), second to the left (T2: no-wound and inoculation), two plants in the center (T3: aerial wounds), T0: plant to the right.

Bolanos-Carriel *et al.*,2017

Pathogenicity test
Rhodococcus fascians

- A44a & A25f strains isolated from host plants using selective and non-selective media.
- Pea seedlings inoculated with these isolates.
- Plants with symptoms of leafy gall and/or shot proliferation used for re-isolations.

Sandberg *et al.*,2011

Pathogenicity tests

C. flaccumfaciens pv. *fluccumfaciens*

- The pathogenic *C. flaccumfaciens* pv. *fluccumfuciens* and non pathogenic *Curtobacterium* species were inoculated onto leaves of both soybean and garden bean plants.
- All except non pathogenic *Curtobacterium* species were pathogenic on both beans and soybeans.

Dunleavy,1989

Pathogenicity tests

C. flaccumfaciens pv. *fluccumfuciens*

- Comparison of symptoms from plants inoculated:
- With pink (left) and
- A standard highly virulent orange (right) isolate (15 days post-inoculation).

Robert M. Harveson

Pathogenicity tests

On alfalfa(*Medicago sativa* also called lucerne)

C. michiganensis subsp. *insidiosus*

- Grow susceptible alfalfa test plants (varieties Europe and Orca seem to be highly susceptible) in pots with enough substrate, at approximately 20-25C (day) and >70% relative humidity in a glasshouse or growth chamber. At least 10 5-6 week old plantlets should be used for each pathogenicity test.
- Use a reference strain (known to be pathogenic) as a positive control to inoculate a series of 10 plantlets for each experiment as well as a series of plantlets inoculated with sterile physiological saline as a negative control.
- Prepare an appropriate volume of approximately 10^9cfu mL^{-1} suspension of the presumptive 24-72 h old isolates and of the reference strain in sterile physiological saline (24-48 h old culture).

OEPP/EPPO Bulletin,2010

Pathogenicity tests

On alfalfa(*Medicago sativa* also called lucerne)

C. michiganensis subsp. *insidiosus*

Continued..

- Three methods can be used for inoculation:
- Method 1: dip a previously disinfected pair of scissors into the bacterial suspension and use them to cut the plantlets. Dip the scissors as many times as required into the suspension to be sure that enough suspension is present.
- Method 2: cut the plantlets with a disinfected pair of scissors and add a drop of bacterial suspension on each wound with a pipette.
- Method 3: dip shortened roots of test plants into the bacterial suspension for 17-18 h (Víchová and Kozová,2004).

OEPP/EPPO Bulletin,2010

Pathogenicity tests

C. michiganensis subsp. *insidiosus* on alfalfa(*Medicago sativa* also called lucerne) Continued..

- Cormack et al. (1957) concluded that root inoculation is more efficient than stem inoculation, at least for breeding tests. But this method is not easy to perform.
- Immediately after inoculation, place a plastic bag over the plantlets or use any other appropriate system to keep the plantlets under high moisture conditions for 24 h, the optimum temperature being 17-24C.
- After this, plantlets can be kept under normal glasshouse conditions.
- Keep the plants under observation for at least 6-8 weeks. From the fourth week, make at least weekly observations for wilting.
- Isolate from wilting plants by removing a 1-cm stem section from 2 cm above the inoculation point and suspending in phosphate buffer.
- Perform dilution plating on King's B or YPGA media.
- Subculture presumptive isolates and undertake identification tests to confirm they are *C. michiganensis* subsp. *insidiosus*.

OEPP/EPPO Bulletin,2010

Pathogenicity tests

On alfalfa

C. m subsp. *insidiosus & P. viridiflava*

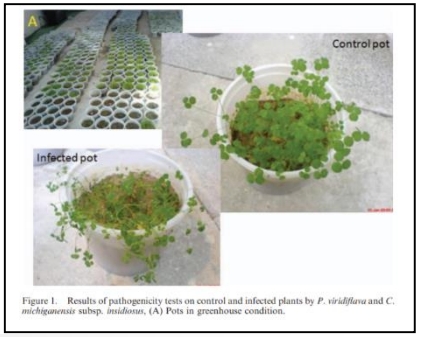


Figure 1. Results of pathogenicity tests on control and infected plants by *P. viridiflava* and *C. michiganensis* subsp. *insidiosus*, (A) Pots in greenhouse condition.

- Healthy alfalfa seeds were selected for pathogenicity test, disinfected with 5% sodium hypochlorite for 10 min and then they were washed thrice in sterile and distilled water.
- Then seeds were planted in pots containing 2 kg of sterile sandi-lumi soil in greenhouse with three repeats. After 45 days, plantlets reached a three-to four-leaved stage; only 15 plantlets per pot were kept.
- Bacterial suspension was prepared by culturing the bacterium in nutrient broth yeast extract (NBY). To recognize pathogenic strains, a bacterial suspension with an OD= 0.1 at 600 nm was injected in stem and crown of plantlets.
- Similarly, the bacterial suspension was added to soil (Lukezic *et al.*,1983). For each strain, one pot was considered as control to which only sterile distilled water was injected or added to the soil.

Heidari and Khodakaramian,2012

Pathogenicity tests

Clavibacter sepedonicus

- Suspensions (10^6 cfu/ml) were injected into stems of ten *Solanum melongena* plants (cv. Black Beauty) at leaf stage 3.
- Control plants were inoculated with sterile water.
- Plants were incubated at 21°C and 70-80 % humidity.
- First symptoms were observed as dark green areas on leaves after ten days followed by wilting and necrosis.
- *C. sepedonicus* was re-isolated from eggplant and identified.

Positive pathogenicity test on eggplant.

Altundağ *et al.*,2008

Preservation
Clavibacteria spp.

- Freeze-drying may sometimes affect virulence of plant pathogens (Servin-Massieu, 1971).
- Virulence of *Clavibacter nebraskensis* and probably other subspecies can be maintained satisfactorily by storage on solid complex media at 6°C for 2 years or by lyophilization.
- Storage of *Clavibacter nebraskensis* in sterile distilled water or phosphate buffer at 6°C or room temperature was unsatisfactory for maintenance of viability and virulence.
- Strains maintained in dried leaves of greenhouse grown plants were viable and virulent up to three months (Vidaver, 1977).
- The bacteria can survive in infected corn, but also in infected kernels or in irrigation water.

Preservation
Clavibacteria spp.

- The bacterium *C. m. insidiosus* can remain infective for more than 3 years.
- As laboratory experiments showed, the bacterium can remain in dried tissue or seed for 10 years (Erwin,1990).

Eichenlaub *et al.*,2006

Identification of the bacterial pathogens *Streptomyces*

Disease diagnosis and pathogen diagnostics

Gram-Positive Bacteria
Plant pathogenic genera

- **Domain or empire: Bacteria**
  Division or Phylum: "Actinobacteria"
  Class: Actinobacteria
  Subclass: Actinobacteridae
  Order: Actinomycetales
  Suborder: Streptomycineae
  Family: Streptomycetaceae
  Genus: *Streptomyces*

J.P. Euzéby,2010

Actinobacteriology
Actinomycetes

- Actinomycetes are non-motile, filamentous, gram positive bacteria.
- They produce branching mycelium which may be of two kinds viz.:

1. Substrate mycelium, and
2. Aerial mycelium.

- Among actinomycetes, the streptomycetes are the dominant.
- The non-streptomycetes are called rare actinomycetes, comprising approximately 100 genera.

The family Streptomycetaceae

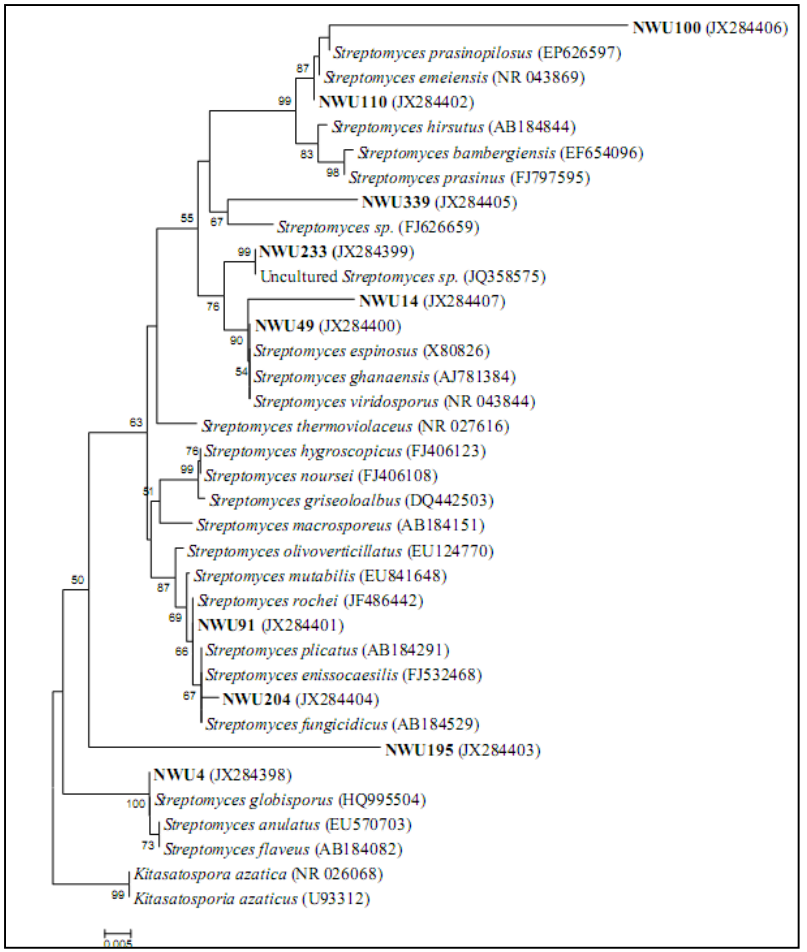
- The family Streptomycetaceae was created by Waksman and Henrici,1943.
- Originally this family harbored only the type genus *Streptomyces*.
- Zhang *et al.*,1997 proposed that the genus *Kitasatospora* be included, and recently, a third genus, *Streptacidiphilus*, was added (Kim *et al.*,2003).

The Prokaryotes (chapter 1.1.7),2006

16S rDNA phylogeny

Genus *Streptomyces*

- Neighbour-joining tree of the bacterial isolates and representative species of the genus *Streptomyces* based on partial 16S rRNA gene sequences.
- Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets.
- Only values greater than 50% are shown.
- The scale bar indicates 0.005 substitutions per nucleotide position.

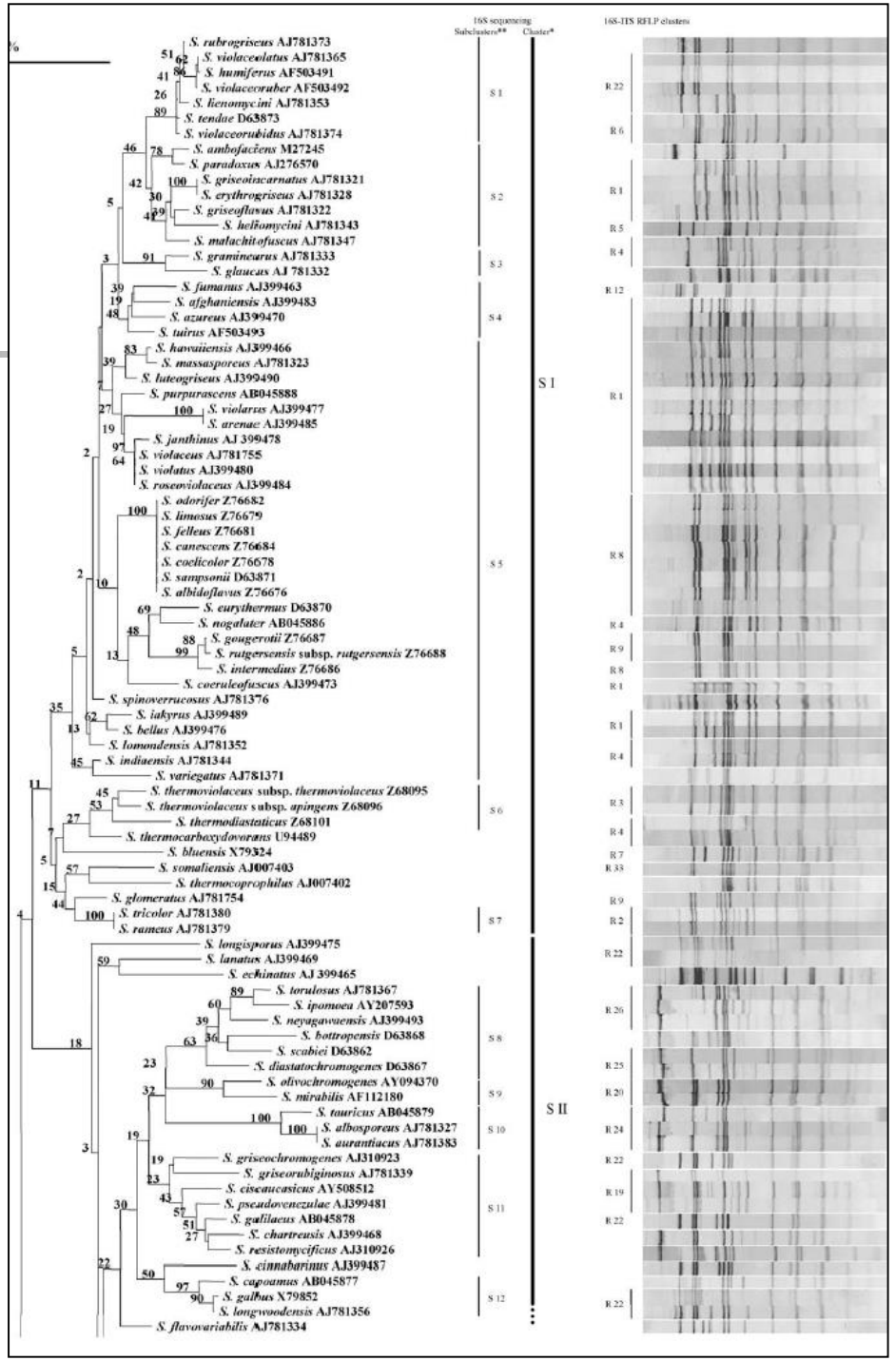


Adegboye and Babalola,2013

Taxonomy

Based on ITS; RFLP; 16S rDNA sequencing

- Neighbor-joining tree based on almost complete 16S rDNA sequences of 158 *Streptomyces* and *Kitasatospora* type strains in correlation with corresponding 16S-ITS RFLP fingerprinting data.



Lanoot *et al.*,2005

Taxonomy

Based on ITS; RFLP; 16S rDNA sequencing (Continued)

* Clusters containing strains with overall similarity above 97%.

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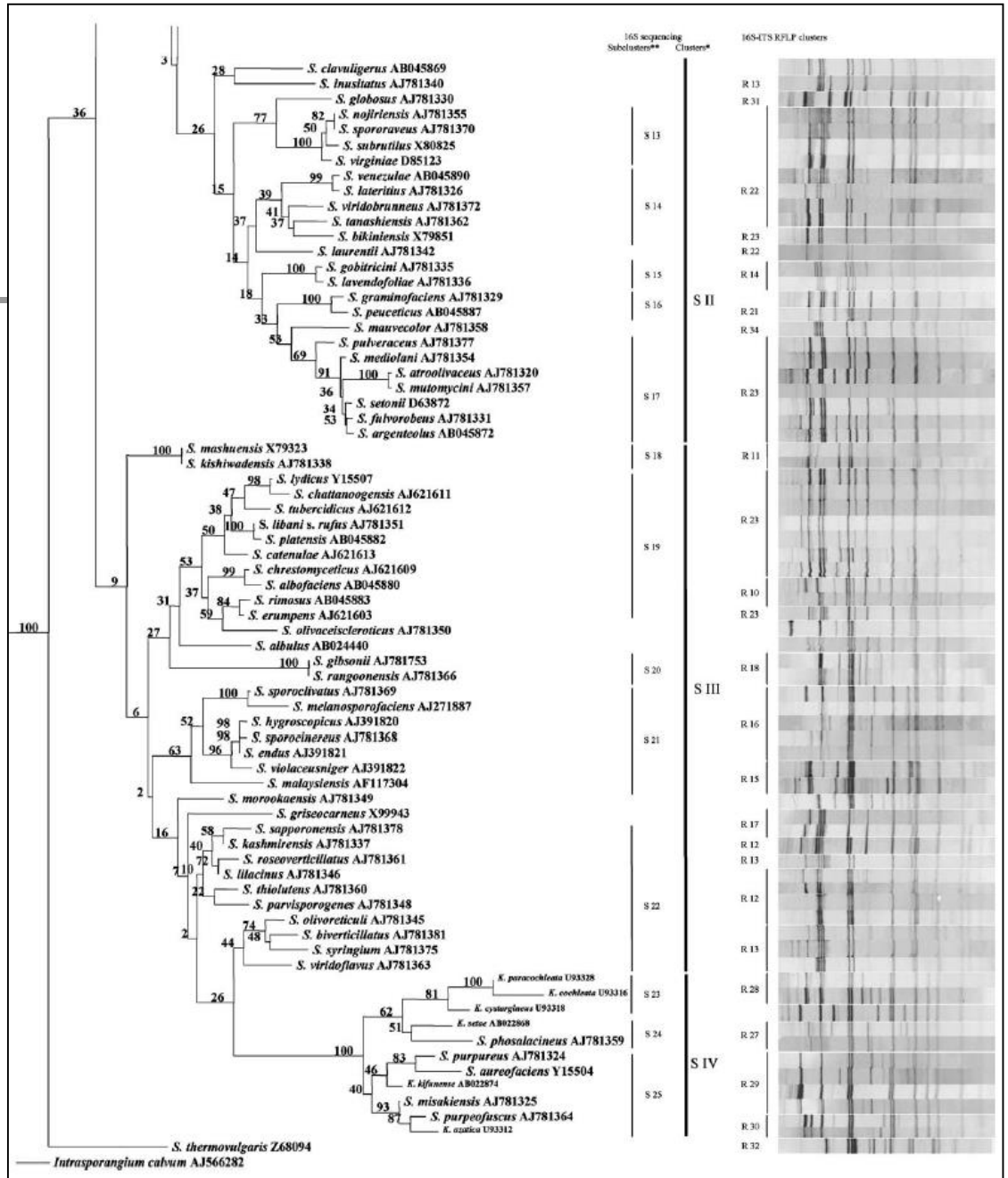

Fig. 1. (Continued).

Description of the family Streptomycetaceae

- The genus *Streptomyces* includes aerobic, Gram-positive, non acid-alcohol fast actinomycetes form an extensively branched substrate mycelium that rarely fragments.
- The aerial mycelium forms chains of three to many spores.
- In some species, aerial hyphae consist of long, straight filaments, which bear 50 or more spores at more or less regular intervals, arranged in whorls (verticils).
- The filamentous bacteria produce well-developed vegetative hyphae (between 0.5-2.0 µm in diameter) with branches.
- The organisms produce a wide range of pigments responsible for the color of the substrate and aerial mycelium.

The Prokaryotes (chapter 1.1.7),2006;..

Description of the family Streptomycetaceae

- *Streptomyces* grow within 5-11.5 pH ranges.
- Fatty acids are complex mixtures of saturated, *iso*- and *anteiso*-fatty acids.
- Mycolic acids are not present.
- The mol% G+C of the DNA ranges generally between 66 and 74%.

The Prokaryotes (chapter 1.1.7),2006

Streptomyces species
Gram positive, filamentous prokaryotes

- An unusual group of Gram positive filamentous bacteria.
- Produce branched filamentous mycelia – Spiral sporophores.
- Produce drought-resistant spores at end of filaments.
- The aerial mycelium at maturity forms chains of three to many spores. The spores are nonmotile.
- Also at maturity produce secondary metabolites:

1. A variety of streptomycin-like antibiotics(streptomycin).
2. Phtotoxins called as thaxtomins.
3. Anti-tumor agents,
4. Immunosuppressants (Loria et al)

Secor,2013;…

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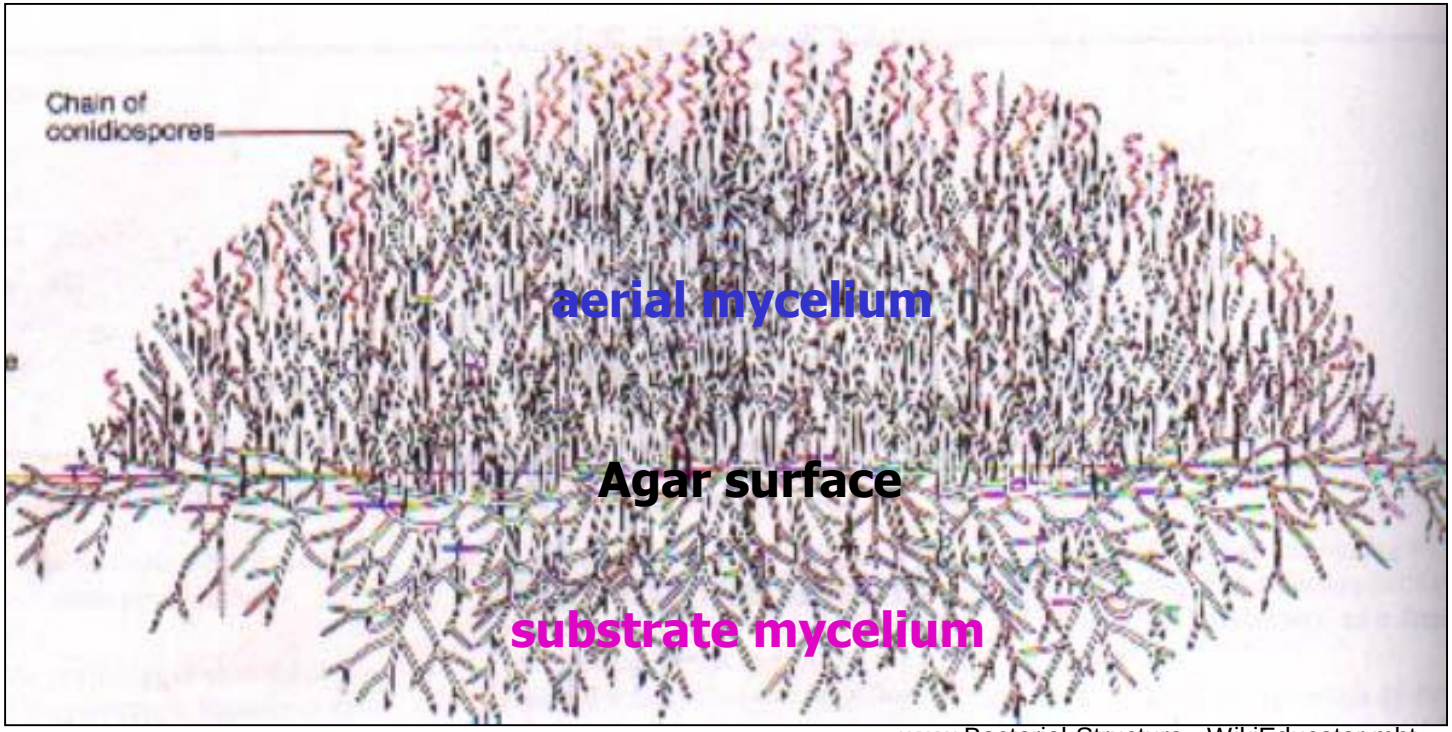
Secor,2013;…

Streptomyces species
Mycelium and spore structures



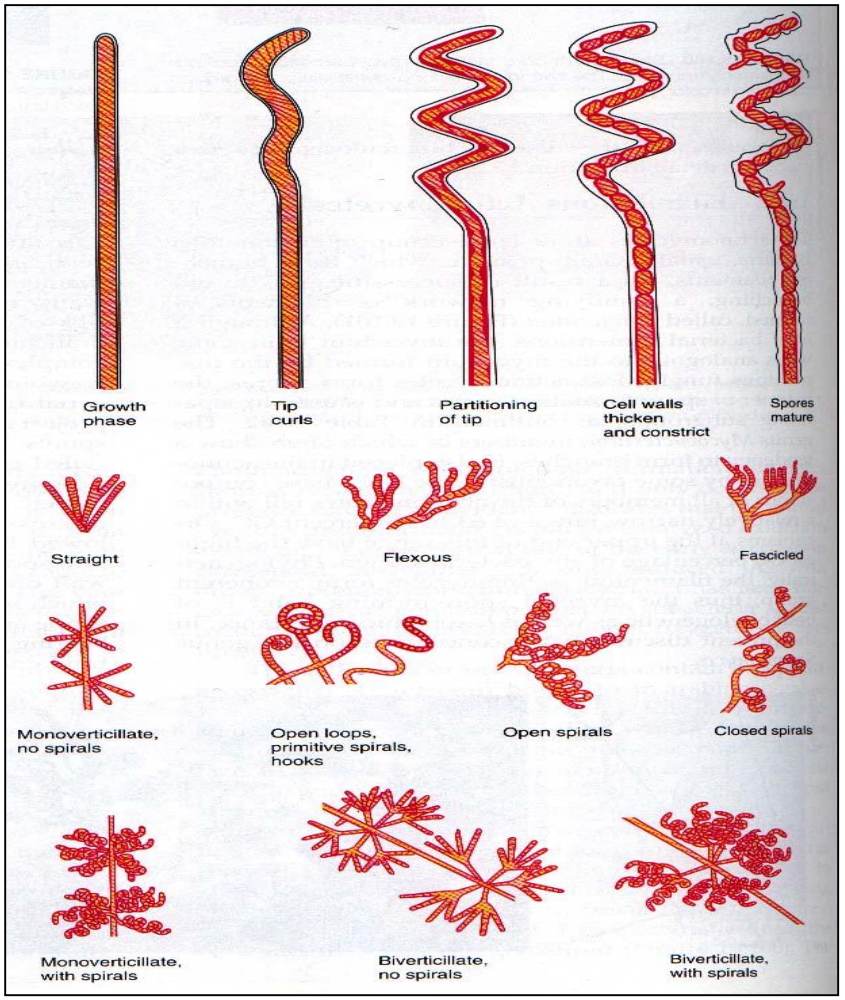
www.Bacterial Structure - WikiEducator.mht

Streptomyces species

Plant pathogenic spp.

- Colonies are slow growing, aerobic, Gram-positive, nonacid-fast, glabrous or chalky, heaped and folded, and white, tan, gray, brown, or black in color.
- Colonies often have an earthy odor.
- Filaments are extensively branched.
- Aerial filaments are abundant and usually produce long chains of spores formed by fragmentation of the filaments.
- Almost all *Streptomyces* spp. are soil-inhabiting saprophytes.
- However, a few species are pathogenic and cause diseases of underground structures of diverse plant species.

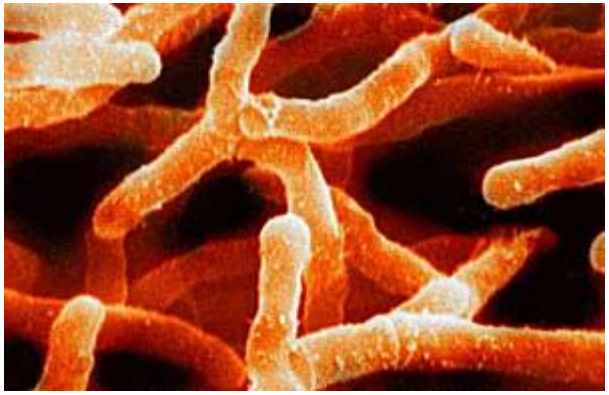
Various types of spore-bearing structures on the streptomyces



www.Actinomycetes Index - Mycology Image Gallery - TML & MSH MicroWeb.mht

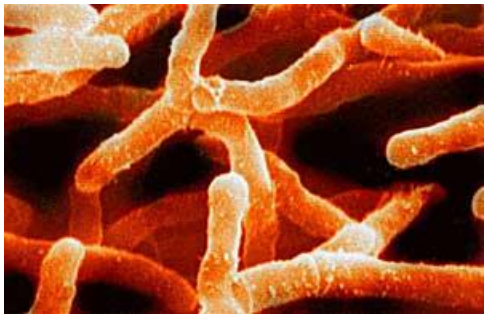
Cell wall structure
Actinomycetes vs. Fungi

- Among actinomycetes, the streptomycetes are the dominant.
- Like fungi, many actinomycetes have long, slender, branching hyphae; like fungi, they produce spores (most bacteria reproduce by cell division); and like fungi, they help to break down the complex, woody organic molecules such as cellulite, lignin and chitin that form cell walls.
- Actinomycetes differ from fungi in the composition of their cell wall.
- They do not have chitin and cellulose which are commonly found in the cell walls of fungi.



Actinobacteria. Source: Wikipedia

Cell wall structure
Actinomycetes vs. Fungi



- Among actinomycetes, the streptomycetes are the dominant.
- Like fungi, many actinomycetes:
- Have long, slender, branching hyphae;
- They produce spores (most bacteria reproduce by cell division);
- They help to break down the complex, woody organic molecules such as cellulite, lignin and chitin that form cell walls.
- Actinomycetes differ from fungi:

1. In the composition of their cell wall.
2. They do not have chitin and cellulose which are commonly found in the cell walls of fungi.

ISPP List,2004

Plant pathogenic *Streptomyces* species

- Huge genus (many hundreds of spp) with perhaps 20 pathogenic spp, some of which are difficult for most labs to differentiate (D. Stead)
- All cause scabs of root crops-potato.
- Several other *Streptomyces* species cause disease, although much less is known about these pathogens than about the three species mentioned previously.
- Unfortunately, there are many unsubstantiated reports in the literature of *Streptomyces* species causing diseases on crops.

- *Streptomyces acidiscabies*
- *Streptomyces albidoflavus*
- *Streptomyces candidus*
- *Streptomyces caviscabies*
- *Streptomyces collinus*
- *Streptomyces europaeiscabiei*
- *Streptomyces intermedius*
- *Streptomyces ipomoeae*
- *Streptomyces luridiscabiei*
- *Streptomyces niveiscabiei*
- *Streptomyces puniciscabiei*
- *Streptomyces reticuliscabei*
- *Streptomyces scabiei*
- *Streptomyces setonii*
- *Streptomyces steliiscabiei*
- *Streptomyces turgidiscabies*
- *Streptomyces wedmorensis*

Symptoms and Signs

- *S. scabies* causes variable symptoms on the surface of potato tubers including erumpent, russet, and pitted lesions.
- Erumpent lesions are raised lesions, russet lesions are defined as superficial corky tissue that covers large areas of the tuber surface and pitted lesions are dark colored sunken areas up to ½ in deep.
- **Scab lesions**
- Scab lesions can occur anywhere on the tuber surface and more than one type of lesion may be present on a single tuber. Scab affects young tubers with the lesions expanding as the tuber matures.
- There are no above ground symptoms of common scab infection.

Symptoms and Signs

- Gram-positive, filamentous bacteria.
- Common in soils worldwide.
- Causes pitted lesions on root and tuber crops.
- Only causes symptoms on growing plant tissue.
- Symptoms due to a toxin that inhibits cellulose synthesis.
- **Two forms of scab occur:**

1. Common scab occurs in all production areas and is most severe in soils with a pH above 5.5.
2. Another less common form, called acid scab, is important in acidic soils (below pH 5.5).

Streptomyces
Common scab

- *fas* genes produce plant hormone cytokinin.
- Causes root cell swelling and in lab can cause galls to form.

Diseases caused by *Streptomyces*

Streptomyces acidiscabies	Common scab of potato and other taproot crops
Streptomyces aureofaciens	Russet scab of potato
Streptomyces caviscabies	Deep pitted scab in potatoes
Streptomyces collinus	Scab of potato
Streptomyces europaeiscabiei	Associated with common scab
Streptomyces ipomoeae	Soil rot of sweet potato
Streptomyces luridiscabiei	Raised corky lesions of potato
Streptomyces niveiscabiei (From Korea)	Potato common scab disease
S. reticuliscabiei	Associated with netted scab of potato
Streptomyces scabiei	Potato common scab disease; Pod wart of peanut
Streptomyces steliiscabiei	Associated with common scab
Streptomyces turgidiscabies	Associated with common scab
Streptomyces sp. (From Japan)	Root tumor of melon

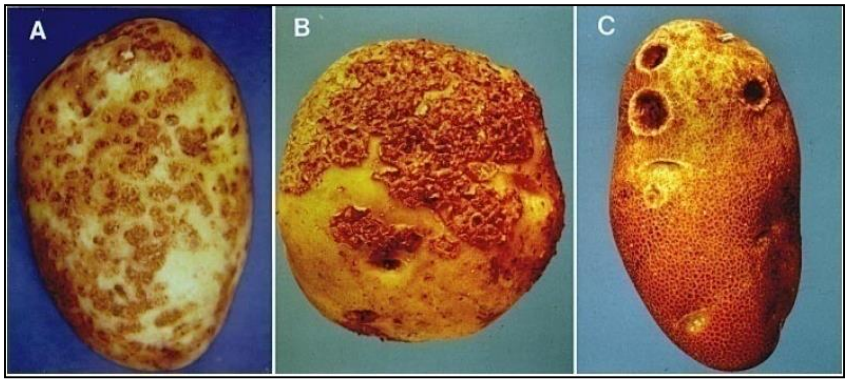
Disease symptoms
Potato scab

- *Streptomyces scabies* can produce a range of symptoms on potato tubers.

A. Superficial lesions are sometimes referred to as russet scab; these and similar symptoms also can be caused by several other *Streptomyces* species.

B. Raised lesions are usually referred to as common scab; however, these symptoms do not differ from those produced by *S. acidiscabies*.

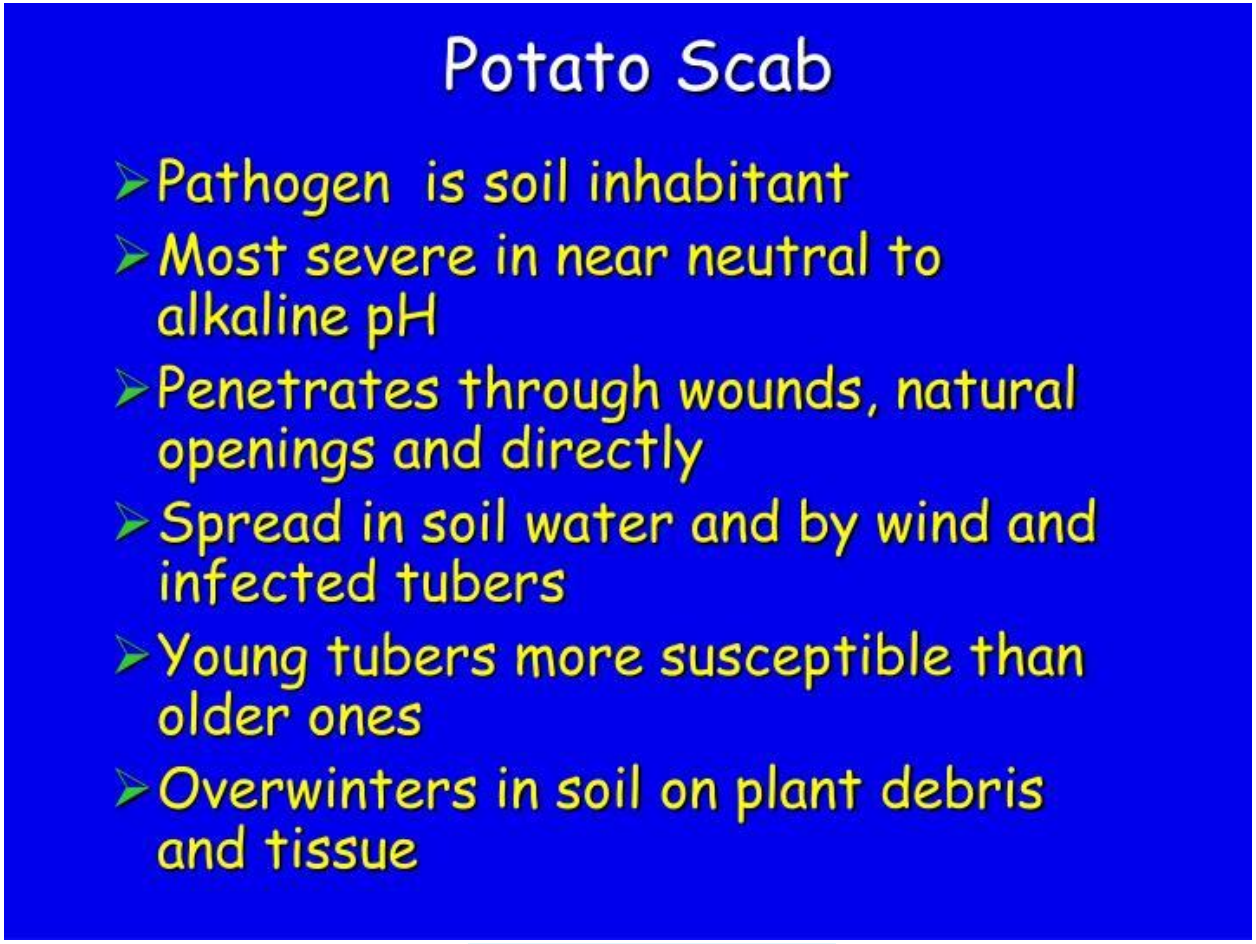
C. Pitted lesions may or may not have a ridge of raised tissue surrounding the pit.



Loria *et al.*,1997

Disease symptoms

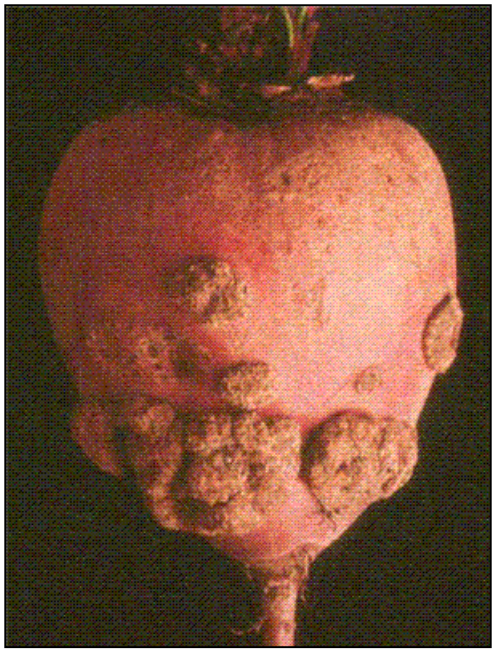
Potato scab



Radish and Beet scab symptoms

Streptomyces scabies

Radish Scab



Beet Scab

Brooke Edmunds

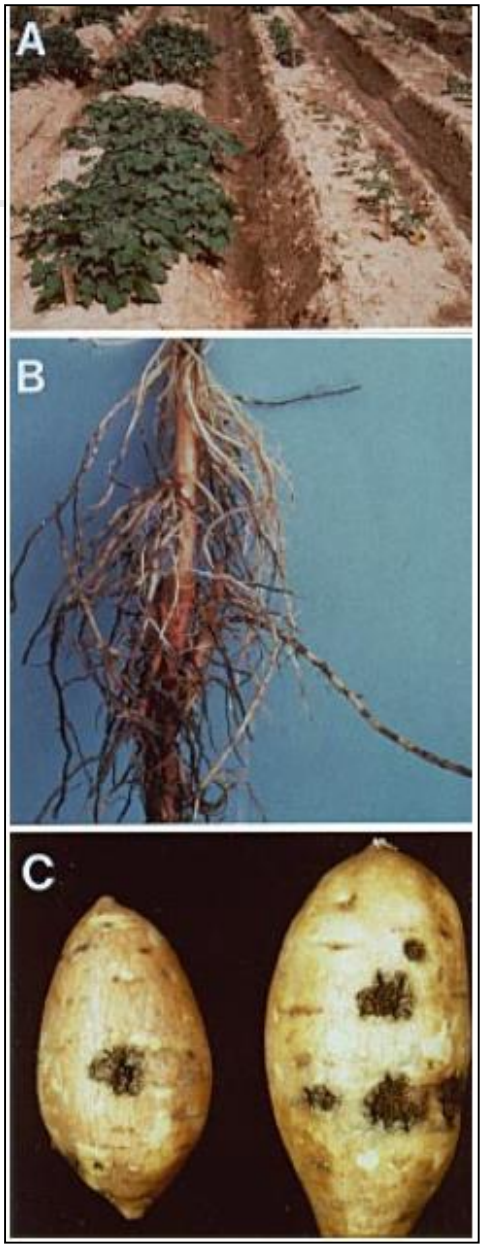
Symptoms
Soil rot of sweet potato

- Soil rot of sweet potato caused by *Streptomyces ipomoeae*.

A. Fibrous root rot causes a reduction in plant stand and vine growth in susceptible (right) compared with resistant (left) cultivars (courtesy C. A. Clark, Louisiana State University, Baton Rouge).

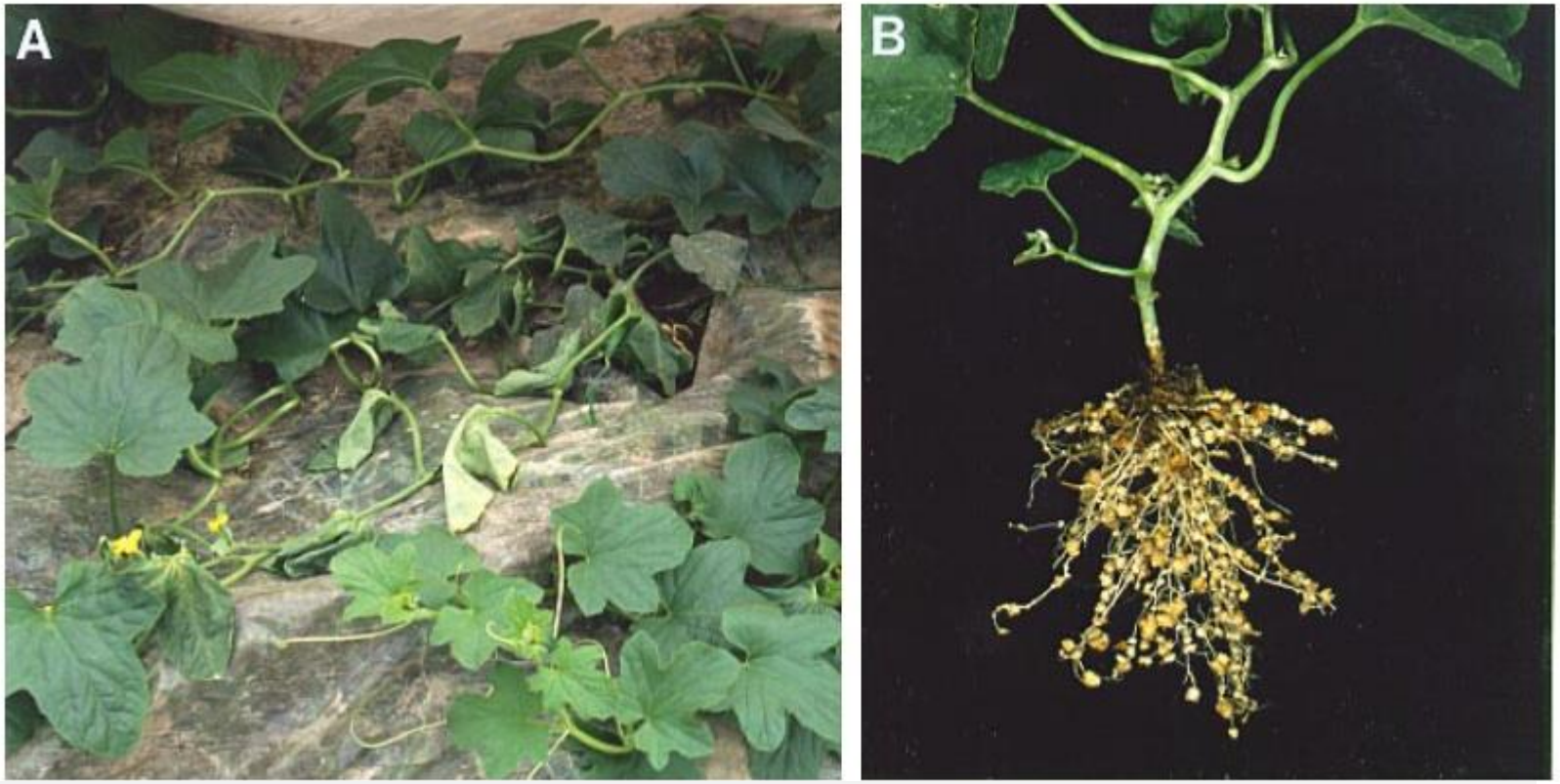
B. Infection of Fibrous, and

C. Storage roots causes tissue necrosis and results in yield loss (courtesy W. J. Martin).



Loria *et al.*,1997

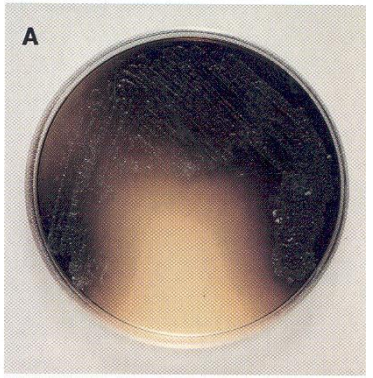
Symptoms
Root tumor of cucurbits



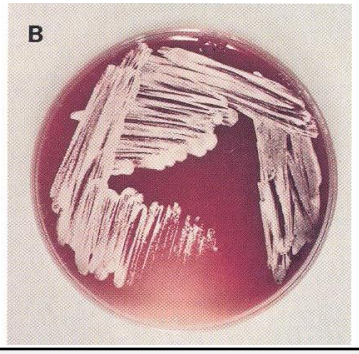
A *Streptomyces* species recently identified from Japan causes root tumor of melon. Aboveground symptoms include (A) reduction in plant growth and wilting of vines that result from (B) galling on fibrous roots.

Pigmentation

- Strains belonging to the genus *Streptomyces* may produce a wide variety of pigments responsible for the color of the vegetative and aerial mycelia.
- In addition, colored diffusible pigments may also be formed.
- Note that the production of pigments largely depends on the medium composition and cultivation conditions.



A. *S. scabies* on peptone yeast extract iron medium.



B. *S. acidoscabies* on modified salts starch agar.

Colors of substrate mycelium and soluble pigment occurring in streptomycetes

Representative species (DSM no.)[a]	Figure (strain no.)[b]	Color
S. aurantiacus (40412); *S. griseoruber* (40275)	4a (40412)	Orange to dark red
S. longispororuber (40599); *S. spectabilis* (40512)		(mainly endopigment)
S. californicus (40058); *S. cinereoruber* (40012)	4b (40058)	Red to blue/violet
S. violaceus (40082); *S. purpurascens* (40310)		(mainly endopigment)
S. coelicolor (40233); *S. cyaneus* (40108)	4c (40163)	Red-violet to blue
S. violaceoruber (40049); *S. lateritius* (40163)		(endo- or exopigment or both)
S. atroolivaceus (40137); *S. canarius* (40528)	4d (40089)	Yellow-orange/greenish-yellow
S. galbus (40089); *S. tendae* (40101)		(endo- and exopigment)
S. flavoviridis (40210); *S. olivoviridis* (40211)	4e (40071)	Green to gray-olive
S. viridochromogenes (40110); *S. nigrifaciens* (40071)		(endo- and exopigment)
"*S. malachiticus*" (40167); "*S. malachitorectus*" (40333)		Green (endopigment)
S. badius (40139); *S. eurythermus* (40014)	4f (40100)	Red-brown to dark-brown
S. phaeochromogenes (40073); *S. ramulosus* (40100)		(endo- and exopigment)
S. alboniger (40043); *S. hygroscopicus* (40578)		Gray-brown to black
S. purpeofuscus (40283); *S. mirabilis* (40553)		(mainly endopigment)

Abbreviations: DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; and ISP, International *Streptomyces* Project.

[a]DSM no. 40XXX = ISP no. 5XXX.

[b]Figures 4a–f show the substrate mycelia of the strain after cultivation on three different media for 7 days; left: starch-casein-nitrate agar, middle: GYM agar, right: oatmeal agar; for compositions, see Tables 10 and 12).

From Korn-Wendisch and Kutzner (1992).

The Prokaryotes (chapter 1.1.7),2006

A–J: Color of the aerial mycelium of *Streptomyces* strains grown on different agar media after 3 weeks of incubation at 28° C.
Left: starch-casein-nitrate agar;
Middle: GYM agar;
Right: oatmeal agar.

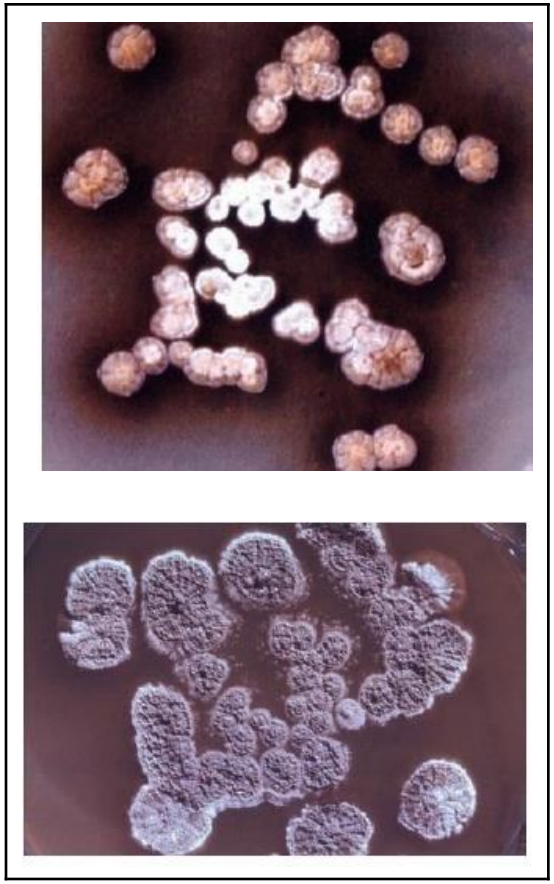
Color of the aerial mycelium of *S. scabiei*

Top: 7-day-old colonies of *S. scabiei* on yeast-malt agar. Start of aerial hyphae formation (white colour).

- The brown colonies are raised, consist of tough mycelial growth and they are difficult to remove from the agar surface.

Bottom: 14-day-old colonies of *S. scabiei* on yeast-malt agar.

- Active spore formation in aerial hyphae that turn into a dusty grey mass.



Janse,2006

Microscopic observation wet mount

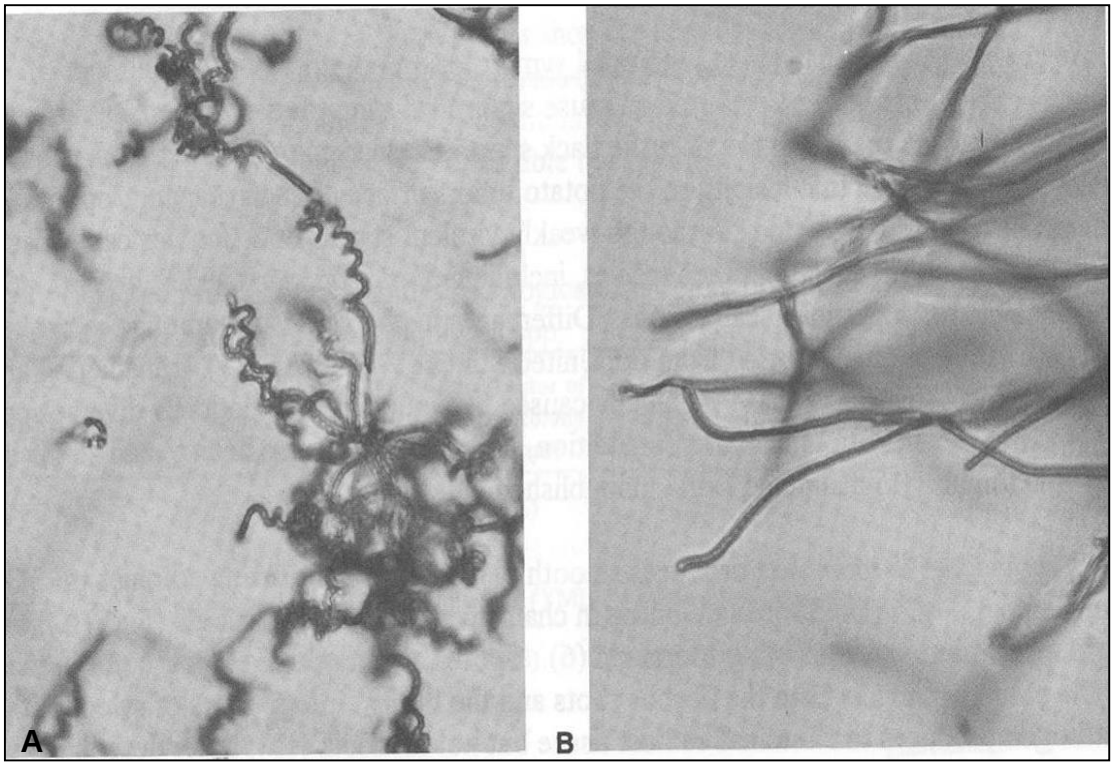
- On clean grease free glass slide, actinomycete colony was suspended in 1-2 drops of water and coverslip was placed then it was observed under microscope.
- It is used to study the shape, size spores, motility etc.

Jayalakshmi *et al.*,2011

Staining spores
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 $(NH_4)_2SO_4$.
- Wash with water, and mount under a microscope.
- The hyphae stain bright yellow, while the spores are blue. Red brown granules can be seen in the hyphae.
- A blue stain may be picked up by some nonsporulating aerial hyphae.

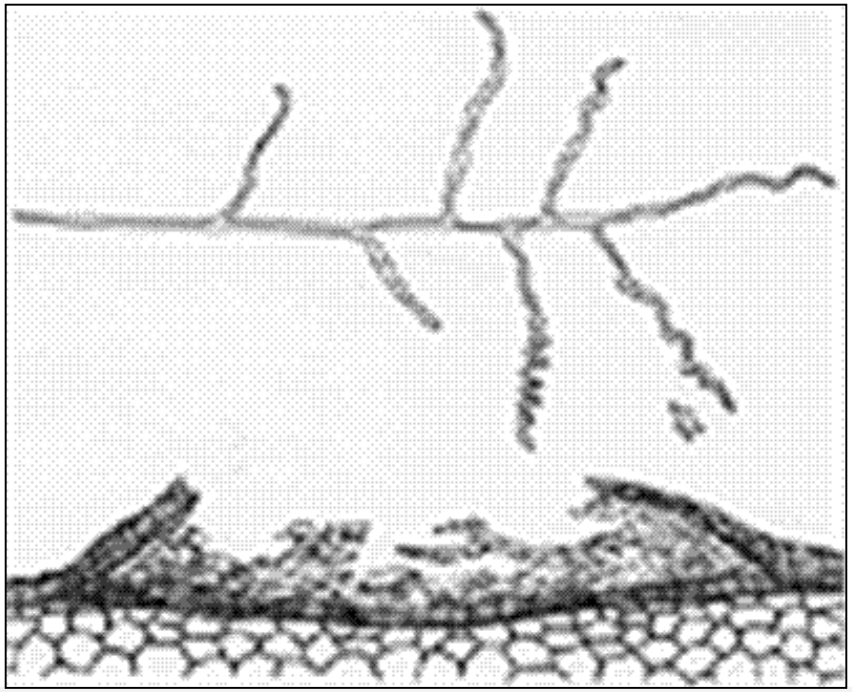
Spore chains of plant pathogenic *Streptomyces* spp.



A. Spiral spore chains of *S. scabies*.
B. Flexuous spore chains of *S. acidiscabies*.

Schaad *et al.*,2001

Characteristic corkscrew mycelium
S. scabies

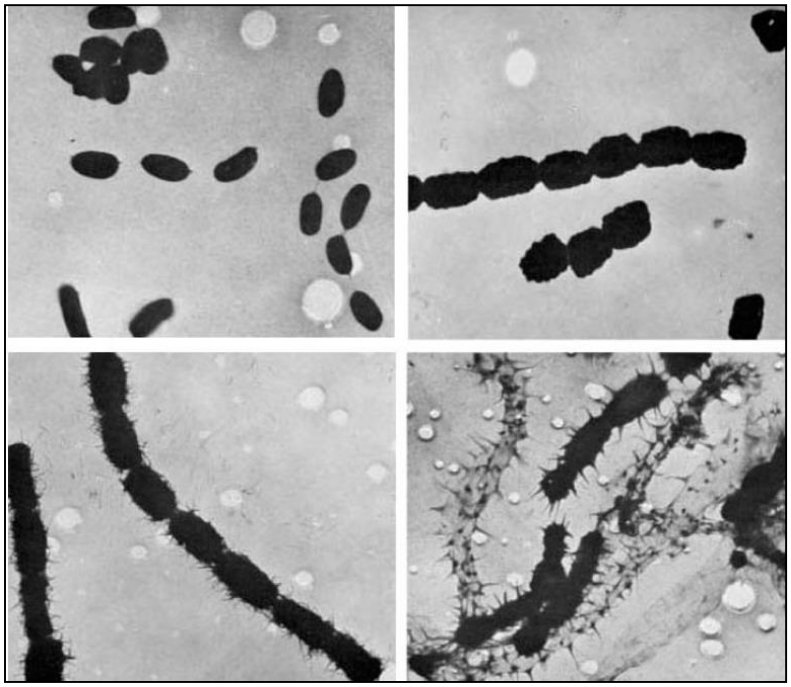


Characteristic corkscrew mycelium produced by *S. scabies*.

Brooke Edmunds

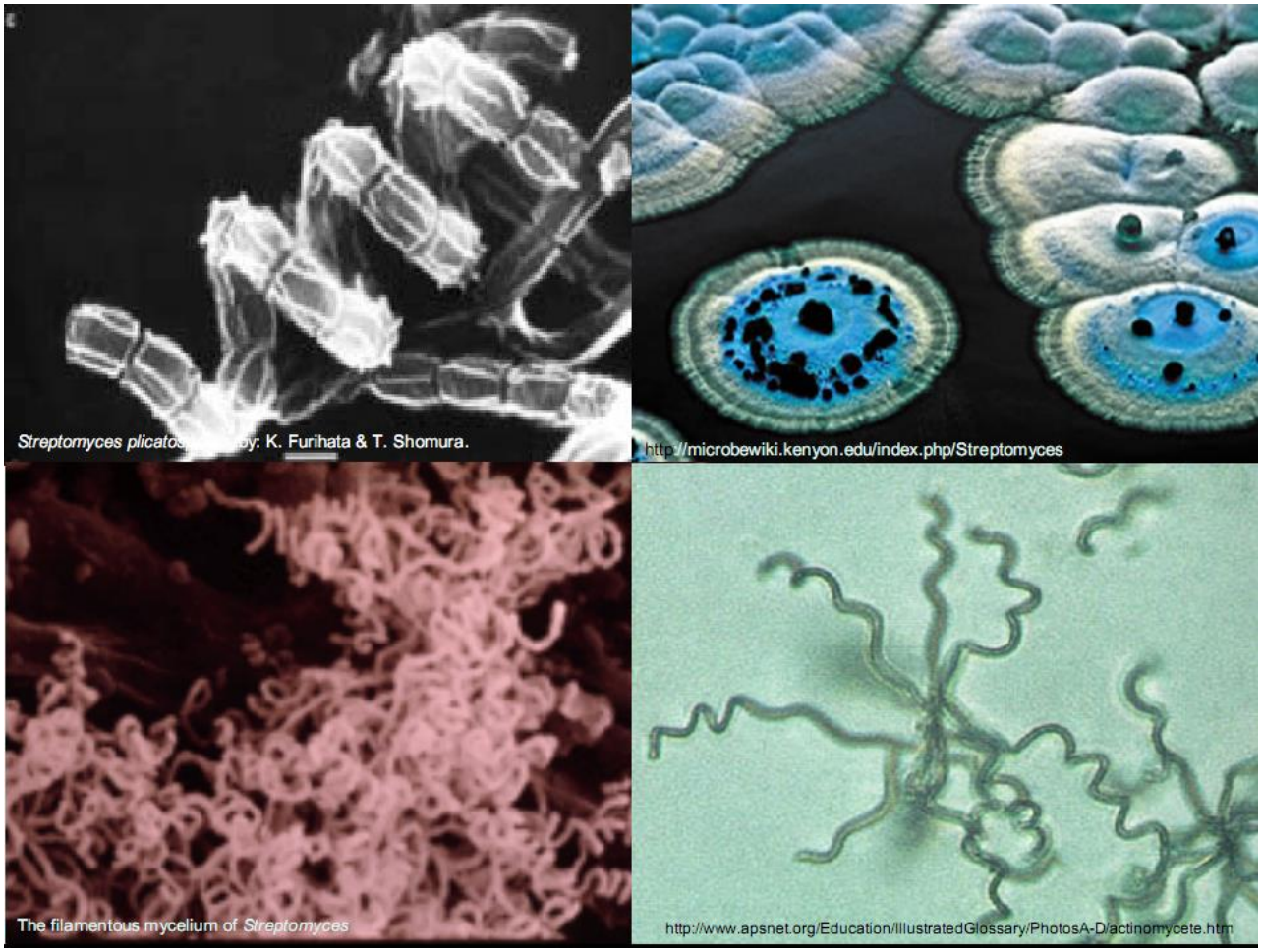
Spore chains of streptomycetes

- Electron micrographs of four types of arthrospores of streptomycetes: smooth, warty, hairy and spiny.
- The spore chains are about 1 m long.



The Prokaryotes (chapter 1.1.7),2006

Spore chains of streptomycetes



Secor,2013

The biodegradative activities of actinomycetes

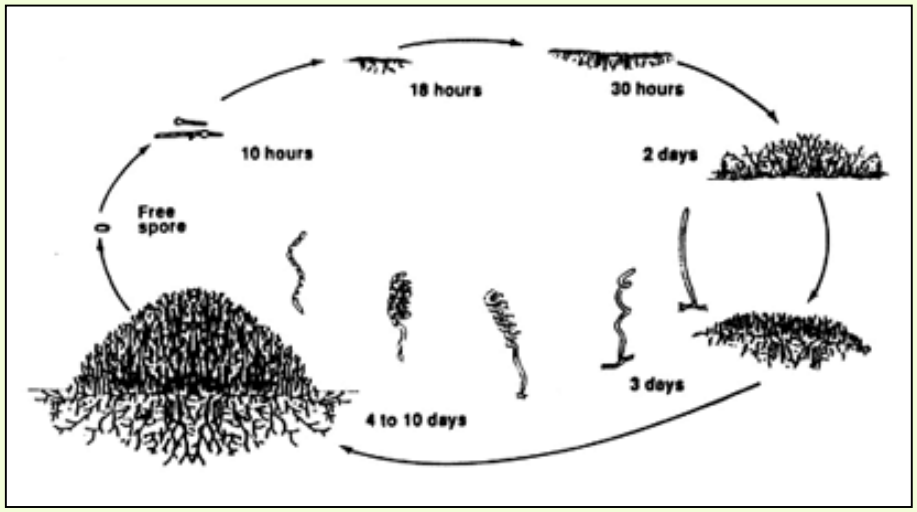
- Most streptomycetes can degrade complex and and aromatic compounds.
- Members of the genus *Streptomyces* are involved in the biodegradation of various polymers abundant in soil owing to their ability to produce extracellular enzymes.
- Streptomycetes are among the very few bacteria able to degrade lignin which occurs in nature.
- Most isolated streptomycetes are nonfastidious; they do not require organic nitrogen sources or vitamins and other growth factors.

Production of antibiotics

- Streptomycetes are the producers of more than 5000 known bioactive compounds (Anderson and Wellington, 2001), and estimates of the total number of antimicrobial compounds produced by representatives of *Streptomyces* screened for new antibiotics are of the order of 100,000 (Watve *et al.*, 2001).
- Many strains produce one or more antibiotics.

Ecology and life cycle
S. scabies

- Members of the genus *Streptomyces* undergo a complex life cycle.
- *S. scabies* survives in the soil as spores in infected tissue and is spread through water, on infected plant material, and in wind-blown soil.
- *S. scabies* infects young tissues (like developing tubers) directly and gains access to older tissue through wounds and natural openings.
- *S. scabies* also produces a toxin, thaxtomin, which is closely involved in the infection cycle.



Streptomyces species

S. scabies

- *S. scabies* is an unusual bacterium that has a filamentous growth form similar to fungi.
- However, *S. scabies* filaments are much smaller than fungal hyphae – *S. scabies* filaments are 1 µm or less in diameter.
- *Streptomyces scabies*, a cause of potato scab and similar diseases of taproot crops, was the first described and is the best studied of he pathogenic species.

Streptomyces species
S. acidiscabies

- *Streptomycin acidiscabies* produces symptoms like those of *S. scabies* (Manzer *et al.*,1977) and has a similar host range (Lambert and Loria,1990), although the two species are not closely related, based on:

1. DNA-DNA homology, and
2. 16S rRNA sequences.

Streptomyces species
S. turgidiscabies

- Another *Streptomyces* sp. that is pathogenic on potato has been described from the island of Hokkaido in Japan (Miyajima *et al.*,1998).
- This species, *S. turgidiscabies*, has a wide host range among root crops and produces symptoms like those of *S. scabies* on potato tubers, suggesting a common mechanism of pathogenicity.
- However, this recently described species is distinctly different from both *S. acidiscabies* and *S. scabies*, based on:

1. DNA-DNA homology,
2. Ribosomal DNA sequence comparisons,
3. Morphological characteristics.

Streptomyces species
Host range

- Potatoes (*Solanum tuberosum*) are the main economic host but other fleshy root crops, including beets, radish, rutabaga, turnip, carrot and parsnips, are affected.
- All phytopathogenic actinomycetes, with the exception of *Nocardia vaccinii*, which causes galls and bud proliferation in blueberry, belong to the genus *Streptomyces* (Locci,1994).

Media used for selective isolation

Table 11. Some media useful for the selective isolation of streptomycetes.

References[a] / Ingredients (g/liter)	1 Starch-casein-KNO_3 agar	2 Glycerol-arginine agar	3 *Actinomyces* isolation agar	4 Chitin agar	5 Raffinose-histidine agar
Chitin (colloidal)	—	—	—	4.0	—
Starch	10.0[b]	—	—	—	—
Glycerol	—	12.5	5.0[c]	—	—
Raffinose	—	—	—	—	10.0
Sodium propionate	—	—	4.0	—	—
KNO_3	2.0	—	—	—	—
Casein	0.3	—	—	—	—
Sodium caseinate	—	—	2.0	—	—
Asparagine	—	—	0.1	—	—
Arginine	—	1.0	—	—	—
Histidine	—	—	—	—	1.0
NaCl	2.0	1.0	—	—	—
KH_2PO_4	—	—	—	0.3	—
K_2HPO_4	2.0	1.0	0.5	0.7	1.0
$MgSO_4 \cdot 7H_2O$	0.05	0.5	0.1	0.5	0.5
$CaCO_3$	0.02	—	—	—	—
$Fe_2(SO_4)_3 \cdot 6H_2O$	—	0.01	—	—	—
$FeSO_4 \cdot 7H_2O$	0.01	—	0.001	0.01	0.01
$CuSO_4 \cdot 5H_2O$	—	0.001	—	—	—
$ZnSO_4 \cdot 7H_2O$	—	0.001	—	0.001	—
$MnSO_4 \cdot H_2O$	—	0.001	—	—	—
$MnCl_2 \cdot 4H_2O$	—	—	—	0.001	—
Agar[d]	18.0	15.0	15.0	20.0	12.0
pH	Adjusted to 7.0–7.5 or lower or higher depending on the flora to be isolated.	Adjusted to 7.0–7.5 or lower or higher depending on the flora to be isolated.	Adjusted to 7.0–7.5 or lower or higher depending on the flora to be isolated.	Adjusted to 7.0–7.5 or lower or higher depending on the flora to be isolated.	Adjusted to 7.0–7.5 or lower or higher depending on the flora to be isolated.

[a]References: Küster and Williams, 1964; El-Nakeeb and Lechevalier, 1963; Hsu and Lockwood, 1975; and Vickers et al., 1948; Difco Laboratories.
[b]Alternatively, glycerol at 10 g/liter can be used.
[c]Not contained in the dehydrated medium; added at the time of preparation.
[d]The different amounts of the agar are due to the varying quality used by the individual authors.

The Prokaryotes (chapter 1.1.7),2006

Isolation method
Diagnostic media

1. **Yeast malt extract medium (YME)**

- Yeast extract 4.0 g/L
- Malt extract 10.0 g
- Dextrose 4.0 g
- Agar 20.0 g
- Adjust to pH 7.0-7.2 with NaOH before adding agar.
- The medium is dissolved in water and autoclaved for 15 min at 15 lb pressure.

2. **Peptone-yeast extract iron (PYI) agar**

- Peptone Iron Agar (Difco) 36.0 g/L
- Yeast extract 1.0 g
- Combine all ingredients, adjust the pH to 7.0-7.2 with NaOH or HC1 if necessary.

Isolation method
Diagnostic media

3. Oatmeal agar (OMA) and oatmeal broth (OMB)

- Oatmeal media are prepared by boiling rolled oats (20 g/1) in distilled water for 20 min., straining through layers of cheesecloth, bringing up the volume to 1 liter and adjusting to pH 7.0-7.2 with 1 M NaOH.
- OMA contains 1.5% agar.

Loria and co-workers

Isolation method
Diagnostic media

4. Modified salts starch agar (MSSA)

Soluble starch	10.0 g/L
$NaN0_3$	10 g
$MgC0_3$	1.0 g
K_2HP0_4	0.3 g
NaCl	0.5 g
Agar	15.0 g

Glycerol may be substituted for starch.

5. *Streptomyces* growth medium(SGM)

Mannitol	20.0 g
K_2HP0_4	0.2 g
$MgS0_4.7H_20$	0.2 g
NaCl	5.0 g
$CaC0_3$	2.0 g
Sodium propionate	1.0 g
Yeast extract	1.0 g
$CoCl_2$	0.11mg
Agar	18.0 g

Loria and co-workers

Isolation method

On Water agar or yeast malt extract medium (YME)

- *s. scabies* can be isolated from infected potato tissue by first surface sterilizing the tuber with 1.0% sodium hypochlorite for 1 min.
- Tissue should be selected from the edge of the necrotic areas (may be a light yellow or straw color). Otherwise, Scab lesions were excised and ground with a pestle and mortar in sterile water (1 g/10 ml).
- A small amount of tissue is homogenized with sterile distilled water and the resulting suspension streaked onto water agar/yeast malt extract medium (YME).
- (NPPC (nystatin, polymyxin, penicillium, cycloheximide) water agar, which contains antibiotics, can be used if bacterial or fungal contaminants are present (see Schaad *et al.*,2001).

Isolation method

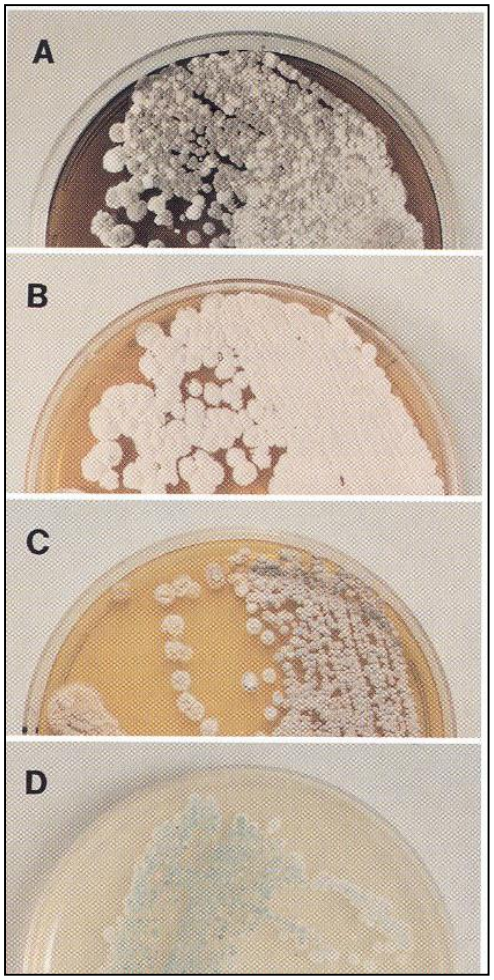
Streptomyces scabies

- Check the plates after a few days for a smooth firm mycelium which later develops aerial hyphae.
- The hyphae will begin to fragment into spores giving the culture a powdery appearance.
- Pure cultures were obtained through three repeated cultures of a single colony on potato-dextrose agar.

Colony morphology

On complex agar media

- On complex agar media, discrete and lichenoid, leathery or butyrous colonies are formed.
- Colonies are initially relatively smooth surfaced, but later they develop an aerial mycelium that may appear floccose, granular, powdery or velvety.



Identification

- Base on:
- Spore color, aerial hyphae form and color, pigment production, biochemical traits and sugar utilization.
- Research is under way to develop rapid diagnostic tests based on PCR identification tools.

Characteristics of the genus *Streptomyces*

- The metabolism is oxidative and chemoorganotrophic.
- The catalase reaction is positive.
- Generally, nitrates are reduced to nitrites.
- Most representatives can degrade polymeric substrates like casein, gelatin, hypoxanthine, starch and also cellulose.
- In addition, a wide range of organic compounds is used as sole sources of carbon for energy and growth.
- The optimum temperature for most species is 25–35°C; however, several thermophilic and psychrophilic species are known.
- The optimum pH range for growth is 6.5-8.0.

Characteristics of four *Streptomyces* species

Adapted from Schaad *et al.*,2001

Species	Hyphae	Aerial myceliu m color	Pigment production in agar	Sugar Utilization	Optimum temp.
S. scabies	Spiral	Gray	Brown	All	28-30°C
S. acidiscabies	Flexous	White/Pink	Red/Yellow	Not raffinose	25-28°C
S. turgidiscabies	Flexous	Gray	None	All	25-28°C
S. ipomeoae*	Spiral	Blue/Green	None	Not galacturonic acid	30-32°C

*host range limited to sweet potato (*Ipomoea batatas*)

Brooke Edmunds

Phenotypic comparison and grouping of the *Streptomyces* spp. isolated from potato tubers

Trait	Group 1a	Group 1b	Group 2	Group 3	Group 4	Group 5	Group 6
Colony color on YME medium	Gray to brown	Gray to brown	Gray to brown	Gray to brown	Tan	Yellow	V
Spore color	Gray	Gray	Gray	Gray	Gray	Gray	V
Sporophore morphology	Spiral	Spiral	Spiral	Flexuous	Flexuous	Flexuous	V
Melanin production	+	+	–	+	–	–	V
Utilization of ISP sugars							
L-arabinose	+	+	+	+	+	V	V
D-fructose	+	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+
D-mannitol	+	V	+	+	+	+	V
raffinose	+	+	+	+	+	V	V
rhamnose	+	+	+	+	+	+	+
sucrose	+	V	+	V	+	V	V
D-xylose	+	V	+	+	+	V	V
meso-inositol	+	+	+	V	+	V	V
Growth at pH = 4.5[b]	–	–	nt	–	+	+	nt
Pathogenicity on potato	34/37[c]	1/5	0/5	1/3	6/38	2/9	0/21

[a] Variable traits within the groups are indicated by V.
[b] Tested only with the pathogenic strains.
[c] Number of pathogenic strains out of the total number of strains.

Lindholm *et al.*,1997

Selected morphological and physiological characteristics and growth conditions of four *Streptomyces* species

Species (reference)	Spore chain type (medium)[1]	Aerial color of mature colony (medium)	Pigment production (medium)	ISP sugar utilization patterns[2]	Growth temperature and medium
S. scabies (19)	Spiral (YME)	Gray (YME)	Brown (PYI)	All sugars	28-30°C (YME)
S. acidiscabies (18)	Rectiflexous (YME)	White/pink (YME)	Red/yellow (MSSA)	All except raffinose	25-28°C (YME)
S. turgidiscabies (26)	Rectiflexous (YME)	Gray (YME)	None	All sugars	25-28°C (YME)
S. ipomoeae (30)	Spiral (SGM)	Blue/green (SGM)	None[3]	Not galacturonic acid	30-32°C (SGM)

[1] Growth media: YME=Yeast malt extract, PYI=Peptone-yeast extract iron agar, MSSA=Modified salt starch agar, SGM=*Streptomyces* growth medium.

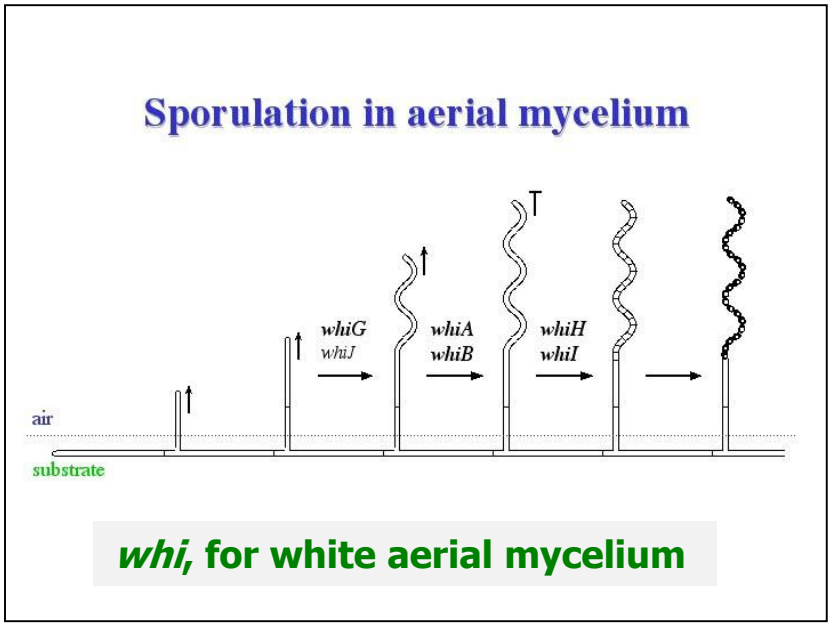
[2] Sugars include: D-Glucose (positive control), L-arabinose, sucrose, D-xylose, I-inositol, D-mannitol, D-fructose, raffinose, cellulose, galacturonic acid; ISP-International *Streptomyces* Project.

Schaad *et al.*,2001

whiG, a gene critical for sporulation of *Streptomyces coelicolor*

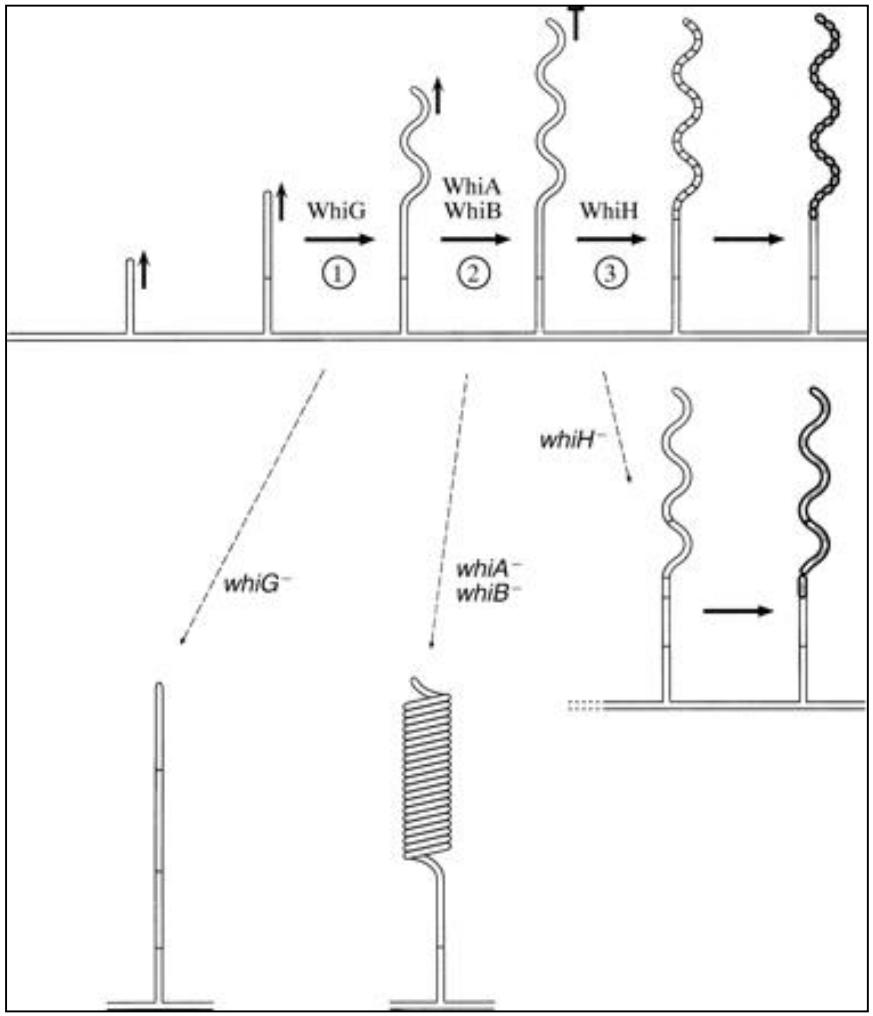
- WhiG protein, is between continued vegetative-type growth and adoption of a type of aerial growth specialized for sporulation.
- WhiA and WhiB proteins, is between continued extension growth and orderly growth cessation, and is a prerequisite for WhiH protein to properly activate:

1. Sporulation septation, and
2. Genome partitioning.



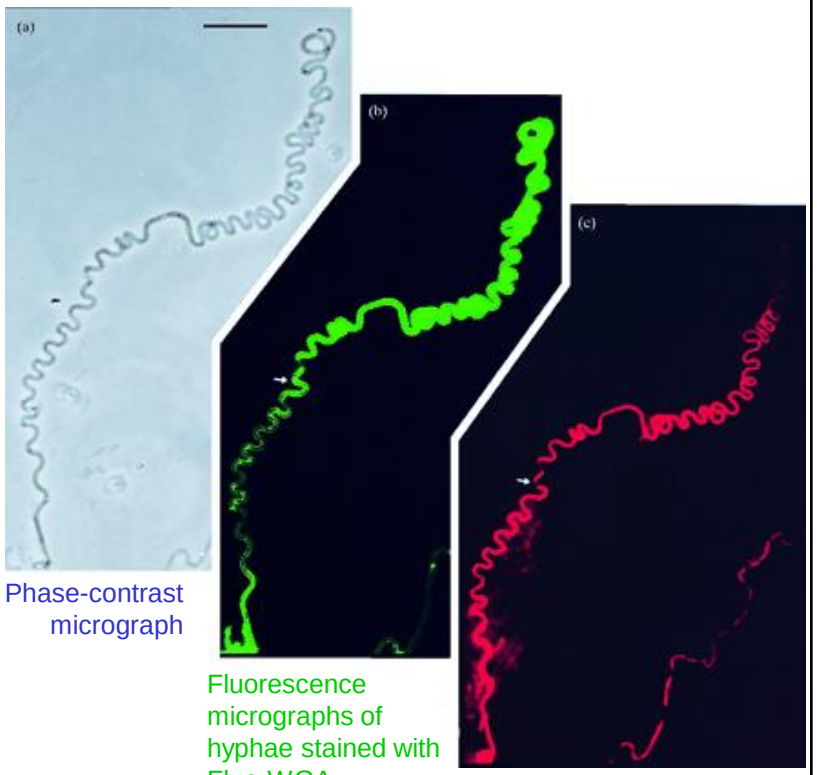
***whi*, for white aerial mycelium**

whiG, a gene critical for sporulation of *Streptomyces coelicolor*



Inactivation of *whiG*, *whiA* and *whiB* completely prevented all traces of sporulation septation and later stages of spore chain development

- The tightly coiled regions of aerial hyphae in J2401 (*whiA*) were virtually devoid of septa, as shown by the absence of Fluo-WGA-staining crosswalls, and by the continuity of the 7-AAD-staining DNA (Fig. 5a-c).
- A very similar pattern was seen in J2402 (*whiB*).
- Although septa were usually absent from the coiled regions of the *whiA* and *whiB* mutants, rare single septa could be found (Fig. 5b-c).



Pathogenicity factors

1. Thaxtomin; main toxin responsible for pathogenicity and symptoms.
   - Nitrated dipeptides A and B
   - Tyrosine: tryptophan (thaxtomin A)
   - Phenylalanine: tryptophan (thaxtomin B)
2. Enzymes
3. Virulence factor; nec1 protein

- Thaxtomin plus the other virulence factors form "pathogenicity islands".

Secor,2013

Mode of action

Pathogenicity islands (PAI)

1. These islands(PAIs) can move among Streptomyces species creating new scab pathogens by converting non-pathogenic Streptomyces to pathogens.
2. May explain variability in symptoms.

Secor,2013

Mode of action
Pathogenicity islands (PAI)

- The PAI contains two characterized virulence determinants:

1. The gene cluster txtAB, txtC
2. An independent virulence gene, nec1 txtAB, code for the peptide synthetase modules in the thaxtomin A biosynthetic pathway.

- It was demonstrated transfer of the PAI from a pathogenic species to the nonpathogen, *S. lividans*, in mating experiments.
- The recipient *S. lividans* strain produces thaxtomin A.

Evolution of pathogenicity
Pathogenicity islands (PAI)

- Recently a pathogenicity island (PAI) in *Streptomyces* species was identified.
- The horizontal transfer of this island from the ancient species *S. scabies* to the newly emerged pathogenic species *S. turgidiscabies* and *S. acidiscabies* appears to be the basis of evolution of pathogenicity in several newly emerged pathogenic species in agricultural systems.

Virulence mechanisms of Gram-positive plant pathogenic bacteria *Streptomyces* spp.

- The lack of a type III protein secretion system (TTSS) in Firmicutes and Actinobacteria immediately raises questions about delivery of virulence proteins across the plant cell wall and membrane.
- *S. scabies*, *Streptomyces turgidiscabies* and *S. acidiscabies* directly penetrate plant cell walls with the help of thaxtomin, a phytotoxin that inhibits cellulose biosynthesis, presumably allowing secretion of proteins at the host cell membrane.
- These pathogens secrete Nec1, a novel protein that is proposed to suppress host defence through an unknown mechanism.

Hogenhout and Loria,2008

Scab-inducing toxins

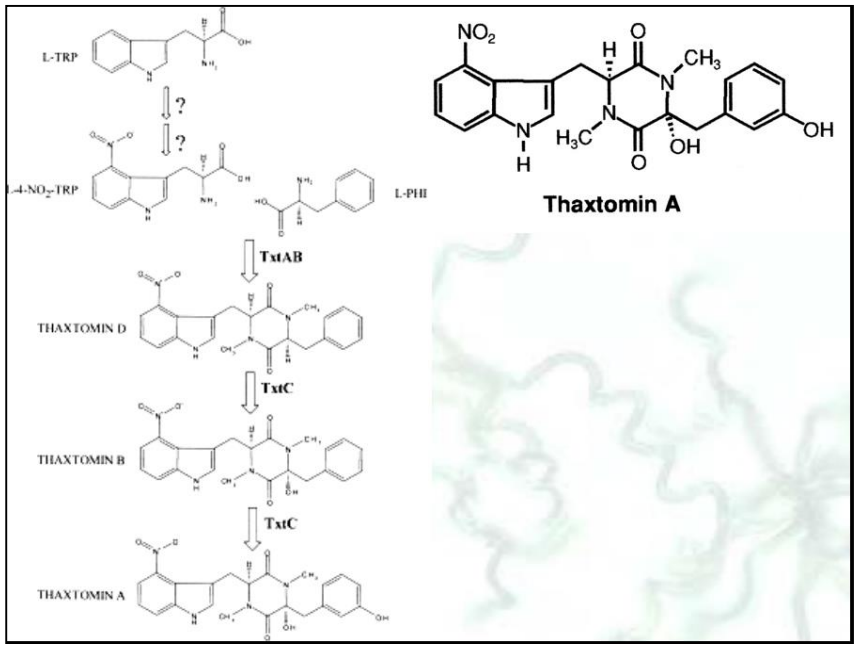
Thaxtomins

Cellulose biosynthesis inhibitors

- Scab-inducing toxins such as the cyclic dipeptide thaxtomin A and B, produced by *Stretomyces scabiei*, causing common scab of potato.
- Demonstration of these toxins can also be used for identification (Kinkel *et al.*,1998).
- Thaxtomins are produced only by plant pathogenic species, cause necrosis and cell hypertrophy on expanding host tissue.
- Production of thaxtomins is perfectly correlated to pathogenicity in all strains tested to date including *S. scabies*, *S. acidiscabies*, and *S. ipomoeae* (King *et al.*,1994), suggesting that thaxtomins may be a common pathogenicity determinant among diverse *Streptomyces* spp. that cause plant disease.

Thaxtomins

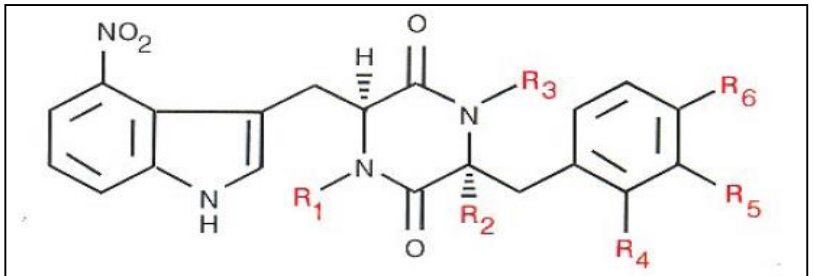
- Thaxtomins, a family of modified dipeptide phytothurs, are produced by *Streptomyces* spp. such as *S. scabies* and *S. acidiscabies*.
- Thaxtomins A and B were the first of the nine members of the family to be described.
- Thaxtomin A is the most abundant of these toxins in potato tuber tissue infected by *S. scabies*.



Raghida *et al.*,1998; Secor,2013

Structures of thaxtomins

- Structures of thaxtomins produced by plant-pathogenic species of *Streptomyces*.
- Thaxtomin A (compound no. 1) is the most abundant of these phytotoxins produced by *S. scabies* in potato tissue.
- Thaxtomin C (compound no. 3) is produced by *S. ipomoeae*.



Compound	R_1	R_2	R_3	R_4	R_5	R_6
1	Me	OH	Me	H	OH	H
2	Me	OH	Me	OH	H	H
3	Me	H	H	H	H	H
4	Me	OH	Me	H	H	H
5	Me	H	Me	H	H	H
6	Me	OH	H	H	H	H
7	Me	OH	Me	H	H	OH
8	Me	OH	Me	H	OH	OH
9	Me	OH	H	H	OH	H
10	H	OH	Me	H	OH	H
11	H	H	H	H	H	H

Structure and function of *nec1*

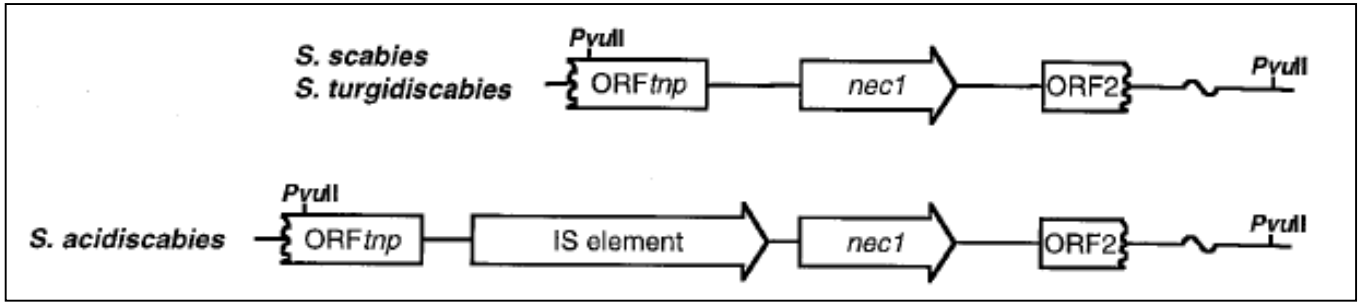
Thaxtomin production

- *nec1* was structurally conserved among all but one of the pathogenic strains examined.
- The function of *nec1* is not presently known.
- But the necrogenic phenotype of *nec1* suggests that this gene plays a role in plant pathogenicity in streptomycetes.
- A previous study with fewer *Streptomyces* strains had found a perfect correlation between the presence of *nec1* and thaxtomin.

Structure and function of *nec1*

Horizontal transfer of *nec1*

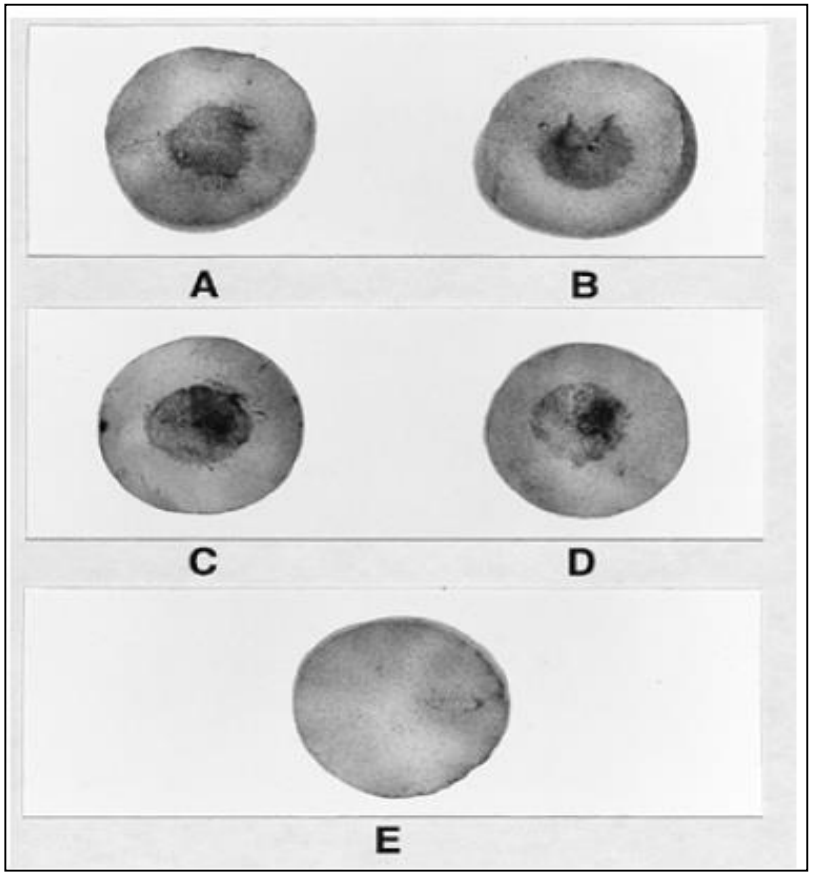
- Arrangement of *nec1*, ORF*tnp*, ORF2, and the IS element in *Strepomyces scabies, S. acidiscabies,* and *S. turgidiscabies*.
- Sequences of *nec1* and ORF*tnp* are identical in strains representing all three species.
- The physical linkage of *nec1* and ORF*tnp* in a large number of genetically and geographically diverse, plant-pathogenic *Streptomyces* strains suggests that ORF*tnp* may have been involved in the horizontal transfer of *nec1* into *Streptomyces*.



Phytotoxin assay

Potato disc assays

- Necrogenic phenotype on potato tuber disks (PTDs) of *Streptomyces lividans* TK24 expressing *nec1* homologs from:
- **A and B**: Two strains of *S. turgidiscabies* Hi-C-13 and Car-8;
- **A and B**: Two wild-type strains Hi-C-13 and Car-8.
- **E:** *S. lividans* TK24 harboring the vector alone is included as a control.



Raghida *et al.*,1998

Plant bioassay

Pathogenicity test on minitubers of potato

- Healthy minitubers of potato cv. Matilda (susceptible to common scab) were produced from leaf-bud cuttings in washed sand in the greenhouse.
- Tubers had a white, thin skin similar to microtubers produced *in vitro* and were formed at the apex of short stolons.
- They were harvested when they were 1–1.5 cm in diameter.
- Actinomycetes were grown in oat meal broth (OMB) (15 ml) for 4 days at 28°C under shaking (150 rpm).
- Three minitubers were soaked in the OMB and incubated on a moist filter paper in a petri dish in the dark for 4 days.
- Minitubers soaked into non-inoculated OMB were used as healthy controls, whereas minitubers inoculated with *S. scabies* ATCC 49173, *S. scabies* SSC101, or strain SSC122 were used as positive controls in all experiments.
- Many strains were re-tested for up to three times.

Plant bioassay

Pathogenicity test on minitubers of potato

- Necrosis induced on minitubers of potato cv. Matilda 3 days after inoculation *in vitro* with strain SSC 122 (left tuber) and strain 376 (*S. scabies*, Group 1a) (right tuber).
- The tuber in the center was treated with noninoculated oatmeal broth.
- The scale is in centimeters.

Lindholm *et al.*,1997

Radish seedling assays

S. scabies, *S. turgidiscabies*, and *S. acidiscabies*

- Radish seeds are surface disinfected with 0.5% NaOCl and 0.1% Tween 20 for 3 min, rinsed twice in sterile distilled water and allowed to germinate by incubating on water agar or moist filter paper overnight at room temperature.
- Germinated seeds with protruding radicals are selected for uniformity and individually placed in glass culture tubes (25 mm diameter) containing 10 ml of 1% water agar or 0.5% gelrite.
- Seeds are then inoculated with 0.1-0.5 ml of undiluted *Streptomyces* cultures grown for 4-6 days in OMB as described in section 4B (no filtration is necessary).
- Seedlings are grown under a 12 h light cycle for 6-10 days at room temperature.
- Pathogenic strains cause brown to black lesions and stunting of the seedling; radial swelling and cell hypertrophy are sometimes visible.

Loria and co-workers

Seedling pathogenicity
Radish and wheat seedlings

- Symptoms caused by two strains each of *Streptomyces scabies* and *S. acidiscabies* on (A) radish and (B) wheat seedlings.

a) Germinated seeds were not treated.

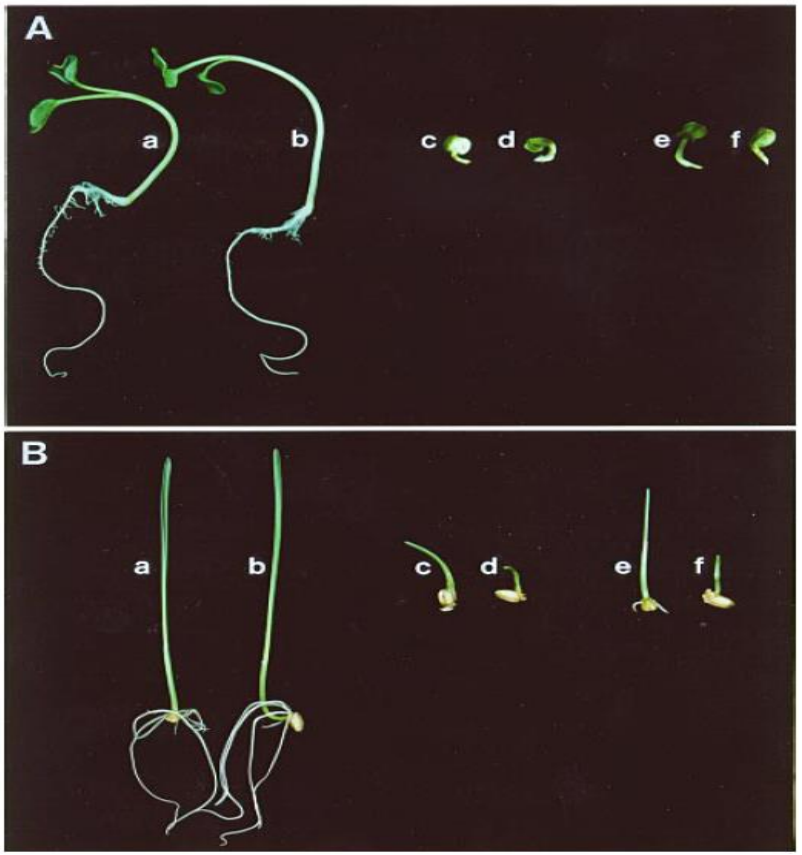
b) Treated with sterile media.

c) Inoculated with *S. scabies* strains 87-22, or

d) *S. scabies* 84-34.

e) Inoculated with *S. acidiscabies* strains 84-110, or

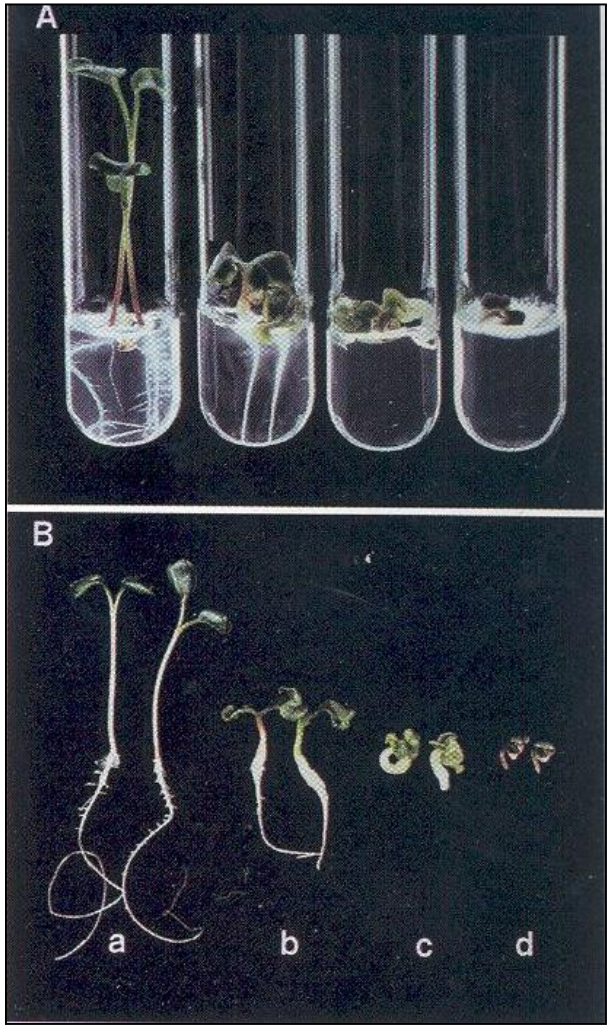
f) *S. acidiscabies* 84-104.



Loria *et al.*,1997

Plant bioassay
Radish seedling assay

- Untreated.
- Treated with pure thaxotamin A.
- Filter-sterilized supernatant of *Streptomyces scabies* (stunting and radial swelling).
- *S. scabies* culture (necrosis and seedling collapse).



Schaad *et al.*,2001

Thaxtomins
Thaxtomin A

- The primary pathogenicity determinant in *Streptomyces* species is the production of thaxtomin phytotoxins, of which, thaxtomin A is the predominant phytotoxin produced by pathogenic species in tuber tissue.
- Thaxtomins inhibit cellulose biosynthesis in higher plants and induce scab symptoms through necrosis and plant cell hypertrophy.
- When purified toxin is applied to plant tissue, it causes necrosis and cell hypertrophy.

Lawrence *et al.*,1990;Cullen and Lees,2006

Radial swelling is due to dramatic cell hypertrophy

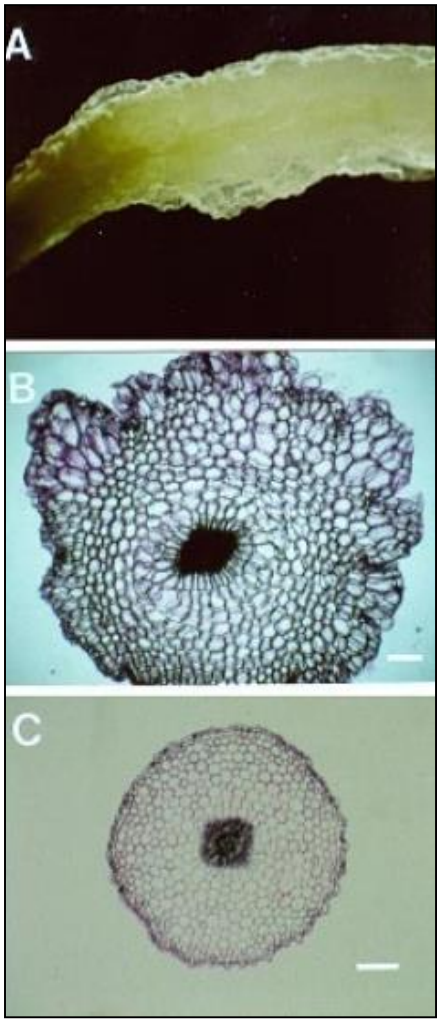
Toluidine blue staining procedure

- **Stain composition:**
- Phosphate buffer:
- Phosphate buffer Solution A (31.2 g/l $NaH_2PO_4.2H_2O$):12.75 ml
- Phosphate buffer Solution B (28.39 g/l Na_2HPO_4):12.25 ml
- Toluidine blue O 0.05 g/l
- Distilled water to make up to 50 ml.
- Toluidine blue O staining procedure (Ghemawat,1977):
- Remove air bubbles from leaf strips by immersing in industrial alcohol for 10 min.
- Rinse off industrial alcohol with a solution of 0.05% toluidine blue O.
- Stain for 15 min in fresh solution of 0.05% toluidine blue O.
- Mount in 0.005% toluidine blue O, examine immediately or keep in a humid chamber for storage of more than 24 h and seal coverslip.

Thaxtomin A

Radial swelling is due to dramatic cell hypertrophy

- Dramatic cell hypertrophy on the hypocotyl of a radish seedling that was treated with thaxtomin A.
- Germinated seeds were incubated on agar containing:
- A and B: thaxtomin A 0.075 µM or
- C: Unamended agar for approximately 7 days before examination.
- Cross sections B and C were approximately 150 µm thick and were stained with toluidine blue.



Loria *et al.*,1997

Thaxtomins

Thaxtomin C

Isolation of *Streptomyces ipomoeae*

- Lesions on sweet potato roots are washed in distilled water.
- Small pieces (1 mm^3) of necrotic tissue are removed and crushed in 0.2 ml of sterile, distilled water containing 0.85% NaCl and 2mM mannitol.
- The suspension is streaked onto *Streptomyces ipomoeae* isolation medium (SUM) and plates are incubated for 3-5 days at 32-36°C in the dark.
- It takes about 5-7 days for blue/green aerial mycelia to develop with *S. ipomoeae* on SGM at 32°C.

Loria and co-workers

Thaxtomins

Thaxtomin C

Sweet potato storage root assays

- *Streptomyces ipomoeae* isolates can be quickly screened for pathogenicity on storage roots and fibrous roots using plugs from 5-7 day-old SGM cultures for inoculum.
- Storage roots are washed, peeled, and 1-2 cm trimmed from each end.
- The storage roots or sweet potato vine segments are then surface disinfected for 10 min in 0.5% NaOCl.
- 1-cm-thick slices which are placed in sterile Petri dish moist chambers.
- A plug of agar is transferred from the culture and placed mycelium side down on the cut surface of the storage root slice.

Loria and co-workers

Thaxtomins

Thaxtomin C

Sweet potato storage root assays

- The slice is incubated at 32°C for 5-10 days after which the callus which develops on the cut surface is gently scraped away with a knife or steel wool.
- Pathogenic isolates cause a dark brown to black necrosis that extends into the storage root tissue.
- Nonpathogenic isolates may grow superficially on the surface or sometimes cause superficial light brown discoloration, but do not induce necrosis extending into the slice.
- The pathogen infects both the fibrous roots and the fleshy storage roots of sweet potato.
- This pathogen produces thaxtomin C in host tissue but not in media; other described thaxtomins are not produced by this species.

Loria and co-workers

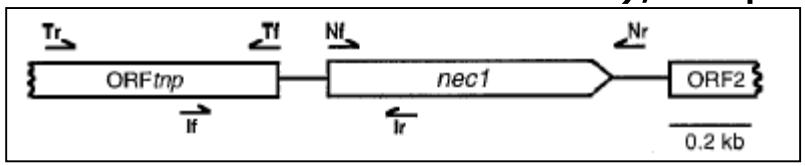
Molecular Diagnosis of *Streptomyces*

- The most recently described spp. are differentiated on DNA homology, 16SrDNA and 16S-23S ITS sequencing supported by some morphological and nutritional differences.
- Genetic fingerprinting, 16SrDNA sequencing and fatty acid analysis do not correspond well with current classification.
- Other gene sequencing, mlst, may solve the problem.

Molecular Diagnosis of *Streptomyces*
PCR Primers for *Streptomyces* spp.

- The primers for *nec1* and ORF*tnp*:

1. Nf (5′- ATGAGCGCGAACGGAAGCCCCGGA-3′) and Nr (5′- GCAGGTCGTCACGAA GGATCG-3′); and
2. Tf (5′- ATGACCGCATCCGACA GTCTGCCC-3′) and Tr (5′- TTGAT GATCCGGCCGCCCTCGC-3′), respectively.



PCR amplification *nec1*-specific oligonucleotide primers amplify the 0.7-kb gene from all the *nec1*-containing strains.

- The primers for the intergenic region:
- If (5′- GTTGTCTTCGGCGAGGGCGTGCAGG-3′) and
- Ir (5′- AGCGGAAGGATT TGCGACCACAACG-3′).

Lindholm *et al.*,1997

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 *Streptomyces*

Genus *Streptomyces*					
Species	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
S. acidiscabies *S. scabiei* *S. turgidiscabies*	**Nf/Nr** *nec1* gene	Conventional	Mycelium (boiled)	Burkhalid *et al.*, 1998	*S. scabies*
S. scabiei *S. turgidiscabies* *S. aureofaciens*	Universal for *Streptomyces* **pA/pH´** Specific for *S. scabies* **ScabI/SacbII** Specific for *S. turgidiscabies* **TurgI/TurgII** Specific for *S. aureofaciens* **AurI/AurII** 16S rRNA gene	Conventional	Bacteria, potato tubers (DNA extraction)	Lehtonen *et al.*, 2004	*S. scabies*
S. acidiscabies *S.scabiei* *S.scabies* var. *achromogenes*	**16S-1F/16S-1R** 16S rRNA gene **Nec1F/Nec1R** *nec1* gene **TxtA1/TxtA2** *txtA* gene (thaxtomin biosynthesis)	Conventional	Bacteria (DNA extraction)	Wanner, 2004	*S.scabies* var. *achromogenes* is not included in the ISPP list.
Streptomyces spp.	**NEC-F2/NEC-R2** (primers) **Probe T** *nec1* gene **Probe IS** Internal standard DNA	Quantitative competitive quenching probe (QCQP)	Potato tubers, soil (DNA extraction)	Koyama *et al.*, 2006	
S. acidiscabies *S. aureofaciens* *S. bottropensis* *S. europaeiscabiei* *S. scabiei* *S. stelliscabiei* *S. turgidiscabies* New *Streptomyces* group	Species and strain-specific 16S rDNA sequences **scab1m/scab2m** **scab1/scab2m** **ASE3/scab2m** *S. scabies* and *S. europaeiscabiei* **Stel3/ T2st2**	Conventional	Bacteria (DNA extraction)	Wanner, 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

Genus *Streptomyces*

	S. stelliscabiei **ASE3/ Aci2** *Streptomyces* newly identified group **Stel3/ Aci2** *S. bottropensis* **Aci1/ Aci2** *S. acidiscabies* **Turg1m/ Turg2m** *S. turgidiscabies* **Aur1/ Aur2** *S. aureofaciens*				
Streptomyces spp.	**NecF1/NecR1 (external)** **NecNF1/NecNR2 (internal)** **NecTqF1/NecTqR1 (primers)** **NecTqP1 (probe)** *nec1* gene	Nested Real-time (TaqMan)	Bacteria, potato tubers and soil (DNA extraction)	Cullen and Lees, 2007	
S. acidiscabies *S. aureofaciens* *S.europaeiscabiei* *S. scabiei* *S. stelliscabiei* *S. turgidiscabies*	**16S-1F/16S 455-435** 16S rDNA **Nf/Nr** *nec1* gene **TxtAB TxtAB1/ TxtAB2** *TxtAB* gene **Tom3/Tom4** *TomA* gene **Species-specific 16s rDNA** **ASE3/Scab2m** (*S. scabies* and *S. europaeiscabiei*) **ASE3/ Aci2** (Newly identified *Streptomyces* group) **Aci1/ Aci2** (*S. acidiscabies*)	Conventional	Bacteria (DNA extraction)	Wanner, 2007	

Palacio-Bielsa *et al.*,2009

Preservation

- Three short-term preservation methods:
- First, agar slope cultures may be stored at 4°C for few months.
- Second, spore suspensions can be mixed with soft water agar and kept at 4°C (Kutzner,1972).
- Third, glycerol can be added to spore suspensions (final concentration, 10%, v/v) and stored at -20°C (Wellington and Williams,1978).
- For long-term preservation, spore suspension was prepared in 20% glycerol and freezing at –20°C.
- Another procedure is the growth of strains in complex media (like trypticase soy broth [TSB] agar), addition of 20% glycerol plus 10% lactose, and storage in the vapor phase of liquid nitrogen.
- In addition, drying on unglazed porcelain beads (Lange and Boyd,1968), followed by soil culture, and lyophilization are used.

Identification of the bacterial pathogens *Bacillus* and *Paenibacillus*

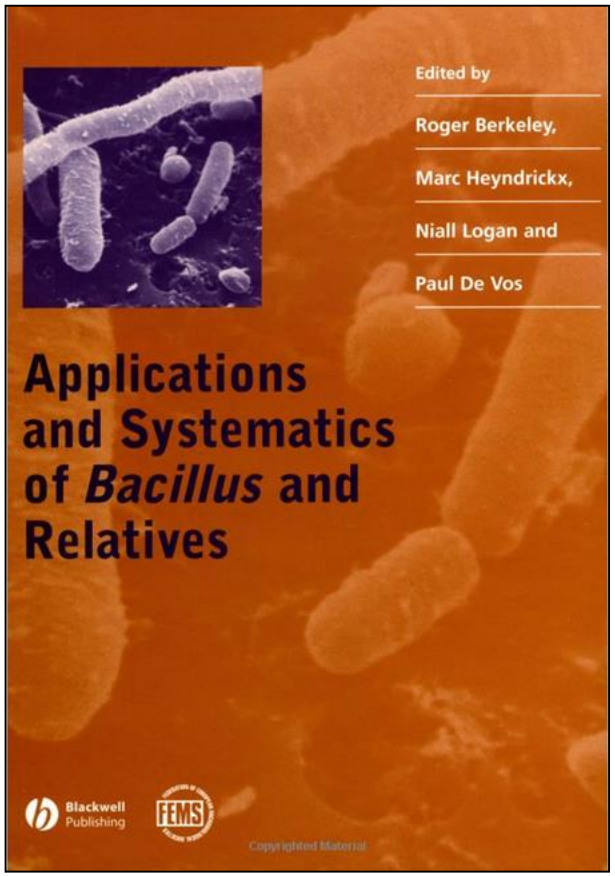
Disease diagnosis and pathogen diagnostics

Applications and Systematics of *Bacillus* and Relatives

- **Applications and Systematics of *Bacillus* and Relatives**
- by Roger Berkeley, Marc Heyndrickx, Niall Logan and Paul De Vos (Editors).

Product Details

- **Hardcover:** 317 pages
- **Publisher:** 2002 by Blackwell Publishing company



Gram-Positive Bacteria
Plant pathogenic genera

- **Domain Bacteria**
  - Phylum "Firmicutes"
    - Class "Bacilli"
      - Order Bacillales
        - Family Bacillaceae (*Bacillus*)
        - Family Paenibacillaceae (*Paenibacillus*)
    - Class "Clostridia"
      - Order Clostridiales
        - Family Clostridiaceae (*Clostridium*)

Nested genera in *Bacillus*

Another example of a large genus with nested genera is the *Bacillus* genus, in which the genera *Paenibacillus* and *Brevibacillus* are nested clades. There is insufficient genomic data at present to fully and effectively correct taxonomic errors in *Bacillus* (Wikipedia,2012).

Numbers of species assigned to the genus *Bacillus* in different editions of *Bergey's Manual* up to 1974 (modified from Gordon 1981)

Bergey's Manual	Year	Number of species
1st edition	1923	75
2nd edition	1925	75
3rd edition	1930	93
4th edition	1934	95
5th edition	1938	146
6th edition	1948	33
7th edition	1954	25
8th edition	1974	Group I: 22 Group II: 26

Berkeley *et al.*,2002

Phylogeny
Bacillaceae family
Base on 16S rRNA and ITS sequences

- Biodiversity of *Bacillus* spp:
- The molecular analysis of Bacillaceae species, using 16S rRNA and the 16S-23S ITS (internally transcribed spacer), to differentiate, in clusters, this bacterial family.
- *Bacillus* species may be divided into five or six groups (groups I–VI), based on 16S rRNA phylogeny or phenotypic features, respectively (Priest, 1993).
- ITS-PCR using the primers L1 and G1 to amplify the 16S-23S intergenic transcribed spacer region.

Økstad and Kolstø,2011; Hunag *et al.*,2012; Alina *et al.*,2015

Phylogeny
Bacillaceae family
Antagonists and PGPR Bacillus groups

- Those *Bacillus* strains that are known to have the potential to protect plants from pathogens or pests or stimulate plant growth are attributed to two groups:

1. the *B. cereus* group, and
2. the *B. subtilis* group.

- The *B. cereus* group includes *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanesis*.
- The *B. subtilis* group includes *B. subtilis*, *B. pumilus*, *B. atrophaeus*, *B. licheniformis* and *B. amyloliquefaciens*.

Hunag *et al.*,2012

Phylogeny

Bacillus species classification based on 16S rRNA sequences

- The phylogenetic studies, based on 16S rRNA sequence, suggest five groups of closely related species.

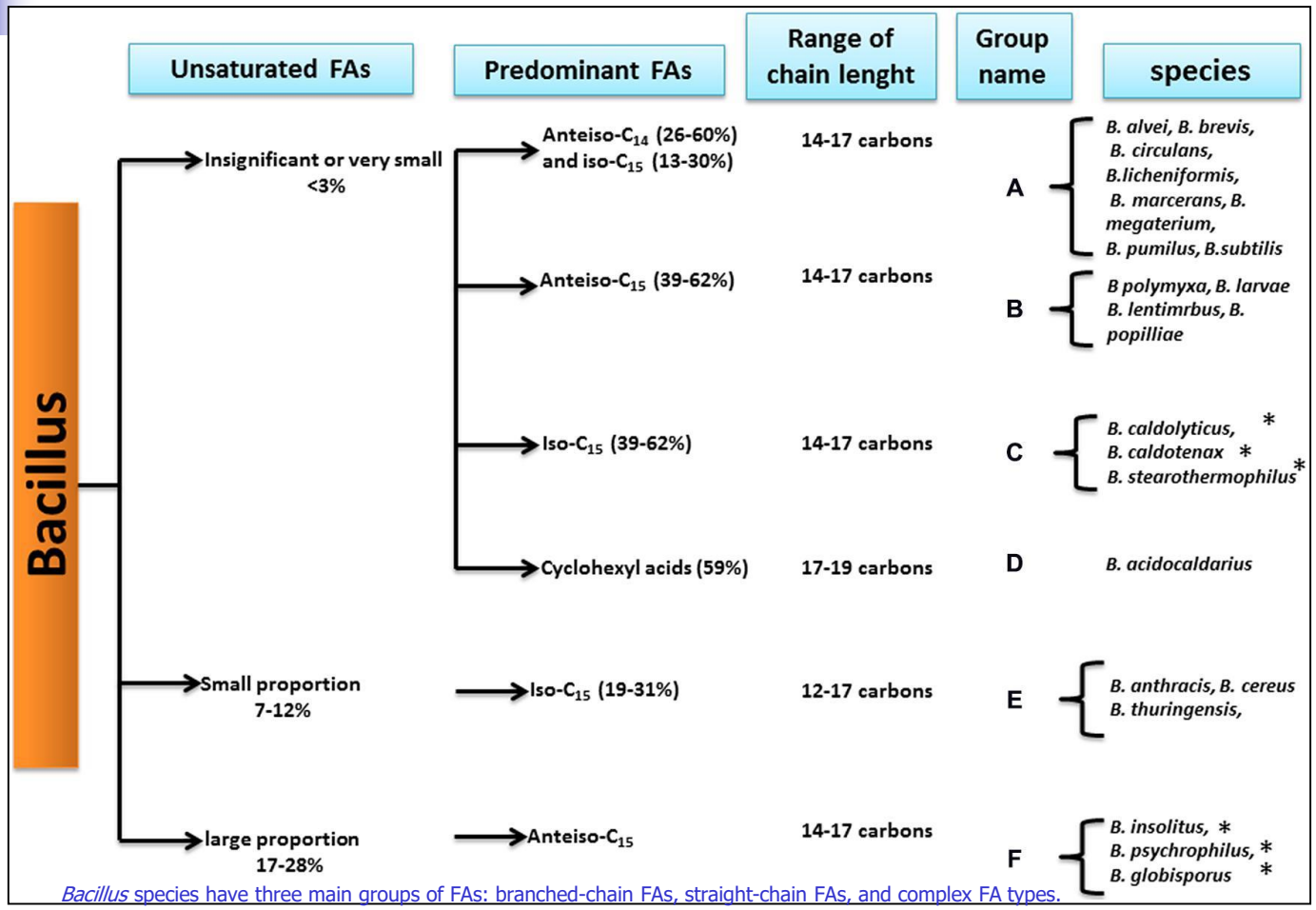
1. *Bacillus cereus*,
2. *B. megaterium*,
3. *B. subtilis*,
4. *B. circulans*, and
5. *B. brevis* group.

- There is also mentioned a group of closely related bacteria, referred to as *Bacillus pumilus* subgroup, as it is included in *Bacillus subtilis* group.

Alina *et al.*,2015

Phylogeny

Bacillus species classification based on the fatty acid patterns

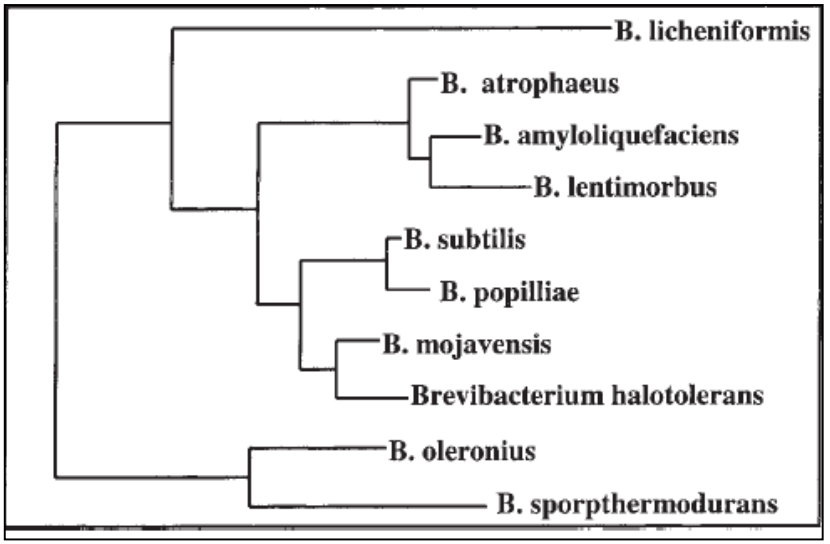


Bacillus species have three main groups of FAs: branched-chain FAs, straight-chain FAs, and complex FA types.

Kaneda,1977; Diomandé *et al.*,2015

Phylogeny
Based on 16S rRNA sequences

- Neighbor joining tree for species within the *Bacillus* group, indicating that they are very closely related to each other by 16S rRNA gene sequence analysis.

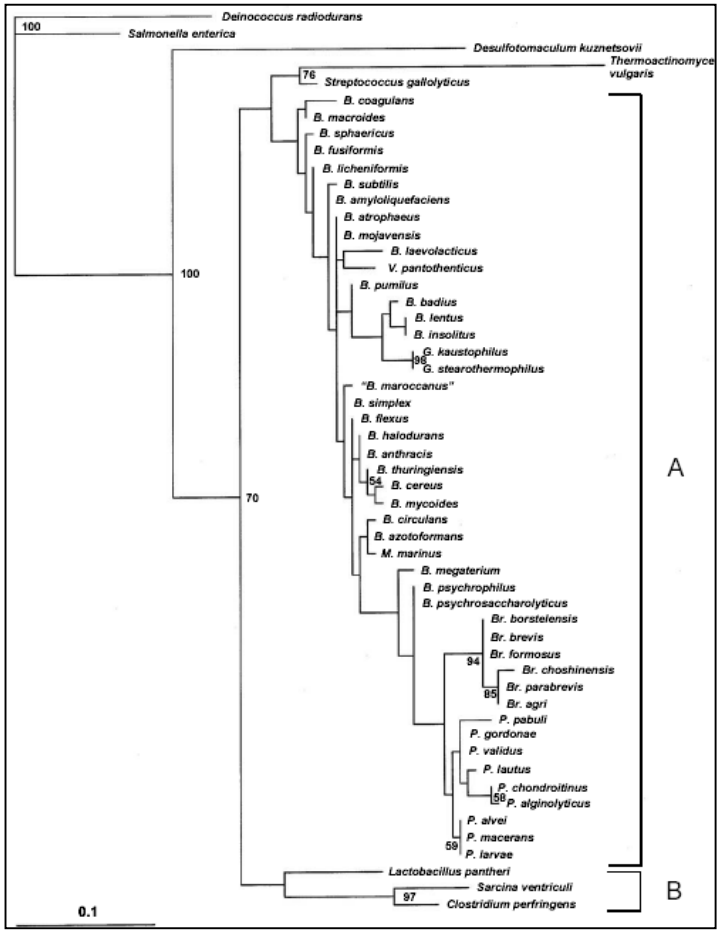


Spencer and Spencer,2004

Phylogeny

Comparative analysis of the 39 end 16S rRNA coding region reveals that at least the last 157 bp share extensive nucleotide identities with all 46 Bacillaceae species

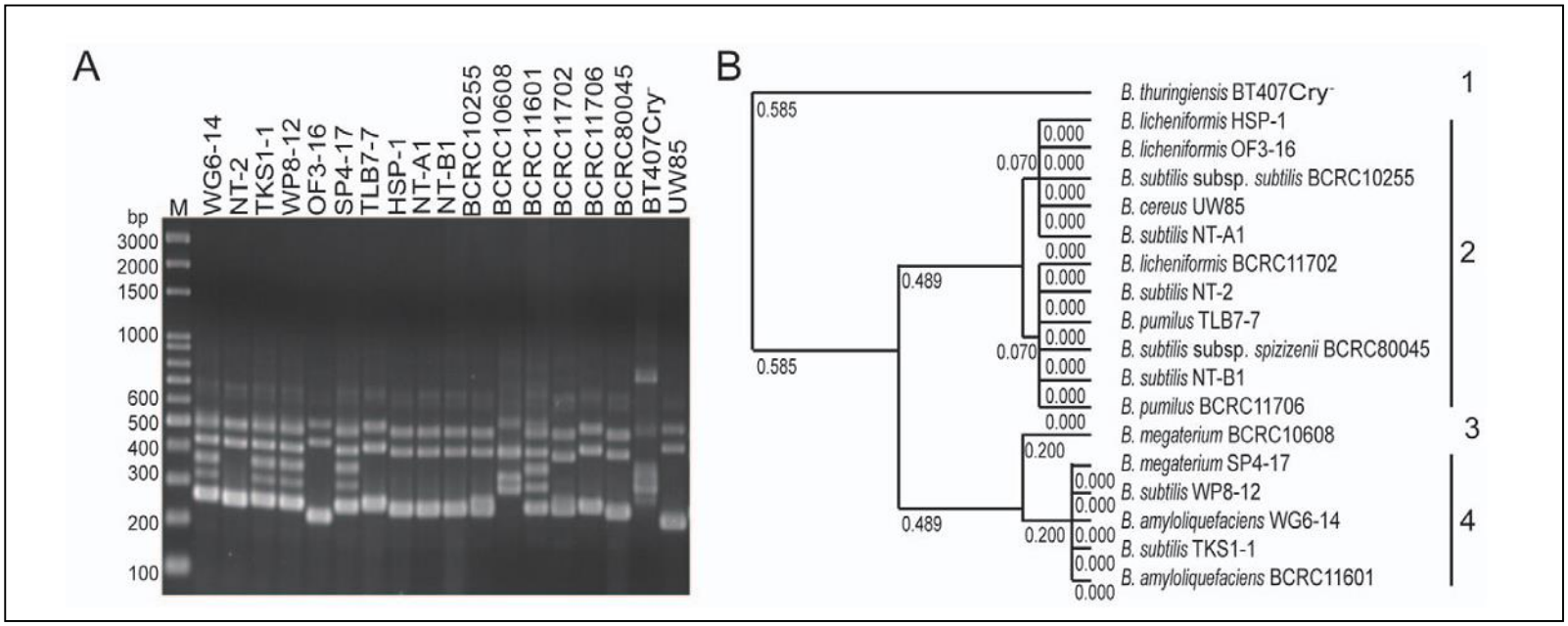
- Phylogenetic relationships of 46 *Bacillus*, *Brevibacillus*, *Paenibacillus*, *Virgibacillus* species, and eight distant species inferred from the alignment of the 157 bp 39 end 16S rRNA coding region.
- Bootstrap values (expressed as percentages of 100 replications) are shown at branch points; values greater than 50% were considered significant.
- The bar represents the unit length of the number of nucleotide substitutions per site.
- Abbreviations:
- B., *Bacillus*;
- Br., *Brevibacillus*;
- G., *Geobacillus*;
- M., *Marinibacillus*;
- P., *Paenibacillus*;
- V., *Virgibacillus*.



Xu and Caté,2002

Phylogeny

ITS-PCR fingerprint and UPGMA cluster analysis of Bacillus species. (A) ITS-PCR fingerprint and (B) UPGMA cluster analysis



ITS-PCR using the primers L1 and G1 to amplify the 16S-23S intergenic transcribed spacer region.

Hunag *et al.*,2012

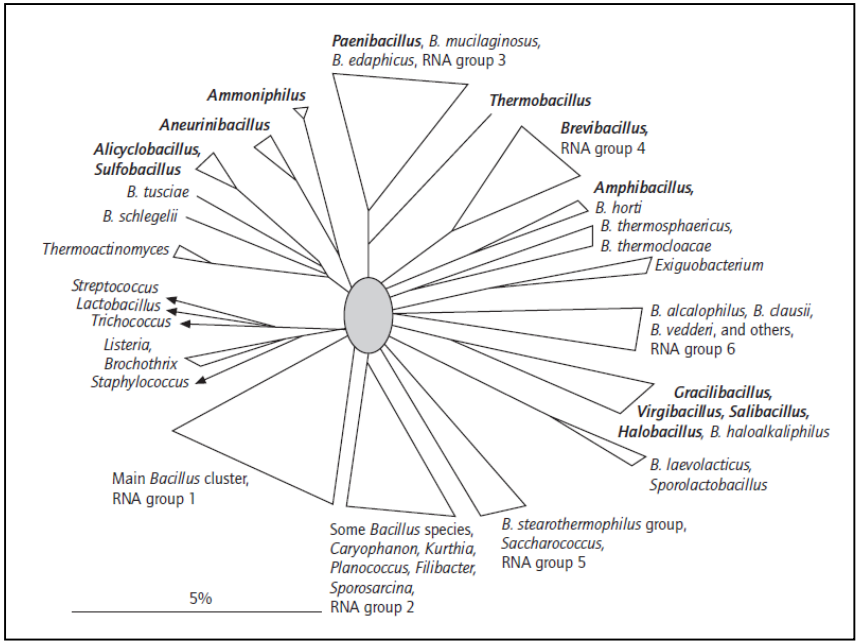
Phylogeny

Bacillus and novel genera originated from genus *Bacillus*

- Schematic outline of the phylogenetic diversity of 16S rDNA of aerobic, rod shaped and spore-forming, Gram-positive bacteria including:

1. Species of *Bacillus*,
2. Genera that originated from the dissection of *Bacillus*, and
3. Species that were affiliated to novel genera because of their distinct phylogenetic positions.

- *Bacillus* species were found to form clusters that have been named RNA groups 1 to 6.



Berkeley *et al.*,2002

Phylogeny

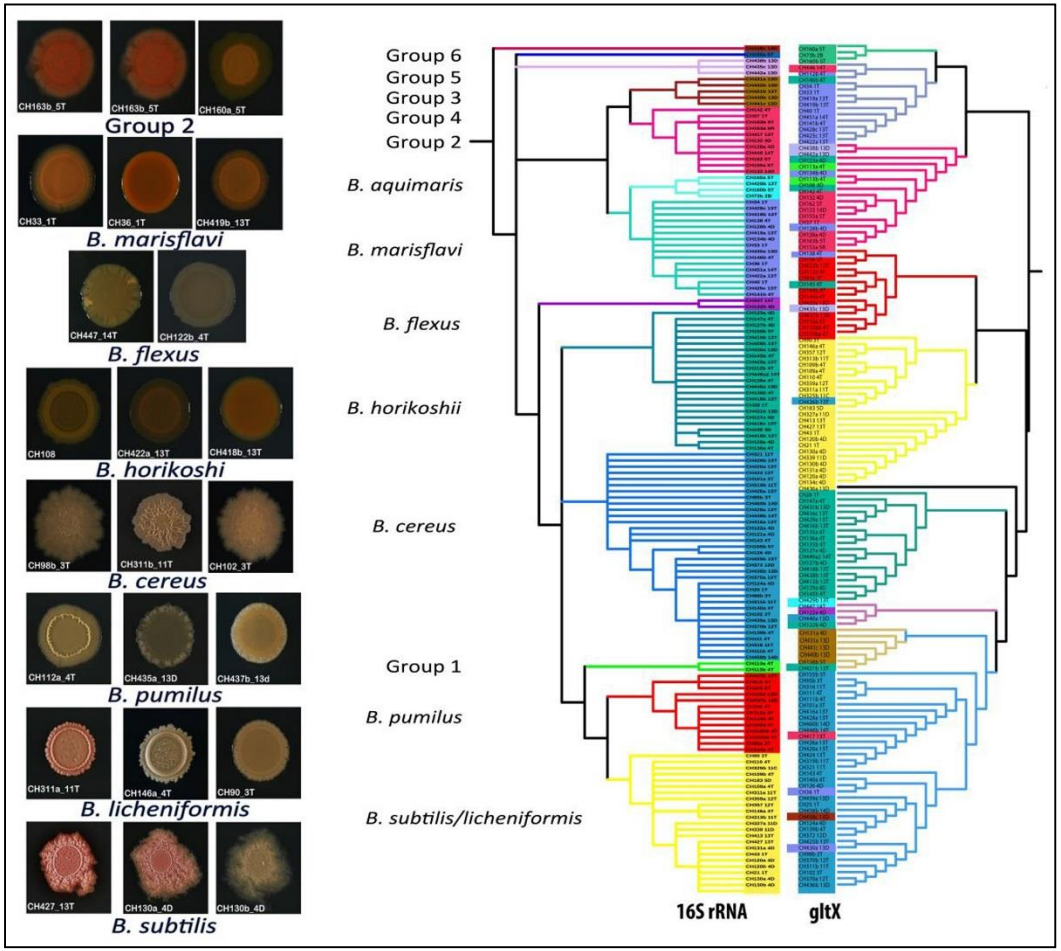
Based on 16S rRNA sequences and *gltX* gene (glutamyl-tRNA synthetase)

- We added one more gene, *gltX*, to increase phylogeny resolution.
- We amplified the 16S rRNA gene by PCR using previously published primer sequences 27F and 1492R(Lane,1991).
- This included regions V1 to V3 (~275 bp of the 5′ end region), considered to be the most informative for the *Bacillus* spp. (Goto *et al.*,2000).
- A 700 bp segment of the *gltX* gene (glutamyl-tRNA synthetase) was amplified using the primers
- gltX-for (5′ CGYGGBGADGAYCAYATYT 3′) and
- gltX-rev (5′ CRATTTCMGCDCCRWARCT 3′) and PCR-amplified by 30 cycles of 94°C for 30 s, 47°C for 1 min, 72°C for 2 min, and then a final elongation step at 72°C for 8 min with a thermocycler Palm-Cycler (Corbett Research).

Rodríguez-Torres *et al.*,2017

Phylogeny

Based on 16S rRNA sequences and *gltX* gene (glutamyl-tRNA synthetase)



Rodríguez-Torres *et al.*,2017

The Genus *Bacillus*
History

- One of the earliest bacteria to be described was "*Vibrio subtilis*" by Ehrenberg in 1835.
- In 1872, Cohn renamed the organism *Bacillus subtilis* (Gordon,1981).
- The family Bacillaceae was first formulated by Fisher in 1895 (Gordon,1981).

Diseases caused by
Plant pathogenic *Bacillus* species

- Plant-associated bacilli are recognized either as plant pathogens, saprophytes, or as biological control agents.
- There are only three known phytopathogenic bacilli:

Bacillus megaterium* pv. *cerealis	**White blotch of wheat**
Bacillus circulans	**Discoloration in heart tissue of mature plants. Also causes a disease in tissue cultures of date palm seedlings**
Bacillus pumilus	**Bacterial blotch of immature balady peach; ginger rhizome rot disease; Association with leaf and twig dieback of Asian pear cause by *P. syringae*.**

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

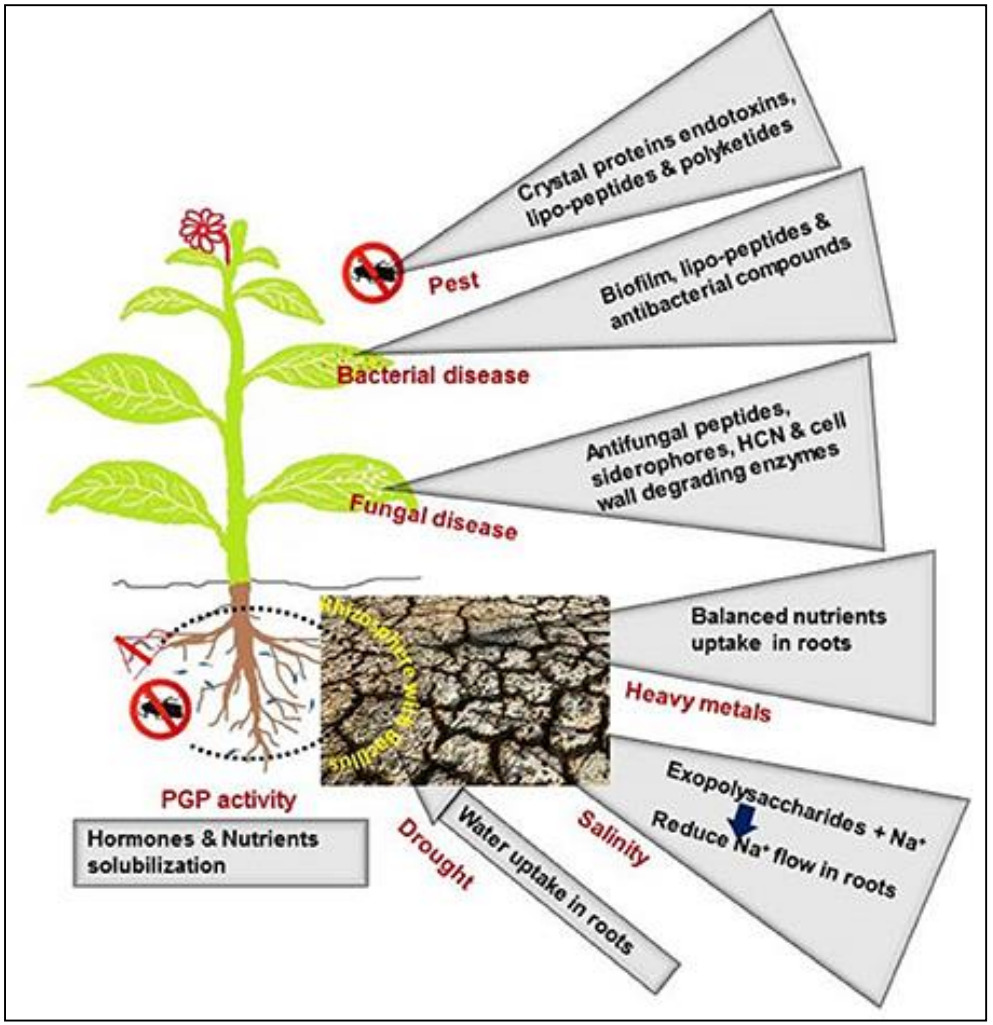
Bacillus species
As biological control agents

- *Bacillus* species are common among the resident microflora of the inner tissues of various species of plants, including cotton, grape, peas, spruce and sweet corn, where these microorganisms play an important role in plant protection and growth promotion plant protection and growth promotion.
- Production of antibiotics and hydrolytic enzymes is a feature of many endophytic bacilli including:
- *Bacillus cereus*
- *Bacillus pumilus*
- *Bacillus subtilis*

Bacillus species

Direct effect of *Bacillus*-secretions on plant protection from adverse environments

The mitigating effects of *Bacillus*-induced physiological changes in plants.



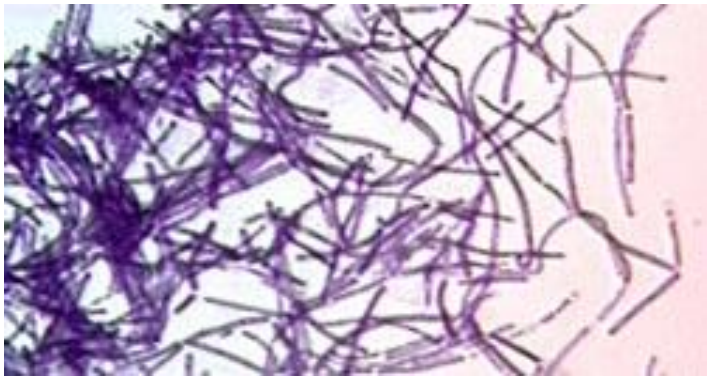
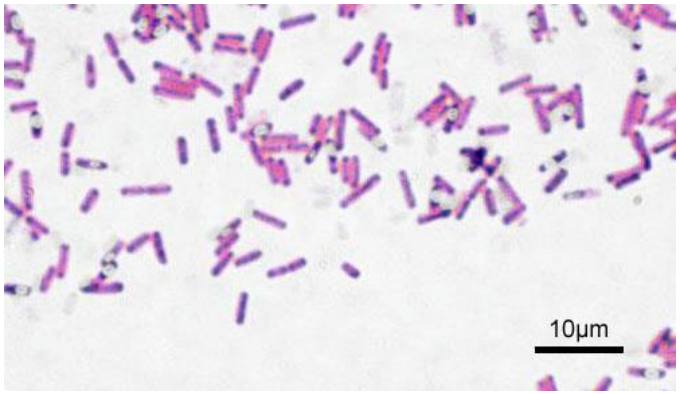
Radhakrishnan *et al.*,2017

Major distinguishing characteristics of the genus *Bacillus*

- The features of the members of the genus *Bacillus* that distinguish it from other Bacillaceae (all endosporeformers e.g. *Sporolactobacillus*, *Clostridium*, etc.) are:
- Their aerobic nature, which may be strict (e.g. *Bacillus subtilis*) or facultative anaerobic (e.g. *Bacillus cereus*).
- *Bacillus polymyxa* ferments lactose and produces gas.
- Rod shape (Large rod in case of *Bacillus cereus*).
- Older cultures (more than 24 hours) of *Bacillus polymyxa* may appear as cocci.
- In both *Bacillus cereus* and *B. subtilis*, older cultures may stain pink, due to the deterioration of cell walls.
- Catalase production.

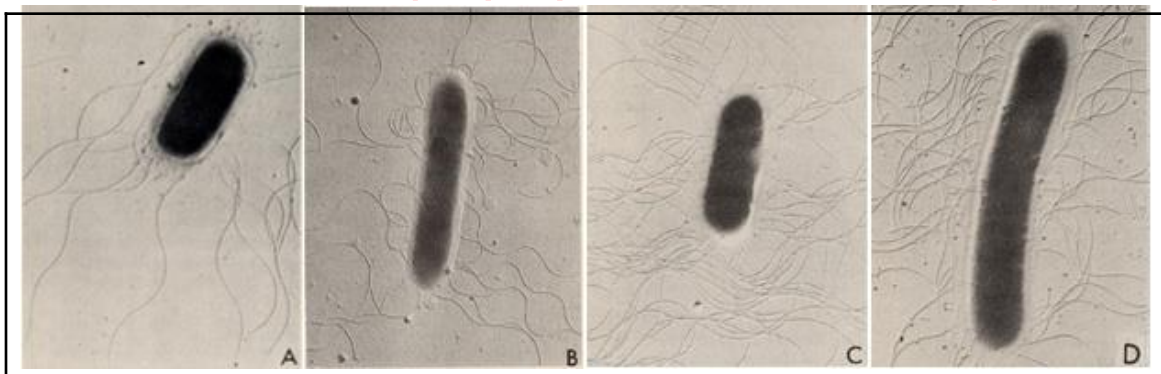
Cell size

The size of bacilli range between 3 to 5 micrometers

	
Bacillus megaterium	***Bacillus subtilis***

Flagella

- Individual cells of motile *Bacillus* species photographed on nutrient agar.
- A. *B. subtilis*; B. *B. polymyxa*; C. *B. laterosporis*; D. *B. alveli*.



About 15,000X magnification

Todar,2003

Surface structures of *Bacillus*

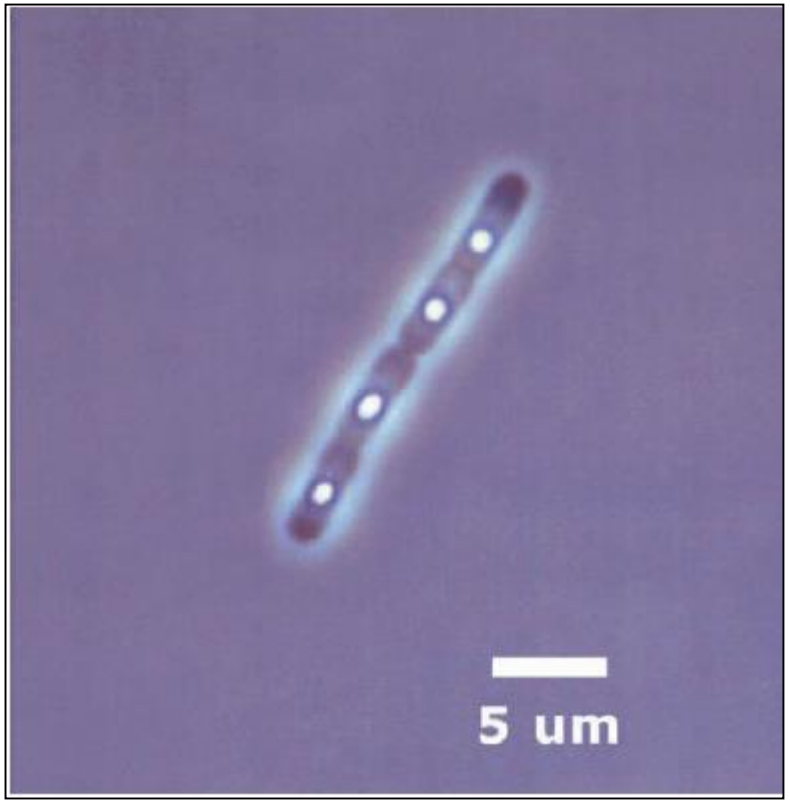
S-Layers

- Crystalline surface layers of protein or glycoprotein subunits, called S-layers, are found in members of the genus *Bacillus*.
- S-layers of individual strains of *Bacillus* have been shown to differ in molecular weight (40-200 kDa), the degree of glycosylation of the subunits, and the geometry of the S-layer lattice.
- Not all *Bacillus* species contain S-layers and some strains within a species may lack such a layer.

The Prokaryotes (chapter 1.2.16),2006

Resting spores or endospores
Bacillus sp.

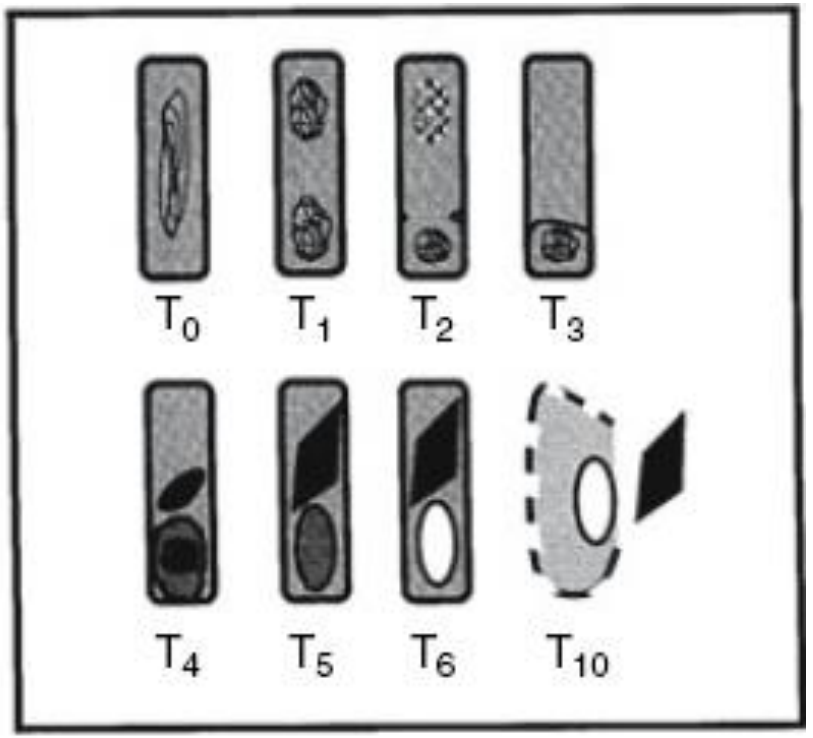
- Thick-walled resting spores or endospores in a *Bacillus* sp.
- Because of the thick wall the spores are much more refractive in phase-contrast microscopy than the surrounding vegetative cell.
- Spores are centrally placed in the cell.



Janse,2006

Sporulation and crystal toxin production

- An artist's conception of growth, sporulation, and crystal toxin production in *B. thuringiensis* subsp. *kurstaki*.
- Endotoxin crystals of *B. thuringiensis* may be detected by phase contrast microscopy or by staining with 0.5% aqueous basic fuchsin or TB carbol fuchsin.
- The other members of the *B. cereus* group do not produce endotoxin crystals that can be detected by staining.
- The cells were grown in liquid GYS medium as described in the text.



The Prokaryotes (chapter 1.2.17),2006

Origins of isolates of *Bacillus* spp.

The Prokaryotes (chapter 1.2.16),2006

Name of *Bacillus* species	Habitats from which isolated
B. subtilis	Soil, water
B. acidocaldarius	Thermal acid water and soil
B. alcalophilus	pH 10 enrichment from soil
B. alvei	Soil, diseased bee larvae
B. amylolyticus	Soil
B. anthracis	Anthrax-diseased animals
B. azotoformans	Soil
B. badius	Feces, foods, marine sources
B. brevis	Soil, foods
B. cereus	Soil, foods
B. circulans	Soil
B. coagulans	Acid foods
B. fastidiosus	Soil, poultry litter
B. firmus	Soil, salt marshes
B. globisporus	Soil, water
B. insolitus	Soil
B. larvae	Diseased bee larvae
B. laterosporus	Soil, water
B. lautus	Soil, feces
B. lentimorbus	Diseased honeybee larvae
B. lentus	Soil, foods
B. licheniformis	Soil
B. macerans	Plant materials, food
B. macquariensis	Subantarctic soil
B. marinus	Marine sediment
B. megaterium	Soil
B. mycoides	Soil
B. pabuli	Soil, fodder
B. pantothenicus	Soil
B. pasteurii	Soil, water, sewage
B. popilliae	Diseased scarabid beetles
B. psychrophilus	Soil, water
B. pumilus	Soil
B. schlegelii	Lake sediment
B. sphaericus	Soil, water sediments, foods
B. stearothermophilus	Soil, hot spring, foods
B. thermoglucosidasius	Soil
B. thuringiensis	Soil, foods
B. validus	Soil

Based on Claus and Berkeley (1986).

Bacillus plasmids

Phenotype associated with plasmid

Bacterium	Plasmid	DNA size (kb)	Phenotype associated with plasmid
B. anthracis	pXO1	168	Exotoxin (lethal factor, edema factor, protective antigen)
	pXO2	85.6	Capsule
B. cereus	pBC7	69	Bacteriocin
	pBC16	4.3	Tetracycline resistance
B. pumilus	pBL10	6.8	Bacteriocin
B. subtilis	pIM13	2.2	Erythromycin resistance
B. subtilis (natto)	pLS19	5.4	Polyglutamate production
	pLS20	55	Self-transmissible plasmid, which also promotes transfer of other plasmids
B. thuringiensis	pXO12	112.5	Production of insecticidal crystal protein, and is a self-transmissible plasmid, which can co-transfer unrelated plasmids
Bacillus species (thermophilic)	pTB19	26	Kanomycin and tetracycline resistance
	pTB20	4.3	Tetracycline resistance

The Prokaryotes (chapter 1.2.16),2006

Production of antibiotics by some *Bacillus* spp.

Species	Antibiotic
B. subtilis	Subtilin[a]
	Surfactin[b]
	Bacilysin[a]
	Difficidin[c]
	Oxydifficidin[c]
	Bacillomycin F[d]
	Mycobacillin[d]
B. brevis	Gramicidin S[a]
	Lincar Gramicidin[a]
	Tyrocidin[a]
B. licheniformis	Bacitracin[a]
	Proticin
B. pumilus	Pumilin[a]
	Tetain
B. mesentericus	Esperin[a]
B. polymyxa	Polymyxin[e]
	Colistin[e]
B. thiaminolyticus	Octopytin[a]
	Baciphelacin[a]
B. circulans	Circulin[e]
	Butirosin[a]
B. laterosporus	Laterospuramine[a]
	Laterosporin[a]
B. cereus	Biocerin[a]
	Cerexin[a]

[a]Anti-Gram-positive bacteria.
[b]Inhibitor of fibrin clotting.
[c]Broad spectrum antibiotic.
[d]Anti-fungal antibiotic.
[e]Anti-Gram-negative bacteria.

The importance of *Bacillus* species in the production of industrial enzymes

- The global market for industrial enzymes is considered to total 1.6 billion US dollars.
- The market is divided as follows:
- Technical enzymes (detergent proteases and amylases and textile): *c.* US$1 billion
- Food enzymes (baking, beverage and dairy): *c.* US$0.5 billion
- Feed enzymes (animal feed and other): *c.* US$0.1 billion.

Berkeley *et al.*,2002

Thioglycollate Agar

Aerobic and Anaerobic Growth

The genera *Bacillus and Clostridium*

- Before inoculation, the medium is steamed (to melt the agar and drive out oxygen), cooled to 45-50°C, and then inoculated.
- Cystine and sodium thioglycollate are included in the medium to maintain a low oxidation-reduction potential.
- Agar decreases the diffusion of oxygen into the medium.
- Resazurin is used as an indicator of oxygen; this compound is pink or red in the oxidized state and colorless when reduced.
- The color may not be seen if good growth is present.
- Glucose is included as a fermentable energy source although some clostridia can ferment amino acids in the yeast extract and casitone; any of these organic compounds may be respired by various aerobes and facultative anaerobes.

Thioglycollate Agar

Component	Amount (grams/L)
Yeast extract	5
Casitone	15
Glucose	5
Sodium chloride	2.5
Cystine	0.5
Sodium thioglycollate	0.5
Resazurin	0.001
Agar	0.75

Thioglycollate Agar
Modified

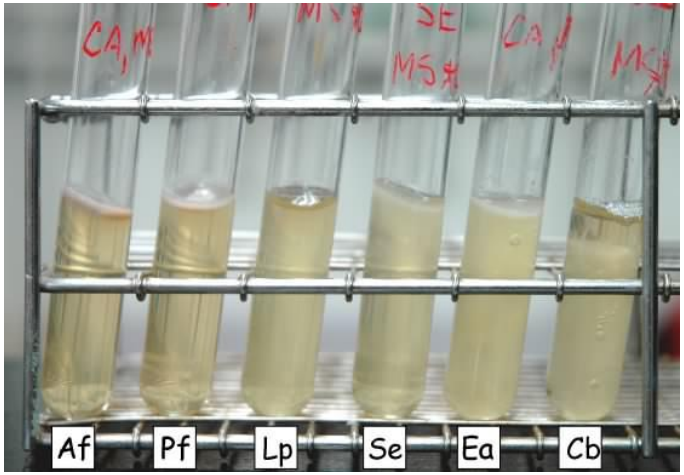
- **Anaerobic Agar**
- Trypticase 20 g
- Glucose 10 g
- Sodium chloride 5 g
- Agar 15 g
- Sodium thioglycolate 2 g
- Sodium formaldehyde sulfoxylate 1 g
- Distilled water 1 liter
- pH=7.2
- Inoculate a tube of anaerobic agar with a small loopful of nutrient broth culture by stabbing to the bottom of the culture tube.
- At incubation temperatures below 45°C the growth should be recorded at 3 and 7 days.

Thioglycollate Agar

Aerobic and Anaerobic Growth

The genera *Bacillus* and *Clostridium*

- Strict aerobes will only grow near the surface of the agar (Af, *Alcaligenes faecalis*, Pf, *Pseudomonas fluorescens*); Aerotolerant anaerobes grow at the same rate in presence or absence of oxygen(Lp, *Lactobacillus plantarum*); Facultative anaerobes will grow throughout the tube, but will display more growth near the top of the tube (Se, *Staphylococcus epidermidis*; Ea, *Enterobacter aerogenes*); Strict aerobes will only grow in the presence of oxygen, at the top of the tube(Cb, *Clostridium butyricum*). Strict anaerobes will only grow in the bottom of the tube where oxygen is absent.



Virtual Microbiology

Media used for the isolation and cultivation of *Bacillus* spp.

B. acidocaldarius	Part A: $(NH_4)_2SO_4$, 0.4; $MgSO_4$, 1.0; $CaCl_2 \cdot 2H_2O$, 0.5; KH_2PO_4, 6.0; distilled H_2O, 1 liter; pH adj. to 4.0 Part B: glucose, 2.0; yeast ext., 2.0 distilled H_2O, 1 liter Combine A and B after sterilization
B. alcalophilus	Part A: glucose, 1.0; peptone, 5.0; yeast ext., 5.0; KH_2PO_4, 10.0 $MgSO_4 \cdot 7H_2O$, 0.2, distilled H_2O, 900ml. Part B: $Na_2CO_3 \cdot 10H_2O$, 20; distilled H_2O, 100ml. Combine A and B after sterilization (final pH = 10.5)
B. azotoformans	Peptone, 10.0; $Na_2HPO_4 \cdot 12H_2O$, 3.6; $MgSO_4 \cdot 7H_2O$, 0.03; $MnSO_4 \cdot H_2O$, 0.05; KH_2PO_4, 1.0; NH_4Cl, 0.5; $CaCl_2 \cdot 2H_2O$, 0.1; distilled H_2O, 1 liter.
B. brevis	K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.02; NaCl, 0.02; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot H_2O$, 0.01; betaine, betaine·HCl or valine, 0.05M; agar, 16.0; distilled H_2O, 1 liter.
B. fastidiosus	K_2HPO_4, 0.8; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.05; $CaCl_2 \cdot 2H_2O$, 0.05; $FeSO_4 \cdot 7H_2O$, 0.015; $MnSO_4 \cdot H_2O$, 0.01; uric acid, 10.0; distilled H_2O, 1 liter.
B. lentus	Peptone, 10.0; meat ext., 10.0; agar, 15.0; distilled H_2O, 1 liter. adj. pH to 7.0–7.5; after sterilization, add 100g urea, steam for 10min.
B. licheniformis	Peptone, 5.0; meat ext., 3.0; KNO_3, 80.0; distilled H_2O, 1 liter; adj. pH to 7.0; fill glass-stoppered bottle to top for anacrobic conditions.
B. marinus	Peptone, 5.0; yeast ext., 1.0; $FePO_4 \cdot 4H_2O$, 0.01; agar, 15.0; aged sea water, 750ml; distilled H_2O, 250ml; adj. pH to 7.6.
B. pantothenicus	Nutrient broth + 4% (w/v) NaCl
B. pasteurii	Nutrient broth + 2% (w/v) urea
B. schlegelii	$Na_2HPO_4 \cdot 2H_2O$, 4.5; KH_2PO_4, 1.5; NH_4Cl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.01; ferric ammonium citrate, 0.005; $NaHCO_3$, 0.5; trace element soln, 5ml ($ZnSO_4 \cdot 7H_2O$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.03; H_3BO_3, 0.3; $CoCl_2 \cdot 6H_2O$, 0.02; $CuCl_2 \cdot 2H_2O$, 0.001; $NiCl_2 \cdot 6H_2O$, 0.02; $Na_2MoO_4 \cdot 2H_2O$, 0.03; distilled H_2O, 1 liter) Other: 65C, atmosphere of 0.05atm. O_2 + 0.01atm. CO_2 + 0.45atm. H_2
B. stearothermophilus	Nutrient agar; incubate cultures at 55°C

[a]Numerical amounts are grams unless specified.
[b]Most other *Bacillus* cultures will grow on nutrient broth and nutrient agar.
Based on Claus and Berkeley (1986).

The Prokaryotes (chapter 1.2.16),2006

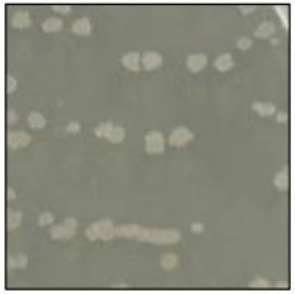
Identification of *Bacillus* spp.

- The identification of *Bacillus* species is difficult, and molecular methods are the basis for distinguishing species within this very large, complex genus.
- Isolates may be identified tentatively to the genus *Bacillus* if they have the ability to hydrolyze gelatin, utilize citrate, reduce nitrate, and ferment L-arabinose and mannitol.
- Any isolate that is positive for all five of these phenotypic tests probably belongs to the *Bacillus subtilis* group of closely related species.
- This may include *B. mojavensis* isolates which are endophyte, nonpathogenic to plants, antagonistic to fungi, and to enhance plant growth.
- Indole production is negative for some species such as *subtilis*, *polymyxa* and *cereus*.

Spencer and Spencer,2004;…

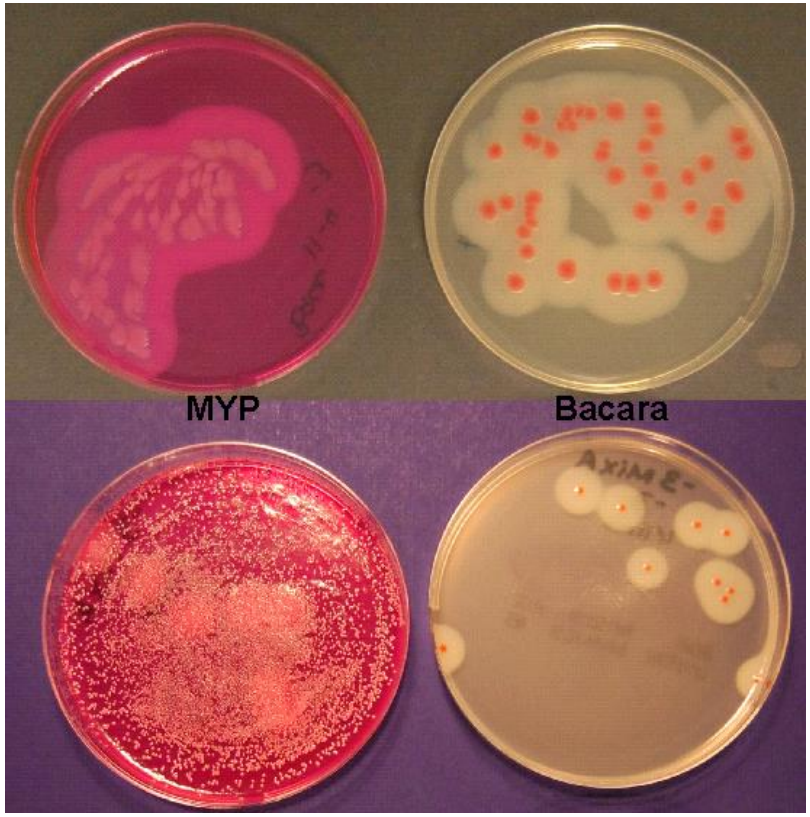
Colony morphology

Bacillus spp.

Bacillus subtilis		The cream colored colonies are larger, but not as large as *B. cereus*. The margin is undulate, with circular form and flat elevation.	*Bacillus cereus*		Colonies are large, irregular and flat with an undulate margin
Bacillus polymyxa		Colonies are white, large, irregular and flat with an undulate margin	*Bacillus megaterium*		White/ round, glossy shade yellow colored colony with smooth and entire margin.

Colony characteristics of *B. cereus*

1. Colonies of *Bacillus cereus* grown on MYP medium are pink and lecithinase positive, but other bacteria are not inhibited and can interfere with isolation of *B. cereus*.
2. Colonies of *B. cereus* grown on Bacara medium are pink-orange and are lecithinase positive, but other organisms are inhibited.



Tallent *et al.*,2012

Characteristics used in the identification of *Bacillus* spp.

Bacillus species/group	*anthracis*	*cereus*	*licheniformis*	*megaterium*	*megaterium* pv. *cerealis*	*subtilis*	*coagulans*	*alvei*	*brevis*	*circulans*	*circulans*[b]	*latero-porus*	*mace-rans*	*poly-mixa*
Gram reaction	+	+	+	+	+	+	+	I	I	I	–	I	I	I
Motility[a]	–	+	+	+	–	+	+	+	+	+	+	+	+	+
Oval spores	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Spore position														
Terminal	–	–	–	–	–	–	+	+	+	+	–	–	+	+
Central	+	+	+	+	+	+	+	+	+	+	–	+	–	+
Subterminal	–	–	–	–	–	–	+	+	+	+	+	–	–	+
Swelling of bacillary body	–	–	–	–	–	–	v	+	+	+	+	+	+	+
Growth at 45 Ċ	–	v	+	v	–	+	+	v	v	v	+	v	v	–
Growth at pH 5.7	+	+	+	+	+	+	+	–	v	v	ND	–	+	+
Growth in 7% NaCl	+	v	+	+	–	+	–	–	–	v	–	–	–	–
Utilization of citrate	v	+	+	+	+	+	v	–	v	–	–	–	–	–
Anaerobic growth in glucose broth	+	+	+	–	–	–	+	+	–	v	+	+	+	+
Acid from:														
Arabinose	–	–	+	v	ND	+	v	–	–	+	+	–	+	+
Mannitol	–	–	+	v	+	+	v	–	v	+	–	+	+	+
Xylose	–	–	+	v	+	+	v	–	v	+	+	–	+	+
VP test	+	+	+	–	–	+	v	+	–	–	–	–	–	+
Starch hydrolysis	+	+	+	+	+	+	+	+	–	+	+	–	+	–

[a] All species may produce non-motile cells

[b] Taken from Leary, J.V and Chun ,W. 1985.

v = Variable ND= Not defined I = Indefinite

Schaad *et al.*,1998

Characteristics used in the identification of *Bacillus* spp.

	Plant Pathogens		Other Species								
	circulans	*megaterium* pv. *cerealis*	*anthacis*	*cereus*	*licheniformis*	*subtilis*	*coagulans*	*alvei*	*brevis*	*laterosporus*	*macerans*
Gram reaction	I	+	+	+	+	+	+	I	I	I	I
Motility[2]	+	-	-	+	+	+	+	+	+	+	+
Spore position											
Terminal	+	-	-	-	-	-	+	+	+	-	+
Central	+	+	+	+	+	+	+	+	+	+	-
Subterminal	+	-	-	-	-	-	+	+	+	-	-
Swelling of bacillary body	+	-	-	-	-	-	V	+	+	+	+
Growth at 45°C	V	-	-	V	+	+	+	V	V	V	V
Growth at pH 5.7	V	+	+	+	+	+	+	-	V	-	+
Growth in 7% NaCl	V	-	+	V	+	+	-	-	-	-	-
Utilization of citrate	-	+	V	+	+	+	V	-	V	-	-
Anaerobic growth in glucose broth	V	-	+	+	+	-	+	+	-	+	+
Acid from:											
Arabinose	+	ND	-	-	+	+	V	-	V	+	+
Mannitol	+	+	-	-	+	+	V	-	V	+	+
Xylose	+	+	-	-	+	+	V	-	V	-	+
Voges-Proskaur test	-	-	+	+	+	+	V	+	-	-	-
Starch hydrolysis	+	+	+	+	+	+	+	+	-	-	0

+, 80% or more strains positive; +[D], 80% or more strains delayed positive; V, between 21-70% of strains positive; -, 80% or more strains negative; ND, not determined.

[1] Adapted from Cowan (6) and Hosford for *Bacillus megaterium* pv. *cerealis* (12) and Leary and Chun for *B. circulans* (15)

[2] All species may produce non-motile cells.

Glucose broth: Nutrient broth+1% glucose

Schaad *et al.*,2001

Table 2. Characteristics useful to differentiate among some species of *Bacillus*.

Bacillus species	Characteristic[a]															
	Rods 1.0 µm wide or wider	Parasporal bodies	Growth in anaerobic agar	Growth at 50°C	Growth at 65°C	Growth in 4% NaCl	Growth in 7% NaCl	Catalase	Voges-Proskauer reaction	Acid from glucose	Acid and gas in glucose	NO_3 reduced to NO_2	Starch hydrolyzed	pH in V-P medium <6.0	Hydrolysis of casein	Lecithinase
anthracis	+	−	+	−	−	+	+	+	+	+	−	+	−	+	+	+
megaterium	+	−	−	−	−	ND	+	+	−	+	−	V	−	V	+	−
cereus	+	V	+	−	−	ND	+	+	+	+	−	+	+	+	+	+
thuringiensis	+	+	+	−	−	+	+	+	+	+	−	+	+	+	+	+
lichenformis	−	−	+	+	−	+	+	+	+	+	−	+	+	V	+	−
subtillis	−	−	−	+	−	+	+	+	+	+	−	+	+	V	+	−
pumilus	−	−	−	+	−	+	+	+	+	+	−	−	−	+	+	−
firmus	−	−	−	−	−	+	+	+	−	+	−	+	+	−	+	−
coagulans	V	−	+	+	−	−	−	+	+	+	−	V	+	+	V	−
polymyxa	−	−	+	−	−	−	−	+	+	+	+	+	+	V	+	−
macerans	−	−	+	+	−	−	−	+	−	+	+	+	+	−	−	−
circulans	−	−	V	+	−	V	V	+	−	+	−	V	+	V	V	−
stearothermophilus	V	−	−	+	+	V	−	V	−	+	−	V	−	+	V	−
alvei	V	−	+	−	−	V	−	+	+	+	−	−	−	+	+	−
laterosporus	−	+	+	+	−	V	−	+	−	+	−	+	−	−	+	+
brevis	−	−	−	+	−	−	−	+	−	+	−	V	−	−	+	−
sphaericus	V	−	−	−	−	V	V	+	−	−	−	−	−	−	V	−
larvae	−	−	+	−	−	−	+	−	−	+	−	V	−	−	+	ND
popilliae	−	+	+	−	−	−	+	−	−	+	−	−	−	−	−	ND
lentimorbus	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	ND
pasteurii	−	−	+	−	−	+	+	ND	−	ND	−	+	−	ND	V	−
pantothenticus	−	−	+	V	−	+	+	+	−	+	−	V	−	+	V	−

[a]+, greater than 85% of strains positive; −, greater than 85% of strains negative; V = variable character; ND, no data available.
From Reboli and Farrar (1988).

TABLE 15-1 Basic Characteristics for Identification of Selected Bacillus Species[a]

Species[b]	Motility	Catalase Production	Parasporal Bodies	Lipid Globules in Protoplasm	Lecithovitellin Reaction	Citrate Utilization	Anaerobic Growth	V-P Reation	pH in V-P Medium < 6.0	Growth at 50° C	Growth at 60° C	Growth in 7% NaCl	Acid from AS Glucose	Acid + Gas from AS Glucose	Nitrate Reduction	Casein Hydrolysis	Strach Hydrolysis	Propionate Utilization
Morphologic group 1																		
B megaterium	v	+	−	+	−	+	−	−	v	−	−	+	+	−	v	+	+	n
B cereus	+	+	−	+	+	+	+	+	+	−	−	+	+	−	+	+	+	n
B cereus subsp *mycoides*	−	+	−	+	+	+	+	+	+	−	−	+	+	−	+	+	+	n
B *anthracis*	−	+	−	+	+	v	+	+	+	−	−	+	+	−	+	+	+	n
B thuringiensis	+	+	+	+	+	+	+	+	+	−	−	+	+	−	+	+	+	n
B licheniformis	+	+	−	−	−	+	+	+	+	+	−	+	+	−	+	+	+	+
B subtilis	+	+	−	−	−	+	−	+	v	v	−	+	+	−	+	+	+	−
B pumilus	+	+	−	−	−	+	−	+	+	v	−	+	+	−	−	+	−	−
B firmus	v	+	−	−	−	−	−	−	−	−	−	+	+	−	+	+	+	−
B coagulans	+	+	−	−	−	v	+	+	+	+	v	−	+	−	v	v	+	−
Morphologic group 2																		
B polymyxa	+	+	−	−	−	−	+	+	v	−	−	−	+	+	+	+	+	n
B macerans	+	+	−	−	−	v	+	−	+	+	−	−	+	+	+	−	+	n
B circulans	v	+	−	−	−	v	v	−	+	v	−	v	+	−	v	v	+	n
B stearothermophilus	+	v	−	−	−	−	−	−	+	+	+	−	+	−	v	v	+	n
B alvei	+	+	−	−	−	−	+	+	+	−	−	−	+	−	−	+	+	n
B laterosporus[c]	+	+	−	−	(+)	−	+	−	+	−	−	−	+	−	+	+	−	n
B brevis	+	+	−	−	−	v	−	−	−	v	v	−	+	−	v	+	−	n
Morphologic group 3																		
B sphaericus	+	+	−	−	−	v	−	−	−	−	−	v	−	−	−	v	−	n

[a] V-P, Voges-Proskauer; AS, ammonium salt; +, more than 85 percent of strains examined by Gordon et al. (See References) were positive; -, more than 85 percent of strains negative; v, variable; n,test not applicable; (+), under colony which must be scraped off to see positive reaction.
[b] Species grouped according to the classification scheme of Gordon et al. Morphologic group 1: sporangium not swollen by spore; spore ellipsoidal or cylindrical, central or terminal; Gram positive. Morphologic group 2: sporangium swollen by ellipsoidal spore; spore central or terminal; Gram variable. Morphologic group 3: sporangium swollen by spore; spore spherical, subterminal, or terminal; Gram variable.
[c] Sporangium and spore have characteristic canoe shape.

Table 6. Summary of the characters used in the simplified key for *Bacillus* species.

	Catalase	V-P reaction	Growth in anaerobic agar	Growth at 50°C	Growth in 7% NaCl	Acid and gas in glucose	NO_3 reduced to NO_2	Starch hydrolyzed	Growth at 65°C	Rods 1.0µm wide or wider	pH in V-P medium <6.0	Acid from glucose	Hydrolysis of casein	Parasporal bodies
B. megaterium	+	−	−	−	+	−	V	+	−	+	V	+	+	−
B. cereus	+	+	+	−	+	−	+	+	−	+	+	+	+	V
B. thuringiensis	+	+	+	−	+	−	+	+	−	+	+	+	+	+
B. licheniformis	+	+	+	+	+	−	+	+	−	−	V	+	+	−
B. subtilis	+	+	−	+	+	−	+	+	−	−	V	+	+	−
B. pumilus	+	+	−	+	+	−	−	−	−	−	+	+	+	−
B. firmus	+	−	−	−	+	−	+	+	−	−	−	+	+	−
B. coagulans	+	+	+	+	−	−	V	+	−	V	+	+	V	−
B. polymyxa	+	+	+	−	−	+	+	+	−	−	V	+	+	−
B. macerans	+	−	+	+	−	+	+	+	−	−	−	+	−	−
B. circulans	+	−	V	+	V	−	V	+	−	−	V	+	V	−
B. stearothermophilus	V	−	−	+	−	−	V	+	+	V	+	+	−	
B. alvei	+	+	+	−	−	−	−	+	−	V	+	+	+	−
B. laterosporus	+	−	+	+	−	−	+	−	−	−	−	+	+	+
B. brevis	+	−	−	+	−	−	V	−	−	−	−	+	+	−
B. larvae	−	−	+	−	+[a]	−	V	−	−	−	−	+	+	−
B. popilliae	−	−	+	−	+[a]	−	−	−	−	−	−	+	−	+
B. lentimorbus	−	−	+	−	−	−	−	−	−	−	−	+	−	−
B. sphaericus	+	−	−	−	V	−	−	−	−	V	−	−	V	+

+, Greater than 85% of strains examined by Gordon, Haynes, and Pang (1973) positive; −, greater than 85% of strains negative; V, variable character.

[a]Growth in 2% NaCl agar.

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Distinguishing phenotypic characteristics of plant-associated *Bacillus* species.

Reva *et al.*,2002

+, >89% strains positive; D, 11–89% strains positive; −, <11% strains positive; numeric values indicate the proportion of the four strains that were positive (strain 12DX was negative in all cases of variable reactions); ND, no data.

Character	*B. insolitus**	*B. licheniformis*†	*B. megaterium*†	*B. pumilus*†	*B. subtilis*†‡	Endophytic isolates
Spore position/ Spore shape	Terminal Round	Central Oval	Central Oval	Central Oval	Central Oval	Terminal Ellipsoidal
Vacuoles in cytoplasm	+	−	+	−	−	+
Cell width >1·0 µm	+	−	+	−	−	+
Motility	D	+	+	+	+	−
Anaerobic growth	−	+	−	−	−	−
Voges–Proskauer	−	+	−	+	+	−
Oxidase	+	+	−	+	+	+
Nitrate reduction	−	+	−	−	+	−
Hydrolysis of:						
Casein	−	+	+	+	+	−
Gelatin	−	+	+	+	+	−
Starch	−	+	+	−	+	−
Urea	−	−	+	−	−	−
Acid from:						
Arabinose	−	+	+	+	−	+
Cellobiose	+	+	+	+	+	−
Galactose	ND	+	+	+	D	−
Glucose	−	+	+	+	+	+
Inositol	ND	+	+	−	+	+
Lactose	−	D	D	D	−	−
Mannitol	−	+	+	−	+	+
Maltose	−	+	+	D	+	+
Mannose	−	+	D	+	+	+
Raffinose	+	+	−	D	+	0·75
Rhamnose	ND	−	−	−	−	+
Sorbitol	ND	−	D	−	+	−
Trehalose	−	+	+	+	+	−
Xylose	−	+	+	+	D	0·75
Use of:						
Acetate	ND	+	+	−	D	−
Citrate	−	+	+	+	+	+
Gluconate	ND	+	+	−	−	+
Propionate	ND	+	−	−	−	−
Arginine dihydrolase	ND	+	−	−	−	−
Phenylalanine deaminase	−	−	+	−	−	−
Growth in:						
5% NaCl	−	+	+	+	+	+
10% NaCl	−	−	−	+	D	+
Growth at 50 °C	−	+	−	−	−	−

* Data from Larkin & Stokes (1967) and Rüger *et al.* (2000).

† Data from Priest *et al.* (1988).

‡ Data from Nakamura *et al.* (1999).

Key differential characteristics of the *Bacillus* spp.

Summary of test results

species	Lecithinase	Motility	Penicillin susceptibility	Crystal formation
Bacillus anthracis	+*	-	S	-
Bacillus cereus	+	+	R	-
Bacillus megaterium	-	+	R	-
Bacillus mycoides	+	-	R	-
Bacillus thuringiensis	+	+	R	+
Bacillus circulans	-	+	R	-
Bacillus coagulans	-	+	R	-
Bacillus licheniformis	-	+	R	-
Bacillus pumilus	-	+	R	-
Bacillus subtilis	-	+	R	-
Bacillus sphaericus	-	+	R	-

* *B. anthracis* may produce narrow lecithinase zones and colony may need to be scraped away to see reaction.

Standards Unit, 2015

Identification of *Bacillus* species

Species	Motility	Catalase	Parasporal bodies	Lipid globules in protoplasm	Lecthovitellin reaction	Citrate Utilization	Anaerobic Growth	V-P Reaction	PH in V-P medium <6	Growth at 50°C	Growth at 60°C	Growth in 7% NaCl	Acid from AS glucose	Acid and gas from AS glucose	Nitrate reduction	Casein Hydrolysis	Starch Hydrolysis	Propionate Utilization
Morphological Group 1																		
B. megaterium	V	+	-	+	-	+	-	-	V	-	-	+	+	-	V	+	+	N
B. cereus	+	+	-	+	+	+	+	+	+	-	-	+	+	-	+	+	+	N
B. cereus mycoides	-	+	-	+	+	+	+	+	+	-	-	+	+	-	+	+	+	N
B. anthracis	-	+	-	+	+	V	+	+	+	-	-	+	+	-	+	+	+	N
B. thuringiensis	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	N
B. licheniformis	+	+	-	-	-	+	+	+	+	+	-	+	+	-	+	+	+	+
B. subtilis	+	+	-	-	-	+	-	+	V	V	-	+	+	-	+	+	+	-
B. pumulis	+	+	-	-	-	+	-	+	+	V	-	+	+	-	-	+	-	-
B. firmus	v	+	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	-
B. coagulans	+	+	-	-	-	V	+	+	+	+	V	-	+	-	V	V	+	-
Morphological Group 2																		
B. polymyxa	+	+	-	-	-	-	+	+	V	-	-	-	+	+	+	+	+	N
B. mascerans	+	+	-	-	-	V	+	-	+	+	-	-	+	+	+	-	+	N
B. circulans	V	+	-	-	-	V	V	-	+	V	-	V	+	-	V	V	+	N
B. stearothermophilus	+	V	-	-	-	-	-	-	+	+	+	-	+	-	V	V	+	N
B. alvei	+	+	-	-	-	-	-	+	+	-	-	-	+	-	-	+	+	N
*B. laterosporus**	+	+	-	-	(+)	-	+	-	+	-	-	-	+	-	+	+	-	N
B. brevis	+	+	-	-	-	V	+	-	-	V	V	-	+	-	V	+	-	N

Valerius de Vos

Identification of *Bacillus* species

Biochemical characteristics of Morphological Group 1

	B. megaterium	*B. cereus*	*B. cereus* var *.mycoides*	*B. anthracis*	*B. thuringiensis*	*B. licheniformis*	*B. subtilis*	*B. pumilus*	*B. firmus*	*B. coagulans*
LV (egg yolk) reaction	-	+	+	+	+	-	-	-	-	-
Citrate utilization	+	+	+	v	+	+	+	+	+	v
Anaerobic growth	-	+	+	+	+	+	-	-	-	+
V-P reaction	-	+	+	+	+	+	+	+	-	v
Nitrate reduction	v	+	+	+	+	+	+	-	+	v
Indole production	-	-	-	-	-	-	-	-	-	-
Growth in 7% NaCl	+	+	+	+	+	+	+	+	+	-
Starch hydrolysis	+	+	+	+	+	+	+	-	+	+
Casein hydrolysis	+	+	+	+	+	+	+	+	+	v
Gelatine hydrolysis	+	+	+	+	+	+	+	+	+	-
Urease activity	v	v	v	-	v	V	v	-	-	-
*Acid from: Glucose	+	+	+	+	+	+	+	+	v	+
Mannitol	v	-	-	-	-	+	+	+	+	v
Xylose	v	-	-	-	-	+	+	+	v	v
Arabinose	v	-	-	-	-	+	+	+	v	v
Haemolysis (blood agar)		+	+	-	+					
Motility		+	-	-	+					
Propionate utilization						+	-			
Parasporal bodies		-	-	-	+					
Tyrosine hydrolysis	+/-	+	+/-	-	+	-	-	-	-/+	-
Growth in 0,001% lysozyme	-	+	+	+	+	-	-/+	+/-	-	-

Valerius de Vos

Lysozyme test

- Inoculate a loopful of a broth culture into a tube of the Lysozyme Resistance Medium and into a control tube of nutrient broth.
- After incubation for up to 7 to 14 days observe for growth or its absence.
- Members of the *B. cereus* group are resistant to lysozyme.
- **Lysozyme Resistance Medium**

  Nutrient broth 99 ml

  Lysozyme solution 1 ml

  (10,000 enzyme units/ml of distilled water)

The members of the *B cereus* group spp. include *B. cereus*, *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus weihenstephanensis*.

Tyrosine test

- Inoculate plates of the Tyrosine Decomposition Agar with one streak of inoculum and incubate.
- Observe at up to 21 days for clearing of the tyrosine crystals around and below the growth.
- *B. cereus* and other members of the *B. cereus* group except for *B. anthracis* readily decompose tyrosine.
- Tyrosine Decomposition Agar:
- L-Tyrosine 0.5 g
- Distilled water 10 ml
- Sterile nutrient agar 100 ml

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Casein decomposition test

- Inoculate plates of milk agar with one streak of inoculum and examine after incubation at 7 and 14 days for clearing of the casein around and underneath the growth.
- Casein Decomposition; Milk Agar
- Agar 1 g in 50 ml of distilled water Skim milk powder 5 g in 50 ml of distilled water.

Fatty acid composition of several species of the genus *Bacillus* rRNA group 1 of Ash *et al.* 1991[a]

Species	Saturated acids								Saturated *iso*-branched acids							Saturated *anteiso*-branched acids					Unsaturated acids					Other acids	Reference
	10:0	12:0	13:0	14:0	15:0	16:0	17:0	18:0	*i*12:0	*i*13:0	*i*14:0	*i*15:0	*i*16:0	*i*17:0	*i*18:0	*a*13:0	*a*14:0	*a*15:0	*a*16:0	*a*17:0	*i*16:1	*a*16:1	*i*17:1	*a*17:1	*i*18:1		
Group 1[b]																											
B. cereus				3	Tr	3				10	2	55	3					3		3	7		9	Tr			Kämpfer (1994)
B. cereus			1	3	5	2				8	2	49	3	6		1		4		1	4		3			8	Shida *et al.* (1997a)
B. thuringiensis				3		4				8	4	32	5	1		1		5		1	14		10	3		9	Kämpfer (1994)
Group 2																											
B. amyloliquefaciens				Tr	Tr	3				Tr	1	34	3	10				36		7	1			2		3	Kämpfer (1994)
B. amyloliquefaciens (mean of 3 strains)						3					1	25	4	14				38		12	1		2				Roberts *et al.* (1996)
B. atrophaeus (mean of 5 strains)						5					1	31	4	9				36		7	3		2				Roberts *et al.* (1996)
B. firmus				1	1	1					3	32	1	2				45		2	5		1	3		3	Kämpfer (1994)
B. flexus				2		3					4	30	Tr	2				47		4	2		2	Tr		4	Kämpfer (1994)
B. fumarioli (mean of 20 strains)												51	6	15				6	4	14			2				Logan *et al.* (2000)
B. jeotgali				1		3					2	49	2	4				9		4	5		7			11	Yoon *et al.* (2001c)
B. lentus				2		4					7	30	3					44		5	5						Kämpfer (1994)
B. licheniformis				Tr		2					1	34	3	11				27		9	1			3		9	Kämpfer (1994)
B. licheniformis (mean of 5 strains)						4					1	29	4	7				38		11	5		1				Roberts *et al.* (1996)
B. megaterium				2		2				Tr	6	28	Tr	1				49		3	4		1	1		3	Kämpfer (1994)
B. megaterium				2	2	2					8	33	1	Tr				45		1						6	Shida *et al.* (1997a)
B. mojavensis (mean of 22 strains)						2					1	22	3	9				43		13	3			3			Roberts *et al.* (1996)
B. oleronius				1		2						47	3	5				25		16							Kunigk *et al.* (1995)
B. pumilus				1		1				Tr	1	43						37		5	2			3		7	Kämpfer (1994)
B. sonorensis (mean of 8 strains)						5					Tr	30	3	9				37		12	1		Tr				Palmisano *et al.* (2001)
B. subtilis				Tr	Tr	1			Tr		1	27	1	8				39		10	1		3	1		8	Kämpfer (1994)
B. subtilis				Tr	2	1						28	2	6				45		7			Tr			9	Shida *et al.* (1997a)
B. subtilis (mean of 5 strains)						3					1	29	2	10				40		9	2		2				Roberts *et al.* (1996)
B. vallismortis (mean of 5 strains)						3					1	25	4	14				37		12	1		2				Roberts *et al.* (1996)
Other species																											
B. badius				2	3	3					4	41	1					10		4	5	16	7	4			Kämpfer (1994)
B. badius				2	10	2					2	60	3	2				8		2						9	Shida *et al.* (1997a)
B. badius				2	2	3					2	56	5	3				6		2	4		6	2		7	Heyndrickx *et al.* (1997)
B. circulans				3	1	3					4	14						47		7	6		1			14	Kämpfer (1994)
B. circulans				4	2	4					4	10	3					57		3						13	Shida *et al.* (1997a)
B. coagulans				2		2					1	10	4	Tr				63		17	3						Kämpfer (1994)
B. coagulans				1	5	1						2	Tr					66		23						2	Shida *et al.* (1997a)
B. simplex				1	1	4					3	10		1				69		3	6		1				Kämpfer (1994)
B. smithii						8						19	6	13				12		42						2	Andersson *et al.* (1995)

[a] Data are given for the type strains unless stated otherwise. The data for some unsaturated and branched fatty acids were combined in this table. Data are given as percentage of total cellular fatty acids. Tr, traces (<1%). Because some of the values were summed off, the total sum of fatty acids is not in all cases 100%. More details about the heterogeneity of fatty acids within some species can be found in the study of Kämpfer (1994). *Bacillus cohnii*, grouped into rRNA group 1 (Nielsen *et al.* 1994), showed 46–54% *i*15:0 and *a*15:0, and a proportion of unsaturated fatty acids from 22 to 27% (Spanka & Fritze 1993).

[b] For *B. anthracis*, fatty acid profiles were given by Lawrence *et al.* (1991). When grown on RCM-medium (a complex medium), *B. anthracis* produced (similar to *B. cereus*) high amounts of 16:0 (>50%) and only 7% of *i*15:0. A cultivation on a synthetic medium (RM-medium) increased the amount of *i*15:0 to 16%. Details are given by Lawrence *et al.* (1991).

Berkeley *et al.*,2002

Primers used for amplification and sequencing of the 16S rRNA gene of *Bacillus anthracis*, *B. thuringiensis*, and *B. cereus*

8F	5′AGT TGA TCC TGG CTC AG 3′
1492R	5′ACC TTG TTA CGA CTT3′
Primers for amplification of the 16S rRNA gene	
67F	5′TGA AAA CTG AAC GAA ACA AAC 3′
1671R	5′CTC TCA AAA CTG AAC AAA ACG AAA 3′
Inner primers used for nested PCR on clinical samples	
23F	5′ACA AAC AAC GTG AAA CGT CAA 3′
136R	5′AAA CGA AAC ACG GAA ACT T 3′
Primers used for sequencing of the 16S rRNA gene	
104F	5′GGA CGG GTG AGT AAC ACG TG 3′
104R	5′CAC GTG TTA CTC ACC CGT CC 3′
1230F	5′TAC ACA CGT GCT ACA ATG 3′
1390F	5′GGG CCT TGT ACA CAC CG 3′
1390R	5′CGG TGT GTA CAA GGC CC 3′
8F	5′AGT TGA TCC TGG CTC AG 3′
357F	5′TAC GGG AGG CAG CAG 3′
357R	5′CTG CTG CCT CCC GTA 3′
530F	5′CAG CAG CCG CGG TAA TAC 3′
530R	5′GTA TTA CCG CGG CTG CTG 3′
790F	5′ATT AGA TAC CCT GGT AG 3′
790R	5′CTA CCA GGG TAT CTA AT 3′
981F	5′CCC GCA ACG AGC GCA ACC C 3′
981R	5′GGG TTG CGC TCG TTG CGG G 3′

Sacchi *et al.*,2002

ISR-PCR from *Bacillus* spp. strains
PCR Amplification

1. *Taq* polymerase and 10X STR buffer (Promega).
2. Thermal cycler (PE 9700, Applied Biosystems, CA).
3. 100 mM Stock solution of primers:

- ISR-1494 (5‘-GTCGTAACAAGG TAGCCGTA-3') and
- ISR-35 (5‘-CAAGGCATCCACCGT-3').

Intergenic spacer region-PCR (ISR-PCR).

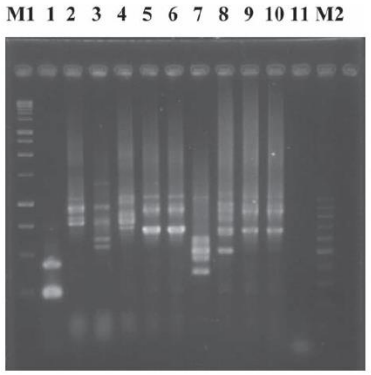
Spencer and Spencer,2004

ISR-PCR from *Bacillus* spp. strains
DNA isolation

1. Lysis buffer TEC-SDS: 10 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 mM NaCl, and 2% (w/v) SDS.
2. 20 mg/mL Proteinase K in distilled water.
3. 3 M Sodium acetate, pH 5.2.
4. TE-buffer-saturated phenol.
5. Chloroform: isoamyl alcohol, 24:1 (v/v).
6. Isopropanol and 70% ethanol
7. RNAase A solution (10 mg/mL stock) in distilled water.

Spencer and Spencer,2004

ISR-PCR from *Bacillus* spp. strains
Agarose gel electrophoresis



- Lanes:
- M1, 1 kb DNA Ladder;
- 1, *B. subtilis* 1 A1;
- 2, *B. halodurans*
- From lanes 3 to 10, *Bacillus* sp. alkaliphilic strains: 3, MIR32; 4, MRL1; 5, MRL2; 6, MRL22; 7, MRL33; 8, MRL4; 9, MRL5; 10, MRL5;
- 11, control reaction without DNA template;
- M2, 100 bp DNA Ladder.
- Band between 200 and 600 bp are obtained for members of *subtilis* and *cereus* groups and patterns including long ISR spacers, generally between 600 and 1200 bp are typical or most alkaliphilic *Bacillus* spp.

Spencer and Spencer,2004

Bacillus spp.

Endoglucanase gene sequences

Specific primers for identification of *B. subtilis*

- Primers ENIF and EN1R from endoglucanase gene were used to amplify a 1311 bp DNA fragment.
- The specificity of the primers was tested with seven reference strains and 28 locally isolated strains of endoglucanase positive *Bacillus* species.
- Two sets of primers:
- EN1F (103–124 bp) 5′-CCAGTAGCCAAGAATGGCCAGC-3′,
- EN1R (1,413–1,393 bp) 5′-GGAATAATCGCCGCTTTGTGC-3′) were designed by analyzing the conserved regions of the aligned sequences.

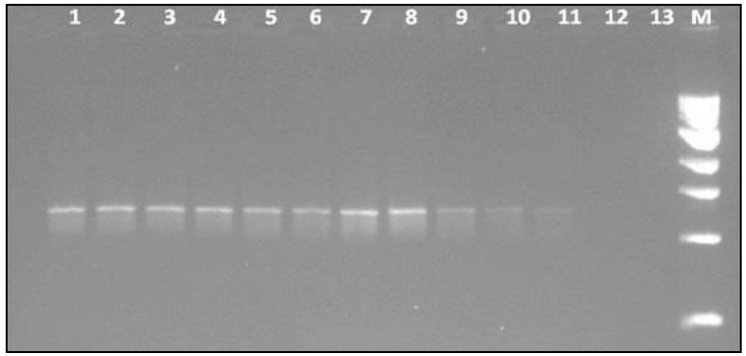
Ashe *et al.*,2014

Bacillus spp.

Endoglucanase gene sequences

Specific primers for identification of *B. subtilis*

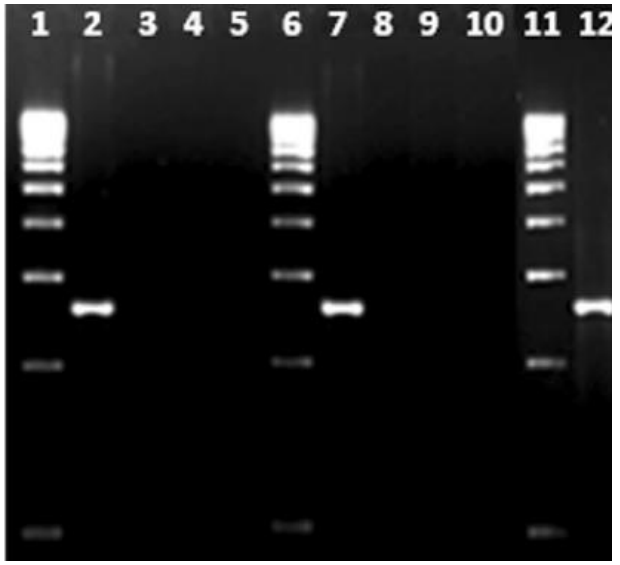
- Limit of detection of endoglucanase gene in different concentration of DNA.
- Lane 1 100 ng, lane 2 50 ng,
- lane 3 10 ng, lane 4 5 ng,
- lane 5 1 ng, lane 6 100 pg,
- lane 7 50 pg, lane 8 10 pg,
- lane 9 5 pg, lane 10 1 pg,
- lane 11 500 fg, lane 12 100 fg,
- lane 13 negative control,
- lane M size marker (1 kb ladder, NEB).



Ashe *et al.*,2014

Bacillus spp.
Endoglucanase gene sequences

- PCR amplification for endoglucanase gene in different *Bacillus* spp.
- Lane 1 size marker (500 bp ladder);
- lanes 2-5 *B. subtilis* ATCC-6,051, *B. cereus*-ATCC 13,061, *B. pumilus* ATCC-14,884, *B. megaterium* ATCC-9,885;
- lane 6 size marker (500 bp ladder);
- lanes 7-10 *B. subtilis* ATCC-11,774, *B. thuringiensis* ATCC-10,792, *B. licheniformis* ATCC-13,061, *B. amyloliquefaciens* CF8;
- lane 11 size marker (500 bp ladder);
- lane 12 *B. subtilis* C11B1.



Ashe *et al.*,2014

Bacillus subtilis group

Bacillus pumilus

Common PCR

- PCR amplification of 16S rDNA from genomic DNA of tested strains.
- A DNA fragment of 16S rDNA was amplified by PCR from 19 tested strain genomic DNA extracted using the method described by Gao et al. (12).
- The PCR amplification was performed by using primer pairs 27f/1492r (27f: 5′-AGAGTTTGATCCTGGCTCAG-3′, 1492r: 5′-GGTTACCTTGTTACGACTT3′) (8) and the following procedure: denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 90 s, and extension at 72°C for 90 s; and a final extension at 72°C for 7 min.
- The PCR product was purified from 1.2% agarose with a a Gel Extraction kit (OMEGA).

Peng *et al.*,2013

Bacillus subtilis group

Bacillus pumilus

Nested PCR

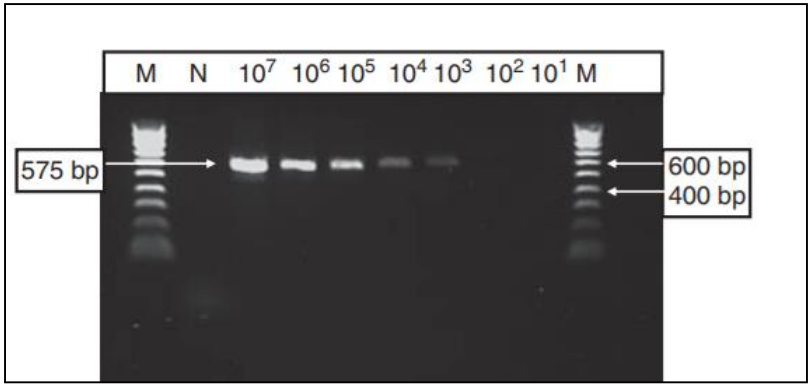
- The PCR amplification was performed by using specific primers OLI-1 (5′-GGGGGTAGCTTGCTACCTGCC-3′) and OLI-2 (5′-CGTCATCCACTCCAGGT-ATTAACCGAA-3′) and the following procedure: denaturation at 94°C for 2 min; followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 68°C for 20 s, and extension at 72°C for 20 s; and a final period of 10 min at 72°C.
- The second-stage PCR was performed by using nested primer pairs JE2 (5′-GTGGGGGATAACTAGTCGAAAGAC-3′)/Y2 (5′-CCCACTGCTGCCTCCCGTAGGAGT-3′) and template from the first reaction product.
- In the second stage, 30 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 20 s and a final period of 10 min at 72°C were used.
- The second PCR product was detected on 2% agarose gel with ethidium bromide (0.5 mg/ml).
- A specific PCR product of 172 bp was predicted.

Peng *et al.*,2013

Bacillus cereus group

motB gene (encoding flagellar motor protein)

- A PCR technique was developed as a reliable and rapid identification method for the *Bacillus cereus* group species, based on a unique conserved sequence of the *motB* gene (encoding flagellar motor protein) from
- *Bacillus cereus*,
- *Bacillus thuringiensis*, and
- *Bacillus anthracis*.



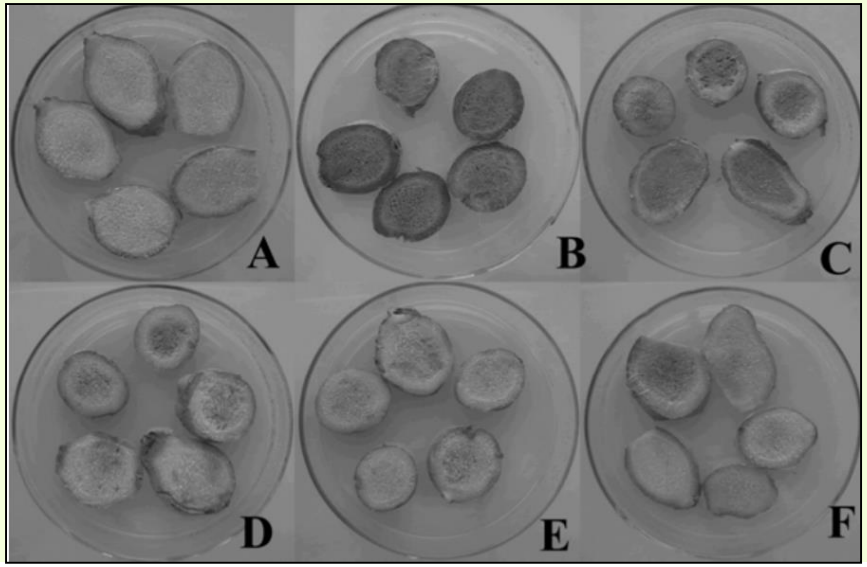
Sensitivity of PCR detection of *Bacillus cereus* DSM 4312 with primers BCFomp1/ BCRomp1. Numbers above lanes indicate the level of inoculation as confirmed by conventional plate count enumeration. N, negative control; M, molecular marker.

Oliwa-Stasiak *et al.*,2010

Pathogeniciy test

Bacillus pumilus, ginger rhizome rot pathogen in China

- Symptoms caused by *Bacillus pumilus* strain GR8 on rhizome slices. A, Healthy rhizome slices that were inoculated with sterile distilled water and incubated in petri dishes for 2 days at 30°C.
- B and C, Severely rotten rhizome slices (symptoms: brown discoloration and water soaking) that were inoculated with a suspension of strain GR8 at 10^9 and 10^8 CFU/ml, respectively.
- D-F, Mild rotten rhizome slices (symptoms: brown discoloration) that were inoculated with a suspension of strain GR8 at 10^7, 10^6 , and 10^5 CFU/ml, respectively.



Peng *et al.*,2013

The genus *Paenibacillus*

- The name *Paenibacillus* is derived from the Latin adverb *paene*, meaning almost; almost a *Bacillus.*
- Isolated from a wide range of sources, the genus *Paenibacillus* comprises bacterial species relevant to humans, animals, plants, and the environment.
- Many *Paenibacillus* species can promote crop growth directly via:

1. biological nitrogen fixation,
2. phosphate solubilization,
3. production of the phytohormone indole-3-acetic acid (IAA), and
4. release of siderophores that enable iron acquisition.

Grady *et al.*,2016

The genus *Paenibacillus*

- Species of *Paenibacillus* were originally included in the genus *Bacillus*, which historically was defined based on morphological characteristics in common with the type species *Bacillus subtilis*, isolated in 1872.
- When 16S rRNA gene sequences were determined for standard strains of 51 species then defined as *Bacillus*.
- Phylogenetic analyses showed that these sequences segregated into at least five distinct clusters, one of which was reassigned to the novel genus *Paenibacillus* in 1993 and includes the type species *Paenibacillus polymyxa*.

Grady *et al.*,2016

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

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.

Some *Paenibacillus* species are known to infect various organisms, including honeybees and the parasite vector *Biomphalaria glabrata*, and occasionally present as opportunistic infections in humans(Grady *et al*.,2016).

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

The genus *Paenibacillus*

Fatty acid composition of species of the genus *Paenibacillus*[a]

	Saturated acids								Saturated *iso*-branched acids							Saturated *anteiso*-branched acids					Unsaturated acids					
Species	10:0	12:0	13:0	14:0	15:0	16:0	17:0	18:0	*i*12:0	*i*13:0	*i*14:0	*i*15:0	*i*16:0	*i*17:0	*i*18:0	*a*13:0	*a*14:0	*a*15:0	*a*16:0	*a*17:0	*i*16:1	*a*16:1	*i*17:1	*a*17:1	*i*18:1	Reference
P. alginolyticus				Tr	1	3					1	5	10	2				70		6						Shida *et al.* (1997a)
P. alvei				2	1	9					1	11	6	5				58		8	4					Shida *et al.* (1997a)
P. alvei (mean of 6 strains)				2	1	8					Tr	11	5	4				57		7	3					Nakamura (1996)
P. amylolyticus				3	Tr	13					2	2	9	3				46		2	Tr					Shida *et al.* (1997b)
P. apiarius				1	2	5					Tr	8	4	5				60		16	1		Tr			Shida *et al.* (1997a)
P. apiarius (mean of 6 strains)				1	1	4					Tr	14	5	6				52		10	3		2			Nakamura (1996)
P. azoreducens				3	Tr	22					Tr	6	9	6				34		20						Meehan *et al.* (2001)
P. azotofixans				2	Tr	18					1	2	7	1				62		5	Tr					Shida *et al.* (1997a)
P. borealis				18	Tr	10				Tr	5	11	10	5		Tr		35		2	1		Tr			Elo *et al.* (2001)
P. chondroitinus				1	2	6					2	2	10	1				70		3						Shida *et al.* (1997a)
P. chibensis				Tr	1	5					1	4	12	3				58		14						Shida *et al.* (1997b)
P. curdlanolyticus				1	1	7					2	2	24	1				56		3						Shida *et al.* (1997a)
P. dendritiformis				1	3	6						6	5	8				43		21	3		1			Tcherpakov *et al.* (1999)
P. glucanolyticus				1	1	11					1	3	14	2				56		8	Tr		Tr			Shida *et al.* (1997a)
P. illinoisensis				2	1	24					2	1	6	1				57		5						Shida *et al.* (1997b)
P. kobensis				1	3	12						2	9	Tr				65		3						Shida *et al.* (1997a)
P. koreensis						28							21					51								Chung *et al.* (2000)
P. larvae (mean of 6 strains)				3	2	28					1	11	3	7				28		9						Nakamura (1996)
P. larvae ssp. *pulvifaciens*				1	1	6						10	5	5				49		21	1					Shida *et al.* (1997a)
P. lautus				1	Tr	20					Tr	3	4	6				37		11	Tr		1			Shida *et al.* (1997b)
P. lautus				2		7					2	6	9					58		9	4					Kämpfer (1994)
P. lautus				1	Tr	16					1	1	7	1				57		10	2		Tr			Shida *et al.* (1997a)
P. macerans				4	Tr	18					8	3	16	1				36		12	Tr					Shida *et al.* (1997a)
P. macquariensis				1	1	3					1	5	3	Tr				81		1	1		Tr			Shida *et al.* (1997a)
B. pabuli				2	1	5					5	5	9					60		3	2					Kämpfer (1994)
P. pabuli				1	Tr	10					1	2	5	1				74		4						Shida *et al.* (1997a)
P. peoriae				1	Tr	11					1	8	7	5				55		10	Tr		Tr			Shida *et al.* (1997a)
P. polymyxa				Tr	Tr	9					Tr	1	6	2				63		17						Shida *et al.* (1997a)
P. thiaminolyticus (mean of 6 strains)				2	Tr	12					Tr	6	3	4				47		16	5		1			Nakamura (1996)
P. thiaminolyticus				1	Tr	11						11	6	6				45		16	5		2			Shida *et al.* (1997a)
P. validus				1	1	11					1	4	12	3				57		7	Tr		Tr			Shida *et al.* (1997a)

[a] Data are given for the type strains unless stated otherwise. The data for some unsaturated and branched fatty acids were combined in this table. Data are given as percentage of total cellular fatty acids. Tr, traces (<1%). Because some of the values were summed off, the total sum of fatty acids is not in all cases 100%. For *P. campinasensis* (Yoon *et al.* 1998), 53% *a*15:0 were reported as the predominant fatty acid.

Berkeley *et al.*,2002

Identification of the bacterial pathogens
Fastidious bacteria

Fastidious and non-culturable xylem- or phloem-limited bacteria

Fastidious bacteria

- Most phytopathogenic bacteria invade their host plants through natural openings or wounds, colonizing intercellular spaces, expressing virulence factors and inducing various host plant responses.
- A few, however, are introduced directly into the sugar-rich phloem sieve tubes or into the water-transporting xylem elements by vascular-feeding insects.
- Their location within living (sieve tubes) or degenerated (xylem elements) plant cells, rather than in intercellular spaces, offers different challenges and opportunities for them to avoid the host plant's defense system.

Fletcher and Wayadande,2002

Fastidious bacteria

- These vascular-colonizing bacteria can be divided into three groups:

1. Wall-less mollicutes (phytoplasmas and spiroplasmas).
2. Walled phloem-inhabiting bacteria(BLOs), and
3. Walled xylem-limited bacteria(*Xylella*).

Identification of the bacterial pathogens *Xylella*

Xylem-limited prokaryotes

Disease diagnosis and pathogen diagnostics

***Xylella*, a small piece of wood, a small log**

Domain: Bacteria
Phylum: Proteobacteria

- Class: Gammaproteobacteria
  - Order: Pseudomonadales
    - Family: Pseudomonadaceae
      - Genus: *Pseudomonas*
  - Order: Xanthomonadales
    - Family: Xanthomonadaceae
      - Genera: *Xanthomonas* and *Xylella*

Deep phylo-taxono genomics reveals *Xylella* as a variant lineage of plant associated *Xanthomonas* and supports their taxonomic reunification along with *Stenotrophomonas* and *Pseudoxanthomonas* (Bansal *et al.*,2021)

Euzéby,2018;CABI,2018

Xylella fastidiosa nomenclature

- *Xylella* Wells *et al*. 1987, gen. nov.
- Type species: *Xylella fastidiosa* Wells, Raju, Hung, Weisburg, Mandelco-Paul and Brenner,1987, 141.
- *Xylella fastidiosa* gen. nov., sp. nov.: Gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp.

The causal agent of PD was isolated from grape in pure culture for the first time in 1978 (Davis *et al*.,1978). However, this xylem-inhabiting, vector-transmitted, Gram-negative, very slow growing bacterium, was only properly described, classified and named *Xylella fastidiosa* in 1987 (Wells *et al*.,1987).

Janse and Obradovic,2010;..

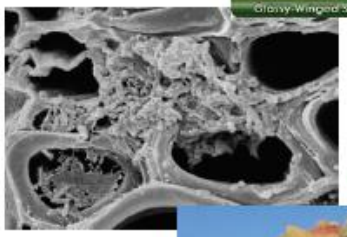
The genera *Xanthomonas* and *Xylella* belong to the same family and are made up of several species

Xylem-inhabiting Fastidious Bacteria

Pierce's disease of grape

Xylella fastidiosa

- Small with rippled cell wall, Gram (-)
- No flagella
- Grows on complex nutritional media
- Transmitted by sharpshooter leafhoppers



Culturable…but slow.

Pierce's disease

- In 1892, Newton B. Pierce, a bacteriologist and state plant pathologist of California, examined grapevines (*Vitis vinifera*) with a scorch and decline of unknown cause.

Newton B. Pierce

Newton B. Pierce, As the Special Agent of Department of Agriculture was Appointed in 1890.

Gould & Lashomb,2005

Habitat

Pathogen, endophyte and commensal

Evolved with plants to exist as a xylem-limited endophyte

- *Xylella fastidiosa* has been found in more than 600 plant species belonging to 63 diverse plant families (Castro *et al.*,2021).
- It may colonizes or infects host plant species.
- *X. fastidiosa* has been proposed to be primarily an endophyte because an interaction does not always result in disease (Chatterjee *et al.*,2008).
- The bacterium colonizes a wide range of host plants, but reports where there is a pathogenic effect of *X. fastidiosa* on its host indicate a much smaller number of species, so Xf is typically considered a commensalist (Sicard *et al.*,2018).
- According to Castro *et al.*,2021, in the vast majority of its hosts, it is considered a benign commensal.

Johnson *et al.*,2021;..

Habitat

Pathogen, endophyte and commensal

Evolved with plants to exist as a xylem-limited endophyte

- There is no apparent specificity between a particular *X. fastidiosa* subspecies and insect vector species.
- In fact, individual glassy-winged sharpshooter (GWSS) (*Homalodisca vitripennis*) can acquire more than 1 *X. fastidiosa* subspecies in its foregut and can potentially transmit these strains to a variety of plants where the bacterium can behave as:

1. Pathogen, or
2. a commensal endophyte.

- However, alternative hosts are an important component of the epidemiology of Xf diseases.

Castro *et al.*,2021;..

Habitat

Pathogen, endophyte and commensal

Evolved with plants to exist as a xylem-limited endophyte

- The bulk of the research on *X. fastidiosa* is biased toward isolates that are pathogenic in economically important hosts.
- The mechanism by which *X. fastidiosa* causes disease only in certain hosts, but not others, has not been fully elucidated, and its interactions with commensal hosts is largely understudied.
- However, it is speculated that compatibility between xylem pit membrane carbohydrate composition and *X. fastidiosa*–secreted cell wall–degrading enzymes mediate disease onset and progression (Ingel *et al.*,2019; Sun *et al.*,2011).

Castro *et al.*,2021

Habitat

Pathogen, endophyte and commensal

Evolved with plants to exist as a xylem-limited endophyte

- In addition, the O antigen is a critical component in evading initial immune recognition in the susceptible grapevine immune system, and it is tempting to speculate that O antigen composition dictates the type of symbiotic association with the plant commensalism versus parasitism (Rapicavoli *et al.*,2018).
- Understanding the mechanisms that underlie how different Xylella–plant host interactions skew toward parasitism or commensalism is an area of research that is ripe for exploration.

Castro *et al.*,2021

Habitat

Insect and plant hosts

Evolved with plants to exist as a xylem-limited endophyte

- It is a destructive disease which attacks a wide range of susceptible, commercially grown crops including:

1. economically important crops (citrus, stone fruits, grapevine and olive), in addition to
2. wild forest trees, shrubs and landscape plants.

- It is known to have a remarkably broad host range, with 359 plant species, from 204 genera and 75 different botanical families recorded.

Shubib and Hamdan,2017; Johnson *et al.*,2021

Habitat

Evolved with plants to exist as a xylem-limited endophyte

Endophytic lifestyle

- *Xylella fastidiosa* is a gram-negative bacterium (family Xanthomonadaceae) that has evolved with plants to exist as a xylem-limited endophyte(Shubib and Hamdan,2017).
- The bacterium is a plant endophyte native to the Americas, which develops in up to 300 plant species including ornamental and agricultural plants (Godefroid *et al*.,2019).
- *Xylella fastidiosa* – a devastating agricultural pathogen with an endophytic lifestyle(Burbank and Roper,2021).

Milestones in the study of *Xylella fastidiosa*

- Xf has been identified as the causative agent of Pierce's disease, which has been causing extensive damage to vineyards in California for almost 130 years.
- However, it was only at the end of the 1970s that this fastidious bacterium could be isolated on solid medium.

Pierce, N.B.; Newton, B. 1892. The California Vine Disease: A Preliminary Report of Investigations; G.P.O.: Washington, DC, USA.

Clavijo-Coppens *et al.*,2010

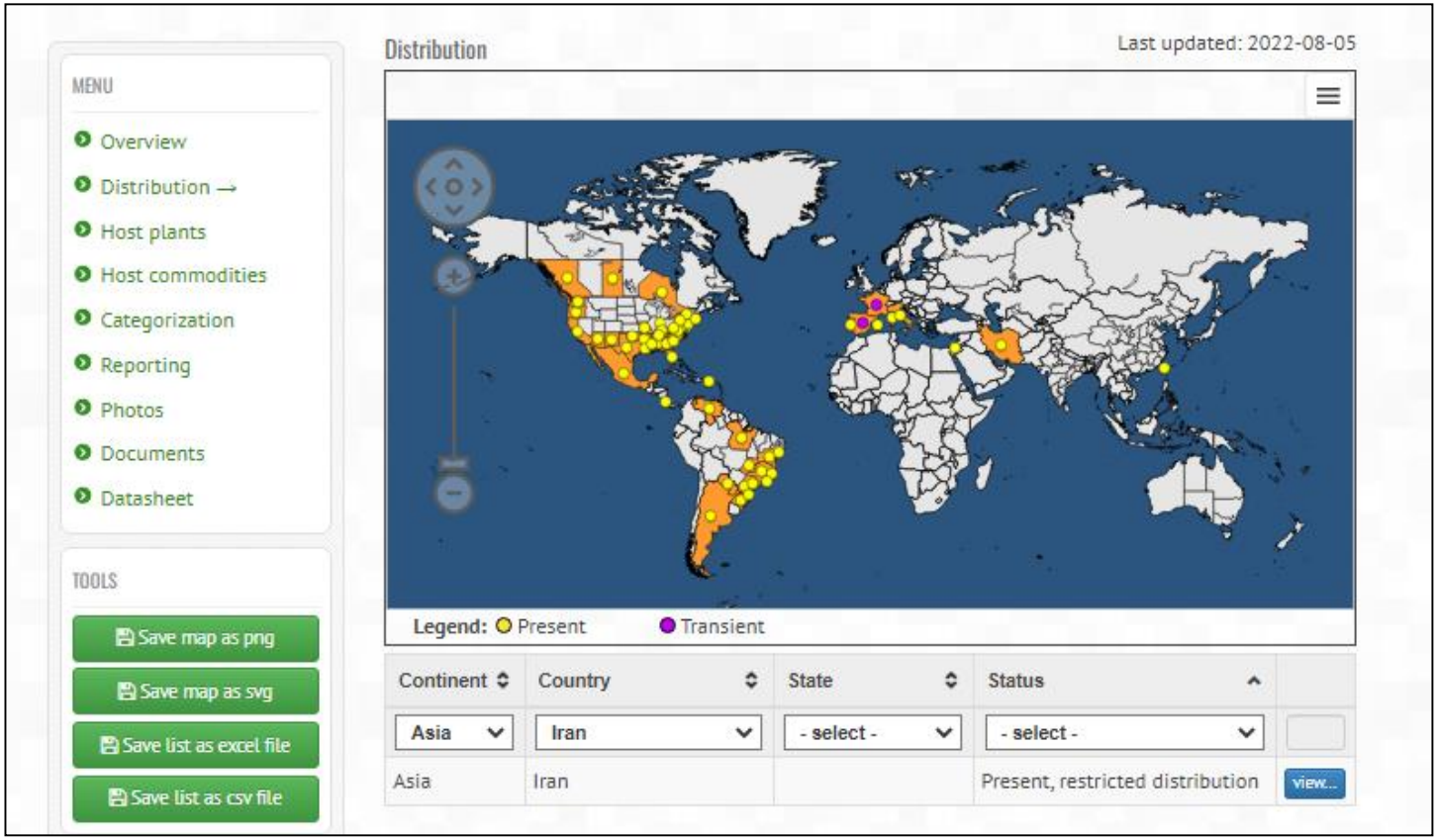
Milestones in the study of *Xylella fastidiosa*

First report of Pierce's disease and phony peach disease(Pierce and Newton,1892)	1890s
Graft transmission of Pierce's disease and phony peach disease	1939
Leafhopper transmission of Pierce's disease and alfalfa dwarf	1946
Tetracycline suppression of Pierce's disease symptoms	1971
Association of rickettsia like bacteria with Pierce's disease and phony peach disease	1973
Isolation of bacterium from infected grapevine	1978
Pierce's disease bacterium associated with leaf scorch	1980
Description of the *Xylella fastidiosa* species	1987

Pierce, N.B.; Newton, B. 1892. The California Vine Disease: A Preliminary Report of Investigations; G.P.O.: Washington, DC, USA.

Lashomb *et al.*,2001

Geographical distribution of *Xylella* spp. In Iran

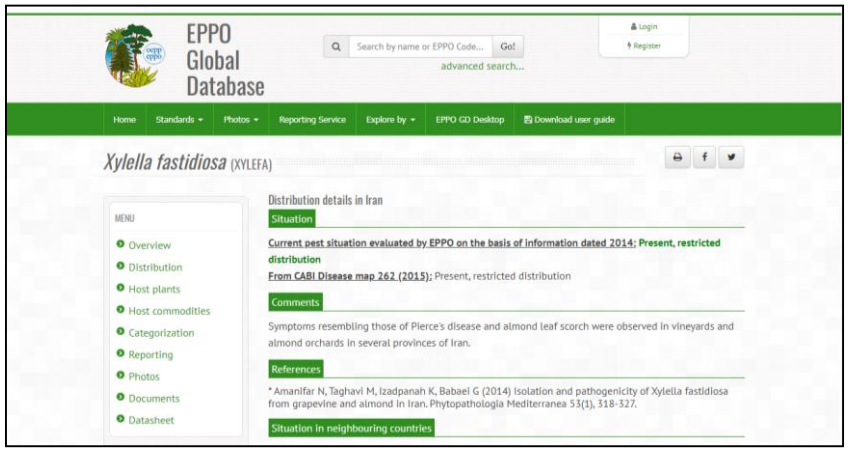


EPPO Global Database,2022

Geographical distribution of *Xylella* spp. In Asia: Iran, Israel and Taiwan

- Symptoms resembling those of Pierce's disease and almond leaf scorch were observed in vineyards and almond orchards in several provinces of Iran.
- Amanifar N, Taghavi M, Izadpanah K, Babaei G (2014). Isolation and pathogenicity of *Xylella fastidiosa* from grapevine and almond in Iran. Phytopathologia Mediterranea 53(1), 318-327.

Asia	India		Absent, unreliable record	view...
Asia	India	Himachal Pradesh	Absent, unreliable record	view...
Asia	Iran		Present, restricted distribution	view...
Asia	Israel		Present, few occurrences	view...
Asia	Lebanon		Absent, invalid record	view...
Asia	Taiwan		Present, restricted distribution	view...



EPPO Global Database,2022

Bacterial Leaf Scorch (BLS)

BLS symptoms vs. Abiotic scorch

- **BLS Symptoms:**
- Irregular, 'tri-color' scorch.
- Older leaves more severely affected.
- **Abiotic scorch:**
- Uniformly affects new and older leaves.

Symptoms caused by fastidious vascular bacteria

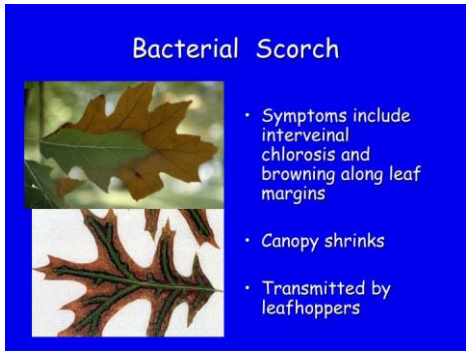
Xylella fastidosa

- Nine *Xylella fastidiosa* symptoms you should know about!

1. Scorching(Leaf scorching, otherwise known as leaf burn or sun scorch, is the browning of the plant tissue);
2. Discoloration;
3. Stunting;
4. Wilting;
5. Premature leaf abscission;
6. Shriveled fruit;
7. Premature fruit abscission;
8. Dieback;
9. Plant death.

Bacterial Leaf Scorch (BLS)

Xylella fastidosa



Leaf scorch

Leaf scorch

Leaf discoloration

Wilting

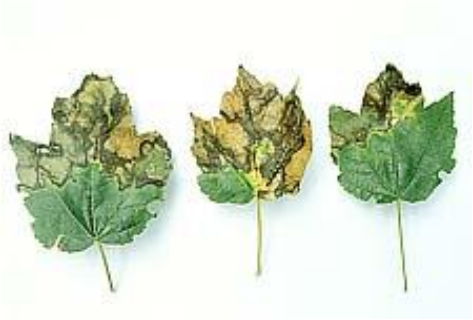
Premature leaf abscission

Shriveled fruit

Gould & Lashomb,2005; SlideServe,2020; BIOVEXO,2020

Bacterial Leaf Scorch (BLS)

Xylella fastidosa

		
Sweet gum	**Shingle oak**	**White mulberry**
		
Oak	**Elm**	**Red maple**

Gould & Lashomb,2005

Bacterial Leaf Scorch (BLS)

Xylella fastidosa

		
Almond	**Blueberry**	**Sweet orange(CVC)**
		
Coffee	**Olive**	**Oleander**

EPPO,2016

Bacterial Leaf Scorch (BLS)

Xylella fastidosa

		
Lavender	**Polygala**	**Medicago**
		
Pear	**Apricot (in mixed infection with phytoplasma)**	**Plane tree (*Platanus* species)**

Bacterial Leaf Scorch (BLS)

Plane(*Platanus* species)

Xylella fastidiosa

1. Leaf scorch, usually occurring at leaf margins first; tissues die, leaves curl inwards. Often no yellow boundary between diseased/healthy tissue as in other hosts.
2. Main veins remain green with remainder of leaf dying and turning brown.
3. Diseased leaves in crown appear scorched, brown and curl inwards.
4. Discoloration/death of foliage and dieback of twigs/branches in crown. These symptoms typically develop at base of crown before moving upwards and outwards as disease progresses.

BRIGIT

Bacterial Leaf Scorch (BLS)

Almond

Xylella fastidiosa subsp. *multiplex*

X. fastidiosa impacts – severe symptoms on almond

- Which area was first infected? How long? What genera of plants affected? How wide spread is *Xylella* in Spain? Investigations ongoing.

Citrus variegated chlorosis(CVC)

A serious disease of citrus caused by the bacterium *Xylella fastidiosa* subsp. *pauca*

- The growth rate of affected trees is greatly reduced, and twigs and branches may wilt.
- Trees in nurseries can show symptoms of variegated chlorosis as do trees aged over 10 years.
- Young trees (1-3 years) become systemically colonized by *X. fastidiosa* faster than older trees.
- Trees more than 8-10 years old are usually not totally affected, but rather have symptoms on the extremities of branches.

Small raised lesions appear on the underside of the citrus leaves.

EPPO,2018

Bacterial Leaf Scorch/Scald

Xylella fastidosa

Plum leaf scald: typical scorched symptom on plum leaf caused by *Xylella fastidiosa*.
Reproduced from Mizell *et al.*,2015.

Leaf scorch symptoms caused by *Xylella fastidiosa* on cherry.
Courtesy D. Boscia, CNR-Institute for Sustainable Plant Protect ion.

EPPO,2016

Oleander leaf scorch

Xylella fastidiosa

oleander leaf scorch (OLS)

leaves first have chlorosis

OLS strain restricted to oleander

leaves have marginal "burn"

Oleander leaf scorch

In Arizona a severe infestation in central Phoenix has killed oleanders; detected elsewhere sporadically

X. fastidiosa infection moves down the row – but NOT by pruning tools; the smoke tree sharp shooter was abundant in these oleanders

Olsen,2008

Pierce's disease

Grapevine twig and fruits

Xylella fastidiosa

- Symptoms of *Xylella fastidiosa* on grapevine showing

A. marginal necrosis surrounded by a chlorotic halo on the leaf and,

B. irregular ripening of bark.

EPPO Global Database

Pierce's disease

Grapevine twig and fruits

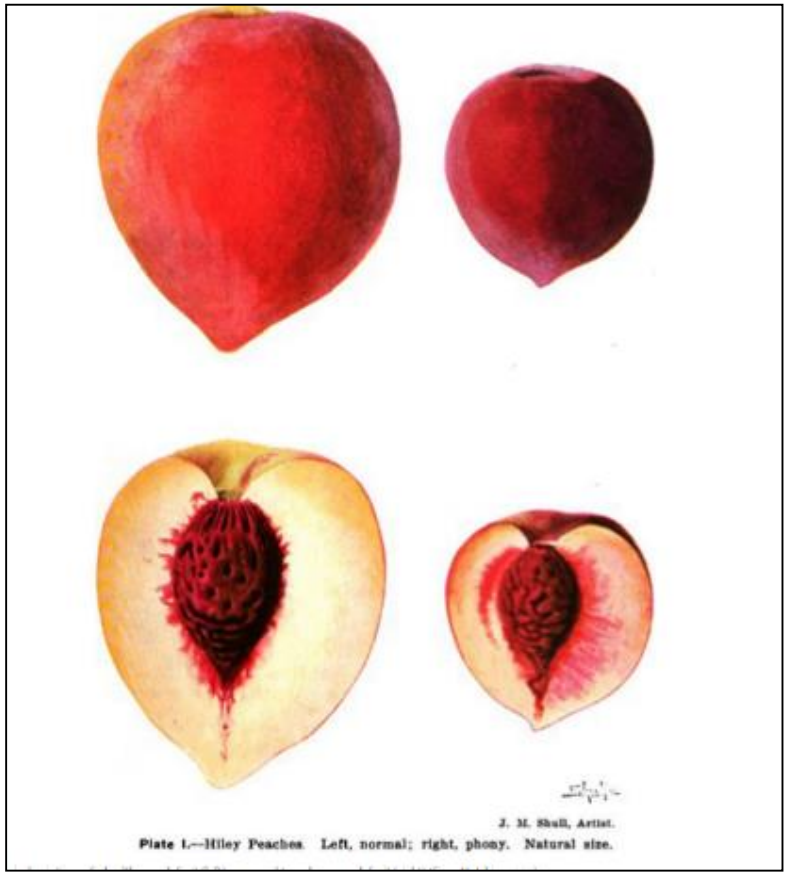
Xylella fastidiosa

	
"Green islands", "match sticks" and leaf necrosis - all characteristic symptoms of Pierce's Disease. Courtesy Thomas A. Miller	Cluster showing delayed development and shriveling. Courtesy Bill Peacock

Peacock,2000

Phony peach disease(PPD)
Xylella fastidiosa subsp. *multiplex* (Xfm)

- Historical paintings of a healthy peach fruit (left) compared to a phony peach fruit (right) (from Hutchins 1933).



Johnson *et al.*,2021

Phony peach disease(PPD)
Xylella fastidiosa subsp. *multiplex* (Xfm)

Symptoms of Phony peach disease on *Prunus persica* (peach), reduced growth of the tree on the left.

Janse and Obradovic,2010

Tree with phony peach disease (foreground) showing advanced petal fall (bloom) and leaf development as compared to healthy trees in the background (still in full bloom and with limited leaf expansion)

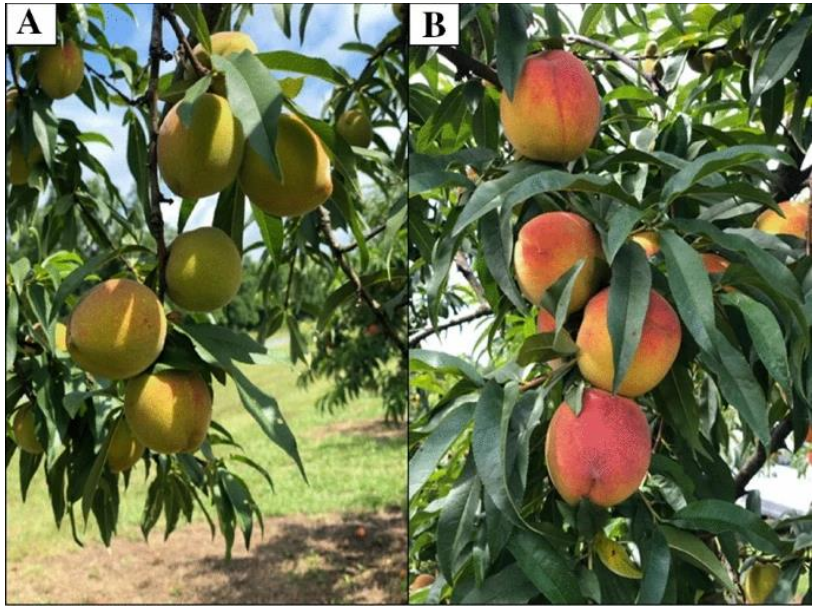
Johnson *et al.*,2021

Phony peach disease(PPD)
Xylella fastidiosa subsp. *multiplex* (Xfm)

- Peach fruit ripeness (color) comparisons on June 23, 2020.

A. Fruit on a tree with phony peach disease are less mature and ripe as compared to

B. fruit on a healthy peach tree.



Johnson *et al.*,2021

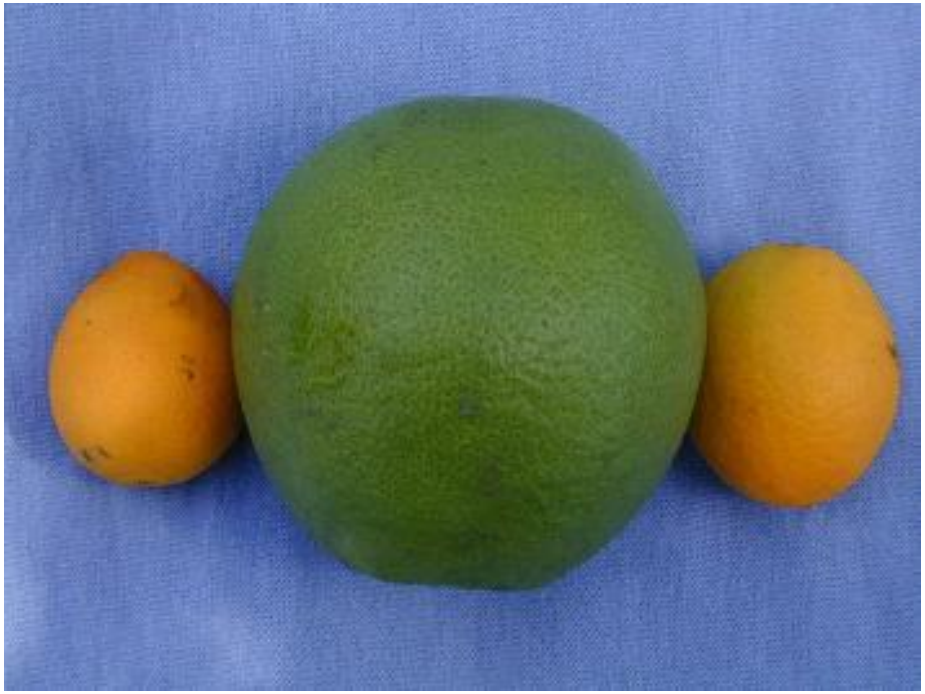
Typical symptoms
Preliminary diagnosis of BLS
Xylella fastidiosa

- Preliminary diagnosis of bacterial Leaf Scorch, BLS is made by interpreting the symptoms described above in late-summer and early fall.
- Especially useful diagnostic criteria include:

1. leaf scorch,
2. premature leaf drop,
3. the random distribution of affected branches around the canopy,
4. thinning of the crown, and the random appearance of the disease within populations of trees.

Gould &Lashomb,2005

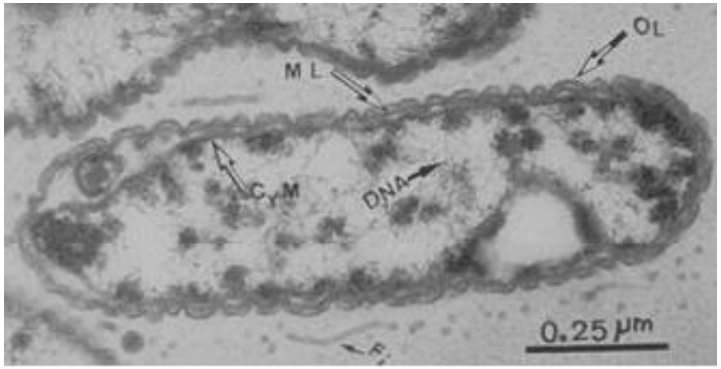
Healthy fruit between diseased citrus fruits
Xylella fastidiosa



Francisco F. Laranjeira

Cell morphology

Xylella fastidiosa



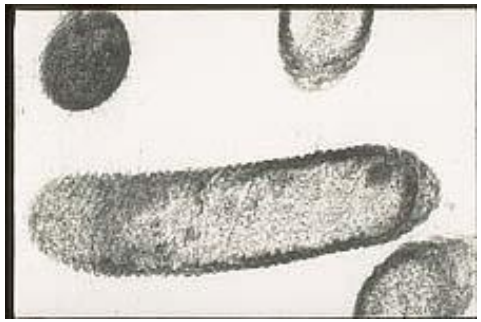
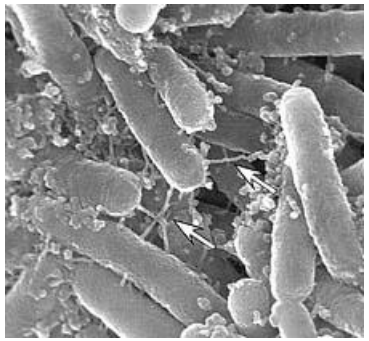
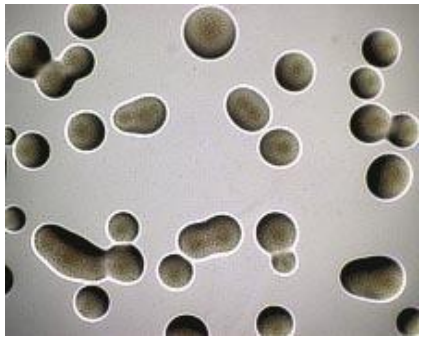
Note wavy outer wall layer.

Under dark field microscopy, the bacterium has a rod-shaped appearance with the following dimensions: 0.2-0.35 µm by 1-4 µm. Under the electron microscope, *X. fastidiosa* shows a characteristic rippled wall.

Scanning electron micrograph photo by E. W. Kitajima, Brazil

Cell morphology

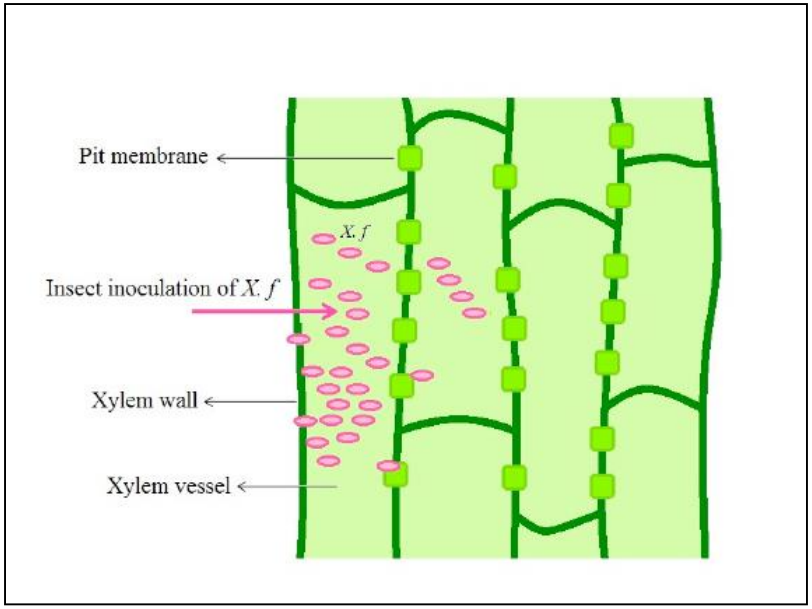
The bacterial cells often possess a rippled (undulating) cell wall

	
The undulating cell wall typical of *Xylella fastidiosa*	**A dividing cell of *Xylella fastidiosa***
	
Cells of *Xylella fastidiosa* from grape. Note the terminal fimbriae (or pili) (arrows)	**Colonies of *Xylella fastidiosa* from oak on periwinkle wilt agar medium**

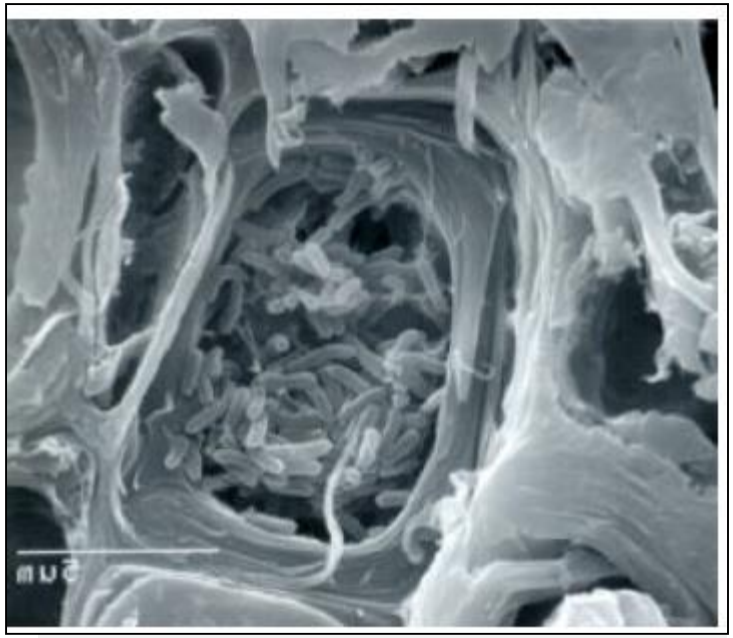
Gould&Lashomb,2005

Disease development

Xylella fastidosa



X. fastidiosa is xylem-limited and can only spread to the neighboring xylem vessel by disrupting the pit membrane (PM). Picture is adapted from (Chatterjee *et al.*, 2008).



Xylem cell occluded with bacterial cells.
(Photograph courtesy of R. Jordan)

Disease development
Xylella fastidiosa

1. No disease is produced in many host plants;
2. Some individual strains are able to infect different hosts, but others do not (Sherald,1993).

Liberato *et al.*,2009;..

Pathogen host range

Xylella fastidiosa: A pathogen of landscape trees

- *Xylella fastidiosa* is a pathogen that causes leaf scorch and related diseases in over 150 plant species, including Pierce's disease in grapevines (PD), phony peach disease (PP), plum leaf scald (PLS), and leaf scorch in almond (ALS), oak (OAK), and oleander (OLS).

Alfalfa dwarf	Oleander leaf scorch
Almond leaf scorch	Phony peach disease
Citrus variegated chlorosis	Pear leaf scorch
Coffee leaf scorch	Periwinkle wilt
Elm leaf scorch	Pierce’s disease of grapevine
Maple leaf scorch	Plum leaf scald
Mulberry leaf scorch	Sycamore leaf scorch
Oak leaf scorch	

Pathogen host range

Xylella subsp. host plant families-the most abundant in species

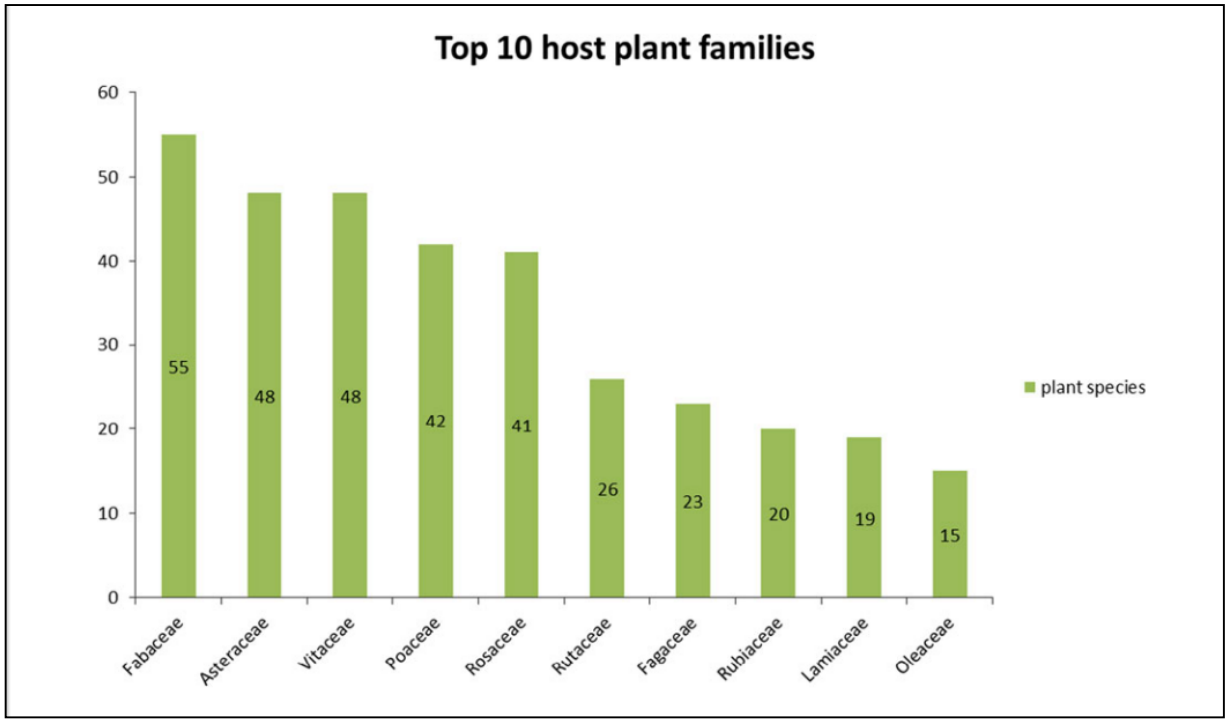
- The total number of positive host plant species of *X. fastidiosa* subspecies (subsp. or ssp.) was counted.
- The total number of plants reported infected by *X. fastidiosa* regardless of the detection method was 563 species, 264 genera and 82 families.

1. Artificial inoculations were positive in 122 plant species.
2. While 234 plant species were reported positive in natural infections.

EFSA (European Food Safety Authority), 2018

Xylella subspecies host plant families- the most abundant in species

Top 10 host plant families



The most studied genera belonged to economically important crops: Vitis, Citrus, Prunus and Olea.

EFSA (European Food Safety Authority), 2018

Pathogen host range

A heterogeneous species/subspecies producing a variety of symptoms

- Genetic and phenotypic variation within the species has long been recognized and host-specific pathotypes have been identified.
- For example
- Strains shown to cause Pierce's disease of grape also cause almond leaf scorch and alfalfa dwarf.
- Similarly, the strain or strains responsible for phony peach disease are also responsible for plum leaf scald.
- Strains causing citrus variegated chlorosis (CVC) are closely related to strains responsible for coffee leaf scorch (CLS).
- Strain relationships become more complex when genetically distinct strains cause similar symptoms in the same host.

Sherald,2007;..

Pathogen host range

Diseases associated with *Xylella fastidiosa*

- Alfalfa dwarf
- Almond leaf scorch (ALSD)
- Bacterial leaf scorch of landscape trees (BLS)
- Citrus variegated chlorosis
- Coffee leaf scorch
- Elm leaf scorch
- Maple leaf scorch
- Mulberry leaf scorch
- Oak leaf scorch
- Oleander leaf scorch
- Olive quick decline syndrome (OQDS)
- Pear leaf scorch
- Peach phony disease (PPD)
- Pecan leaf scorch
- Periwinkle wilt
- Plum leaf scald (PPD)
- Pierce's disease of grape (PD)
- Sycamore leaf scorch

Sherald,2007;..

Pathogen host range

Tree hosts of *Xylella fastidiosa*

- *Albizia julibrissin* (Silk tree)
- *Acer negundo* (Boxelder)
- *A. rubrum* (Red maple)
- *A. saccharinum* (Silver maple)
- *A. saccharum* (Sugar maple)
- *Cercis occidentalis* (Western redbud)
- *Chitalpa tashkinensis* (Chitalpa)
- *Cornus florida* (Flowering dogwood)
- *Celtis occidentalis* (Hackberry)
- *Fagus crenata* (Japanese beech (bonsai)
- *Ginkgo biloba* (Maidenhair tree)
- *Jacaranda mimosifolia* (Jacaranda)
- *Juglans* (Walnut)
- *Liquidambar stryaciflua* (Sweetgum)
- *Magnolia grandiflora* (Southern magnolia)
- *Morus alba* (White mulberry)
- *Olea europea* (Olive)
- *Phoenix reclinata (Senegal date palm)*
- *Platanus occidentalis* (American sycamore)
- *P. mexicana* (Mexican sycamore, Mexican plane)
- *P. oaxacana* (Oaxaca plane)
- *P.* x *acerifolia* (London plane)
- *Prunus cerasifera* (Ornamental palm)
- *Pyrus communis* (Pear)

Note: The genus *Plantanus* are often known in English as planes or plane trees. Some North American species are called sycamores (especially *Platanus occidentalis*). Plane trees are a potential host.

Sherald,2007;Wikipedia,2019;..

Pathogen host range

Tree hosts of *Xylella fastidiosa*

- *Quercus alba* (White oak)
- *Q. bicolor* (Swamp oak)
- *Q. coccinea* (Scarlet oak)
- *Q. falcata* (Southern red oak)
- *Q. imbricaria* (Shingle oak)
- *Q. incna* (Bluejack oak)
- *Q. laevis* (Turkey oak)
- *Q. laurifolia* (Laurel oak)
- *Q. macrocarpa* (Bur oak)
- *Q. nigra* (Water oak)
- *Q. paulustris* (Pin oak)
- *Q. phellos* (Willow oak)
- *Q. prinus* (Chestnut oak)
- *Q. rubra* (Northern red oak)
- *Q. shumardii* (Shumard oak)
- *Q. stellata* (Post oak)
- *Q. velutina* (Black oak)
- *Q. virginiana* (Live oak)
- *Ulmus americana* (American elm)
- *U. glabra* (Wych elm)
- *U. pumila* (Siberian elm)

Sherald,2007

Major diseases

Partial list of species in the *Xylella* genus by host plant

X. fastidiosa subsp. *fastidiosa*	*X. fastidiosa* subsp. *multiplex*	*X. fastidiosa* subsp. *pauca*	*X. fastidiosa* subsp. *sandyi*	*X. fastidiosa* subsp. *morus*	*Xylella taiwanensis*
Alfalfa	Almond	Almond	Coffee	Mulberry	Pear
Almond	Asparagus	Citrus	Daylily		
Coffee	Blueberry	Coffee	Magnolia		
Citrus	Crepe myrtle	Hibiscus	Oleander		
Grapevine	Elm	Oleander			
Lupin	Gingko	Olive			
Maple	Lavender	Peach			
Oleander	Maple	Wattle			
Rosemary	Oak	Westringia			
	Oleander				
	Olive				
	Peach				
	Pear				
	Plum				
	Sunflower				
	Wattle				
	Westringia				

Three subspecies of *sandyi*, *morus* and *tashke* are associated with diseases of less economic interest and with a limited host spectrum.

The recognized *Xylella* spp. and subspecies

Xylella fastidiosa and *Xylella taiwanensis*

- Schaad *et al.*, 2004 proposed three subspecies based on:

1. Pathogenicity;
2. Phylogenetic characteristics;
3. DNA relatedness.

- **Two main species:**

1. *Xylella fastidiosa* subsp. *fastidiosa* (inaccurately named as *X. f.* subsp. *piercei*)(Schaad *et al.*,2004),

I. *X. fastidiosa* subsp. *multiplex* (Schaad *et al.*,2004),

II. *X. fastidiosa* subsp. *pauca* (Schaad *et al.*,2004),

III. *X. fastidiosa* subsp. *sandyi* (Randal *et al.*,2009),

IV. *X. fastidiosa* subsp. *tashke* (Randal *et al.*,2009),

V. *X. fastidiosa* subsp. *morus* (Nunney *et al.*,2014),

2. *Xylella taiwanensis* (Su *et al.*,2016).

Schaad *et al.*,2004; Randall *et al.*,2009; EFSA Journal,2022

Diseases caused naturally by *Xylella fastidiosa* is divided into six subspecies

- Initial research on the molecular genetic diversity of PD strains and other Xf pathotypes was published in 2001 (Hendson *et al*.,2001), reporting a division of Xf species at a subspecies or pathovar level.
- Currently six Xf subspecies determined by:

1. internal transcribed spacer (ITS) sequences, and
2. multilocus sequencing through partial sequences of 11 housekeeping genes (Yuan *et al*., 2010; Su *et al*., 2013; Jolley *et al*.,2018).

Johnson *et al*.,2021

Diseases caused naturally by *Xylella fastidiosa* is divided into six subspecies

Xylella fastidiosa* subsp. *fastidiosa (erroneously named *X. f.* subsp. *piercei*)	**Grape vines, citrus, coffee, alfalfa, almond, and maple, *Nerium oleander*, rosemary,..**
X. fastidiosa* subsp. *morus	**White mulberry(*Morus alba*), red mulberry(*Morus rubra*), mulberries(*Morus* sp.), heavenly bamboo/nandina(*Nandina domestica*)**
X. fastidiosa* subsp. *multiplex (primarily associated with forest trees or *Prunus* spp.)	**Britain's native pedunculate oak (*Quercus robur*), wych elm *(Ulmus glabra*), northern red oak (*Q. rubra*), peach, plum, pigeon grape, almond, sycamore (plane),..**
X. fastidiosa* subsp. *pauca (well-known because of citrus variegated chlorosis, CVC, and olive quick decline syndrome, OQDS)	**Citrus(CVC), olive quick decline syndrome(OQDS), coffee, oleander, almond;..**
X. fastidiosa* subsp. *sandyi	**Oleander plants (*Nerium oleander*),..**
X. fastidiosa* subsp. *tashke	**strains from the ornamental tree chitalpa tree (*Chitalpa tashkentensis*)**
Xylella taiwanensis	**Pear leaf schorch diseae**

Diseases caused artificially by *Xylella fastidiosa* subspecies

Xylella fastidiosa* subsp. *fastidiosa	**Pear (*Pyrus communis*), oak (*Quercus petraea*), white willow(*Salix alba*)**
X. fastidiosa* subsp. *multiplex	**Pear (*Pyrus communis*), Apple (*Malus domestica*), oak (*Quercus petraea*), willow(*Salix alba*)**
X. fastidiosa* subsp. *pauca	**Pear (*Pyrus communis*), Apple (*Malus domestica*), oak (*Quercus petraea*), willow(*Salix alba*)**
X. fastidiosa* subsp. *sandyi	**Pear (*Pyrus communis*), Apple (*Malus domestica*)**
X. fastidiosa* subsp. *tashke	**ornamental tree chitalpa tree (*Chitalpa tashkentensis*)**
Xylella taiwanensis	**No new host plants were reported for the pathogen species *Xylella taiwanensis* that so far was recorded only in pear plants.**

Xylella fastidiosa subspecies and host range

Sequence types (STs)

- Every subspecies is subdivided into sequence types (STs), each with different host ranges (Sicard *et al.*, 2018; Nunney *et al.*,2019).
- Sequence types found in almond trees (*P. dulcis*) and location (Jeger *et al.*, 2018; Amanifar *et al.*,2019; Bahar *et al.*,2019; EPPO, 2019; Saponari *et al.*, 2019; EFSA, 2020; www.pubmlst.org).
- EFSA (European Food Safety Authority), Delbianco A, Gibin D, Pasinato L, Boscia D, Morelli M, 2023. Update of the *Xylella* spp. host plant database – systematic literature search up to 30 June 2022. EFSA Journal 2023;21 (1):7726, 90 pp. https://doi.org/10.2903/j.efsa.2023.7726

Greco *et al.*,2021; EFSA Journal,2023

The subspecies categorization of *X. fastidiosa*

Sequence types (STs)

- *X. fastidiosa* can be subdivided into sequence types (STs) using a multilocus sequence typing (MLST) approach based on seven housekeeping genes.
- MLST is a portable and robust platform that is widely used to assign strains to genetic groups while providing information on host plant species that may be susceptible to particular strains.
- In case of ALSD(almond leaf scorch disease) strains belonging to *X. fastidiosa* subsp. *multiplex*, the strains were subdivided into two different genotypes (ALSI and ALSII) below the subspecies by random amplification of polymorphic DNA (RAPD) analysis.

Greco *et al.*,2021

Xylella fastidiosa subspecies

Sequence types (STs) and locations

- Current distribution of *X. fastidiosa* subspecies (subspecies *pauca*, *fastidiosa* and *multiplex*) was reported, together with the identification of several STs in EU.

Country	Region	*X. fastidiosa* subspecies	Sequence type (ST)
France	Corse, PACA Region	*multiplex*	ST6
France	Corse, PACA Region	*multiplex*	ST7
France	PACA Region	*pauca*	ST53
Italy	Apulia	*pauca*	ST53
Italy	Tuscany	*multiplex*	ST87
Portugal	Área metropolitana do Porto	*multiplex*	ST7
Spain	Balearic Islands	*fastidiosa*	ST1
Spain	Balearic Islands, Alicante province, Autonomous Region of Madrid	*multiplex*	ST6
Spain	Balearic Islands	*multiplex*	ST7
Spain	Balearic Islands	*pauca*	ST80
Spain	Balearic Islands	*multiplex*	ST81

Bragard *et al.*,2019

Xylella fastidiosa subsp. *fastidiosa*, *multiplex* and *pauca*

Sequence types (STs) and locations

Subspecies	Sequence type	Location
Fastidiosa	ST1 (ALSI, Tulare, M23, G-genotype, STL)	USA (California), Spain (Majorca), Israel (Hula Valley)
Multiplex	ST6 (ALSII)	USA (San Joaquin County-California), Spain (Majorca, Alicante), France (Corsica)
	ST7 (M12, A-genotype)	USA (Kern County-California), Spain (Majorca), France (Corsica)
	ST27	USA
	ST81	Spain (Majorca), Spain (Menorca)
	ST87	Italy (Tuscany)
	-	Iran (Chahar Mahal-va-Bakh-tiari, West Azerbaijan, Semnan)
Pauca	ST53	Italy (Apulia), France (Corsica)
	ST80	Spain (Ibiza)
	ST78	Argentina

According to Amanifar *et al.*,2019, in Iran, there are two subspecies of this plant pathogen after considering gene sequencing and differences in biological and morphological traits of bacterial colonies, namely, subsp. *fastidiosa* isolated from grapes and subsp. *multiplex* isolated from pistachios and almonds (Amanifar *et al.*, 2014, 2016). However, more gene sequencing is necessary to determine the sequence types present in Iran.

Greco *et al.*,2021

Xylella fastidiosa subsp. *fastidiosa*, *multiplex* and *pauca*

Sequence types (STs) and locations

- Sequence types found on peach (*P. persica*), European plum (*P. domestica*), and Japanese plum (*P. salicina*) trees and location (Della Coletta-Filho et al., 2017; EFSA, 2020; www.pubmlst.org).

Host	Subspecies	Sequence type	Location
Peach	*Multiplex*	ST26	USA (Riverside County-California)
		ST10	USA (Georgia, Florida, Orange County-California)
	Pauca	ST53	France (Corsica)
European plum	*Multiplex*	ST6	Spain (Majorca)
		ST10	USA (Georgia)
		ST26	USA (Riverside County-California)
		ST63	Brazil
		ST81	Spain (Majorca, Menorca)
	Paucapauca	ST71	Brazil
Hybrid plum	*Multiplex*	ST41	USA (Georgia)

Peach and plum trees can be infected by subsp. *pauca*, while subsp. *multiplex* is the etiological factor of PPD and PLS in countries where these diseases have long represented a serious phytosanitary problem.

Greco *et al.*,2021

Xylella fastidiosa subsp. *fastidiosa, multiplex* and *pauca*

Sequence types (STs) and locations

- Sequence types found in other *Prunus* species and locations (EFSA, 2020; www.pubmlst.org).

Host	Subspecies	Sequence type	Location
Apricot (*P. armeniaca*)	*Multiplex*	ST6	Spain (Alicante)
		ST46	USA (Riverside County-California)
		ST26	USA (Riverside County-California)
Purple leaf plum (*P. cerasifera*)	*Multiplex*	ST6	France (Corsica)
		ST7	France (Corsica)
		ST15	USA (Riverside County-California)
		ST34	USA (Riverside County-California)
		ST40	USA
Cherry (*P. avium*)	*Fastidiosa*	ST1	USA (San Bernardino-California), Spain (Majorca)
	Pauca	ST53	Italy (Apulia)
Prunus sp. (decorative prunus)	*Multiplex*	ST26	USA (Riverside County-California)

lists the sequence types found in other species of the *Prunus* genus. Of these, an uncommon disease caused by *X. fastidiosa* is leaf scorch of purple-leafed plum (*P. cerasifera*), which was observed during a survey conducted between 2003 and 2004 in southern California (United States). The symptoms of this disease are leaf scorching and plant decline, similar to those of ALSD and PLS

Greco *et al.*,2021

Nomenclature of *X. fastidiosa* subsp. *pauca* pau'ca L. fem. adj. pauca few

- As more *pauca* strains become studied, their common and peculiar genetic characteristics are being described in increasing detail.
- This information enables a better understanding of intra and inter-subspecies evolution in *X. fastidiosa*, as well as their relationship with host range and geographic distribution.

Safady *et al.*,2009; de Souza *et al.*,2020

Host plant species naturally infected
Olive Quick Decline symptoms (OQDS)
X. fastidiosa subsp. *pauca*

- The highest number of plant species naturally infected is recorded for:

1. *X. fastidiosa* subsp. *multiplex* (203 hosts), followed by
2. *X. fastidiosa* subsp. *pauca* (57 hosts), and
3. *X. fastidiosa* subsp. *fastidiosa* (53 hosts).

- A dramatic outbreak of Xf subsp. *pauca* (Xfp) strain ST53, namely CoDiRO (Complesso del Disseccamento Rapido dell'Olivo, meaning OQDS), decimating olive trees was discovered in 2013 in Apulia, Southern Italy(Saponari *et al.*,2014 and Cariddi *et al.*,2014).

Host plant species naturally infected
Olive Quick Decline symptoms (OQDS)
X. fastidiosa subsp. *pauca*

- Olive quick decline syndrome (OQDS) has been reported in the Salento area (Apulia region, southern Italy)(Saponari *et al.*,2014 and Cariddi *et al.*,2014).
- Strains from subspecies *pauca* have been recently isolated from olive trees with olive quick decline syndrome(OQDS) symptoms in Brazil (Safady *et al.*,2019) confirming its status of emerging pathogen in different regions and crops.

EFSA Journal 2022; Safady *et al.*,2009; de Souza *et al.*,2020

Predisposing Factors for "Olive Quick Decline Syndrome(OQDS)

Synergistic action on *X. fastidiosa* subsp. *pauca*

- Indeed, a number of environmental factors can interact with the host and the pathogen either to predispose and enhance host colonization and infection by the pathogen or to incite microorganism pathogenicity in terms of virulence and aggressiveness.
- Associated with the syndrome, several phytopathogenic fungi were detected in the rhizosphere, endosphere and phyllosphere of the trees, along with the phytopathogenic bacterium *Xylella fastidiosa* subsp*. pauca*.

Scortichini,2020; Nuti *et al.*,2021

Predisposing Factors for "Olive Quick Decline Syndrome(OQDS)

Synergistic action on *X. fastidiosa* subsp. *pauca*

- Together with *Xylella fastidiosa* subsp. *pauca*, some pathogenic fungi such as *Phaeoacremonium* spp. have been found associated with the disease.
- The main predisposing factors to the disease seem to be:
- local cultivar susceptibility, depletion of some micronutrients in the soil that could be related to some agronomical practices favoring the depletion of soil fertility, an incorrect pruning cycle, climatic changes that result in increased soil waterlogging, and frost and drought events.

Scortichini,2020; Nuti *et al.*,2021

Predisposing Factors for "Olive Quick Decline Syndrome(OQDS)

Synergistic action on *X. fastidiosa* subsp. *pauca*

- *X. fastidiosa* subsp. *pauca*, along with several phytopathogenic fungi such as *Phaeoacremonium* spp. plus abiotic factors causes dramatic damage to olive trees, with symptoms of leaf scorching and scattered desiccation of twigs and branches.
- Severe wilting in leaves, twigs, and branches. Often the disease progression results in plant death.

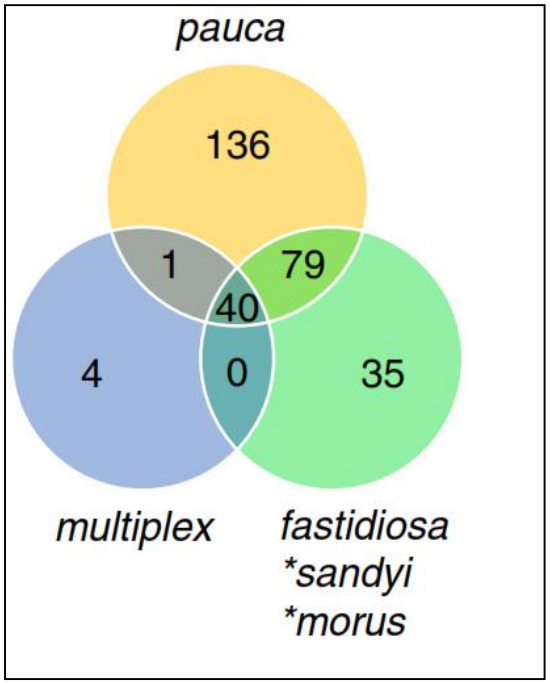
Scortichini,2020

Nomenclature of *X. fastidiosa* subsp. *pauca*

Relationships between three groups (*pauca*, *multiplex* and the third one resulting from the grouping of subsp. *fastidiosa*, *sandyi* and *morus*)

- Using SkIf, a revised taxonomy of *X. fastidiosa* into three major clades defined by:

1. the subspecies *pauca* (clade I),
2. *multiplex* (clade II), and
3. The combination of *fastidiosa*, *morus* and *sandyi* (clade III).



SkIf (Specific k-mers Identification) is a robust and rapid software, freely available, that can be dedicated to the comparison of sequence datasets and is applicable to any field of research. Here it was designed and exploited for comparative genomics on a dataset of 46 *X. fastidiosa* genomes, including seven newly sequenced individuals.

Denancé *et al.*,2019

Host plant species naturally infected *X. fastidiosa* subsp. *pauca*

No.	Plant hosts	No.	Plant hosts
1	(*Acacia saligna*)	13	(*Dimorphotheca fruticosa*)
2	(*Acacia* sp.)	14	(*Dodonaea viscosa*)
3	(*Amaranthus retroflexus*)	15	(*Eremophila maculate*)
4	(*Asparagus acutifolius*)	16	(*Erigeron bonariensis*)
5	(*Catharanthus roseus*)	17	(*Erigeron* sp.)
6	(*Chenopodium album*)	18	(*Erigeron sumatrensis*)
7	(*Cistus albidus*)	19	(*Euphorbia chamaesyce*)
8	(*Cistus creticus*)	20	(*Euphorbia terracina*)
9	(*Cistus sinensis*)	21	(*Grevillea juniperina*)
10	(*Citrus* sp.)	22	(*Hebe* sp.)
11	Arabic coffee (*Coffea arabica*)	23	(*Heliotropium europaeum*)
12	(*Coffea* sp.)	24	(*Hibiscus rosa-sinensis*)

Delbianco *et al.*,2022

Host plant species naturally infected *X. fastidiosa* subsp. *pauca*

No.	Plant hosts	No.	Plant hosts
25	Mallow (*Hibiscus* sp.)	37	(*Pelargonium* sp.)
26	(*Laurus nobilis*)	38	(Periwinkle)
27	(*Lavandula angustifolia*)	39	(*Phillyrea latifolia*)
28	(*Lavandula dentata*)	40	Pistachio (*Pistacia vera*)
29	(*Lavandula* sp.)	41	(*Polygala myrtifolia*)
30	(*Lavandula stoechas*)	42	(*Polygala* sp.)
31	(*Myoporum insulare*)	43	Cherry (*Prunus avium*)
32	(*Myrtus communis*)	44	(*Prunus domestica*)
33	(*Nerium oleander*)	45	(*Prunus dulcis*)
34	(*Olea europaea*)	46	(*Prunus* sp.)
35	(*Olea europaea* subsp. *sylvestris*)	47	(*Prunus persica*)
36	(*Pelargonium fragrans*)	48	(*Rhamnus alaternus*)

Delbianco *et al.*,2022

Host plant species naturally infected *X. fastidiosa* subsp. *pauca*

No.	Plant hosts	No.	Plant hosts
49	Sage (*Salvia officinalis*)		
50	*Salvia rosmarinus*		
51	*Salvia* sp.		
52	*Spartium junceum*		
53	Holly oak (*Quercus ilex*)		
54	*Ulex parviflorus*		
55	Periwinkle (*Vinca minor*)		
56	*Westringia fruticosa*		
57	*Westringia glabra*		

Delbianco *et al.*,2022

Host plant species naturally infected *X. fastidiosa* subsp. *sandyi*

No.	Plant hosts	No.	Plant hosts
1	Arabic coffee (*Coffea arabica*)		
2	*Coffea* sp.		
3	*Coffea canephora*		
4	*Hemerocallis* sp		
5	*Jacaranda mimosifolia*		
6	*Magnolia grandiflora*		
7	*Nandina domestica*		
8	Oleander or nerium (*Nerium oleander*)		
9	Myrtle-leaf milkwort (*Polygala myrtifolia*)		

Delbianco *et al.*,2022

Host plant species artificially infected *X. fastidiosa* subsp. *pauca*

1. *Bidens pilosa*
2. *Brachiaria decumbens*
3. *Brachiaria plantaginea*
4. *Catharanthus roseus*
5. *Citrus reticulata*
6. *Citrus sinensis*
7. *Citrus* sp.
8. Citrus x *nobilis*
9. *Coffea arabica*
10. *Coffea* sp.
11. *Echinochloa crus-galli*
12. *Jasminum azoricum*
13. *Medicago sativa*
14. *Nerium oleander*
15. *Nicotiana clevelandii*
16. *Nicotiana tabacum*
17. *Ocimum basilicum*
18. *Olea europaea*
19. *Polygala myrtifolia*
20. *Solanum americanum*
21. *Arabidopsis thaliana*
22. Periwinkle (common name)
23. *Chenopodium album*
24. *Digitaria horizontalis*
25. *Malus domestica*
26. *Prunus avium*
27. *Prunus domestica*
28. *Prunus dulcis*
29. *Pyrus communis*
30. *Quercus petraea*
31. *Salix alba*
32. *Salvia rosmarinus*
33. *Vitis vinifera*

Host plant species naturally infected *X. fastidiosa* subsp. *morus*

No.	Plant hosts	No.	Plant hosts
1	White mulberry (*Morus alba*)		
2	Red mulberry (*Morus rubra*)		
3	Mulberries (*Morus* sp.)		
4	Nandina/heavenly bamboo (*Nandina domestica*)		

Host plant species naturally infected *X. fastidiosa* subsp. *multiplex*, the causal agent of phony peach disease(PPD)

Acacia dealbata (ACADA)	Host
Acacia saligna (ACASA)	Host
Acer pseudoplatanus (ACRPP)	Host
Anthyllis hermanniae (AYLHE)	Host
Artemisia arborescens (ARTAO)	Host
Artemisia sp. (ARTSS)	Host
Asparagus acutifolius (ASPAC)	Host
Calicotome spinosa (CCOSP)	Host
Calicotome villosa (CCOVI)	Host
Carya illinoinensis (CYAIL)	Host
Cercis siliquastrum (CCSSI)	Host
Cistus creticus (CSTIC)	Host
Cistus monspeliensis (CSTMO)	Host
Cistus salviifolius (CSTSA)	Host
Cistus x incanus (CSTIS)	Host
Convolvulus cneorum (CONCN)	Host
Coprosma repens (CPMRE)	Host
Coronilla valentina (CZRVL)	Host
Coronilla valentina subsp. glauca (CZRVG)	
Cytisus scoparius (SAOSC)	Host
Cytisus villosus (CZSVI)	Host
Dimorphotheca ecklonis (OSPEK)	Host
Elaeagnus angustifolia (ELGAN)	Host
Erigeron karvinskianus (ERIKA)	Host
Euryops chrysanthemoides (EYOCH)	Host
Euryops pectinatus (EYOPE)	Host
Ficus carica (FIUCA)	Host
Fraxinus angustifolia (FRXAN)	Host
Genista corsica (GENCO)	Host
Genista ephedroides (GENEP)	Host
Genista x spachiana (GENSA)	Host
Grevillea juniperina (GREJU)	Host
Hebe (1HBEG) Host	
Hebe elliptica (HBEEL)	Host
Helichrysum italicum (HECIT)	Host
Helichrysum sp. (HECSS)	Host
Helichrysum stoechas (HECST)	Host
Ilex aquifolium (ILEAQ)	Host

Lavandula angustifolia (LAVAN)	Host
Lavandula dentata (LAVDE)	Host
Lavandula stoechas (LAVST)	Host
Lavandula x heterophylla (LAVHE)	Host
Medicago arborea (MEDAR)	Host
Medicago sativa (MEDSA)	Host
Metrosideros excelsa (MTDEX)	Host
Myrtus communis (MYVCO)	Host
Olea europaea (OLVEU)	Host
Pelargonium graveolens (PELGV)	Host
Perovskia abrotanoides (PEKAB)	Wild/Weed
Phagnalon saxatile (PGASA)	Host
Phlomis fruticosa (PLMFR)	Host
Pistacia vera (PIAVE)	Host
Polygala myrtifolia (POGMY)	Host
Prunus armeniaca (PRNAR)	Host
Prunus cerasifera (PRNCF)	Host
Prunus cerasus (PRNCE)	Host
Prunus domestica (PRNDO)	Host
Prunus dulcis (PRNDU)	Host
Prunus persica (PRNPS)	Host
Quercus suber (QUESU)	Host
Rhamnus alaternus (RHAAL)	Host
Robinia pseudoacacia (ROBPS)	Host
Rosa canina (ROSCN)	Host
Rosa Cluster-flowered bush hybrids (ROSXF)	
Salvia rosmarinus (RMSOF)	Host
Santolina chamaecyparissus (SNTCH)	Host
Spartium junceum (SPUJU)	Host
Strelitzia reginae (STZRE)	Host
Ulex europaeus (ULEEU)	Host
Ulex minor (ULEMI)	Host
Vaccinium (1VACG) Host	
Vaccinium corymbosum (VACCO)	Host
Vaccinium virgatum (VACVG)	Host
Vitis aestivalis (VITAE)	Host
Westringia fruticosa (WESRO)	Host

Johnson *et al.*,2021

From EPPO Global Database,2021.

Diseases caused artificially by *Xylella fastidiosa* subspecies

- Within the XF-ACTORS project, the susceptibility of
- Pear (*Pyrus communis*) to subspecies *fastidiosa*, *multiplex*, *pauca* and *sandyi*),
- Apple (*Malus domestica*) to subspecies *multiplex*, *pauca* and *sandyi*),
- Oak tree (*Quercus petraea*) to subspecies *fastidiosa*, *multiplex* and *pauca*), and
- White willow (*Salix alba*) to subspecies *fastidiosa*, *multiplex* and *pauca*) has been proved in artificial infections by needle inoculation.
- Although in these experiments conducted under greenhouse conditions, the pathogen was detected at some distance from the inoculation point (e.g. 40 cm, as reported in the case of pear and apple).
- Further studies will need to prove whether these new hosts are likely to sustain infection under natural conditions.

Host plant species of *Xylella* spp.

The updated numbers of host plant species, genera and families (according to the different categories)

- Category A: Plant species positive with at least two detection methods (among: symptoms observation on the test plant in experimental vector transmission, ELISA, other immunological techniques, PCR-based methods, sequencing and pure culture isolation) or positive with one method (between: sequencing, pure culture isolation).
- Category B: The same as point A, but also including microscopy: plant species positive with at least two detection methods (among: microscopy, symptoms observation on the test plant in experimental vector transmission, ELISA, other immunological techniques, PCR-based methods, sequencing and pure culture isolation) or positive with one method (between: sequencing, pure culture isolation).

Host plant species of *Xylella* spp.

The updated numbers of host plant species, genera and families (according to the different categories)

- Category C: Plant species positive with at least one detection method (among: symptoms observation on the test plant in experimental vector transmission, ELISA, other immunological techniques, PCR-based methods, sequencing and pure culture isolation).
- Category D: Plant species positive with at least one detection method including microscopy (microscopy, symptoms observation on the test plant in experimental vector transmission, ELISA, other immunological techniques, PCR-based methods, sequencing and pure culture isolation).
- Category E: All positives plant species reported, regardless of the detection methods (positive records but without the detection method specified, symptom observations, microscopy, symptoms observation on the test plant in experimental vector transmission, ELISA, other immunological techniques, PCR-based methods, sequencing, pure culture isolation).

Host plant species of *Xylella* spp.

The updated numbers of host plant species, genera and families (according to the different categories)

- Number of host plant species, genera and families of *Xylella* spp. according to categories A, B, C, D, E (based on the detection methods applied:

	A	B	C	D	E
Number of host plant species	412	417	648	657	664
Number of host plant genera	190	191	298	298	299
Number of host plant families	68	68	88	88	88

Host plant species of *Xylella* spp.

The updated numbers of host plant species, genera and families (according to the different categories)

- Number of host plant species, naturally infected, susceptible to the different *X. fastidiosa* subspecies according to categories A, B, C, D, E.

Category	*fastidiosa*	*morus*	*multiplex*	*pauca*	*sandyi*	*tashke*	Unknown
A	40	2	19	20	5	0	89
B	41	2	19	21	5	0	94
C	78	2	31	33	11	1	202
D	78	2	31	33	11	1	208
E	79	2	32	33	11	1	216

Host plant species of *Xylella* spp.

The updated numbers of host plant species, genera and families (according to the different categories)

- The highest number of plant species naturally infected is recorded for *X. fastidiosa* subsp. *multiplex* (203) according to category A, up to 210 for category E), followed by subsp. *pauca* and subsp. *fastidiosa*.

Category	*fastidiosa*	*fastidiosa_sandyi*	*morus*	*multiplex*	*pauca*	*sandyi*	*tashke*	Unknown
A	50	2	4	203	53	7	1	168
B	50	2	4	203	53	7	1	173
C	53	2	4	210	57	8	1	368
D	53	2	4	210	57	8	1	374
E	53	2	4	210	57	8	1	385

Strains differentiation

Based on pathogenicity, nutritional requirements, and genetic homology

- Strains differ in characteristics such as:

1. Host range (the ability to cause disease/pathogenicity);
2. Nutritional requirements;
3. Genetic homology.

Characteristics differentiating *Xylella* from *Xanthomonas*

Characteristic	*Thermomonas haemolytica*[b]	*Xanthomonas campestris*[c]	*Pseudoxanthomonas broegbernensis*[d]	*Stenotrophomonas maltophilia*[e]	*Luteimonas mephitis*[f]	*Xylella fastidiosa*[g]
Temperature optimum (°C)	37–50	28	28	35	28	26–28
Nitrate reduction	−	−	−	+	−	−
Esculin hydrolysis	−	+	−	+	−	−
Susceptibility to:						
Ampicillin	+	−	−	−	−	+
Penicillin G	+	−	−	−	−	−
Erythromycin	+	−	−	−	−	
Kanamycin	+	+	−	+	V+	+
Neomycin	+	−	+	−	V−	
Streptomycin	+	+	−	−	−	−
Predominant fatty acid:						
$C_{15:0\ iso}$	+	+	+	+	+	
$C_{15:0\ anteiso}$		+	+	+		+
$C_{16:0}$			+			
$C_{16:0\ iso}$	+					
$C_{16:1}$		+	+			+
$C_{17:0}$					+	+
$C_{17:0\ iso}$						
$C_{17:1}$					+	
$C_{17:1\ iso}$			+			
Hydroxy fatty acid:						
$C_{10:0\ 2OH}$						+
$C_{11:0\ iso\ 2OH}$			+			
$C_{11:0\ iso\ 3OH}$	+	+	+	+	+	
$C_{12:0\ 3OH}$		+		+		
$C_{13:0\ 2OH}$				+		
$C_{13:0\ iso\ 3OH}$		+		+		
Major polar lipids.[h]						
Diphosphatidylglycerol	+	+		+		
Phosphatidylethanolamine	+	+		+		
Phosphatidylglycerol	+	+		+		
Phosphatidylmonomethylethanolamine		+				
Unidentified phospholipid		+				
Quinone system	Q-8	Q-8	Q-8	Q-8	Q-8	
Major polyamines.[i]						
Cadaverine				+		
Spermidine	+	+		+		

[a]Symbols: +, positive for all strains; −, negative for all strains; nd, no data; V+, most strains are resistant; V−, most strains are susceptible.

[b]Data from Busse et al. (2002).

[c]Data from Oyaizu and Komagata (1983), Busse and Auling (1988), Auling et al. (1991), Yang et al. (1993b, d).

[d]Data from Finkmann et al. (2000).

[e]Data from Oyaizu and Komagata (1983), Palleroni (1984), Busse and Auling (1988), Stead (1992), Yang et al. (1993b, d), Palleroni and Bradbury (1993), Vauterin et al. (1995, 1996b), Finkmann et al. (2000).

[f]Data from Finkmann et al. (2000).

[g]Data from Wells et al. (1987).

[h]Data on polar lipids for *Xanthomonas campestris* and *Stenotrophomonas maltophilia* from Busse et al. (2002).

[i]No data for *Pseudoxanthomonas broegbernensis*, *Luteimonas mephitis*, and *Xylella fastidiosa*.

Bergey's Manual of Systematic Bacteriology,2005

Initial Xf strain differentiation into Pierce's disease (PD) group strains and phony peach (PP) group strains research

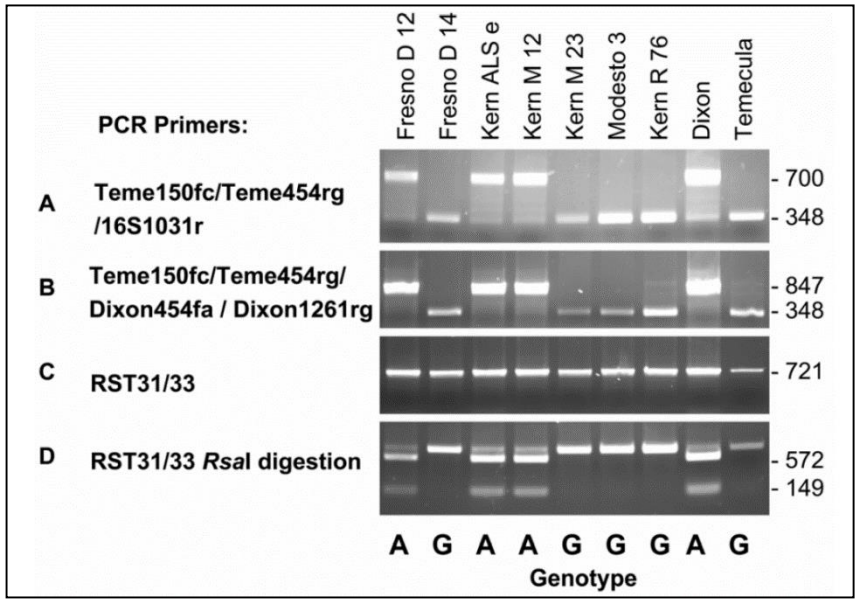
Identification test	PD group strains[a]	PP group strains[b]
Growth on:		
Nutrient glucose agar	+	
PD2 agar	+	+
PW, BCYE, and CS-20 agar		
Intensity of ELISA reaction with antisera to:[c]		
Pierce's disease strain	+++	+
Phony peach strain	+	+++
Digestion ofPCR amplification product by *RsaI*[d]		+

[a] *Xylella fastidiosa* strains that produce symptoms on *Vitis vinifera* grapevines and grow on PD2 agar.
[b] Diverse group of *X fastidiosa* strains that will not produce symptoms on grapevine and will not grow on PD2 agar, probably several different, uncharacterized pathotypes.
[c] Relative intensity of the absorbance from the enzyme-linked immunosorbent assays of antisera with strains from the two groups.
[d] The amplification product (733 bp) of the gene rpoD gene which encodes sigma 70 factor using primer pairs of RST31/RST33, is further digested with *RsaI* restriction enzyme in order to differentiate two pathotype groups of *X fastidiosa* strains, the Pierce's disease group and phony peach group.

Hendson *et al.*,2001; Schaad *et al.*,2001

Initial Xf strain differentiation into Pierce's disease(PD) group strains and phony peach(PP) group strains research

- Panel C is the 733 bp amplified rpoD gene which encodes sigma 70 factor.
- Panel D is the RsaI digestion of RST31-RST33 amplicons. Numbers on the right are DNA fragment size in base pairs.
- Overnight RsaI restriction enzyme incubation, rather than 1 h, resulted in complete DNA digestion.
- Two DNA fragments (149 and 572 bp) were evidenced.



Chen *et al.*,2005

Characters useful for differentiating subspecies of *Xylella fastidiosa*

- Figures are mean percent.
- +/–, very slow growth, 10-12 days for visible colonies; +, slow growth, 8–10 days for visible colonies; ++, relatively fast growth, 5-7 days for visible colonies;
- PD2, Pierce's disease medium; PW, periwinkle medium; taken from Hopkins.
- Relative intensity; +, weak; +++, strong; taken from Hopkins.
- Serology tests differentiate subsp. *pauca* from subsp. *piercei* and subsp. *multiplex*.

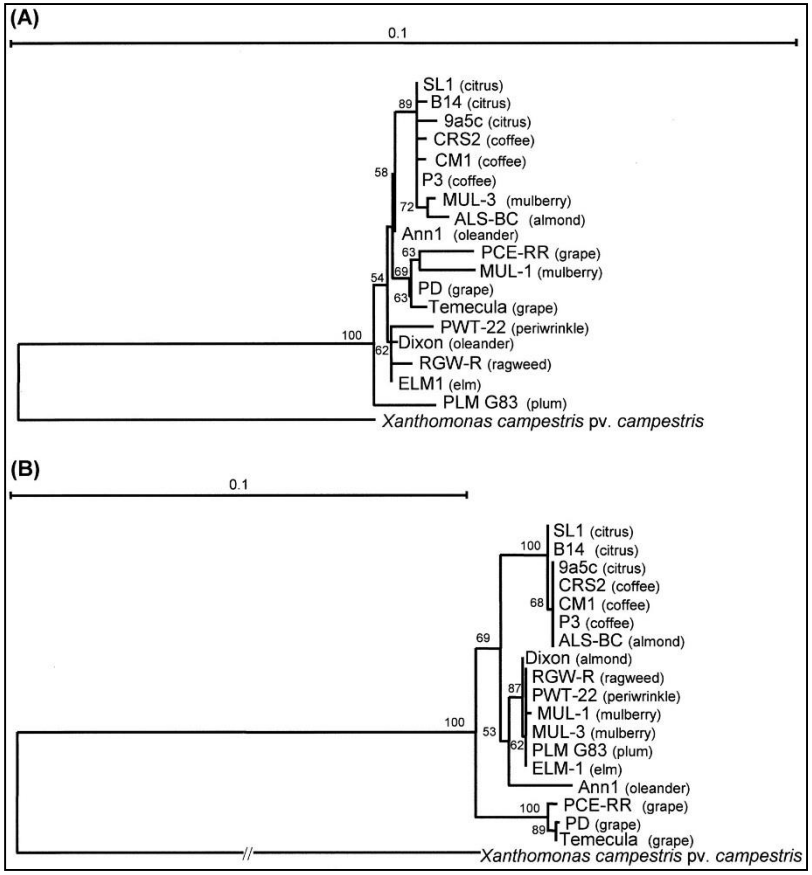
Character	Subspecies		
	piercei	*multiplex*	*pauca*
DNA/DNA relatedness to:[1]			
piercei	85	58	41
multiplex	58	84	45
pauca	41	45	87
ITS similarity to:[1]			
piercei	100	98.7	97.9
multiplex	98.7	100	99.2
pauca	97.9	99.2	100
Growth on:[2]			
PD2 medium	++	+/–	+/–
PW medium	++	++	+
Susceptibility to:			
Penicillin	low	high	high
Carbenicillin	medium	low	low
ELISA, antisera to:[3]			
piercei	+++	+	ND
pauca	+	+++	ND
Hosts	Grape, almond, alfalfa, maple	Peach, plum, almond, elm sycamore, pigeon grape	citrus

Schaad *et al.*,2004

Phylogenetic relationships among *X. fastidiosa* strains

Using 16SrRNA and *gyrB* sequences

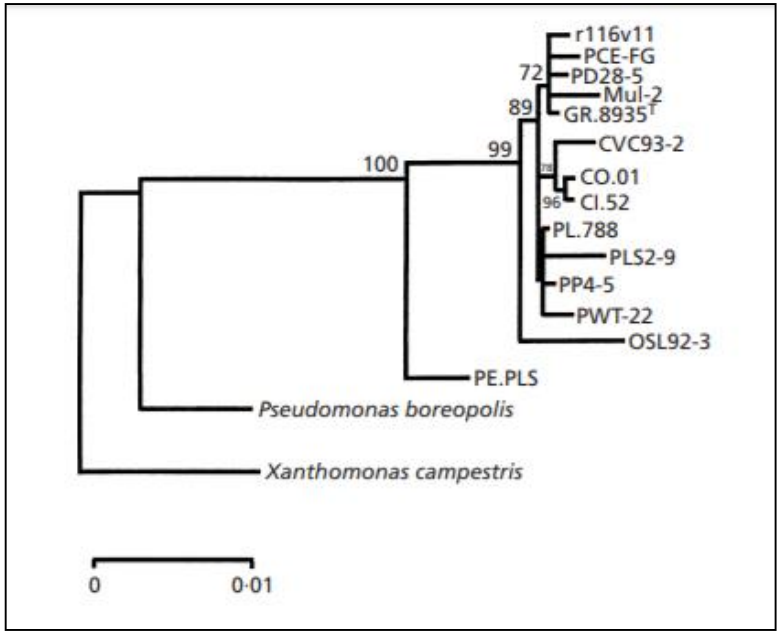
- Phylogenetic trees based on the nucleotide sequences of the 16S rRNA (A) and *gyrB* (B) genes of *X. fastidiosa* strains.
- All *X. fastidiosa* strains yielded nearly complete 16S rRNA gene sequences of 1,452 bp.
- When only one accession number is shown, it represents a *gyrB* sequence.



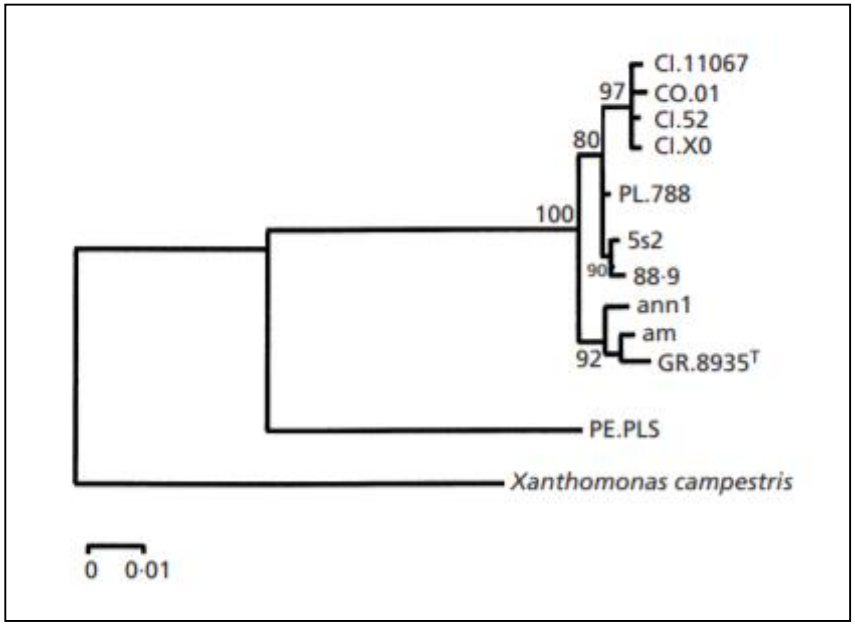
Rodrigues *et al.*,2003

Phylogenetic relationships of *Xylella fastidiosa* strains from different hosts, based on 16S rDNA and 16S-23S intergenic spacer sequences

The citrus, coffee, peach and plum strains were closely related and separate from grapevine strains



Phylogenetic tree constructed using the neighbourjoining method, based on 16S rDNA sequence data for *Xylella fastidiosa* and *Pseudomonas boreopolis*, with *Xanthomonas campestris* as the outgroup. Gaps and missing information excluded from the analysis. The numbers above the branches are bootstrap values obtained for 1000 replications (expressed as percentages; only values greater than 70% are shown). Bar, 1% sequence divergence.

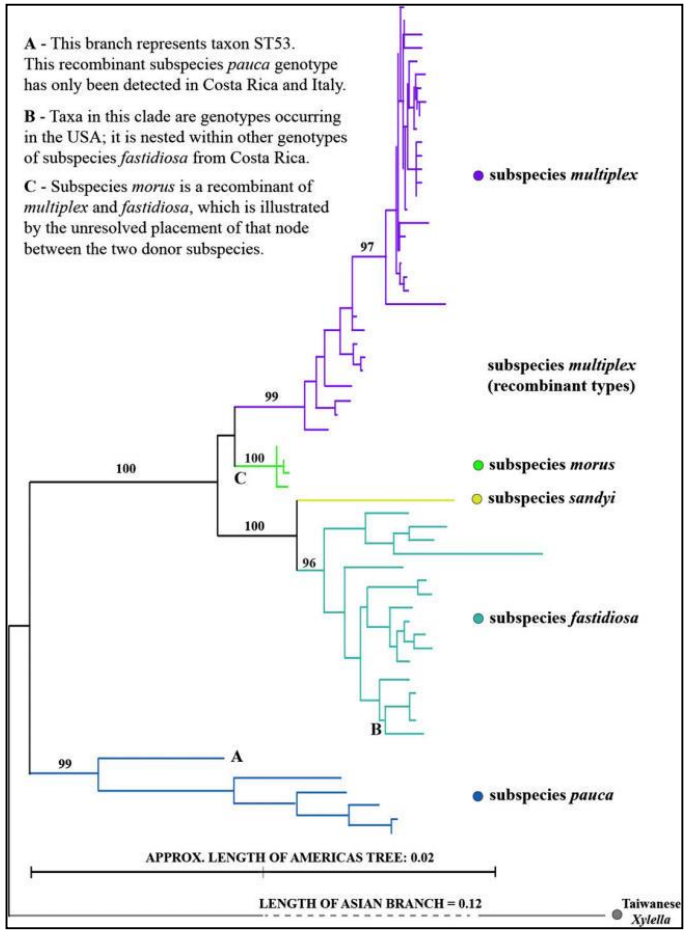


Phylogenetic tree constructed using the neighbourjoining method, based on 16S–23S intergenic spacer sequence data for *Xylella fastidiosa*, with *Xanthomonas campestris* as the outgroup. Gaps and missing information were excluded from the analysis. The numbers above the branches are bootstrap values obtained for 1000 replications (expressed as percentages; only values greater than 70% are shown). Bar, 1% sequence divergence.

Mehta and Rosato,2003

A phylogenetic tree based on genetic distances of all available *Xylella fastidiosa* sequence types identified using multilocus sequence typing

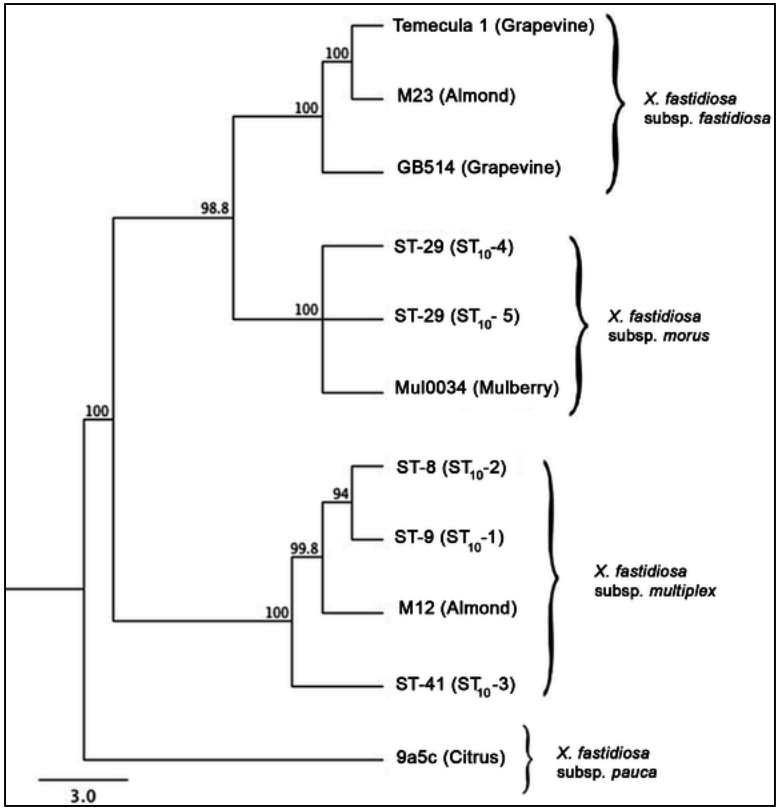
- The five currently and generally accepted subspecies are labeled with different colors, while the Taiwanese genotype causing pear leaf scorch is shown in gray at the bottom of the tree, with a not-to-scale branch due to its dissimilarity to the other taxa.
- We highlight three specific branches of interest with letters A, B, and C, with associated descriptions on the figure itself.



Almeida and Nunney,2015

The phylogeny of *Xylella fastidiosa*
Distance tree of sequence types found infecting urban trees

- A distance tree was constructed with 7,416 bp of concatenated sequence data for each *X. fastidiosa* sequence type.
- The two mulberry strains form a clade that represents the newly described subspecies *morus*, while amenity tree strains nest closely within the subsp. *multiplex* clade.
- Percentages represent bootstrap support from the re-sampling distribution.
- Values in parenthesis represent the ten locus sequence types in this analysis.



Harris and Balci,2015

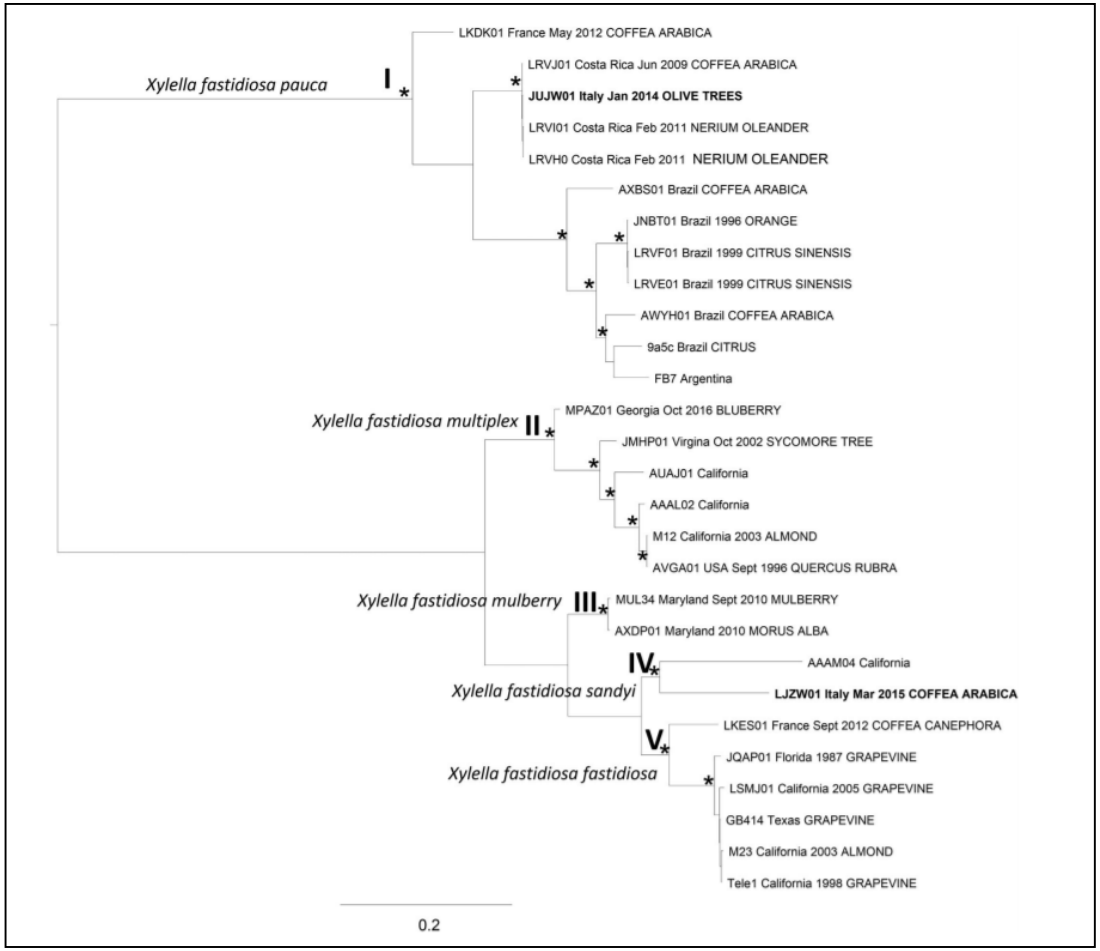
The phylogeny

Maximum-likelihood of *Xylella fastidiosa* spp. SNPs(single nucleotide polymorphisms) alignment. * along the branches indicating a statistical value from bootstrap ≥99%. Clades I–V are highlighted. The Italian strains are in bold

All the genomes available for *Xylella fastidiosa* spp. were downloaded from NCBI. A phylogeographic analysis was performed using BEAST.

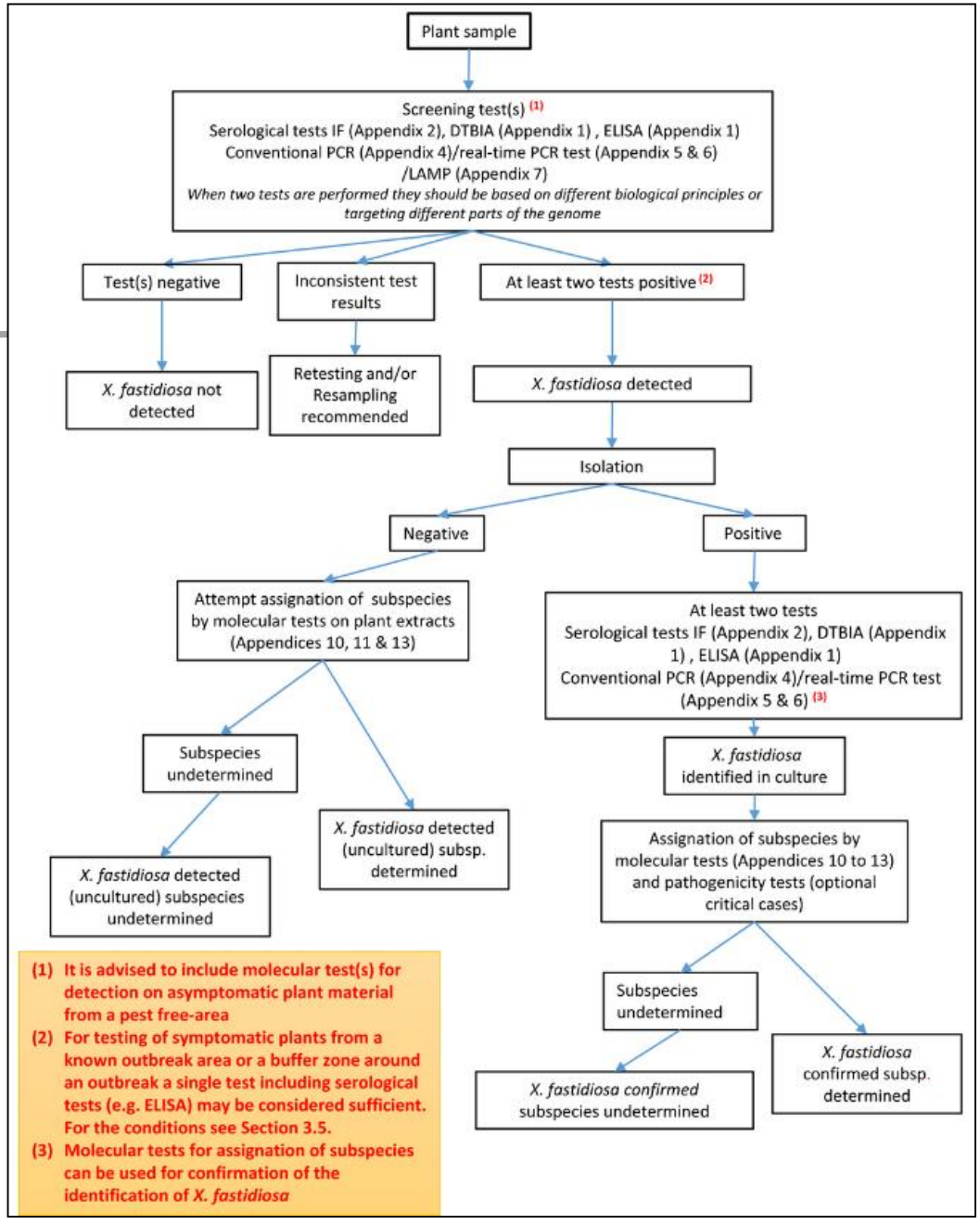
SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments.

This dataset included complete genomes from USA (California, Texas, Florida, Maryland and Virginia) n = 14, Argentina n = 1, France n = 3, Italy n = 2, Costa Rica n = 3, Brazil n = 5.



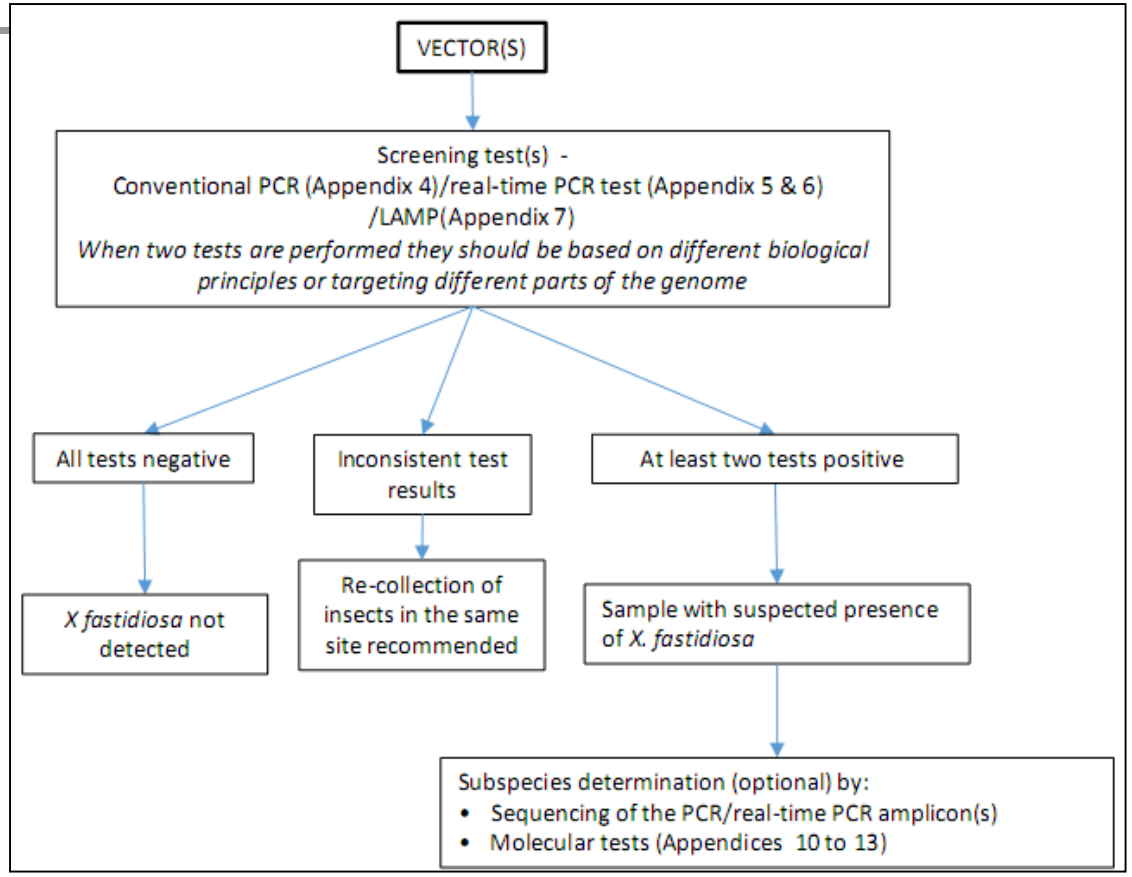
Cella *et al.*,2018

Flow diagram for the diagnostic procedure for *Xylella fastidiosa* in plant material.



EPPO,2016 and 2018

Flow diagram for the diagnostic procedure for *Xylella fastidiosa* in vectors.



EPPO,2016 and 2018

Advantages and disadvantages of diagnostic techniques used to detect *X. fastidiosa*

Diagnostic Test	Advantages	Disadvantages
ELISA fast	fast, inexpensive	false positive possible, false negative possible
PCR	most sensitive	does not distinguish dead from live bacteria, expensive
Culture	false positive not possible	takes several weeks

Varela *et al.*,2001

Sampling of plant material and sample preparation in the laboratory

Sampling period for symptomatic or asymptomatic plants and insects

- To maximize the likelihood of detection, sampling should be performed during the period of active growth of the plants.

1. For outdoor plants in Europe this active growth period is usually from late spring to autumn.
2. For tropical plant species grown indoors such as coffee plants, sampling may be performed all year round.

- Laboratory samples = branches/cuttings with attached leaves including mature leaves preferably be collected from a single plant.
- Young growing shoots should be avoided.

1. Symptomatic plants: branches/cuttings representative of the symptoms seen on the plant
2. Asymptomatic plants: the sample should be representative of the entire aerial part of the plant.

- Sampling for insects should preferably be done from late spring until early autumn.

Loconsole,2016;..

Sampling of plant material and sample preparation in the laboratory

Sampling period for symptomatic or asymptomatic plants

- The concentration of the bacterium in a plant depends upon environmental factors, strains and the host plant species or cultivars.
- Sampling should be performed during the period of active growth of the plants (Hopkins, 1981).
- For tropical plant species grown indoors, such as coffee plants, sampling may be performed all year round.
- For outdoor plants in Europe this active growth period is usually from late spring to autumn.
- In autumn 2013, the presence of *Xylella fastidiosa* was detected in olive stands (Sapnari *et al.*,2017.

EPPO,2018

Sampling of plant material and sample preparation in the laboratory

Sampling period for symptomatic or asymptomatic plants

- Another studies were conducted during unusually mild winters.
- But usually winters with more severe sub-freezing temperatures might reduce the survival rate of *X. fastidiosa* (Purcell,1981).
- Perhaps even in evergreen plants such as California blackberry and periwinkle (Purcell and Saunders,1990).

Sampling of plant material and sample preparation in the laboratory

Sampling process

- Samples should be processed as soon as possible after arrival.
- If the plant samples originate from areas where infected vectors may occur, it is recommended to check whether insects are present in the sample before opening the bags.
- If any insects are present, samples should be stored in the refrigerator for approximately 12 h.
- For isolation, samples may be kept refrigerated for up to 3 days.

EPPO,2018

Sampling of plant material and sample preparation in the laboratory

Sampling process

1. Samples should be inspected for symptoms and, if present, symptomatic leaves (including their petioles) should be selected and processed (removing the necrotic and dead tissue).
2. If no symptoms are noted, leaves should be representative of the entire sample received in the laboratory.

- Dirty samples should be cleaned.

Petioles of suspected grapevines were placed into Ziploc bags, and placing those bags on ice. Once on ice, the samples must be transported to cold storage (4°C or -20°C) where they can be stored for a short period of time because nucleic acid extraction and freezing needs to occur before samples lose their viability(Jones *et al.*,2016).

EPPO,2018

Laboratory sample

Minimum number of leaves (including their petioles) to be used and approximate weight of the laboratory sample

Type of sample	Host plants/type of tissue	Minimum number of leaves per laboratory sample	Approximate weight of the laboratory sample
Samples from individual plants with leaves (symptomatic or asymptomatic)	Petioles and/or midribs or leaves of large size such as *Coffea* sp., *Ficus* sp., *Vitis* sp., *Nerium oleander*	5	0.5-1 g
	Petioles and/or midribs of leaves of small size such as *Polygala myrtifolia* and *Olea* sp.	25	0.5-1 g
	Plant species without petioles or with small petiole and midrib	25	0.5-1 g
Dormant plants or cuttings	Xylem tissue	N.A(not applicable)	0.5-1 g
Other cuttings	Stem	N.A(not applicable)	1 g
Composite sample from asymptomatic plants from several plants (Bergsma, coffee, olive)	Samples collected from, e.g., imported consignments or nursery monitoring	100-200	Up to 10 g (per sample or sub sample) or 10-50 g

EPPO,2016 and 2018; Diagnostic protocols for regulated pest,2018

Plant sample transport and storage in the laboratory

- Once samples are collected, they should be kept cool (e.g. 4-15°C) and transported to the laboratory as quickly as possible.
- Lower temperatures can reduce sample deterioration.
- However, *X. fastidiosa* does not survive well in cold temperatures and for **culture isolation work it is better to process samples immediately rather than refrigerate**.

1. If necessary, however, samples for isolation may be kept refrigerated (e.g. 4°C) for up to three days.
2. For other tests, samples may be refrigerated for up to one week.
3. For longer term storage, samples may be stored at -20°C or -80°C for molecular or serological detection.

Isolation procedure

Surface sterilization

Plant tissue segments

- It is very important to surface sterilize the sample in order to avoid contaminants, because *X. fastidiosa* grows very slowly (up to 30 days) and can be readily overgrown by other microorganisms.
- Petiole or midrib samples are surface sterilized by immersion in 70% ethanol for 1 min and flaming, or in 1% bleach for 2 min, followed by two rinses in sterile distilled water. Surface-sterilized plant tissue segments are cut in the middle, squeezed with flame-sterilized needle-nose pliers, and the sap that exudes can be blotted directly onto media (Hopkins, 2001).

Isolation procedure
Surface sterilization
Plant tissue segments

- Alternatively, tissue is ground in PBS at ratios of 1:10 and 1:100 with a mortar and pestle or a homogenizer (e.g. Homex) and then plated onto two different types of specific media (e.g. PD2, BCYE, PWG).
- The application of ultrasonication during the extraction process has been shown to improve isolation from asymptomatic *Coffea arabica* plants.
- After tissue is ground in PBS, the crushed plant material is ultrasonicated for 30-60s at 40 kHz.

Isolation procedure

Culture defined media

Plant tissue segments

- The surface-sterilized twigs (thin slices of 1-year-old twigs, 1-2 mm thick) can be sliced tangentially(peripherally) with a sterile scalpel and the slices can be directly placed on the agar medium in Petri dishes.
- The plates have to be incubated at 28°C for 8-10 days. The plates are kept in plastic bags to prevent desiccation.

POnTE Project

Isolation procedure

Surface sterilization

Plant tissue segments

- The plating process consisted of removing the terminal 5 mm of each piece of petiole and dissecting the tissue into 3-5mm segments.
- Each segment was squeezed in the center using forceps or pliers and the fluid which was discharged from each end of the segment was blotted onto PW.
- A total of 10-12 attempts per sample.

Torres,2012

Isolation procedure

Surface sterilization

Insect vectors

- Insect vectors are surface sterilized as above and the heads are severed from the body and homogenized in 2 ml PBS.
- Drops of the insect tissue are plated onto specific media as above i.e. PD2, BCYE, PWG.

Isolation procedure

Sample preparation for culturing, PCR analysis and ELISA test

Olive samples

- Olive twigs (at least 10) collected from a sampled tree.

1. Leaves showing leaf scorching and symptomless leaves (N. 8-10 corresponding to 0.5-1 gr) selected for the sample preparation,
2. petioles and midveins excised for the extraction.

Loconsole,2016;..

Isolation procedure

Sample preparation for culturing, PCR analysis and ELISA test

Almond and cherry samples

- Leaves of almond(up) and cherry (down) collected in late summer showing leaf scorch symptoms.

1. The petioles and the basal parts of the almond (up) and
2. cherry (down) leaves used for extraction.

Loconsole,2016;..

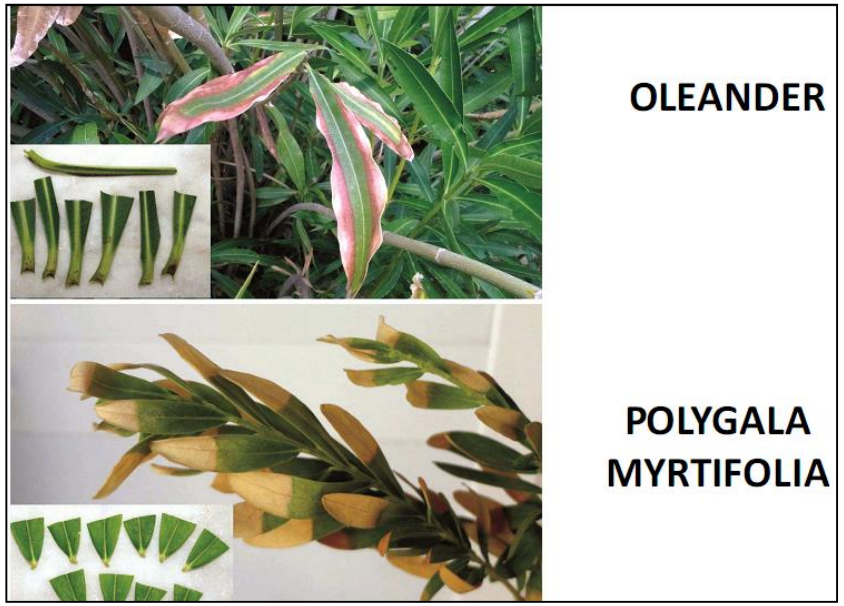
Isolation procedure

Sample preparation for culturing, PCR analysis and ELISA test

Oleander and Myrtle-leaf milkwort (*Polgala myrtifolia*) samples

- **Samples and tissues selected for**

1. **Oleander (up),**
2. ***Polygala myrtifolia* (down).**



Loconsole,2016;..

Isolation procedure

Culture defined media

From grapevine

- Necrotic tissue with yellow or burgundy red margins developed at the edge of the leaves and then coalesced.
- Severely affected leaves became fully necrotic and fell prematurely, leaving matchstick-like petioles attached to the cane.
- Affected twigs and branches declined and plant dieback was observed within 1 to 5 years.

Su *et al.*,2013

Isolation procedure
Culture defined media
From grapevine

- Isolation can be performed from leaf veins, petiole, small twigs or roots.
- In any case, it is critical to properly surface sterilize the sample and to dilute the plant extract in sample buffer.
- The xylem sap obtained either from the crushing of leaf vein, petiole or small twig or from extraction with vacuum infiltration of small twigs and roots can be streaked on to CS20, PD2, PD3 or B.CYE media.

Isolation procedure

Culture defined media

From grapevine leaves

1. For recovering the bacterium from the leaf, 1.0 g of petiole or leaf vein is first surface sterilized in 0.5% of sodium hypochloride for 10 min and then rinsed in four changes of sterile distilled water and dried in a laminar flow cabinet.
2. Subsequently, it is ground in a sterile mortar containing 5 ml of sample buffer (SB) (disodiumsuccinate 1.0 g/l; trisodium citrate 1.0 g/l; K_2HPO_4 1.5 g/l; KH_2PO_4 1.0 g/l; 0.02 M of sodiumascorbate; 5% of acid-washed insoluble polyvinylpyrrolidone; pH 7.0), or
3. grape sample extraction buffer provided by Agdia (Tris (hydroxymethyl) aminomethane 60.5g/l; sodium chloride 8.0g/l; polyvinylpyrrolidone (PVP), MW 24-40 20.0g/l; polyethylene glycol 10.0g/l; sodium azide 0.2g/l; tween-20 0.5g/l; pH 8.2).

Isolation procedure

Culture defined media

From grapevine twig or root

- In case of twig or root, they have to be cut into 1-2 cm sections, stripped of bark, surface sterilized for 5 min in 0.5% of sodium hypochloride with 3% of ethyl alcohol added.
- Then the pieces have to be rinsed in four changes of sterile distilled water and crushed in a sterile mortar containing 5 ml of sample buffer (SB).

Isolation procedure

Culture defined media

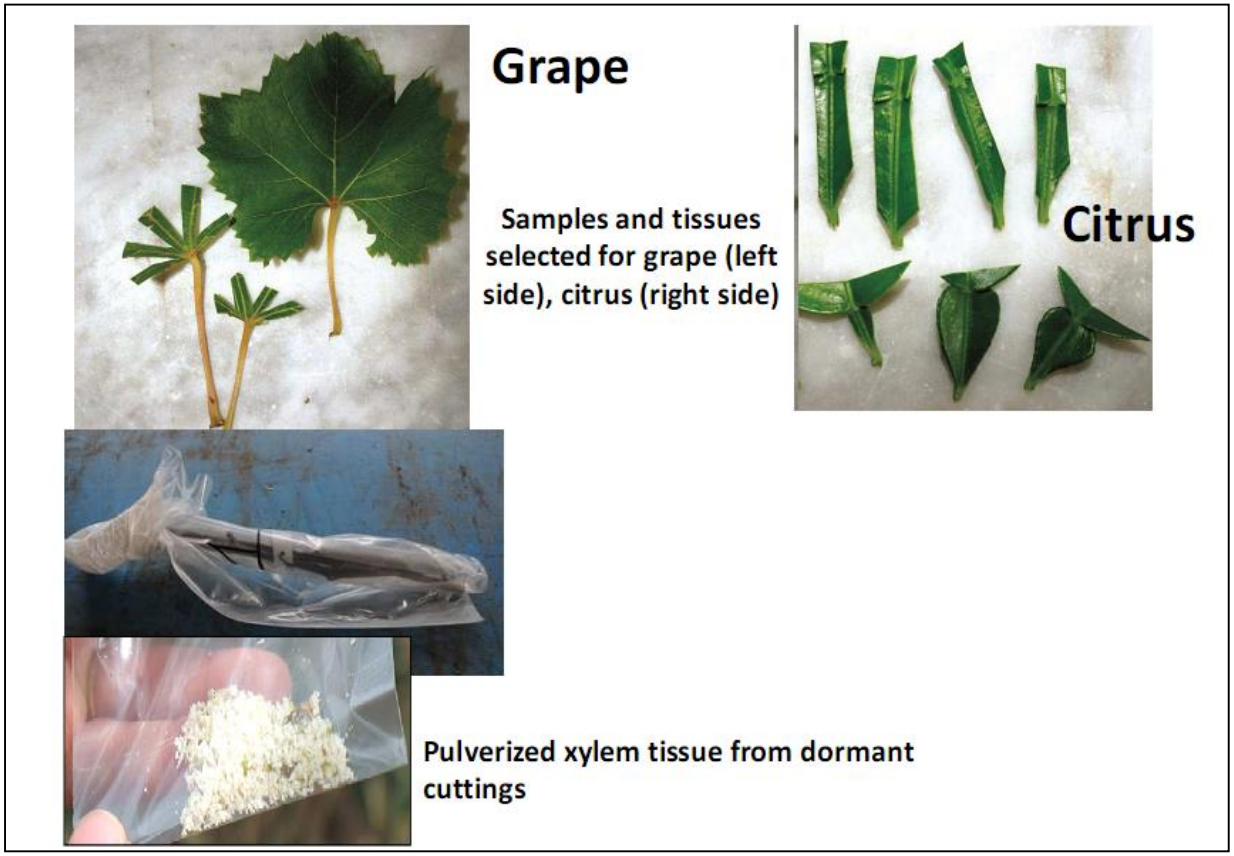
From grapevine twig or root

- In case of twig or root, they have to be cut into 1-2 cm sections, stripped of bark, surface sterilized for 5 min in 0.5% of sodium hypochloride with 3% of ethyl alcohol added.
- Then the pieces have to be rinsed in four changes of sterile distilled water and crushed in a sterile mortar containing 5 ml of sample buffer (SB).

Isolation procedure

Sample preparation for culturing, PCR analysis and ELISA test

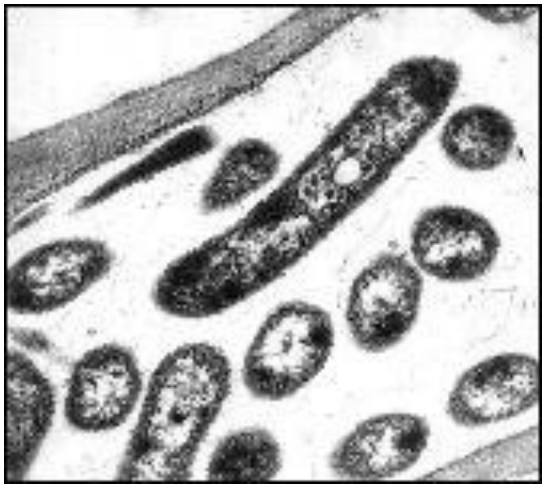
Grape and citrus samples



Loconsole,2016;..

Citrus variegated chlorosis

Xylella fastidiosa subsp. *pauca*

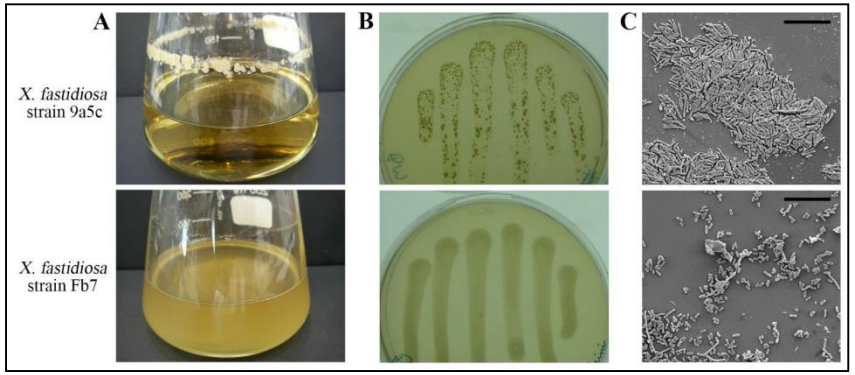
	
Leaf chlorosis between veins caused by Citrus Variegated Chlorosis (CVC).	***Xylella fastidiosa* bacterium, causing Citrus Variegated Chlorosis (CVC).**

Chung and Brlansky,2009

Autoaggregation and intense biofilm formation when cultured *in vitro* *Xylella fastidiosa* subsp. *pauca*

A. The reference CVC strain 9a5c forms large aggregates and compact biofilm structures, while strain Fb7 remains mostly planktonic and forms very little biofilm on glass under agitation.

B. Cellular suspensions spotted on PW agar plates and drained to the other side of the plates forming the columns also show a distinct biofilm formation between the strains after 7 days of growth, with a wax-like texture for 9a5c and a gum-like texture for Fb7.



de Souza *et al.*,2020

Isolation procedure

Culture defined media

From citrus leaves

1. Symptomatic leaves are surface-sterilized with 10% bleach for 5 min, followed by two rinses in sterile distilled water.
2. Midribs and petioles are aseptically excised and placed on to sterile Petri dishes containing 1-2 ml of PBS.

i. Sections of 2-3 mm are obtained with a sterile scalpel.

ii. The sections are grinded and the sap is streaked on to PW or SPW medium.

- The plates are incubated at 28°C for 21 days.

Isolation procedure
Culture defined media
From citrus roots and stems

- For isolation from roots and stem, after their surface-disinfection to be performed as described above, segments of 4-12 mm in diameter and 2-3 cm long are vacuum infiltrated with succinate-citrate-phosphate buffer (1.0 g/l disodium succinate, 1.0 g/l trisodiumcitrate, 1,5 g/l K_2HPO_4, 1.0 g/l K_2HPO_4, pH 7.0) as described for grapevine.
- Then, the vacuum extract (3-4 ml per sample) is centrifuged at 4.500 *g* for 15 min and resuspended in 0.8 ml of buffer.
- One drop (5 µl) is, subsequently, placed on to PW, SPW, CVC1 or CVC2 media.
- The plates are incubated at 27-30°C and they are kept in plastic bags to prevent desiccation. The plates are observed for colony development at weekly intervals for a month with a binocular microscope.

Isolation procedure

Enrichment media

Grape Xylem sap collection

- Thirty ml of xylem sap from 3 different European grape cultivars (*Vitis vinifera*); Cabernet Sauvignon, Pinot Noir, and Sylvaner were pooled together in a 50 ml beaker.
- Six ml of the xylem sap from the 50 ml beaker was placed into a 10 ml beaker and had the pH adjusted to 4.0.
- After pH adjustment the xylem sap was sterilized by placing it in a sterilized 0.13 mm syringe with a sterilized 0.22 µm filter cap, and discharged into a sterilized 10 ml beaker.
- Then 145 µl of "bacteria into SCP" is combined with the sterilized xylem sap and dispersed into the 96 well plate, at 200 µl per well.
- This process is repeated again for pH's 5, 6, and 7, with the 6 ml of xylem sap coming from the originally pooled xylem sap in the 50 mL beaker.
- The pH was lowered with hydrochloride acid or raised with sodium hydroxide.
- PW media with *X. fastidiosa* was used as the positive control.
- The negative control was SCP.

SCP creation 1.0g Na_2 Succinate, 1.5g K_2HPO_4, 1.0g KH_2PO_4, 1.0g Na_3 Citrate, dissolved in 1 liter of deionized water, then 2 ml of the solution was placed into test tubes which were autoclaved for 15 minutes at 140 degrees Celsius prior to use.

Zintzun,2006

Isolation procedure
Culture defined media
PD2, BCYE or PWG

- *Xylella fastidiosa* is very difficult to isolate and grow in axenic culture, even from symptomatic plants.
- The bacterium does not grow on most common culture media and requires specific media.
- PD2, BCYE or PWG are widely used for the isolation from different host species.
- The use of at least two different media is recommended, in particular when isolation is attempted for new hosts or in the case of a first detection.
- Whenever possible, isolation and achieving Koch's postulates is considered to be the 'gold standard' (EFSA, 2016b).

EPPO,2018

Isolation procedure

Culture defined media

BCYE and PWG

- Based on the experience of different laboratories PWG is considered the best isolation media for samples from most plants.
- Samples from Olive plants are best isolated on BCYE.
- It is very important to **surface disinfect the sample to avoid growth of saprophytes** because *X. fastidiosa* grows very slowly (the colonies can take up to 28 days to be visible) and can be readily overgrown by other microorganisms in the plates.

EPPO,2018

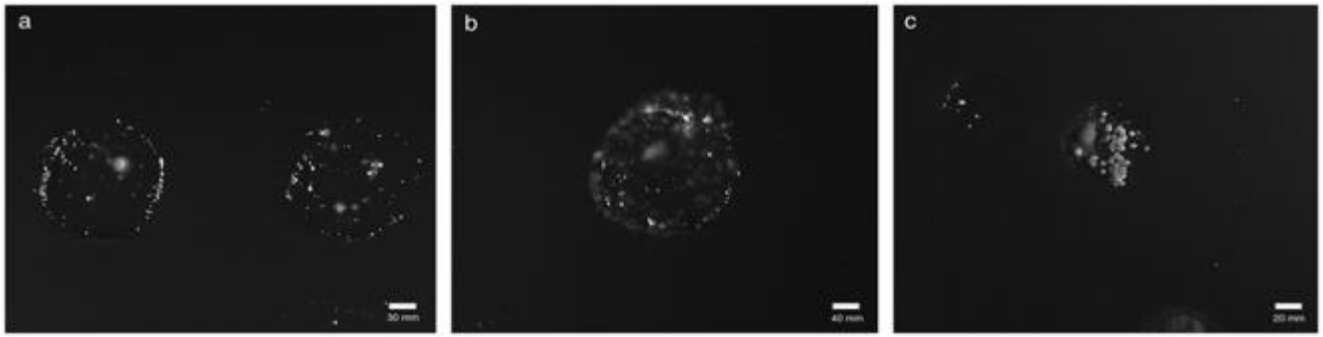
Isolation procedure
Imprint method
BCYE medium

- Stem cuttings from 58 different olive trees were cut into pieces 8 to 10 cm long, washed under tap water, surface-sterilized in 2% sodium hypochlorite for 2 min, soaked in 70% ethanol for 2 min and rinsed three times in sterile water.
- Each piece was cut in half and squeezed at one end with a plier, while the other end was gently pressed on a buffered charcoal yeast extract (BCYE) growth medium to make 2-3 imprints.
- For each sample three BCYE plates were spotted (ca. 20-30 spots per plate), incubated for 3-4 weeks at 28°C and periodically inspected for the growth of *Xylella* colonies.

Saponari *et al.*, 2017

Isolation procedure
Imprint method
BCYE medium

- *Xylella fastidiosa* colonies on BCYE agar medium growing in different stem-prints obtained after imprinting the fresh cut surface of the olive cuttings on the medium.



a. Shows a low number of colonies per spot;
b. and c. show the high number of colonies growing mostly together.

Saponari *et al.*, 2017

Isolation procedure
Culture defined media
Incubation period

- Petioles from grapes were cut and ground in 1ml of distilled water and dilutions (10^{-2} and 10^{-3}) of this suspension were made.
- Aliquots of 300µL of each dilution were inoculated by triplicate in liquid PW media.
- All cultures were incubated in the dark at 28°C under static conditions. After 15 days PW broths were tested by DAS ELISA and only those positive for *X. fastidiosa* were used in the isolation procedure
- Solid PW broths were inoculated with 100µL aliquots of liquid PW broth.
- Plates were incubated at 28°C and observed for bacterial colony growth for 12 days.
- Colonies were tested by Gram staining, catalase and oxidase.

Aguilar *et al.*,2008

Isolation procedure
Interpretation of isolation results

- Colonies are usually visible after 2-3 weeks but the plates should be observed for up 6 weeks.
- The isolation is positive if bacterial colonies with growth characteristics and morphology similar to *X. fastidiosa* are observed within the above-mentioned period on at least one medium.
- The reference culture should also have grown on the media used.
- The presumptive identification of *X. fastidiosa* colonies should be confirmed by serological or molecular tests.

EPPO,2016

Culture defined media

Common and enrichment media

NA, NAG, CS20, PD1,5 PD2,6 PD3, and PW

- It has long been considered that *X. fastidiosa* is fastidious and unable to grow on standard bacteriological media, despite reports that Nutrient Agar can support growth.
- Nutrient agar has been shown to support *X. fastidiosa* growth for grape and plum isolates.
- Bacterial colonies obtained on PD2, BCYE, CS20, are transferred to nutrient glucose agar (NGA) and PW medium or the medium used in the isolation(Schaad *et al.*,2001).
- Note that bacterial colonies obtained on PD2, BCYE, CS20, or PW medium that will not grow on NGA, or grow very grow very slowly on NGA(Schaad *et al.*,2001).

Schaad *et al.*,2001;Almeida *et al.*,2004;..

Culture defined media

Common and enrichment media

CS20, PD1,5 PD2,6 PD3, and PW

- A range of enrichment media such as CS20, PD1, PD2, PD3, and PW have been used successfully to culture *X. fastidiosa*.

1. These are rich in carbon and nitrogen sources.
2. Peptones, soybean, or yeast extracts are used as major nutrient sources;
3. amino acids are added as supplements; all have an additional iron source (bovine hemin chloride or ferric pyrophosphate).

Almeida *et al.*,2004;..

Common defined media
Common and enrichment media

- In addition to these amino acids, *Xylella fastidiosa* appears to have a demand for glucose or organic acids, although the relative importance of each source of C is still under dispute.
- Peptones, soybean, or yeast extracts are used as major nutrient sources; amino acids are added as supplements; all have an additional iron source (bovine hemin chloride or ferric pyrophosphate).

Almeida *et al.*,2004;..

Common defined media
Common and enrichment media

- It has long been considered that *X. fastidiosa* is fastidious and unable to grow on standard bacteriological media, despite reports that Nutrient Agar can support growth (Chang *et al.*,1990; Fry *et al.*,1990).
- Nutrient agar has been shown to support *X. fastidiosa* growth for grape and plum isolates.
- *Xylella fastidiosa* can grow well on a simple, chemically defined medium containing mainly amino acids (especially glutamine) and salts of weak organic acids such as succinate or citrate.

Almeida *et al.*,2004; Purcell,2007

Common defined media
Common and enrichment media

- The bacterium also grew on media with citrate and L-glutamine as the only carbon and nitrogen sources.
- Potato starch was not essential for bacterial growth, but no growth was observed on media without hemin chloride.
- Agar inhibited bacterial growth when used as the gelling agent.

Almeida *et al.*,2004

Common defined media
Common and enrichment media

- *Xylella fastidiosa* grows exclusively in the xylem and although the xylem sap contains a diversity of compounds such as amino acids, organic acids, and inorganic nutrients, they are usually found in low concentrations, limiting bacterial growth.
- Glutamine (Gln) and asparagine (Asn) are the main amino acids in the xylem sap of plants and for this reason they have been included in several artificial media for Xf growth.

Sterilize Glutamine stock solution(4%) by membrane filtration using a 0.22 µm Millipore. Upon autoclaving L-glutamine was structurally modified into 5-oxo proline and 3-amino glutarimide (α-amino glutarimide).

Purcino *et al.*,2007; Sandal *et al.*,2011

Common defined media

Common and enrichment media

CS20, PD1, PD2, PD3, and PW

- A range of enrichment media such as CS20, PD1, PD2, PD3, and PW have been used successfully to culture *Xylella fastidiosa*.
- These are rich in carbon and nitrogen sources.
- It is important to remind that all of the ingredients have to be dissolved in the order given.
- It is recommended to use more than one single medium for the primary isolation.
- The plates are kept in plastic bags to prevent desiccation.

EPPO;..

Common defined media

Common and enrichment media

CS20, BCYE, PD2, PWG, and PW

- The PW or PWG media are well suited for bacterial isolation from CVC symptomatic tissues (petioles or branches of citrus).
- On these media, small (~0.30 mm of diameter), white, and convex colonies are observed under a dissecting microscope after approximately 10 days of growth at 27°C–30°C.
- Other media like BCYE, CS20, and PD2 also support cell growth, but it may take over 20 days for colonies to be observable.

Almeida *et al.*,2014

Common defined media
PD2

- For the isolation of *X. fastidiosa* from several host plants including grapevine.
- Deionized distilled water 1.0 L
- Soy peptone 2.0 g
- Bacto tryptone 4.0 g
- Disodium succinate 1.0 g
- Trisodium citrate 1.0 g
- K_2HPO_4 1.5 g
- KH_2PO_4 1.0 g
- Hemin chloride stock solution (0.1% in 0.05 N NaOH) 10.0 ml
- Bacto agar 15.0 g
- $MgSO_4.7H_20$ 1.0 g
- Bovine serum albumin fraction V (20% w/v)* 10.0 ml
- pH 6.9
- Autoclave at 121°C for 15 min.

*Bovine serum albumin is filter sterilized and added to the rest of the medium at 50°C.

Schaad *et al.*,2001;EPPO,2016

Bovine serum albumin
Preparation Note

- Bovine serum albumin (BSA or "Fraction V") is a serum albumin protein derived from cows.
- Animal serum or albumin is routinely added to culture media as a source of nutrients.
- **Preparation Note**
  BSA is normally dissolved in buffers such as PBS, TBS, PBS-T, and TBS-T.
- Note 1: BSA might foam substantially when dissolved in any solvent; thus, one must wet the BSA powder first and gradually add the solvent to properly dissolve the powder. Some might even suggest to just leave the BSA powder to dissolve without shaking at r.t.p. for 10 min as it would dissolve quite easily in due time.
- **What is the difference between albumin and serum albumin?**
- They are both proteins made by the liver, however, and both have been used historically to evaluate nutritional status.
- Note 2: not all albumins have the same efficacy in culture media.

Common defined media
PD3

- PD3 (Davis *et al.*,1980)
- It is the same as PD2 but bovine serum albumin is replaced with soluble potato starch (2 g/l).
- So, all of the ingredients can be directly sterilized in autoclave.

Extraction of Starch from Yellow Skin Potato

The experimental values of extracting starch from yellow skin potato indicate the processing conditions at 3000 rpm and 15 min as optimum for the highest yield of extracted starch.

Method in details: Six hundred grams of potatoes were washed thoroughly, peeled, sliced, and chopped into small chunks. The distilled water was added to the chopped potato and the extraction process was carried out through the use of a centrifuge at different speeds (1000, 2000, 4000) rpm for different periods of time (5, 10, 15 min). Thereafter, the centrifuged samples were filtered using Whatman no. 1 and the supernatant was neglected to obtain wet starch. The wet starch was dried at room temperature for 5 h, then crushed into a fine powder and stored in sealed containers for later use(Altemimi,2018).

Common defined media
CS20

- For the isolation of *X. fastidiosa* from several host plants including grapevine.

Deionized distilled water:	1.0 L
Soy peptone	2.0 g
Bacto tryptone	2.0 g
Hemin chloride stock solution (0.1% in 0.05 N NaOH)	15.0 ml
$(NH_4)_2HPO_4$	0.8 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.4 g
Phenol red stock solution (0.2%)	5.0 ml
L-glutamine	6.0 g
Dextrose	1.0 g
L-histidine-HCl	1.0 g
Potato starch soluble	2.0 g
Bacto agar	12.0 g

- pH 6.6
- Autoclave at 121°C for 15 min.

1. To prepare Hemin chloride 0.1%, dissolve 1.2 g of NaOH in 600 ml of distilled water and add 0.6 g of Hemin Cl and dissolve. Keep in a dark container.
2. To prepare Phenol red 0.2%, dissolve 0.6 g of phenol red in 30 drops of 20% NaOH and bring the volume up to 300 ml with distilled water. Store in the refrigerator.

Schaad *et al.*,2001;EPPO,2013

Common defined media

BCYE (Buffered charcoal-yeast extract agar)

Used for isolation of XF from citrus, coffee and olive

- **BCYE medium (Wells *et al.*,1981)**
- Deionized distilled water 1 L
- Yeast extract 10.0 g
- Activated charcoal 2.0 g
- L-cysteine HCl.H_2O 0.4 g
- Ferric pyrophosphate (soluble) 0.25 g
- ACES buffer (N-2-acetamido-2-aminoéthane sulfonic acid) 10.0 g
- Bacto agar 17.0 g
- L-cysteine HCl and Ferric pyrophosphate are dissolved, filter sterilized (0.2 µm filter), and added to the autoclaved basal media, pH 6.9.

Due to the difficulty of dissolving and re-suspending the individual components it is recommended that ingredients are dissolved in the following order. ACES buffer is first rehydrated in 500 ml distilled water at 50 °C before addition of the yeast extract, activated charcoal and agar. Before adding the agar, the pH is adjusted to 6.9 by the addition of approximately 40 ml 1 M KOH. The medium is autoclaved and then cooled to 50 °C. Both the cysteine hydrochloride (0.4 g) and ferric pyrophosphate (0.25 g) are resuspended in 10 ml distilled water, filter sterilized and added to the cooled sterile medium. The ferric pyrophosphate needs to be heated, under agitation, at 75°C for approximately 15–20 min (EPPO, 2018b).

Schaad *et al.*,2001;EPPO,2016; Diagnostic protocols for regulated pest,2018

Common defined media

BCYE (Buffered charcoal-yeast extract agar)

Used for isolation of XF from citrus, coffee and olive

- Activated charcoal or activated carbon is nontoxic in nature and is used as an adsorbent to remove broad spectrum of pollutants in air, water and soil.
- The media contains charcoal, which acts as detoxicant and activated charcoal decomposes hydrogen peroxide, a metabolic product toxic to bacteria such as *Xylella* and *Legionella* species. activated charcoal may also collect carbon dioxide and modify surface tension.
- Yeast extract acts as a rich source of vitamins, nitrogen as well as carbon.
- ACES Buffer maintains optimal pH for growth while L-cystine hydrochloride; ferric pyrophosphate stimulate growth of the bacteria.

Common defined media

BCYE modified

Components	Quantity
Yeast extract	10 g
(Vetec) Activated charcoal	2.0 g
L-cysteine HCl (Sigma)*	0.4 g
(Sigma)** Ferric pyrophosphate	0.25 g
(N-2-acetamido-2-aminoéthane sulfonic acid) ACES buffer	10 g
Difco Bacto agar	17 g
Deionized distilled water	940 ml
KOH solution 1M	40 ml

1. Warm (≈50°C) the Aces buffer in 500 ml of distilled water;
2. Add 40 ml KOH in 1N in 440 ml distilled water;
3. Add the active charcoal in step-2 solution;
4. Pool the step 1 and 3 solutions;
5. Add the yeast extract to pooled solution;
6. Adjust the pH to 6.85;
7. Add agar and autoclave;
8. Cool down the medium at 50°C and add pre- filtered L-cysteine and Ferric pyrophosphate.

* L-cysteine diluted in 10ml sterile water;
** Ferric pyrophosphate needs to be diluted in 10ml sterile water.

XF ACTORS; Ponte,2017

Common defined media

The modified PCYE medium was as effective as BCYE for isolation, growth, and quantification of *X. fastidiosa*

- Higher frequencies of positive isolations were obtained for PCYE for the isolation of *X. fastidiosa* from citrus seedlings (36 of 38 versus 40 of 43 in BCYE) and coffee trees (35 of 39 versus 33 of 42 in trees (35 of 39 versus 33 of 42 in BCYE). The level of agreement of both media was 86.8% for citrus seedlings, 100.0% for citrus trees, and 87.2% for coffee trees.

Culture medium and positive or negative isolation	Sample of greenhouses, citrus seedings (%)	Sample of field citrus trees (%)	Sample of field coffee trees (%)
BCYE+/PCYE+	33 (86.8)	9 (100)	30 (76.9)
BCYE+/PCYE–	2 (5.3)	0	0
BCYE–/PCYE+	3 (7.9)	0	5 (12.8)
BCYE–/PCYE–	0	0	4 (10.3)

Comparing the efficacy of Buffered charcoal–yeast extract agar (BCYE) and modified medium named as phosphate buffered charcoal-yeast extract medium (PCYE). The latter, was found as effective, less expensive, and easier to prepare than BCYE.

Lopes and Torres,2006

Common defined media

PW

Periwinkle wilt medium

- PW (For isolation of *X. fastidiosa* from several host plants including *Citrus* spp.)(Davis *et al.*,1983)
- Deionized distilled water 1.0 l
- Soytone 4.0 g
- Bacto Tryptone 1.0 g
- Hemin chloride stock solution (0.1% in 0.05N of NaOH) 10.0 ml
- $MgSO_4.7H_2O$ 0.4 g
- K_2HPO_4 1.2 g
- Phenol red stock solution (0.2%) 10.0 ml
- L-glutamine 4.0 g
- Bovine Serum Albumin fraction V (20% w/v) 30.0 ml
- Bacto agar 12.0 g
- pH 6.6
- Bovin Serum Albumin is filter sterilized and added to the rest of the medium at 50°C.

Schaad *et al.*,2001; EPPO,2013

Common defined media

PWG

A modified periwinkle medium (PW)

- The PWG substituted 9 gram of Gelrite for the agar used in PW and the amount of 20%(W/V) bovine serum albumin solution was reduced from 30 to 15 ml per liter.
- Deionized distilled water 1.0 l
- Soytone 4.0 g
- Bacto Tryptone 1.0 g
- Hemin chloride stock solution (0.1% in 0.05N of NaOH) 10.0 ml
- $MgSO_4.7H_2O$ 0.4 g
- K_2HPO_4 1.2 g
- Phenol red stock solution (0.2%) 10.0 ml
- L-glutamine 4.0 g
- Bovine Serum Albumin fraction V (20% w/v) 20.0 ml
- Gelrite 9.0 g
- pH 6.6
- Bovin Serum Albumin is filter sterilized and added to the rest of the medium at 50°C.

Hill and Purcell,1995

Common defined media

Modified periwinkle-gelrite medium(PWG-M agar)

Periwinkle wilt gelrite or periwinkle wilt GelRite

Gelrite gellan gum (Sigma)	9.0 g
Phytone peptone (e.g. BD BBL)	4.0 g
Bacto tryptone (e.g. Oxoid)	1.0 g
Phenol red stock solution (0.2%)	10 ml
L-glutamine (Sigma)	4 g
Hemin chloride stock solution (0.1% in 0.05 N NaOH)	10 ml
BSA (stored in refrigerator)	3.0 g
$MgSO_4.7H_2O$	1.0 g
K_2HPO_4	1.5 g
KH_2PO_4	1.0 g
Distilled water to a final volume of a 1 liter	

L-glutamine, hemin chloride stock solution and BSA are added after autoclaving. Bovine serum albumin (3 g) is dissolved in 15 ml distilled water, and 4 g L-glutamine is dissolved in 100 ml distilled water over a low heat (c. 50°C) on a hot plate at low heat. Do not boil. Hemin chloride stock is 0.1% bovine hemin chloride dissolved in 0.05 N NaOH. These three solutions are filter sterilized (0.2 µm membrane) and added to the cooled sterile basal medium.

EPPO; Diagnostic protocols for regulated pest,2018; Janse *et al.*,2012

Common defined media

SPW

Supplemented PW

- SPW (For stimulating the growth of *X. fastidiosa* to be isolated from sweet orange) (Hartung *et al.*, 1994).
- It is the same as PW supplemented with:

Malt extract	5.0 g
Sucrose	10.0 g
Myo-inositol	0.1 g
Thiamine chloride	0.01 g
Pyridoxine chloride	0.01 g
Nicotinic acid	0.005 g
Glycine	0.002 g

EPPO,2003

Common defined media
CVC1

- CVC1 (it has been developed for the isolation of *X. fastidiosa* from *Citrus* spp.) (Chang *et al.*,1993)
- Deionized water 970 ml
- Bacto-peptone 4.0 g
- Tryptone 1.0 g
- K_2HPO_4 1.2 g
- KH_2PO_4 1.0 g
- $MgSO_4.7H_2O$ 0.4 g
- Phenol red stock solution (0.2%) 10.0 ml
- Agar 12.0 g
- After autoclaving the following filter-sterilized compounds are added:
- Glutamine stock solution (8%) 50 ml
- Bovine serum albumin stock solution Frac V (10%) 60 ml
- pH 6.5

EPPO,2013

Common defined media
CVC2

- CVC2 (Chang *et al.*,1993)
- It is the same as CVC1 plus 10.0 ml of hemin chloride stock solution (0.1%) to be added before autoclaving.

Culture media

XfD-series media

XFD1-XFD9, XfD5-c, XfD5-aa and XfD2-hc

- A simple defined solid medium containing citrate and succinate, three amino acids (L-glutamine, L-asparagine, and L-cysteine), hemin chloride, potato starch, gellan gum (GelRite), and mineral salts supported the growth of grape strains of *Xylella fastidiosa*, the bacterial pathogen that causes Pierce's disease of grape.
- All XfD media defined here had phenol red (10 mL, 0.2% in H_2O), $K_2HPO_4.3H_2O$ (1.5 g), KH_2PO_4 (1.0 g), and $MgSO_4.7H_2O$ (0.5 g).
- XfD media were prepared with deionized water (final volume brought to 1 L) and autoclaved.
- In preliminary tests we found no difference between using deionized and distilled water.

Almeida *et al.*,2004

Culture media

XfD-series media

XFD1-XFD9, XfD5-c, XfD5-aa and XfD2-hc

	XfD No.											
	1	2	3	4	5	6	7	8	9	2-hc	5-c	5-aa
Amino acids												
L-glutamine (anhydrous) (g)	3.0	3.0	3.0	4.0	4.0	4.0	4.0	4.0	4.0	3.0	4.0	
L-asparagine (anhydrous) (g)	1.0	1.0	1.0							1.0		
L-cysteine (anhydrous) (g)	0.5	0.5	0.5							0.5		
Carbon source												
Trisodium citrate (g)	1.5	1.5	3.0	1.5	3.0	3.0	3.0	3.0	3.0	1.5		3.0
Disodium succinate (g)	1.5	1.5		1.5						1.5		
Others												
Hemin chloride (0.1% in 0.05% NaOH) (mL)	10	10	10	10	10		10				10	10
Iron pyrophosphate (g)									0.25			
Potato starch (g)	3.0	3.0	3.0	3.0	3.0	3.0			3.0	3.0	3.0	3.0
Gelling agent												
GelRite (g)		10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Agar (g)	15.0											
X. fastidiosa growth[a]	+	++	+	+	+	−	+	−	−	−	−	−

[a] ++ = single colonies visible within 2 weeks; + = single or confluent colonies observed within 4 weeks; − = no visible growth within 4 weeks.

Note: All XfD media suggested here had phenol red. New *X. fastidiosa* defined media(XfD) and respective reagents used (g/L). We used modified PD3 and PWG by the addition of phenol red dye to the media. XfD2 was the best of our defined media for *X. fastidiosa* growth. *X. fastidiosa* multiplied slowly on XfD1 (3-4 weeks), and we could not observe individual colonies on it without a microscope.

Almeida *et al.*,2004

Salient characteristics

Colony morphology

Colony and cell size and shapes

- The bacterium grows slowly on selective medium to form small colonies that appear white to yellow.
- Gram-negative, rod-shaped bacterium with dimensions of 0.25 to 0.35 μm in radius and 0.9 to 3.5 μm in length.
- Cell replication is by binary fission.
- The bacterial cells often possess a distinctive rippled (undulating) cell wall, composed of three layers.
- The wall consists of an outer, an inner (each comprised of 3-layered unit membrane structure), and a middle peptidoglycan layer.

Colonial characteristics

Grapevine isolates

On PD2, PD3, CS20 and BCYE media

- Colony morphology and microscopical observation grapevine after 10 days of incubation at 28°C on PD2, PD3, CS20 or BCYE media.
- *X. fastidiosa* yields colonies 0.5-2.0 mm in diameter, circular, with entire margins and convex elevation.
- Sometimes colonies are produced that are also circular with undulate margins with an umbonate or flat elevation.

1. Colonies on CS20 were opalescent, creamy in color, circular in form and had entire margins.
2. Colonies on PD2 were opalescent, bronze colored, circular in form, convex and exhibited twitching motility.

EPPO,2004

Colonial characteristics

Citrus isolates

On SPW, PW, CVC1 and CVC2 media

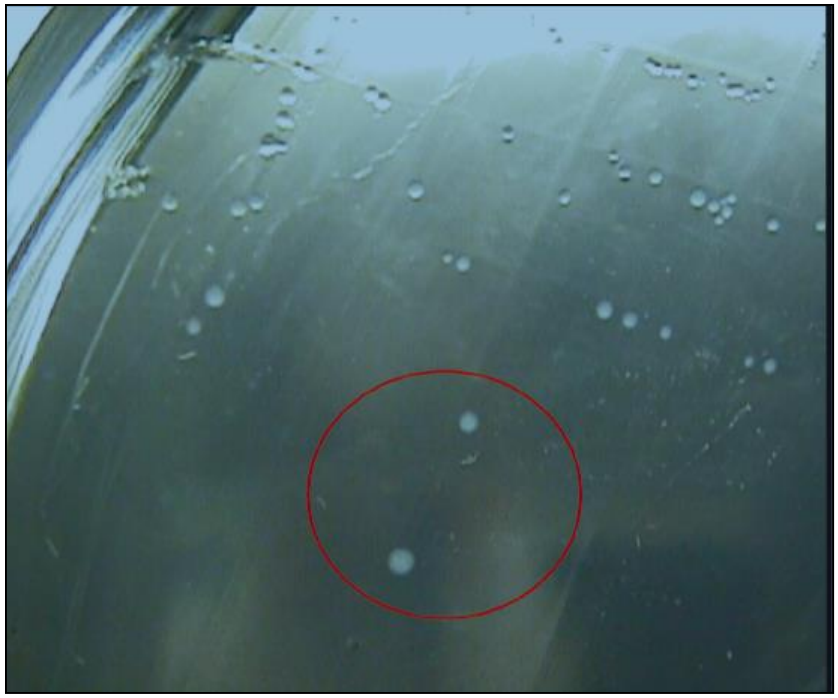
- On SPW medium, isolated colonies are visible within 7 days after streaking. After 21 days of incubation at 27°C, their diameter is 0.35 mm.
- On PW medium, colonies appear 10-14 days after isolation.
- On CVC1 and CVC2 media, colonies develop 25-30 days after isolation.

Xf was first isolated from grape and was successfully cultured on a selective medium called Pierce's Disease 2 (PD2) (Davis *et al.*,1980). Other derivative media also supported growth of Xf (PD3), sugarcane (SC-20) and periwinkle wilt (PW) media were developed by Davis *et al.*,1981.

EPPO,2004;Johnson *et al.*,2021

Colonial characteristics
On PW and PWS media

- Colonies of *X. fastidiosa* isolated from symptomatic grapevine plants.
- Both PW and PWS media were adequate for primary isolations.
- In subsequent attempts of primary isolation, PW was preferred because SPW showed higher rates of contamination than PW.



Colonies of *X. fastidiosa* on periwinkle wilt agar medium PW.

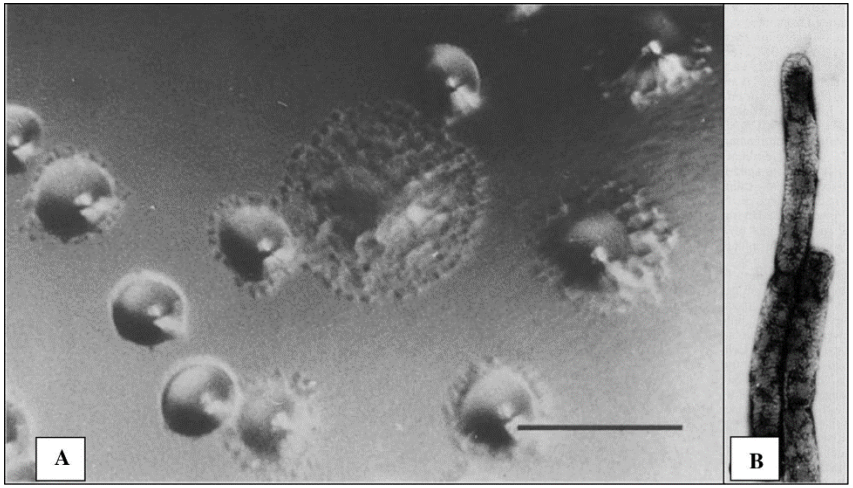
Aguilar *et al.*,2008

Colonial characteristics
On PW agar

A. Colonies of *Xylella fastidiosa* subspecies *multiplex* (Xfm, causal of phony peach disease, PPD) bacterium on PW agar as seen by relected light through a dissecting microscope (bar = 0.05 mm) (Davis *et al.*,1981).

B. Negatively stained bacteria isolated from peach infected with Xfm.

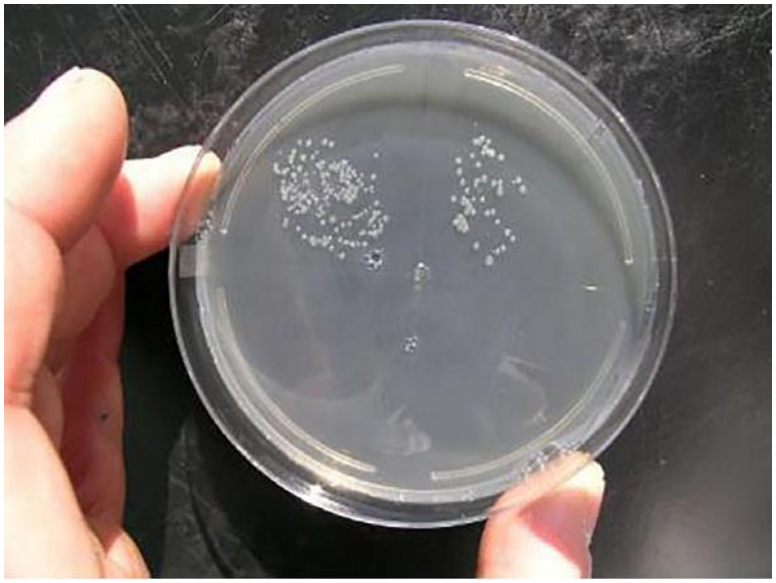
- The rods are 0.25 to 0.27 by 1.2 to 2.4 µm in size and have furrowed cell walls (magnification × 21,000) (from Wells *et al.*,1987).



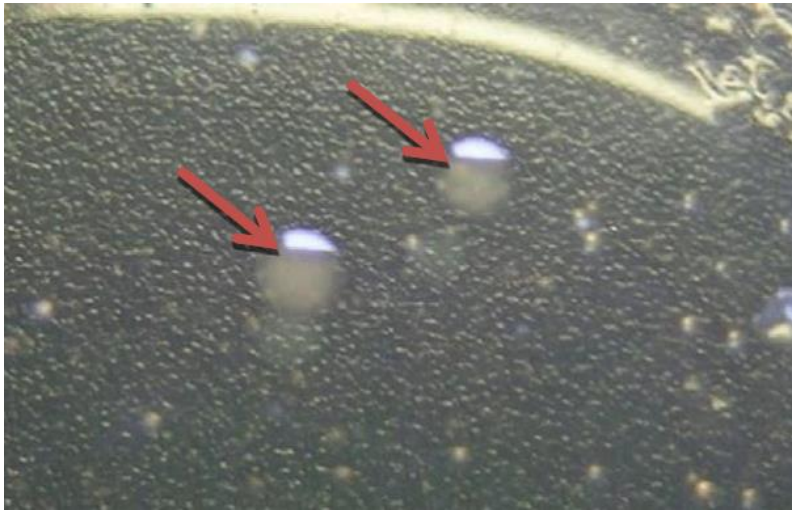
Johnson *et al.*,2021

Colonial characteristics

On modified periwinkle wilt medium (PWG-M agar)



Xylella fastidiosa subsp. *fastidiosa* isolated from *Coffea canephora* on modified PWG (size < 2 mm after 3 weeks).



Xylella fastidiosa subsp. *pauca* isolated from *Coffea arabica* on modified PWG (size <2 mm after 3 weeks) (the background is a sheet of black paper below the plate).

EPPO,2016

Colonial characteristics

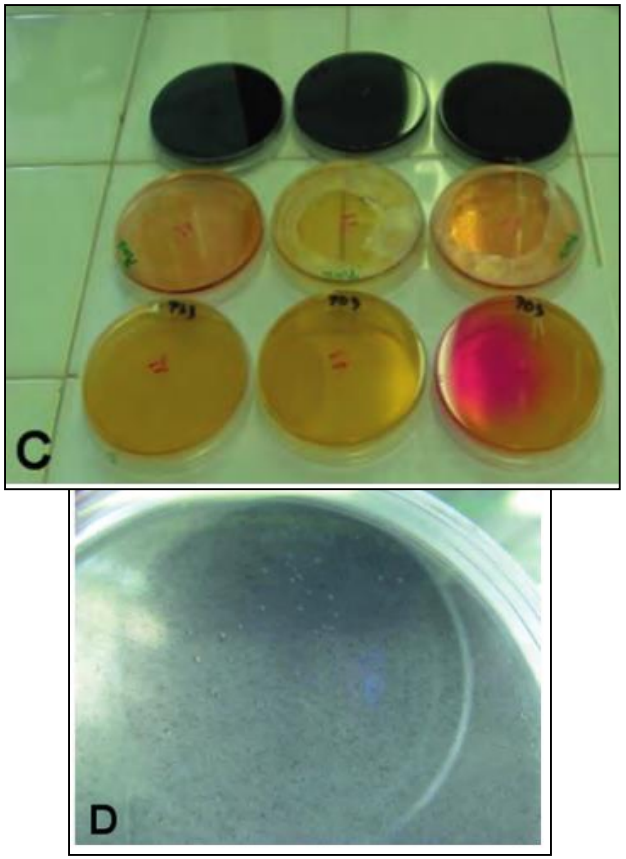
On modified periwinkle wilt medium (PWG-M agar without phenol red)

- Xf strains were cultured at 28°C on modified periwinkle wilt modified medium (PWG-M agar without phenol red) for up to seven days.
- Then, the cultures were transferred to a new plate containing PWG-Magar or B-CYE medium and incubated for up to seven days at 28°C.
- When required, for liquid cultures, Xf strains were incubated at 28°C under 160 rpm agitation in PD2 broth.

Clavijo-Coppens *et al.*,2021

Colonial characteristics
On LMG, PWS and PD3 media

- C. *Xylella* selective media:
- Top row: LMG activated carbon (black) medium;
- Central row: PWG medium with gelrite;
- Lower row: PD3 selective medium (Davis *et al.*,1981).
- D. LMG charcoal medium.
- Pink colour indicates presence of contaminants.

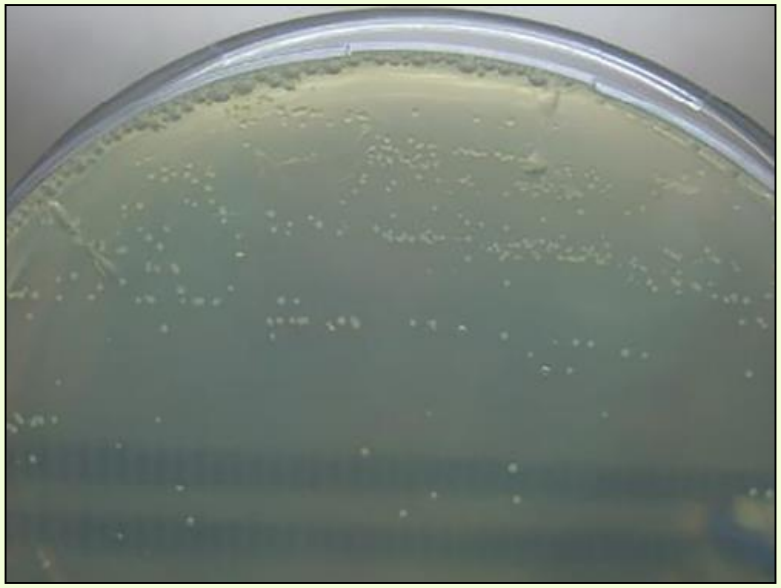


Janse *et al.*,2012

Colonial characteristics

On PD2 medium, a selective medium called Pierce's Disease 2 (PD2) (Davis *et al.*,1980)

- The colonies of *Xylella fastidiosa* subsp. *fastidiosa* cultured at 28°C on solid PD2 medium are opaque. Size < 2 mm after 3 weeks.
- When required, PD2 medium was supplemented with 10 µg/ml gentamicin (Gm) and 10 µg/ml chloramphenicol (Cm).



EPPO,2018;Shi and lin,2018

Colonial characteristics

On buffered charcoal-yeast extract medium (BCYE)

- Two colony types can be observed:
- Smooth and rough, both are circular and opalescent.
- When grown on BCYE1 medium, colonies are small, 0.6 mm in diameter after 10 days at 27°C, expanding to 1.5 mm after 30 days.
- On this medium, rough colonies exhibit green or red margins when viewed under reflected light.

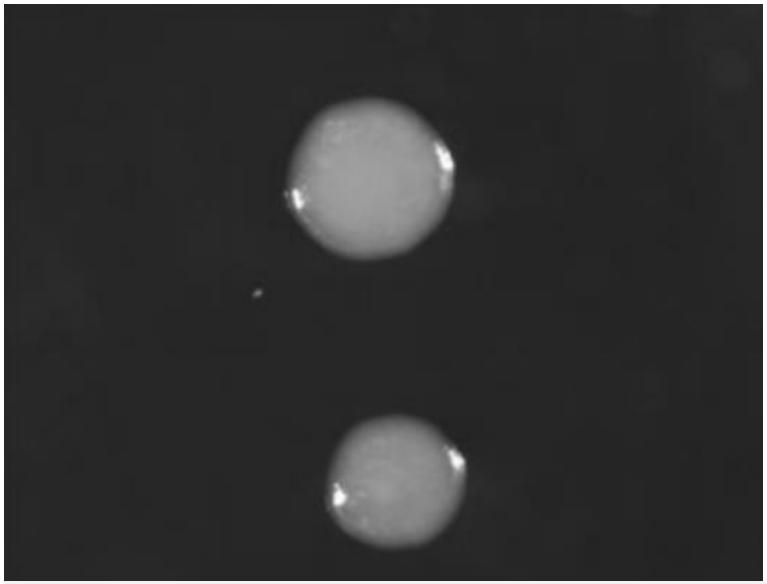
Collection strain of *Xylella fastidiosa* subsp. *fastidiosa* ATCC 35879 on BCYE (size < 2 mm after 3 weeks).

EPPO,2016

Colonial characteristics

On buffered charcoal-yeast extract medium (BCYE)

- Purified colonies of an isolate *X. fastidiosa* subsp. *pauca* Salento-1, grown on BCYE agar for 20 d at 28°C were slightly convex, white, opales-cent, mucoid when touched with a loop, circular with entire margins, with a diameter of about 1.2-1.5 mm and a smooth surface.



Colonies of *Xylella fastidiosa* subsp. *pauca* Salen-to-1, after incubation at 28°C for 20 d on BCYE agar plates.

Bleve *et al.*,2016

Colonial characteristics

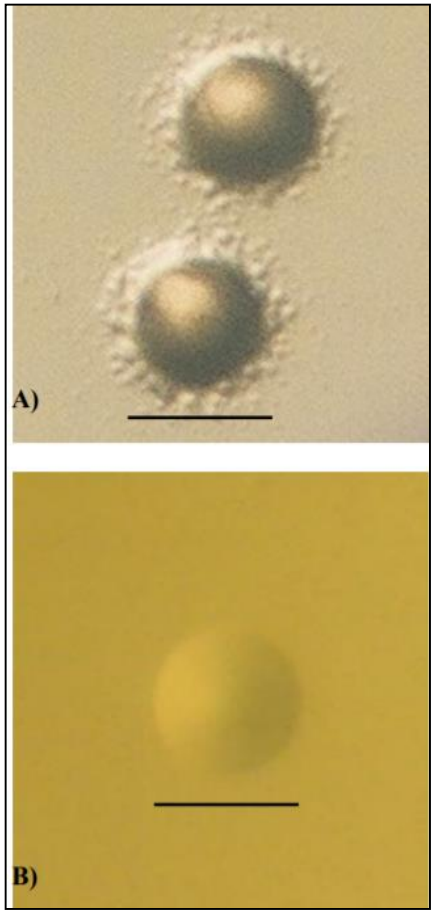
On PD2, and CS20 agar media

Leaf scorch of blueberry(BLSB)

- Two major colony morphotypes: smooth and "pit"-like *Xylella fastidiosa* colonies on: PD2, and CS20 agar media.

A. Colonies on PD2 resembled the pit-like colonies of A-type strains grown on PWG agar media.

B. The G-genotype strains were consistently (>99%) associated with smooth colony morphotypes.



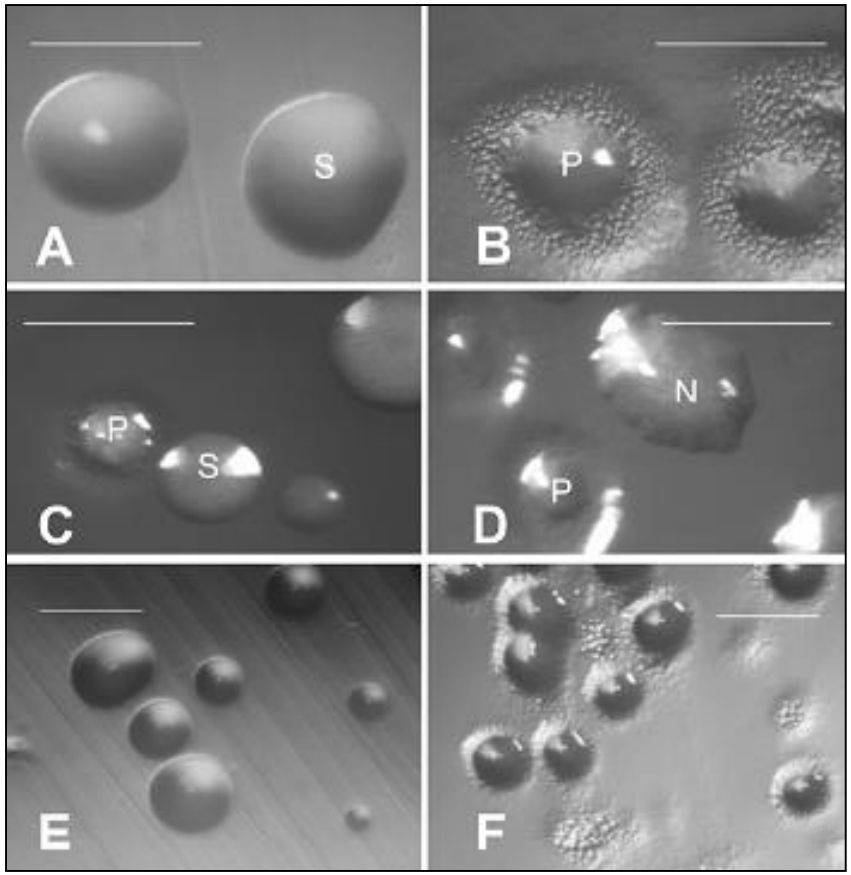
Nissen *et al.*,2010; Chen *et al.*,2007

Colonial characteristics

Almond leaf scorch (ALS)

Two major colony morphotypes: smooth and pit-like

- Two genotypically distinct types of *X. fastidiosa* strains, G-type and A-type, coexist simultaneously in the same infected almond orchard.
- A, Smooth type;
- B, "pit"-like type;
- C, "pit"-like and smooth type;
- D, "pit"-like and non-"pit"-like A-type; and
- E and F, triple-cloned colonies from a single almond petiole (E, smooth G-type; and
- F, "pit"-like A-type).



Chen *et al.*,2005

Diagnostics methods

- Genetic diversity were found amongst *X. fastidiosa* strains.
- The differences between pathogenic populations can be observed using:
- Traditional methods,
- Monoclonal antibodies,
- Random amplified polymorphic DNA (RAPD),
- Arbitrary-primed (AP-PCR),
- Restriction fragment lenght polymorphism (RFLP),
- Variable number of tandem repeated (VNTR),
- Repetitive sequence based-PCR (rep-PCR),
- PCR-RFLP,
- Intergenic spacer (ITS) 16S-23S rDNA sequencing,
- Clamped homogeneous electric field – CHEF,
- Plasmid profile.

Summary of detection methods for *Xylella fastidiosa*

Assay	Number of samples comfortably accommodated	Sensitivity[Y] (number of bacterial cells)	Cost	Labor
Culture	10-100s	1000	Low	Low
ELISA	100-1000s	100,000	Med	Med
PCR	100s	100	High	Med
IC-PCR[Z]	100s	<100	High	High

[Y]Lowest approximate number of bacterial cells that the assay can detect.
[Z]Immunocapture-PCR

Lashomb *et al.*,2002

Diagnostics methods

Salient characteristics

Xylella fastidiosa

- Single, straight rods, 0.25-0.35×0.9–3.5 μm, that can form long filamentous strands under some cultural conditions.
- Gram negative.
- Nonmotile, lacking flagella.
- Endospores not produced.
- Aerobic.
- Colonies are non-pigmented: cream to white.
- Two colonies types occur: umbonate and rough with finely undulate margins, or convex, smooth and entire (Bradbury,1991).
- Indole production, denitrification negative.
- Oxidase negative and catalase positive.
- Optimum temperature for growth, 26-28°C; optimum pH for growth, 6.5-6.9. Nutritionally fastidious.
- Found mainly in the xylem of plant tissue.
- The mol% GC of the DNA is: 51.0-52.4 (Tm); 52-53.1 (Bd).

Bergey's Manual of Systematic Bacteriology,2005

Diagnostics methods

Some more biochemical characteristics

Xylella fastidiosa

- Gelatin is hydrolyzed and gelatinase is produced.
- Many strains produce β-lactamase. Beta-lactamase was detected with penicillin-starch paper strips.
- β-galactosidase, coagulase, lipase, amylase, and phosphatase negative.
- Indole and H_2S are not produced.

Diagnostics methods

Key biochemical and physiological characteristics of *X. fastidiosa*

Catalase	+
Oxidase reaction	-
Gelatin liquefaction	+
Indole production	-
H_2S production	-
DL-lactate	+
Glucose fermentation	-
Temperature optimum	26 to 28°C
pH optimum (*X. fastidiosa* is very sensitive to variations in pH)	6.5 to 6.9

Diagnostic protocols for regulated pest,2018

Beta-lactamase test

1. Preparation of penicillin-starch paper strips

- Beta-Lactamase Test is a means of detecting the enzyme beta-lactamase, which confers penicillin resistance to various bacterial organisms by cleaving the beta-lactam ring of penicillins and cephalosporin antibiotics.
- **Preparation of penicillin-starch paper strips:**
- Strips of Whatman no. 3 filter paper (approximately 5 by 1 cm) are immersed in a solution of 0.2% soluble starch (BBL) and 1% penicillin (potassium penicillin G for injection).
- The strips are then allowed to dry at room temperature for approximately 2 h on a perforated metal rack.
- Dried strips are then stored at -20°C, which ensures their stability for at least 1 year.

Jorgensen *et al.*,1977; Hardy Diagnostics,2017

Beta-lactamase test

2. Performig the Beta-lactamase test

- To perform the test, a strip is removed from the freezer and placed in a disposable plastic petri dish.
- The strip is thoroughly moistened with either Gram or Lugol iodine, producing a deep purple color, and excess liquid is then poured off.
- Approximately 10 colonies of the primary growth of the test organism on agar media are then applied to the center of the moistened strip by rubbing it with an inoculating loop in a circular fashion, describing a circle approximately 5 mm in diameter.

1. If penicillinase is produced, the deep purple color of the strip becomes white within 1 min in the area where the organisms were applied.
2. If penicillinase is not produced, the strip remains purple or becomes slightly yellow at the immediate site of inoculation.

Jorgensen *et al.*,1977

Mechanism of disease development
The fastidian gum(EPS)

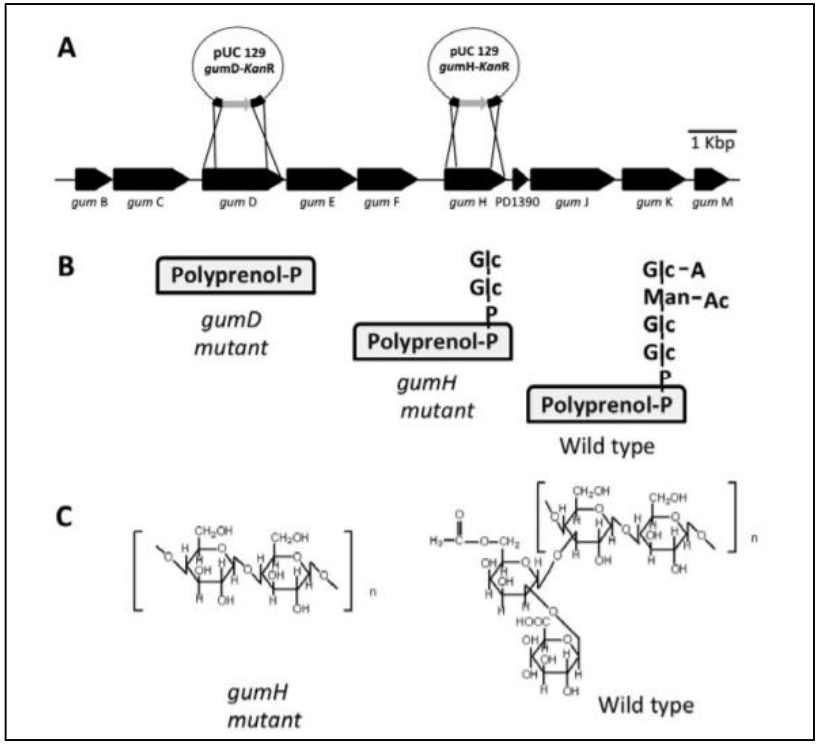
- The fastidian gum(EPS) may be linked directly to the pathogenicity of this bacterium.
- Terminal fimbriae (also called type IV pili) are important for biofilm formation.
- In addition, although the bacterium lacks flagella for motility, terminal fimbriae aid in a type of incremental movement called "twitching motility" which enables bacterial cells to move against the xylem stream (transpirational flow in xylem).
- Expression levels of the *X. fastidiosa* gum genes *gumC*, *gumD*, and *gumJ* are affected by cell density, suggesting that *X. fastidiosa* EPS production could be regulated by a quorum-sensing mechanism.

Mechanism of disease development
The fastidian gum(EPS)

A. Genetic map of the *Xylella fastidiosa* gum operon; plasmids and targeted genes for disruption are indicated.

B. Repeating units in gum polymer expected from *gum*D and *gum*H mutants compared with the wild type, as added to the polyprenol.

C. Putative exopolysaccharide structure expected for the *gum*H mutant compared with the wild type; *gum*D not shown because no additional residues are expected to be added to polyprenol.



Killiny *et al.*,2013

The fastidian gum(EPS)

Biofilm assessment

1. On glass slides

- Slides were affixed to the inner surface of the wide-mouthed glass jars (cat. # 02-911-420; Fisher Scientific) with polydimethylsiloxane (Sylgard 184).
- Following sterilization by autoclaving, 20 mL of the PD2 and/or sap media were added to the jars, followed by seeding with Xf to an initial OD_{600} nm of 0.05, and incubation at 28°C with agitation at 100 r.p.m. for up to 10 days.
- Subsequently, the glass slides were removed, cleaned of extraneous cells on the backside and the biofilms imaged by scanning with an Epson Perfection 4870 Photo scanner at 600 dpi resolution and managed with ADOBE PHOTOSHOP.
- For scanning electron microscopy (SEM), the same set-up was used with the addition of coverslips attached to the glass slides with a small quantity of polydimethylsiloxane. Once removed from the jar and then from the glass slides, coverslips were prepared for SEM according to Meng *et al.*, 2005.

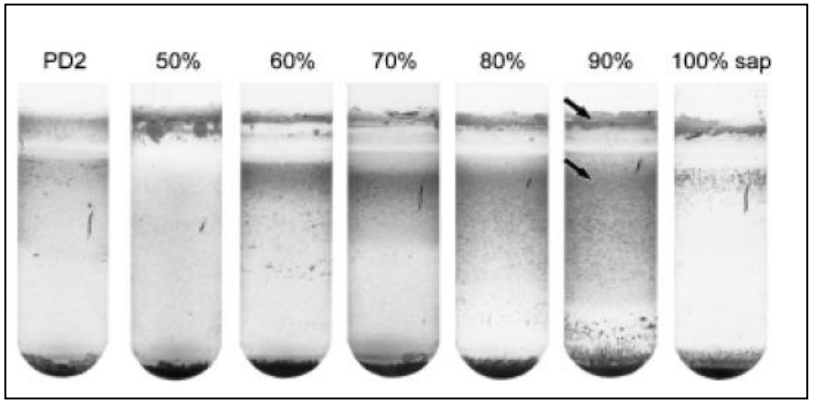
Zaini *et al.*,2009

The fastidian gum(EPS)

Biofilm assessment

On glass slides(continued)

- Formation of biofilms on glass surfaces by *Xylella fastidiosa*.
- Cells were cultured at 28 1C and 200 r.p.m. and photographs taken after 7 days.
- Arrows indicate the biofilm formed at the air-liquid interface and submerged regions.
- Highest growth and more robust biofilms are attained at high sap: PD2 ratios.



Zaini *et al.*,2009

The fastidian gum(EPS)

Biofilm assessment

2. In 96-well polystyrene culture plates

- Biofilm development was also assessed in 96-well polystyrene culture plates. Xf cells suspensions were made to an initial OD_{600} nm of 0.1 in various PD2:sap mixtures and 150 mL were added to wells of 96-well polystyrene plates (Falcon 35-1177; Becton Dickinson). Cultures were maintained for 5 days at 28 1C with agitation at 200 r.p.m.
- A Synergy 2 plate reader (Biotek) was used to quantify the OD_{600} nm in each well, and this was considered the 'total' measurement.
- One hundred microliters of the supernatant of each well was transferred to wells in new plates and once again absorbance measured. This was considered the 'planktonic cell' measurement.
- Original plates were then rinsed three times with water using a plate washer (Biotek). This step removes planktonic cells and any cells loosely attached or deposited in the bottom of the well.

Zaini *et al.*,2009

The fastidian gum(EPS)

Biofilm assessment

In 96-well polystyrene culture plates(continued)

- To determine attached cells, 200 mL of aqueous 0.1% crystal violet was added to each well and plates kept at room temperature (*c.* 23°C) for 20 min.
- Plates were washed three times as before with water, followed by the addition of 200 mL of 6:4 acetone: ethanol and agitated for 5 min.
- The acetone: ethanol–dye solution was measured at OD_{600} nm.
- This was considered the 'attached cell' measurement.
- A total of 44 samples for each media condition were used.
- Ten microliters from the 'planktonic' suspensions were serial diluted and 10 mL of each dilution dropped onto PW agar plates without spreading to determine the number of CFU.
- Independent dilutions were prepared with samples from three 96-well plates and the number of colonies for each condition was averaged.

Zaini *et al.*,2009

The fastidian gum(EPS)

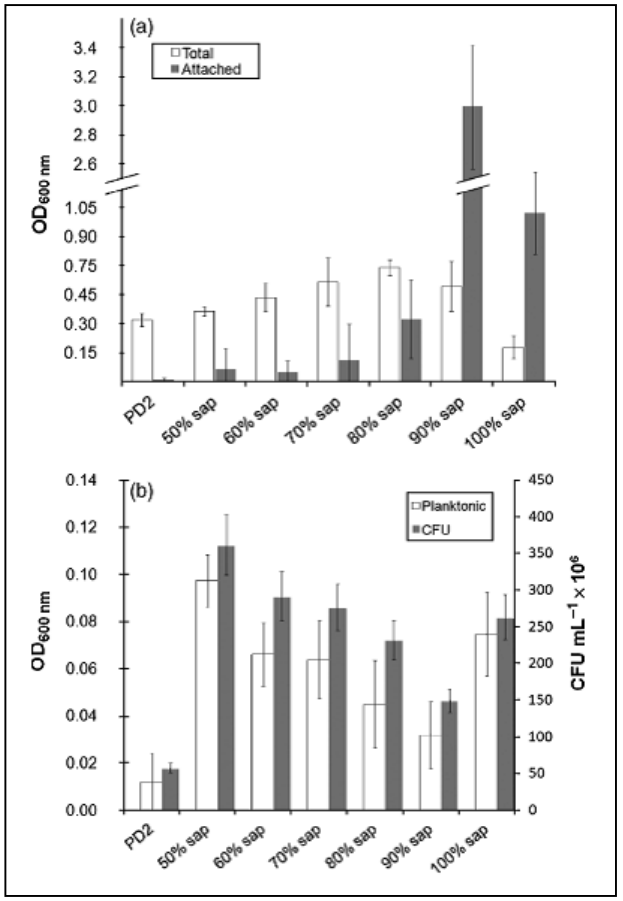
Biofilm assessment

In 96-well polystyrene culture plates(continued)

- Effect of xylem sap on growth and biofilm formation on polystyrene surfaces by *Xylella fastidiosa* .

A. After 4 days of growth at 281C and 200r.p.m. in 96-well plates, OD of total and attached cells were measured.

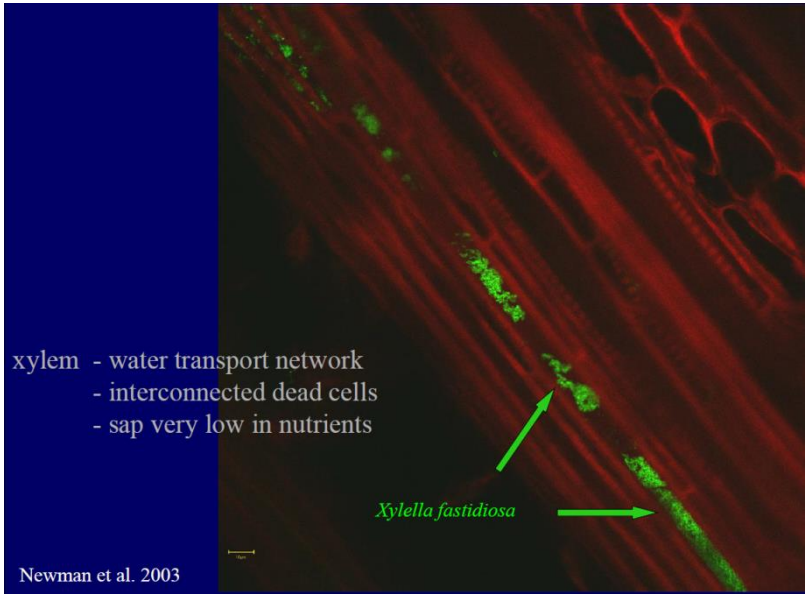
B. OD of planktonic cells were also measured and numbers of CFU counted.



Zaini *et al.*,2009

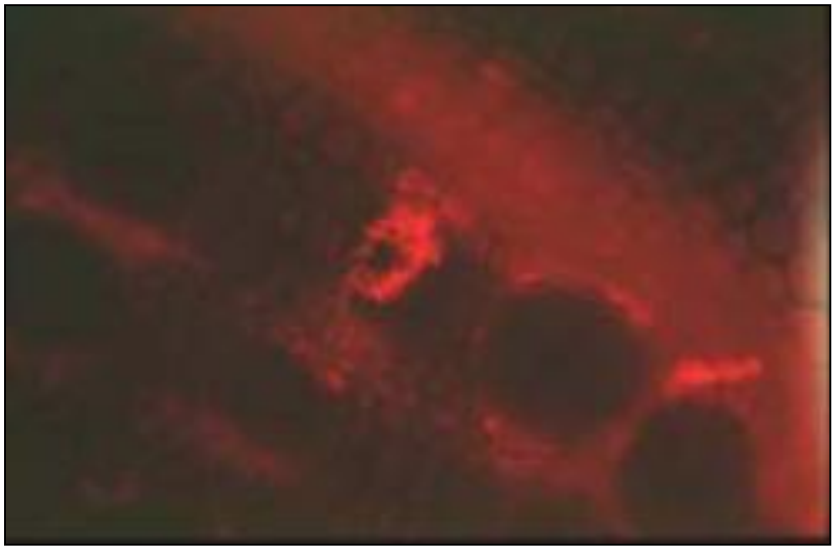
Diagnostics methods
Green fluorescent cell staining

- **Longitudinal sections of infected citrus petioles.**
- Plant xylem is depicted in red or blue.
- *X. fastidiosa* cells are green.
- xylem - water transport network.
- interconnected dead cells.
- sap very low in nutrients.



Diagnostics methods
Immunostaining

- This section of an inoculated young grape stem cuts across three water-conducting vessels.
- One of the three contains *Xylella fastidiosa* cells which are seen as the red fluorescent ring revealed by immunostaining with the Texas red-anti-rabbit antibody conjugate.



Labavitch *et al.*,2001

Serological diagnosis
Serological methods vs. molecular tests

- In general, the accuracy of the results obtained in each laboratory for the ELISA tests was lower than the accuracy values obtained using the molecular tests.
- The majority of the deviations were recorded for the samples containing the lowest bacterial concentration ($5x10^4$ CFU/ml), with several replicates testing negative, i.e. producing negative deviations.
- However, excluding from the analysis these replicates and considering the results obtained for the *Xylella* contaminated samples containing 5x10^6 CFU/ml and 5x10^5 CFU/ml and the *Xylella*-free samples, all laboratories were proficient with an accuracy of 100%.

EPPO,2017

Serological diagnosis

Indirect double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) test

- Antiserum preparation:
- Polyclonal antiserum Female New Zealand white rabbits weighing 2kg were endovenously inoculated with standardized bacterial suspension at 1×10^6 *X. fastidiosa* cells/mL in PBS, and this procedure was repeated three times at 14 day intervals.
- Ten days after the last injection, the blood was collected through cardiac puncture and the serum titer and specificity were determined by indirect enzyme-linked immunosorbent assay (ELISA) (Clark *et al.*,1986) and pre immune serum was used as a negative control.

Peroni *et al.*,2008

Diagnostics methods

Double antibody sandwich (DAS)-ELISA test

Specificity of indirect ELISA assay

- Specificity of antiserum:
- The reactivity of the *X. fastidiosa* antiserum was tested by indirect ELISA, and the average of the results was expressed as the optical density at 405 nm as a function of the concentration of antigens.
- The antiserum had a robust reactivity and its detection ranged from 10^6 to 10^4 bacterial cells, and detected *X. fastidiosa* with similar magnitude in sample plant extracts.

Peroni *et al.*,2008

Diagnostics methods

Double antibody sandwich (DAS)-ELISA test

Kits for serological detection of *X. fastidiosa*

- All the collected samples from olive, citrus and grapevine trees were tested by ELISA, using specific polyclonal antibodies to *X. fastidiosa* (Loewe Biochemica, Germany), according to the manufacturer's instructions.
- In the presence of PBS-buffer, extracts were obtained from leaf petioles and midveins, using mortars and pestles.
- Absorbance was measured after 30, 60, 120 and 180 minutes (min) with a microplate reader (ELX800, BioTek) at 405 nm.

Afechtal *et al.*,2020

Diagnostics methods

Double antibody sandwich (DAS)-ELISA test

Specificity of indirect ELISA assay

- **Specificity of antiserum:**
- These polyclonal antibodies are highly specific for *X. fastidiosa*, since it has no cross-reactivity in ELISA with other endophytic citrus bacteria such as *Methylobacterium mesophilicum*, *Methylobacterium extorquens*, *Curtobacterium flaccumfaciens*, *Bacillus* sp. and *Pantoea agglomerans*.
- The cross-reactivity can interfere in the specificity, making the diagnosis useless, due to false positive results even in the absence of the *X. fastidiosa*.

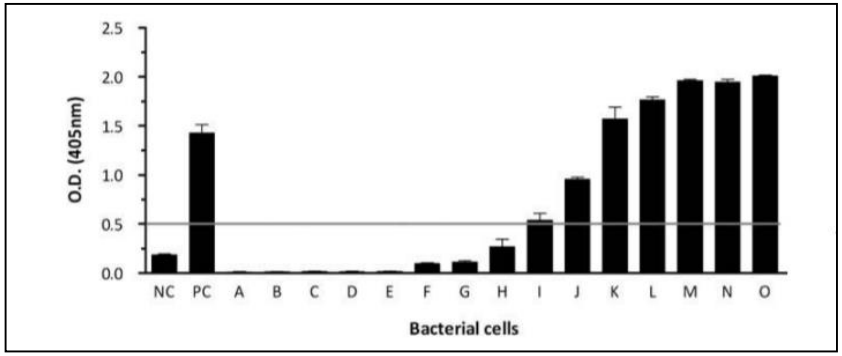
Peroni *et al.*,2008

Diagnostics methods

Double antibody sandwich (DAS)-ELISA test

Specificity of indirect ELISA assay

- Detection limit of CVC by Indirect ELISA.
- (NC) **Negative control** (without X. fastidiosa); (PC**) positive control** (1×10^6 *X. fastidiosa* cells in PW medium); 1×10^6 bacterial cells of:
- (A) *Methylobacterium mesophilicum*,
- (B) *Methylobacterium extorquens*,
- (C) *Curtobacterium flaccumfaciens*,
- (D) *Bacillus* sp.,
- (E) *Pantoea agglomerans*;
- (lanes F to O) 10-fold serial dilutions of *X. fastidiosa* ranging from 10^{10} to 10^1 cells.
- Therefore, the ELISA sensitivity ranges between 10^4 and 10^6 bacterial cells, similar to PCR sensitivity that can amplify around 10^6 cells per sample.



Peroni *et al.*,2008

Diagnostics methods

Double antibody sandwich (DAS)-ELISA test

Kits for serological detection of *X. fastidiosa*

- Kits for serological detection of *X. fastidiosa* can be supplied by different companies.
- The ELISA kits from Agritest and Loewe have been validated for olives, oleander, almond, citrus, oak, grape and other species (i.e. weeds) (Loconsole *et al.*,2014).

EPPO,2016;2017

Diagnostics methods

Double antibody sandwich (DAS)-ELISA test

Xylella fastidiosa (Xfas) ELISA Kit

- Contents of Kit:
- ##96 test format##

1. Antibody (IgG): 0.1 ml
2. Antibody-AP-conjugate: 0.1 ml
3. Positive Control: 10 tests
4. Negative Control: 10 tests
5. Coating Buffer: 1 liter
6. Wash Buffer: 1 x 5 liter
7. Conjugate/Sample Buffer: 1 x 1 liter
8. Substrate Buffer (5x): 1 x 25 ml
9. Substrate Tablets: 4 x 5 mg
10. Tween 20: 10 ml
11. High-binding ELISA plates: 12x8 wells
12. Sealing Cover: 1

- Storage:
- Our ELISA reagents are standardized for use at a dilution of 1:200 and a test volume of 200 µl/well. The products must be kept refrigerated (ca. 4°C) upon receipt. Once opened, we recommend using the reagents within 5 months.

Diagnostics methods

Double antibody sandwich (DAS)-ELISA test

Kits for serological detection of *X. fastidiosa*

- **Qualitative results:**
- The ELISA test worked properly: the positive control of the kit reacted positively, whereas no color change was observed with the negative control of the kit.
- Loconsole *et al.*, 2014 performed several laboratory tests, in which the reactivity of different commercially available ELISA kits was compared, and showed that a kit from Loewe (Biochemica GmbH, Germany) detected a higher number of known positive samples with reactions occurring within 2 hours, following manufacturer's instructions and using the controls supplied with the kit
- Thus, this kit was used for this preliminary survey on the presence of *X. fastidiosa* in Morocco.

Afechtal *et al.*,2020

Diagnostics methods

Double antibody sandwich (DAS)-ELISA test

Kits for serological detection of *X. fastidiosa*

- **Qualitative results (ELISA absorbance values at 405 nm wavelength):**
- A sample is assessed as "positive" when the OD_{405} value is at least three times higher than the OD_{405} value of the negative control, conversely it is categorized as negative when the value is below this threshold.
- A test was considered positive if the specified test well emitted a blue color and generated an absorbance reading >0.3000 using 620 nm as the measurement wavelength on the SPECTRAFlour ELISA Reader.

EPPO,2016 and Torres,2012

Diagnostics methods

Double antibody sandwich (DAS)-ELISA test

Kits for serological detection of *X. fastidiosa*

- Performance values recovered in each laboratories for the ELISA test performed using the kits Agritest and Loewe. Results of the homogeneity and stability tests for the ELISA tests. OD 405 values are indicated for each replicate.

HOMOGENEITY TEST									
		AGRITEST				LOEWE			
		5X 10^6 cells/ml	5X 10^5 cells/ml	5X 10^4cells/ml	healthy	5X 10^6 cells/ml	5X 10^5 cells/ml	5X 10^4cells/ml	healthy
Replicate 1	repetition 1	2,525	1,722	0,197	0,037	2,510	1,449	0,148	0,040
Replicate 2		2,522	1,830	0,170	0,037	2,555	1,443	0,174	0,037
Replicate 3		2,649	1,777	0,168	0,013	2,488	1,391	0,157	0,010
Replicate 1	repetition 2	2,491	1,745	0,197	0,040	2,471	1,345	0,141	0,033
Replicate 2		2,485	1,835	0,169	0,028	2,533	1,447	0,174	0,019
Replicate 3		2,524	1,903	0,160	0,017	2,476	1,411	0,153	0,011
Quantitative homogeneity		yes	yes	no	yes	yes	yes	no	yes
Qualitative homogeneity		yes	yes	yes	yes	yes	yes	yes	yes
STABILITY TEST									
		AGRITEST				LOEWE			
		5X 10^6 cells/ml	5X 10^5 cells/ml	5X 10^4cells/ml	healthy	5X 10^6 cells/ml	5X 10^5 cells/ml	5X 10^4cells/ml	healthy
Replicate 1	repetition 1	2,733	1,557	0,111	0,045	2,730	1,602	0,130	0,045
Replicate 2		2,762	1,415	0,105	0,040	2,720	1,450	0,101	0,037
Replicate 3		2,796	1,643	0,099	0,020	2,747	1,629	0,123	0,025
Replicate 1	repetition 2	2,728	1,552	0,108	0,047	2,740	1,603	0,127	0,042
Replicate 2		2,768	1,423	0,105	0,036	2,715	1,445	0,103	0,030
Replicate 3		2,805	1,637	0,104	0,023	2,735	1,637	0,128	0,021
Quantitative stability		no	no	no	yes	no	no	no	yes
Qualitative stability		yes	yes	yes	yes	yes	yes	yes	yes

Diagnostics methods

1. Plant sample preparation for ELISA test

- Tissue sources for ELISA tests can be leaves (including petioles), twigs or canes.
- Samples can be prepared by macerating the leaves in extraction buffer (1:10, w:v) using a mortar and pestle or tissue homogenizer (e.g. Polytron, Homex, etc.).
- Samples can be frozen in liquid nitrogen for homogenization.
- For twigs and canes, the bark is removed, and pieces of stem can be cut and minced with a razor blade and ground as described above.
- Comment: It should be noted that for some hosts species (e.g. *Quercus*, *Platanus*) or some samples (due to the microbiota) high background signals resulting in false-positive reactions (not confirmed with molecular tests) can occur.
- In some cases, surface sterilization of the samples may help to overcome this problem.

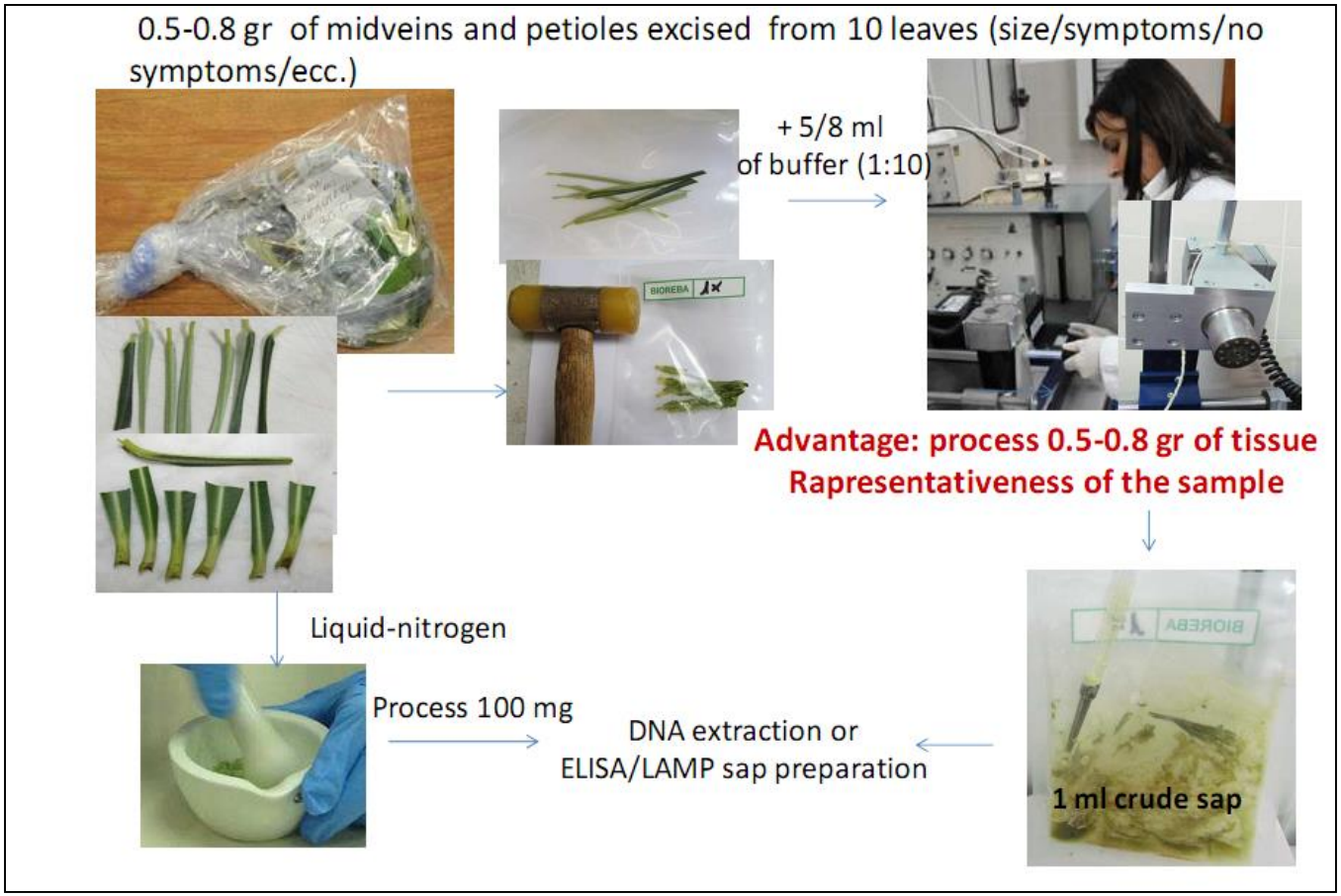
EPPO,2018

Diagnostics methods
Plant sample preparation for ELISA test

- Leaf peduncles and midribs excised from mature leaves are the most suitable tissues for *X. fastidiosa* detection in perennial crops.
- For annual herbaceous plants stem and leaf peduncles and veins from basal leaves should be used.
- For each sample, at least 0.5-0.8 gr of tissue are recovered from 5-10 leaves (according to the leaf size and consistency) and used for DNA extraction or ELISA sap preparation.
- Samples should be inspected for symptoms and if present symptomatic leaves (showing leaf scorching and necrosis), selected and processed, removing the necrotic and dead tissue.

Detection procedure

Plant sample preparation for ELISA test



Loconsole,2016;..

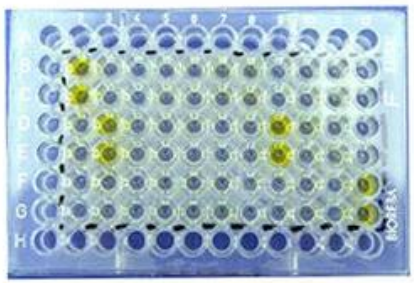
Detection procedure

Plant sample preparation for ELISA test

ELISA test

ELISA sap

leaf tissues + extraction buffer (1:10)



➢ Preliminary tests

- reactivity of different commercially available ELISA kits compared
- kit from LOEWE (Biochemica GmbH, Germany) detected a higher number of known positive samples

KIT LOEWE was used throughout the validation and monitoring program in 2013-2014

Absorbance measured after 30, 60, 120 and 180 min with a microplate reader at 405 nm.

REACTION POSITIVE = after 120 min, the absorbance 3 times greater than the mean absorbance of healthy control samples.

Loconsole,2016;..

Diagnostics methods

ELISA test

2. Colony suspensions were also tested by DAS ELISA

- Petioles from grapes were cut and ground in 1ml of distilled water and dilutions (10^{-2} and 10^{-3}) of this suspension were made.
- Aliquots of 300µl of each dilution were inoculated by triplicate in liquid PW media.
- All cultures were incubated in the dark at 28°C under static conditions.
- After 15 days PW broths were tested by DAS ELISA and only those positive for *X. fastidiosa* were used in the isolation procedure.
- Solid PW broths were inoculated with 100µl aliquots of liquid PW broth.
- Plates were incubated at 28°C and observed for bacterial colony growth for 12 days.
- Colony suspensions were also tested by DAS ELISA.

Aguilar *et al.*,2008

Diagnostics methods

ELISA test

Colony suspensions were also tested by DAS ELISA

- For each isolate two cell suspensions were prepared for species confirmation with double antibody sandwich- enzyme linked immunosorbant assay (DAS-ELISA).
- Using a 3mm inoculating loop, two clumps of bacteria were removed from the PD3-G^{+} media plates and thoroughly vortexed in a 1.5 ml micro tube to suspend cultures in 1 ml of General Extraction Buffer as provided in the Agdia DAS-ELISA kit.
- A test was considered positive if the specified test well emitted a blue color and generated an absorbance reading >0.3000 using 620 nm.

A positive reaction was determined to be greater than the mean of the absorbance at 490 nm of the negative controls plus three times the standard deviation (Aguilar *et al.*,2008).

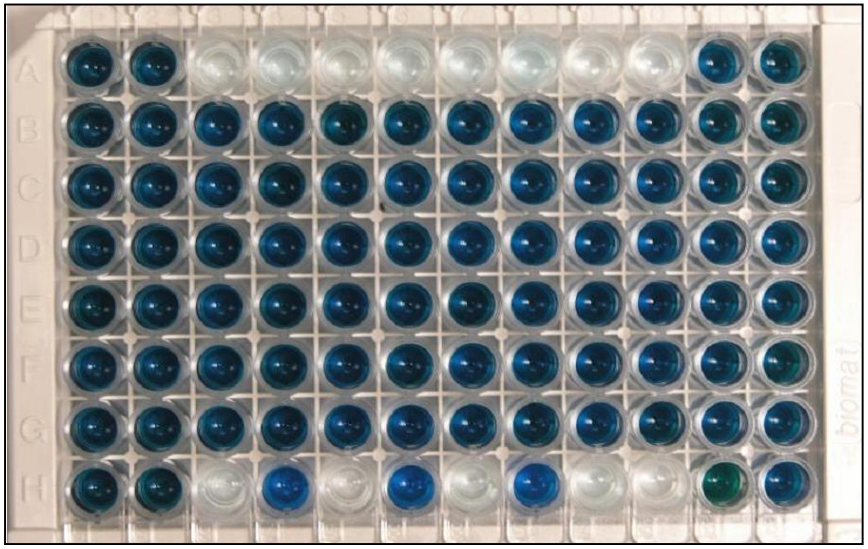
Torres,2012

Diagnostics methods

ELISA test

Colony suspensions were also tested by DAS ELISA

- DAS-ELISA of isolated cultures.
- Photograph depicts results of DAS-ELISA assay used to identify suspected cultures as *X. fastidiosa*.
- Blue color indicates a positive test where clear wells indicate a negative test.
- All clear wells in this photograph correspond to the negative controls.



Torres,2012

Diagnostics methods
Principle of the test

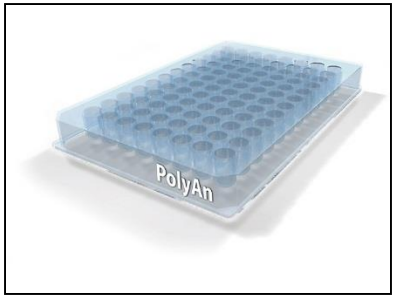
1. During the first step of the assay the surface of a microtiter plate is coated with the antigen-specific coating-antibody (IgG).
2. When an antigen-containing sample is added during the second step, the antigen binds to the immobilized IgG, forming an antibody-antigen complex.
3. This complex reacts with the enzyme-labelled antibody-AP-conjugate during the third step by forming a double-antibody sandwich.
4. During the fourth step the alkaline phosphatase (AP) reacts with the substrate 4- nitrophenylphosphate (PNP) in an enzymatic reaction, resulting in yellow coloured 4-nitrophenol as product.

- This colour development can be evaluated visually or measured in a spectrophotometer at 405 nm after 1 and 2 hours.

The products must be kept refrigerated (ca. 4°C) upon receipt.
Once opened, we recommend using the reagents within 5 months.

CD, Cat. No: DEIAPB5

Diagnostics methods
The procedure for the ELISA test



- The following steps must be followed:
- 1. Coat the plate:
- Dilute the IgG (anti-*Xf*.-IgG) or capture antibody/coating antibody 1:200 (Loewe)/1:500 (Agritest) in coating buffer in 1.5-2.0 mL microvials e.g. Eppendorf tube (mix the prepared capture antibody solution thoroughly and use immediately) and load 100 or 200 µl to each well of the microtiter plate.
- Prepare a humid box by lining an airtight container with a wet paper towel. Keeping test wells in a humid box during incubation will help prevent samples from evaporating.
- Cover the plate tightly and place it in a humid box.
- Incubate the plate at 37°C for 4 h.
- 2. Washing step:
- Remove the sap from the wells (use a quick flipping motion to empty the wells into a sink or waste container) and wash 4 times the plates using the washing buffer, remove any liquid by blotting the plate on paper towels.

Note: All antibodies and enzyme conjugates should be prepared in a container made of a material such as polyethylene or glass that does not readily bind antibodies. Do not use polystyrene, polypropylene or polycarbonate.

Diagnostics methods
The procedure for the ELISA test

- 3. Plant sap preparation and Antigen incubation:
- Homogenize the samples in extraction buffer 1:10 (w/v): weigh at least 0.5 g of leaf petioles and basal portion of the leaves, cut in small pieces using a razor blade (while processing the samples, sterilize the blade between samples).
- Transfer the plant tissue into the extraction bags and add 5 ml of extraction buffer; crush with a hammer and grind by a semi-automated homogenizer (i.e. Homex).
- Transfer 1 ml of sap into a microcentrifuge tube that store at 4°C until use, allowing plant debris precipitation.
- Load 100 or 200 µl of plant extract to each well of the microtiter plate.
- Include two wells per plate for both the positive and negative controls (see next slide).
- Cover the plate and incubate at 4°C overnight in a humid box.

Diagnostics methods
The procedure for the ELISA test
Positive and negative controls

- Positive and negative controls should be prepared as described below:
- **Positive controls**:
- Obtain a 48-h culture of a virulent strain of the target bacterium on Nutrient Agar medium or another suitable general medium and suspend in PBS buffer to obtain a cell density of approximately 10^8 cells ml^{-1}
- This is usually obtained by a faintly turbid suspension equivalent to an optical density of 0.1 at 600 nm.
- **Negative controls**:
- Healthy plant extract (for detection) or a suspension of the heterologous bacterial species (for identification) should be used as negative controls. The healthy plant should be the same species and preferably the same variety and the same plant part at the same growth stage for better comparison with the samples to analyse.
- Aliquots/extracts of the same host plant which previously tested negative for the target bacterium can be used as negative controls.

EPPO Standard in 2010-09

Diagnostics methods
The procedure for the ELISA test
Continued…

- 4. Washing step:
- Repeat step 2.
- 5. Add the detection antibody (always make enzyme conjugate solution within 10 minutes before use):
- Dilute enzyme-conjugated antibodies (anti-Xf.-AP conjugate) 1:200 (Loewe)/1:500 (Agritest) in conjugate buffer. Add 100 or 200 µl to each well of the microtiter plate. Cover the plate and incubate at 37°C for 4h in a humid box.
- 6. Washing step:
- Repeat step 2.
- 7. Add Substrate:
- Dissolve the p-nitrophenylphosphate(PNP) (0.6-1 mg/ml) in substrate buffer and add 100 or 200 µl per well. Incubate at room temperature (18-25°C) till the yellow color reaction start to develop and read the plate at 60-120-180 min (if necessary, prolong the reaction over-night) using a plate reader at λ =405 nm.
- The enzymatic reactions can be stopped by adding 25 µl 3 M NaOH (Sodium Hydroxide) to each well.

Diagnostics methods

The procedure for the ELISA test

Buffers required for ELISA (with no sterilization)

- **PBS (pH 7.4):**

NaCl	8 g
KH_2PO_4 anhydrous	0.2 g
Na_2HPO_4 anhydrous	1.15 g
KCl	0.2 g
NaN_3(optional)	0.2 g

Bring final volume to 1L with distilled water.

- **Washing buffer (PBST):**

PBS	1 L
Tween-20	0.5 ml

Store at room temperature.

- **Coating buffer (1 L; pH 9.6):**

Na_2CO_3 anhydrous	1.59 g
NaHCO3	2.93 g
NaN_3 (optional)	0.2 g

Store at 4°C.

Diagnostics methods

The procedure for the ELISA test

Buffers required for ELISA(with no sterilization)

- **Extraction buffer/Conjugate buffer (1 L; pH 7.4):**

PBST	1 L
Polyvinylpyrrolidone (PVP-25)	20 g
Bovin serum albumin (BSA)	2 g

  Store at 4°C.

- **Substrate buffer (1 L; pH 9.8):**

Diethanolamine	97 ml
$MgCl_2$ x $6H_2O$	0.2 g
NaN_3 (optional)	0.2 g

  Bring to final volume of 1 L with distilled water. Adjust pH to 9.8 with 1N HCl.
  Store refrigerated at 4°C.

Molecular Diagnosis of *Xylella*
PCR approaches to *X. fastidiosa* study

- The most commonly used PCR approaches to *X. fastidiosa* study, ranging from classical PCR, to several PCR-based detection methods:

1. random amplified polymorphic DNA (RAPD),
2. quantitative real-time PCR (qRT-PCR),
3. nested PCR (N-PCR),
4. immunocapture PCR (IC-PCR),
5. short sequence repeats (SSRs, also called VNTR),
6. single nucleotide polymorphisms (SNPs), and
7. multilocus sequence typing (MLST).

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Molecular Diagnosis of *Xylella*

PCR approaches to *X. fastidiosa* study

The most commonly used PCR

Molecular method	Advantages	Disadvantages	References
Classic PCR	High sensitivity, specificity and accurate results for the detection of *Xylella* and its subspecies also in non-axenic conditions. Many applications in molecular analysis. Easy diagnostic interpretation	Unable to quantify the target DNA, only qualitative test. Some metabolites or contaminants in the sample can interfere with PCR performance. PCR conditions must be optimized in each host and environment for better performance	Firrao and Bazzi, 1994; Minsavage et al., 1994; Pooler and Hartung, 1995a; Banks et al., 1999; Rodrigues et al., 2003; Huang and Sherald, 2004; Chen et al., 2005; Travensolo et al., 2005; Hernandez-Martinez et al., 2006; Martinati et al., 2007; Morano et al., 2008; Huang, 2009; Livingston et al., 2010; Melanson et al., 2012; Guan et al., 2015; Bleve et al., 2016
RAPD	Useful to study unknown species where there is not *apriori*, knowledge of sequencing. Quite useful to detect high polymorphisms. Limited cost, Simple and rapid. Small amount of template DNA required. The amplification products can be further characterized	Markers are dominant. Reproducibility can be low among labs and with different polymerases and facilities, especially when not using random primers with high annealing temperature. In many cases standardization of the protocol for each lab is required. Nowadays this technique is considered obsolete	Denny et al., 1988; Hartung and Civerolo, 1989; Grajalmartin et al., 1993; Chen et al., 1995, 2002; Pooler and Hartung, 1995a; Albibi et al., 1998; Rosato et al., 1998; Banks et al., 1999; Hendson et al., 2001; Lacava et al., 2001; Qin et al., 2001; Su et al., 2008
RFLP	It was one of the first methods used for genetic fingerprinting. The basic RFLP analysis is no longer used. Variations exist such as terminal restriction fragment length polymorphism (TRFLP), which may still have applications related to the characterization of bacteria. By PCR-RFLP, the hybridization step can be skipped	Obsolete technique. Relatively large amount of DNA is required. RFLP approach is tedious and requires numerous steps that may take weeks to yield results. Relatively high cost and low polymorphism	Chen et al., 1992; Rosato et al., 1998; Mehta et al., 2001; Qin et al., 2001; Picchi et al., 2006
qRT-PCR	It allows not only the identification, but also the quantification of bacteria in real time. High sensitivity, specificity and reproducibility. Relatively fast method. It is possible to use also variations in melting temperature to differentiate strains of bacteria	Expensive equipment and reagents are required. Setting up and optimization of the protocol require specific technical skills as well the interpretation of results	Oliveira et al., 2002; Schaad et al., 2002; Bextine et al., 2005; Francis et al., 2006; Bextine and Child, 2007; Choi et al., 2010; Harper et al., 2010; Brady et al., 2012; Guan et al., 2013; Li et al., 2013; Ionescu et al., 2016
SSR	Simple lab procedure, relatively low costs to start, based on PCR termocycler. High level of polymorphism and relatively low amount of target DNA required. Co-dominant markers. The reproducibility is quite good	Previous knowledge of the genomic sequence is required to design specific primers, thus SSRs are limited primarily to economically important species. Point mutations at the site of primer annealing could lead to occurrence of null alleles	Della Coletta-Filho et al., 2001; Coletta and Machado, 2003; Lin et al., 2005, 2013, 2015; Montero-Astua et al., 2007; Montes-Borrego et al., 2015; Francisco et al., 2017
MLST	Highly discriminatory nucleotide sequence based method of characterization based on the sequencing of approximately 450-bp internal fragments of seven housekeeping genes amplified by PCR. This approach is particularly helpful for the typing of bacterial pathogens. The system is very sensitive to discriminate *X. fastidiosa* subspecies and strains in rapid real time reactions. The major advantage of MLST is the possibility to compare the results obtained in different studies. It may also be used to address basic questions about evolutionary and population biology of bacterial spp.	The analysis of only seven loci may limit the sensitivity, especially when close strains are analyzed. Sequencing of the PCR products using an automated sequencer is required. For that, MLST is not always suitable for routine infection controls or outbreak investigation due to relatively high cost and lack of broad access to high-throughput DNA sequencing	Scally et al., 2005; Schuenzel et al., 2005; Almeida et al., 2008; Yuan et al., 2010; Brady et al., 2012; Nunney et al., 2012, 2013, 2014a,b,c; Parker et al., 2012; Elbeaino et al., 2014; Harris and Balci, 2015; Marcelletti and Scortichini, 2016a; Bergsma-Vlami et al., 2017; Coletta-Filho et al., 2017; Denancé et al., 2017; Kandel et al., 2017
Multiplex PCR	Costs are reduced when compared to standard PCR as well as reaction volumes. It allows rapid detection also of multiple strains simultaneously. Close tube system limits the risk of contamination	Primer design is the critical point, they can interfere each other giving false negative (genes or bacteria undetected). Skilled personnel is required to perform the test	Rodrigues et al., 2003; Choi et al., 2010; Myers et al., 2010; Lopes et al., 2014; Jacques et al., 2016
Nested PCR	Improved sensitivity and specificity when compared with classical PCR methodology. Useful technique for studying molecular epidemiology in the field	The protocol may be a little more difficult to optimize than for standard PCR. More time consuming and expensive than normal PCR. Unable to quantify the target DNA	Pooler et al., 1997; Buzkan et al., 2003; Ciapina et al., 2004; Huang, 2007; Silva et al., 2007; Lopes et al., 2014

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Molecular Diagnosis of *Xylella*

PCR approaches to *X. fastidiosa* study

The most commonly used PCR

Forward primer	Reverse primer	Size	Target	Hosts	Citations WoS - G-Scholar	References
RST31: GCGTTAATTTTCGAAGTGATTCGA	**RST33:** CACCATTCGTATCCCGGTG	733	7.4 kb EcoR1 restriction fragment	Grapevine, citrus, oak, red oak, sycamore, plum, goldenrod	193–285	Minsavage et al., 1994
XF1-F: CAGCACATTGGTAGTAATAC	**XF6_R:** ACTAGGTATTAACCAATTGC	400	16s rDNA	Grapevine, almond, plum, American elm, citrus	(nd 2004) – 16	Firrao and Bazzi, 1994
208-1: ACGGCCGACCATTACTGCTG	**208-2:** ACGGCCGACCCGGAGTATCA	320	RAPD fragment	Citrus, grapevine, mulberry, almond, plum, elm, oak, ragweed, periwinkle	119–180	Pooler and Hartung, 1995a
203-1: CACGGCGAGTATCGGCTTTC	**203-2:** CACGGCGAGTCGACGTAAAT	2,000	–	–		
204-1: TTCGGGCCGTCAATGTGTTG	**204-2:** TTCGGGCCGTTTACTCAAAG	800	–	–		
230-1: CGTCGCCCATCAACGCCAAA	**230-2:** CGTCGCCCATTGTGCTGCAG	700	–	–		
272-1: AGCGGGCCAATATTCAATTGC	**272-2:** AGCGGGCCAAAACGATGCGTG	700	–	–		
272-1-int: CTGCACTTACCCAATGCATCG	**272-2-int:** GCCGCTTCGGAGAGCATTCCT	500	–	–		
CVC-1: AGATGAAAACAATCATCGAAA	**272-2:** AGCGGGCCAAAACGATGCGTG	600	–	Citrus		
CVC-1: AGATGAAAACAATCATCGAAA	**272-2-int:** GCCGCTTCGGAGAGCATTCCT	500	–	Citrus (specific)		
XF176f: AAACAATCACAGGGGACTGC	**XF954r:** CTATCCGAACACTTCCTATG	779	PD-specific RAPD fragment (PD1-1-2)	Grapevine (Pierce's disease)	20–29	Banks et al., 1999
XF176f: AAACAATCACAGGGGACTGC	**XF686r:** ATATTCATAGATTCCGTCGA	511	–	Citrus, mulberry, oak, periwinkle, peach, plum (Non-Pierce's disease)		
0067-a-S-19: CGGCAGCACATTGGTAGTA	**1439-a-A-19:** CTCCTCGCGGTTAAGCTAC	1,348	16S rDNA	Citrus, grapevine, mulberry	43–70	Rodrigues et al., 2003
0067-a-S-19: CGGCAGCACATTGGTAGTA	**0838-a-A-21:** CGATACTGAGTGCCAATTTGC	745	–	–		
0838-a-S-21: GCAAATTGGCACTCAGTATCG	**1439-a-A-19:** CTCCTCGCGGTTAAGCTAC	603	–	–		
FXYgyr499: CAGTTAGGGGTGTCAGCG	**RXYgyr907:** CTCAATGTAATTACCCAAGGT	429	b-subunit polypeptide of the DNA gyrase (gyrB)	–		
G1: GAAGTCGTAACAAGG	**L1:** CAAGGCATCCACCGT	522	16S rDNA	Porcelain berry, wild grape, Mulberry		Huang and Sherald, 2004
Teme150fc: TCTACCTTATCGTGGGGGAC	**Teme454rg:** ACAACTAGGTATTAACCAATTGCC	348	16S rDNA	Almond	54–78	Chen et al., 2005
Teme150fc: TCTACCTTATCGTGGGGGAC	**Xf16s1031r:** AAGGCACCAATCCATCTCTG	700	–	–		
Dixon454fa: CCTTTTGTTGGGGAAGAAAA	**Dixon1261rg:** TAGCTCACCCTCGCGAGATC	847	–	–		
REP1-R: IIIICGICGIATCCIGGC	**Xf-1:** CGGGGGTGTAGGAGGGGTTGT	350	Amplified genomic fragment	Citrus, grape, almond, mulberry, oak, periwinkle, plum, coffee, elm, ragweed	3–7	Travensolo et al., 2005
XF1968-L: GGAGGTTTACCGAAGACAGAT	**XF1968-R:** ATCCACAGTAAAACCACATGC	638	Gene XF1968	Almond, oleander	21–42	Hernandez-Martinez et al., 2006

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Molecular Diagnosis of *Xylella*

PCR approaches to *X. fastidiosa* study

The most commonly used PCR

Forward primer	Reverse primer	Size	Target	Hosts	Citations WoS - G-Scholar	References
XF2542-L:TTGATCGAGCTGATGATCG	**XF2542-R:** CAGTACAGCCTGCTGGAGTTA	412	Gene XF2542	Grape, almond, Spanish broom, *Brassica* spp.		
ALM1: CTGCAGAAATTGGAAACTTCAG	**ALM2:** GCCACACGTGATCTATGAA	521	Gene ALM1	Almond (specific)		
16S-23SF: GATGACTGGGGTGAAGTCGT	**16S-23SR:** GACACTTTTCGCAGGCTACC	650	16S-23S intergenic spacer	Citrus, coffee, grapevine, mulberry, almond elm, ragweed, periwinkle	1–2	Martinati et al., 2007
307 BBF: GCAAGTCAGGGTAGCGTCTC	**943 BBR:** GGCTTCTCTGTCGATTTTCG	nd	mopB	Grape, Sea myrtle, Redspike Mexican hat, others	5–10	Morano et al., 2008
HQ-OLS08: TGTACGTCCTGAAACCATCTTG	**HQ-OLS05:** TTCTGGAAGCTTTGAGTAAGGG	274	RAPD fragment	Oleander (specific)	7–10	Huang, 2009
D056L: AACAAGGGACCTTCCATGC	**D858R:** AGCAATCGCTGCACCTAAAT	842	Succinyl-CoA synthetase alpha subunit (sucD)	Almond	0–0	Livingston et al., 2010
G151f: GATCCGGAAAGTGGGGAGATTACTATC	**G368r:** GCCATTGCAAAAGCAGTACGCTCA	217	DNA polymerase III subunit beta (dnaN)	–		
A151f: GATGCGGAAAGCGGGGAGATTACTATT	**A518r:** GCCTTTACGCGGCAAAATAATCTGA	367	DNA polymerase III subunit beta (dnaN)	–		
A97f: CATGCTGGTGGTAAGTTCGACGATAAC	**A476r:** CAACAATGCCGTTGTGCTCACCG	379	b-subunit polypeptide of the DNA gyrase (gyrB)	–		
G223f: CGGTGGCGAAACGGTAATCC	**G705r:** GGAGAAATGTTTGGCAAAGACAGGC	482	mdh Malate dehydrogenase	–		
A345f: TTTTTCAATGTTGGCGACAGGCTTACT	**A705r:** GGAGAAATGTTTGGCAAAGACAGGT	360	mdh Malate dehydrogenase	–		
B001L: TTAGGTGGCAAGGATCGAAT	**B462R:** GGGCCGATCAAAATCAATCT	491	ppiB Peptidyl-prolyl cis-trans isomerase	–		
A284f: GACGAGTTTGCCAAGTTTGATGATGAAATC	**A680r:** GCCAGTCGAACCCACCAAG	396	gltA Citrate synthase	–		
I068L: CGTGGGTCACGAGTCATAAA	**I386R:** TCACACAAAACTACGGCACTG	358	rpsl 30S ribosomal protein S9	–		
PspB-256f: TGAGTGCCTGCGGTGGTA	**PspB-256r:** CGAAACTTGGCAGCTAACG	256	pspB Serine protease	–		
XFPglA_Fw: GCCTCCGGTGCGACTGCTTC	**XFPglA_Rv:** GCTGCGATTGGACACACATTG	nd	PglA	Pecan, Grapevine, Oleander, sycamore	10–14	Melanson et al., 2012
Mul-15040-F: ATTTTCGCGATTTTGGAGTT	**Mul-15040-R:** TTCTTGTGTACTCCGCCTCA	312	Hypothetical protein with putative bacillithiol system oxidoreductase, YpdA family	Mulberry and olive (specific)	2–2	Guan et al., 2015
Xfa-rpod-F4: ACTGAGGTTGTCGTTGGCTT	**Xfa-rpod-R4:** CCTCAGGCATGTCCATTTCC	988	RNA polymerase sigma-70 factor (rpoD)	Olive, citrus, coffee	1–2	Bleve et al., 2016
Xfa-dnaA-2F: TTCCATCAAATTGACGCGCT	**Xfa-dnaA-2R:** CGGCAAGCATGTAACACTGT	650	Chromosomal replication initiator protein DnaA (dnaA)	–		

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PCR detection and identification of plant pathogenic bacteria

Name of primers and target DNA used

Genus *Xylella*					
Species / hosts	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
X. fastidiosa / citrus	*X. fastidiosa* citrus strains specific **CVC-1/272-2-int** RAPD fragment *X. fastidiosa* strains (general) **272-1-int/272-2-int**	Conventional	Bacteria, sap (DNA extraction)	Pooler and Hartung, 1995	
	XF2542-L/XF2542 XF2542 fimbrial protein gene **ALM1/ALM2** Genomic DNA (unknown)				
X. fastidiosa	**272-1/272-2** (external) **272-1-int/272-2-int** (internal) RAPD fragment	Nested + Immunomagnetic separation (IMS)	Immunomagnetic separation of insects tissue extracts	Pooler *et al.*, 1997	
X. fastidiosa	**S-S-X.fas (sets A, B, C)** 16S rRNA gene **FXYgyr499/RXYgyr907** *gyrB* gene	Multiplex	Plant tissue, vector insects (DNA extraction)	Rodrigues *et al.*, 2003	
X. fastidiosa	**RST31/RST33** Genomic DNA (unknown) **G1/L1** ITS region	Conventional Immunocapture and conventional	Plant (DNA extraction)	Costa *et al.*, 2004	
X. fastidiosa	**RST31/RST33** Genomic DNA (unknown) **HL5/HL6** Genomic DNA (unknown) Probe **5´6FAM-labeled** **3´BHQ1™ labeled**	Conventional Real-time (TaqMan)	Plant and insect vectors (DNA extraction)	Francis *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

X. fastidiosa / citrus	Primers **CVC-1/CCSM-1** Probe **TAQCVC 5´6FAM-labeled 3´TAMRA labeled** Genomic DNA (unknown)	Real-time (TaqMan)	Bacteria, leaf (DNA extraction)	Oliveira *et al.*, 2002	
X. fastidiosa / grapevine	**RST31/RST33** Genomic DNA (unknown)	Conventional	Bacteria, leaf (DNA extraction)	Berisha *et al.*, 1998	
X. fastidiosa / grapevine	**XfF1/XfR1** ITS region **XfF2/XfR2** 16S rRNA gene Probes **5´6FAM-labeled 3´TAMRA labeled** (ITS) **5´6FAM-labeled 3´TAMRA labeled** (16S)	Real-time (TaqMan) Multiplex-Real-time (TaqMan)	Sap and macerated chips of secondary trunks of vines xylem (untreated)	Schaad *et al.*, 2002	
X. fastidiosa / grapevine	**XfF1/XfR1** ITS region **Probe 5´6FAM-labeled 3´TAMRA labeled ITS**	Real-time (TaqMan) BIO (Agar absorption)	Xylem sap (DNA extraction) Leaf and petiole (directly or previous plating)	Baumgartner and Warren, 2005 Fatmi *et al.*, 2005	
X. fastidiosa / citrus and grapevine	**RST31/RST33** Genomic DNA (unknown)	Conventional	Plant and xylem fluid (PVPP and sodium ascorbate addition)	Minsavage *et al.*, 1994	
X. fastidiosa / citrus and grapevine	*X. fastidiosa* grapevine strains **RST31/RST33** Genomic DNA (unknown) *X. fastidiosa* citrus strains **CVC-1/272-2-int** RAPD fragment	Conventional	Plant and xylem fluid (DNA extraction)	Minsavage *et al.*, 1994; Pooler and Hartung, 1995; Anon., 2004b	Recommended in the EPPO protocol.
X. fastidiosa / citrus and coffe	**JB-1/JB-2** RAPD fragment	Conventional	Plant tissue (DNA extraction)	Ferreira *et al.*, 2000	Strains from various hosts amplified at annealing 64°C. Only citrus and coffe related strains amplify at 68°C.
X. fastidiosa / grapevine and oleander	**RST31/RST33** Genomic DNA (unknown)	Conventional	Bacteria, xylem sap or plant (DNA extraction)	Bextine and Miller, 2004	
X. fastidiosa / grapevine, almond, oleander	**XF1968-L/1968-R** XF1968 methyltransferase gene	Conventional Multiprimer	Bacteria, plant tissue (DNA extraction)	Hernandez-Martinez *et al.*, 2006	

Palacio-Bielsa *et al.*,2009

Molecular Diagnosis of *Xylella*

PCR approaches to *X. fastidiosa* study

The most commonly used PCR

Primer Name	Sequence	Reference	Application
272-1-INT	CTGCACTTACCCAATGCATCG	Pooler and Hartung (1995)	Detection
272-2-INT	GCCGCTTCGGAGAGCATTCCT	Pooler and Hartung (1995)	Detection
27f	AGAGTTTGATCMTGGCTCAG	Lane (1991)	Detection
143r	GTCCCCCACGATAAGGTAGA	Lane (1991)	Detection
S-S-X.fas-0067-a-S-19	CGGCAGCACATTGGTAGTA	Rodrigues et al. (2003)	16S Sequencing
S-S-X.fas-0838-a-S-21	GCAAATTGGCACTCAGTATCG	Rodrigues et al. (2003)	16S Sequencing
S-S-X.fas-0838-a-A-21	CGATACTGAGTGCCAATTTGC	Rodrigues et al. (2003)	16S Sequencing
S-S-X.fas-1439-a-A-19	CTCCTCGCGGTTAAGCTAC	Rodrigues et al. (2003)	16S Sequencing
1525r	AAGGAGGTGWTCCARCC	Lane (1991)	16S Sequencing
1439-a-S-19	GTAGCTTAACCGCGAGGAG	Modified from Rodrigues et al. (2003)	16S-23S Sequencing
Xf 16S 1430F	AGCAGGTAGCTTAACCGCGA	Mundell, this study	16S-23S Sequencing
23S uni 322	GGTTCTTTTCGCCTTTCCCTC	Honeycutt et al. (1995)	16S-23S Sequencing
G1	GAAGTCGTAACAAGG	Jensen et al. (1993)	16S-23S Sequencing
23S-	TAC GGC CCT TTC GGA TAC AG	Mundell, this study	16S-23S Sequencing
23S-A-1998	ACCAGGTTCGCCTCCTTGAG	Mundell, this study	16S-23S Sequencing
23r	GTGCCAAGGCATCCACC	Li and de Boer (1995)	16S-23S Sequencing
FXYgyr499	CAGTTAGGGGTGTCAGCG	Rodrigues et al. (2003)	*gyrB* Sequencing
RXYgyr907	CTCAATGTAATTACCCAAGGT	Rodrigues et al. (2003)	*gyrB* Sequencing
LH18SRNAf	CGCGGTAATTCCAGCTCC	Mundell, this study	LH18SRNA
LH18SRNAr	CGGTGTGTACAAAGGGCAGG	Mundell, this study	LH18SRNA
LH28SRNAf	GAGAGTTCAAGAGTACGTGA	Mundell, this study	LH28SRNA
LH28SRNAr	CAGCTCTGACGATCGATTG	Mundell, this study	LH28SRNA

Primer Set	Forward Primer	Reverse Primer	Application	Product Length (bp)	Positive Tm Range (°C)	Annealing Temperature (°C)
27f-143r	27f	143r	Detection	116	83.01-84.3	60
272-int	272-1-int	272-2-int	Detection	472	79.4-80.9	62
16S Set B	S-S-X.fas-0067-a-S-19	S-S-X.fas-0838-a-S-21	16S Sequencing	771	82.05-84.28	56
16S Set C	S-S-X.fas-0838-a-A-21	S-S-X.fas-1439-a-A-19	16S Sequencing	619	83.63-84.48	56
16S 2nd	S-S-X.fas-0838-a-A-21	1525r	16S Sequencing	707	83.45-83.74	56
*gyr*B	FXYgyr499	RXYgyr907	*gyr*B Sequencing	429	79.72-80.27	54
LH18SRNA	LH18SRNAf	LH18SRNAr	Leafhopper 18S rDNA	1156	82.76-83.63	62
LH28SRNA	LH28SRNAf	LH28SRNAr	Leafhopper 28S rDNA	800	89.6-89.95	62

Mundell,2005

Molecular Diagnosis of *Xylella*
Conventional PCR(C-PCR)
DNA extraction for plant material

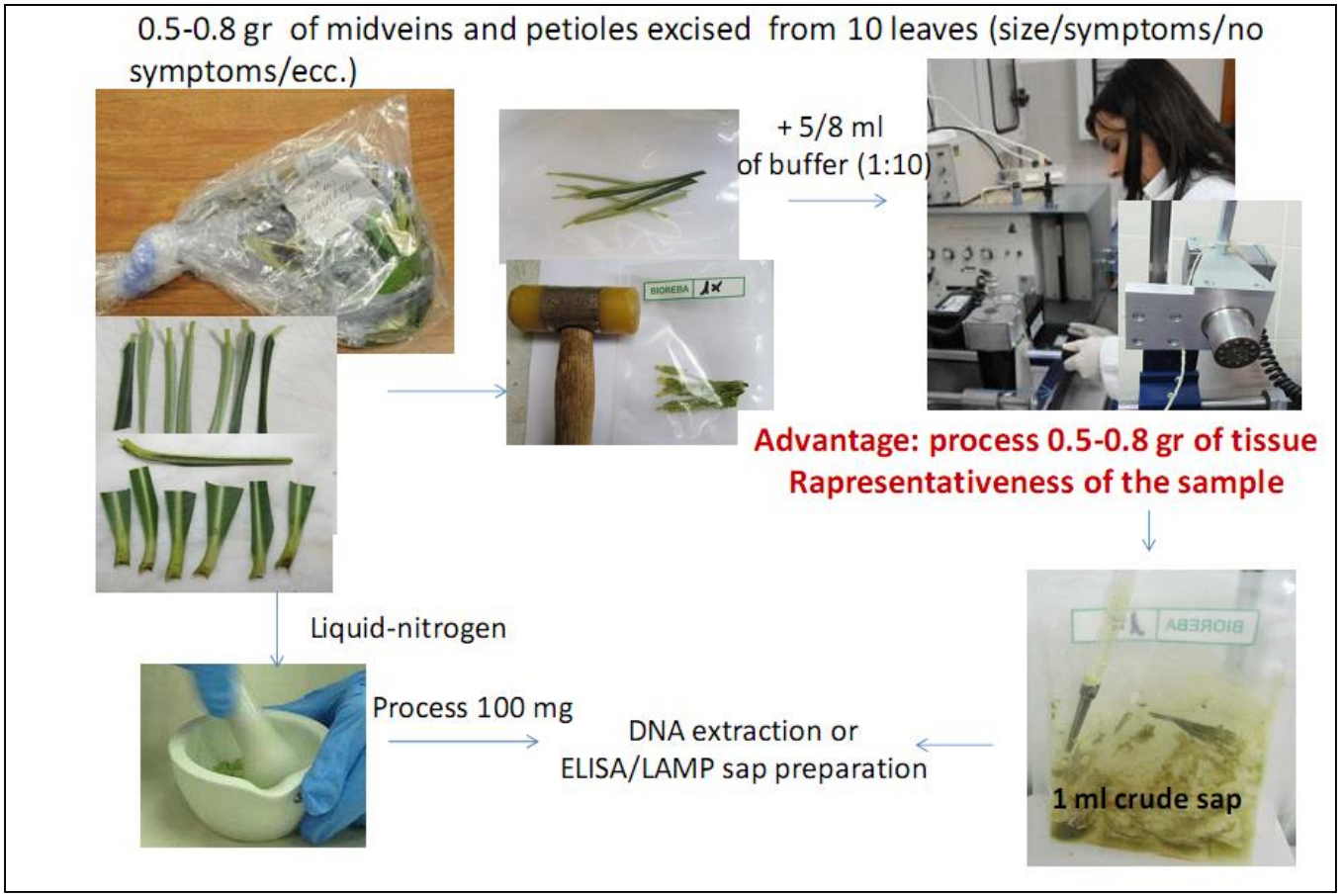
- **CTAB-based extraction**
- 0.5-1 g of fresh small pieces of midribs, petioles, leaf basal part or twigs (1/4 of the indicated amount, if lyophilized) should be weighed, put into the extraction bags or into suitable tubes with 5 mL of CTAB buffer and homogenized using a homogenizer (e.g. Homex, Polytron, etc.).
- 1 mL of extract should be transferred into a 1.5-mL micro-centrifuge tube and the sample should be heated at 65°C for 30 min and then centrifuged at 16 000 *g* for 5 min. 1 mL of the supernatant from centrifugation should be transferred to a new 2-mL micro-centrifuge tube, with care being taken not to transfer any of the plant tissue debris. 1 mL of chloroform:isoamyl alcohol (24:1) should be added and the sample should be mixed well by shaking.
- After centrifugation at 16 000 *g* for 10 min, 700 µL of the supernatant should be transferred to a 1.5-mL micro-centrifuge tube and 490 µL (approximately 0.7 volumes) of cold 2-propanol should be added. After mixing by inverting twice, the tube should be incubated at -20°C for 20 min.
- Centrifugation of the samples at 16 000 *g* for 20 min will allow recovery of a pellet that should be washed with 1 mL of 70% ethanol. An additional centrifugation at 16 000 *g* for 10 min and decantation in 70% ethanol should be performed. Sample should be air or vacuum-dried. The pellet should be resuspended in 100–150 µL of TE buffer or RNase- and DNase-free water.

EPPO Standards, 2016

Detection procedure

Conventional PCR(C-PCR)

Sample preparation DNA extraction



Loconsole,2016;..

Molecular Diagnosis of *Xylella*

Conventional PCR(C-PCR)

Nucleic acid extraction from pure cultures

- For pure cultures, a single colony of fresh pure culture is suspended in approximately 1 mL of molecular-grade water; lysis should be performed at 100°C for 5 min.
- Extracts of total nucleic acids can be stored at 4°C for immediate use or at −20°C for further use.

EPPO Standards,2016,2018,2019 and 2023

Molecular Diagnosis of *Xylella*

Conventional PCR(C-PCR)

Primers used for detection of ribosomal RNA (16S rDNA)

- Primers were designed based on the sequence of the 16S rRNA gene (XFr04) of *X. fastidiosa* strain 9a5c, isolated from sweet orange in Brazil.
- The primers, designated
- XFr04af (5′-TAAGTG AAG AGT TTG ATC CTG GC-3′), and
- XFr04ar (5′-AAAGGA GGT GAT CCA GCC-3′), directed the amplification of the entire gene, producing an amplicon of 1,545 bp.

Montero-Astúa *et al.*,2007

Molecular Diagnosis of *Xylella*

Conventional PCR(C-PCR)

Primers used for detection of ribosomal RNA (16S rDNA)

Forward primer	Reverse primer	Size	Target	Hosts	References
XF1-F: CAGCACATTGGTAGT AATAC	XF6-R: ACTAGGTATTAACCAATTGC	400	16s rDNA	Grapevine, almond, plum, American elm, citrus	Firrao and Bazzi, 1994
0067-a-S-19: CGGCAGCACATTGGT AGTA	1439-a-A-19: CTCCTCGCGGTTAAGCTAC	1,348	16s rDNA	Citrus, grapevine, mulberry	Rodrigues *et al.*, 2003
S-S-X.fas-0838-a-S-21(Forward): GCAAATTGGCACTCA GTATCG	S-S-X.fas-1439-a-A-19(Rrverse): CTCCTCGCGGTTAAGCTAC	603	16s rDNA		Rodrigues *et al.*, 2003
0067-a-S-19: CGGCAGCACATTGGT AGTA	0838-a-A-21: CGATACTGAGTGCCAATTTGC	745	16s rDNA		Rodrigues *et al.*, 2003
G1: GAAGTCGTAACAAGG	L1: CAAGGCATCCACCGT	522	16s rDNA	Porcelain berry, wild grape, Mulberry	Huang and Sherald, 2004

Baldi and La Porta,2017;..

Molecular Diagnosis of *Xylella*

Conventional PCR(C-PCR)

Primers used for detection of ribosomal RNA (16S rDNA)

Forward primer	Reverse primer	Size	Target	Hosts	References
Teme150fc: TCTACCTTATCGTGGGGGAC	Teme454rg: ACAACTAGGTATTAACCAATTGCC	348	16s rDNA	Almond	Chen *et al.*, 2005
Teme150fc: TCTACCTTATCGTGGGGGAC	Xf16s1031r: AAGGCACCAATCCATCTCTG	700	16s rDNA		Chen *et al.*, 2005
Dixon454fa: CCTTTTGTTGGGGAAGAAAA	Dixon1261rg: TAGCTCACCCTCGCGAGATC	847	16s rDNA		Chen *et al.*, 2005

Baldi and La Porta,2017;..

Molecular Diagnosis of *Xylella*

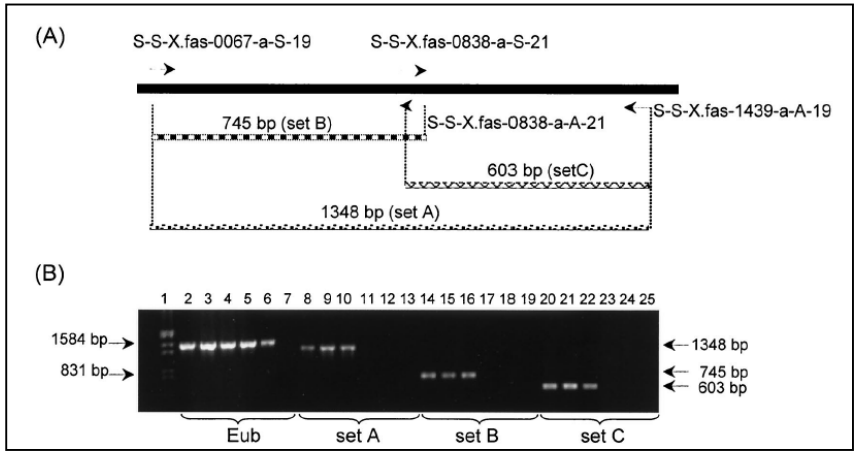
Conventional PCR

***X. fastidiosa*-specific (sets A to C) primers target ribosomal RNA (16S rDNA)**

A. Scheme of relative position of the 16S rRNA gene-specific primers for *X. fastidiosa*.

B. Ethidium bromide-stained agarose gel of PCR products after amplification of the 16S rRNA gene with eubacterial (Eub) or *X. fastidiosa*-specific (sets A to C) primers.

- The expected sizes for specific primer sets A, B, and C are 1,348, 745, and 603 bp, respectively.



Rodrigues *et al.*,2003

Molecular Diagnosis of *Xylella*

Conventional PCR(C-PCR)

Primers used for detection of the target genes RNA polymerase sigma factor (RST) and ribosomal RNA (16S rDNA)

- The primer sets tested in this work have previously been used for generic detection of *X. fastidiosa* by targeting conserved genomic regions.
- The list includes primers RST31/33 targeting RNA polymerase sigma-70 factor (rpoD)(Minsavage *et al.*,1994), which are widely accepted for the detection of the bacterium in quarantine programs (EPPO, 2004), as well as primers targeting the 16S rDNA genomic region (Firrao *et al.*,1994; Rodriguez *et al.*,2003), which are more suitable for accurate detection of a wider number of genetically diverse strains of *X. fastidiosa* (Harper *et al.*,2010).

Target gene	Primers	Amplicon size (bp)	Sequence (5' ®3')	References
RNA polymerase sigma factor	RST-31 RST-33	733	GCGTTAATTTTCGAAGTGATTCGATTGC CACCATTCGTATCCCGGTG	Minsavage *et al* ., 1994
16S rDNA	XF-1 XF-6	404	CAGCACATTGGTAGTAATAC ACTAGGTATTAACCAATTGC	Firrao and Bazzi, 1994
16S rDNA	S-S-X.fas-0838-a-S-21 S-S-X.fas-1439-a-A-19	603	GCAAATTGGCACTCAGTATCG CTCCTCGCGGTTAAGCTAC	Rodrigues *et al.*, 2003

Loconsole *et al.*,2014

Molecular Diagnosis of *Xylella*

Conventional PCR(C-PCR)

Primers used for detection of the target genes RNA polymerase sigma factor (RST) and ribosomal RNA (16S rDNA)

- The primer sets tested in this work have previously been used for generic detection of *X. fastidiosa* by targeting conserved genomic regions.
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Loconsole *et al.*,2014

Molecular Diagnosis of *Xylella*

Conventional PCR(C-PCR)

Primers used for detection of the target genes RNA polymerase sigma factor (RST) and ribosomal RNA (16S rDNA)

- PCR reactions for amplification of 16S rDNA and RNA polymerase sigma factor (RST) were performed in 1X GoTaq buffer (Promega, USA) in a final volume of 25 µl containing 2 µl TNA, 160 µM dNTPs, 0.2 µM of each primer and 1.25 U of GoTaq DNA polymerase (Promega, USA).
- PCR conditions were different depending on the primer set used.
- They initially consisted of a denaturation step at 95°C for 5 min followed by 35 cycles of 30 sec at 94°C, 30 sec at 50°C for XF1-F/XF6-R or 55°C for RST31/RST33 and S-S-X.fas-0838-a-S21/S-S-X.fas-1439-a-A-19, and 40 sec at 72°C.
- All reactions were finally extended at 72°C for 7 min and visualized on agarose gel.

Loconsole *et al.*,2014

Molecular Diagnosis of *Xylella*

Conventional PCR(C-PCR)

Primers used for detection of the target genes RNA polymerase sigma factor (RST) and ribosomal RNA (16S rDNA)

- Gel electrophoresis of PCR products recovered from the samples XF1-XF12.
- Target gene/primers:

1. RNA polymerase sigma factor RST-31/RST-33 (733 bp)
2. 16S rDNA XF-1/XF-6 (404 bp)
3. 16S rDNA S-S-X.fas-0838-a-S-21/S-S-X.fas-1439-a-A-19 (603 bp)

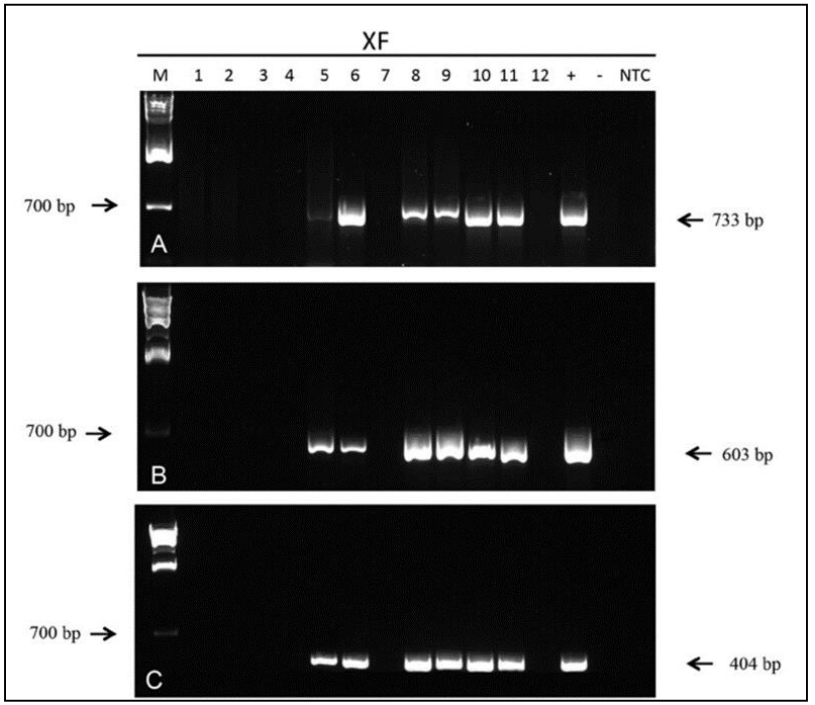
M. DNA ladder;

A. + = positive control;

B. - = negative control;

C. NTC = non-template control.

- The primer set XF1/XF6 and S-S-X.fas-0838-a-S-21S-S-X.fas-1439-a-A-19 gave comparable results, while primers RST31/RST33 amplified DNA bands showing a lighter signal.



Target gene	Primers	Amplicon size (bp)	Sequence (5' ®3')	References
RNA polymerase sigma factor	RST-31 RST-33	733	GCGTTAATTTTCGAAGTGATTCGATTGC CACCATTCGTATCCCGGTG	Minsavage *et al* ., 1994
16S rDNA	XF-1 XF-6	404	CAGCACATTGGTAGTAATAC ACTAGGTATTAACCAATTGC	Firrao and Bazzi, 1994
16S rDNA	S-S-X.fas-0838-a-S-21 S-S-X.fas-1439-a-A-19	603	GCAAATTGGCACTCAGTATCG CTCCTCGCGGTTAAGCTAC	Rodrigues *et al*., 2003

Loconsole *et al*.,2014

Molecular Diagnosis of *Xylella*

Conventional PCR(C-PCR)

PCR primers

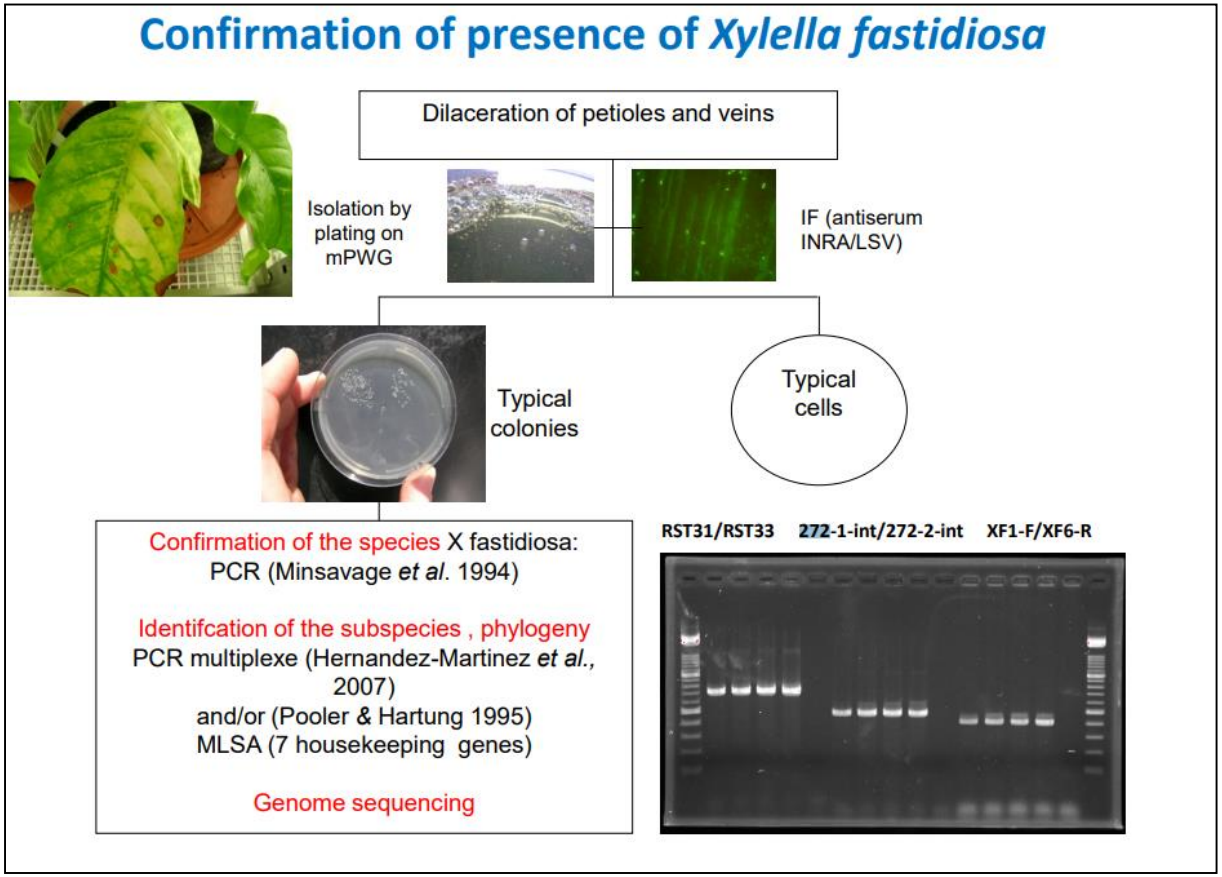
- PCR using universal primer set RST31-RST33 generated an expected 721-bp amplicon, confirming the *X. fastidiosa* identity of the Almond leaf scorch disease(ALSD) strains. Target part of the *rpoD* gene (sequence is located in the 3′ end of the gene *rpoD*) coding for an RNA polymerase sigma-70 factor.
- RST31F: 5′-GCGTTAATTTTCGAAGTGATTCGATTGC-3′
- RST33R: 5′-CACCATTCGTATCCCGGTG-3′
- 16S rRNA gene sequences are highly conserved at the species level, making them good candidates for species identification.
- XF1-F: 5′-CAGCACATTGGTAGTAATAC-3′
- XF6-R: 5′-ACTAGGTATTAACCAATTGC-3′
- Universal primers 272-1-int and 272-2-int, targeting hypothetical protein HL gene generate a PCR product of 500/600 bp may also be used to to detect all known strains of *X. fastidiosa* including citrus as well as for grapevine.
- 272-1-int (forward): 5′-CTG CAC TTA CCC AAT GCA TCG-3′
- 272-2-int (reverse): 5′-GCC GCT TCG GAG AGC ATT CCT-3′

Huang and Sherald,2004; EPPO,2016; ISPM 27,2018

Molecular Diagnosis of *Xylella*

Conventional PCR(C-PCR)

PCR primers: RST31F/RST33R, XF1-F/XF6-R and 272-1-int/272-2-int



Legendre *et al.*,2015

Molecular Diagnosis of *Xylella*

Conventional PCR(C-PCR)

PCR primers

- Hypothetical protein HL gene:
- Despite a relatively high number of nucleotide sequences of *X. fastidiosa* deposited for many genes, including those described before, a very low number of sequences of hypothetical protein (HL) still exist in all public nucleotide databases.

Target Gene	Primers pairs	Oligonucloetide sequence (5′→3′)	Approx. amplicon size (bp)	References
Hypothetical protein (HL)	HL5 HL6	AAGGCAATAAACGCGCACTA GGTTTTGCTGACTGGCAACA	221	Francis *et al.*, 2006

Mang *et al.*,2016

Molecular Diagnosis of *Xylella*
Conventional PCR(C-PCR)
PCR primers

- The 16S-23S rRNA intergenic regions of the porcelain berry, wild grape, and mulberry strains of *X. fastidiosa* was amplified by PCR with primers:
- G1 (5′-GAAGTCGTAACAAGG-3′) and
- L1 (5′-CAAGGCATCCACCGT-3′)
- CVC specific primers:
- Primers CVC-1 and 272-2-int, which generate a PCR product of 500 base pairs, are used for the detection of *X. fastidiosa* causing Citrus Variegated Chlorosis (CVC).
- DNA fragments are used (Pooler & Hartung,1995):
- CVC-1: 5′-AGATGAAAACAATCATGCAAA-3′
- 272-1-int: 5′-GCCGCTTCGGAGAGCATTCCT-3′.

EPPO,2004; Huang and Sherald,2004

Molecular Diagnosis of *Xylella*

Conventional PCR(C-PCR)

PCR primers

Assay	Primers name	Target gene Name	Sequence (5′-3′)	References
PCR	RNA polymerase sigma factor	RST-31F	GCGTTAATTTTCGAAGTGATT CGATTGC	Minsavage *et al.*, 1994
		RST-33R	CACCATTCGTATCCCGGTG	
	16S rRNA	S-S-X.fas-0838-a-S-21	GCAAATTGGCACTCAGTATCG	Rodriguez *et al.*, 2003
		S-S-X.fas-1439-a-A-19	CTCCTCGCGGTTAAGCTAC	
	gyrB	FXYgyr499	CAGTTAGGGGTGTCAGCG	Rodriguez *et al.*, 2003
		RXYgyr907	CTCAATGTAATTACCCAAGGT	
	Internal transcriber spacers (ITS)	HL5	AAGGCAATAAACGCGCACTA	Francis *et al.*, 2006
		HL6	GGTTTTGCTGACTGGCAACA	

Waliullah *et al.*,2019

Conventional PCR(C-PCR)

Master mix composition, cycling parameters and amplicons for conventional PCR using the 16S rRNA gene-targeted primers of Rodrigues *et al.*,2003

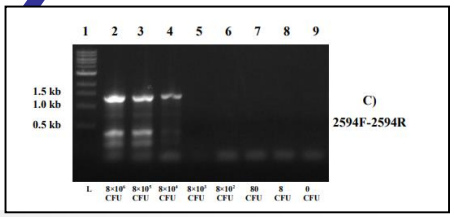
PCR primers

- Set A:
- S-S-X.fas-0067-a-S-19 (forward): 5′-CGG CAG CAC ATT GGT AGT A-3′
- S-S-X.fas-1439-a-A-19 (reverse): 5′-CTC CTC GCG GTT AAG CTA C-3′
- Primer set A amplifies a product of 1348 bp.
- Set B:
- S-S-X.fas-0067-a-S-19 (forward): 5′-CGG CAG CAC ATT GGT AGT A-3′
- S-S-X.fas-0838-a-A-21 (reverse): 5′-CGA TAC TGA GTG CCA ATT TGC-3′
- Primer set B amplifies a product of 745 bp.
- Set C:
- S-S-X.fas-0838-a-S-21 (forward): 5′-GCA AAT TGG CAC TCA GTA TCG-3′
- S-S-X.fas-1439-a-A-19 (reverse): 5′-CTC CTC GCG GTT AAG CTA C-3′
- Primer set C amplifies a product of 603 bp.

Molecular Diagnosis of *Xylella*

Conventional PCR(C-PCR)

PCR primers



- OLIGO Primer Analysis Software was used to facilitate the design of 2594F and 2594R, which flank FX2594 in the M12 genome.
- Designed to amplify a 1323-bp DNA fragment from A-type strains of *X. fastidiosa*.
- The 2594F-2594R primer pair was approximately 1000-fold less sensitive than the RST32-RST33 primer pair.

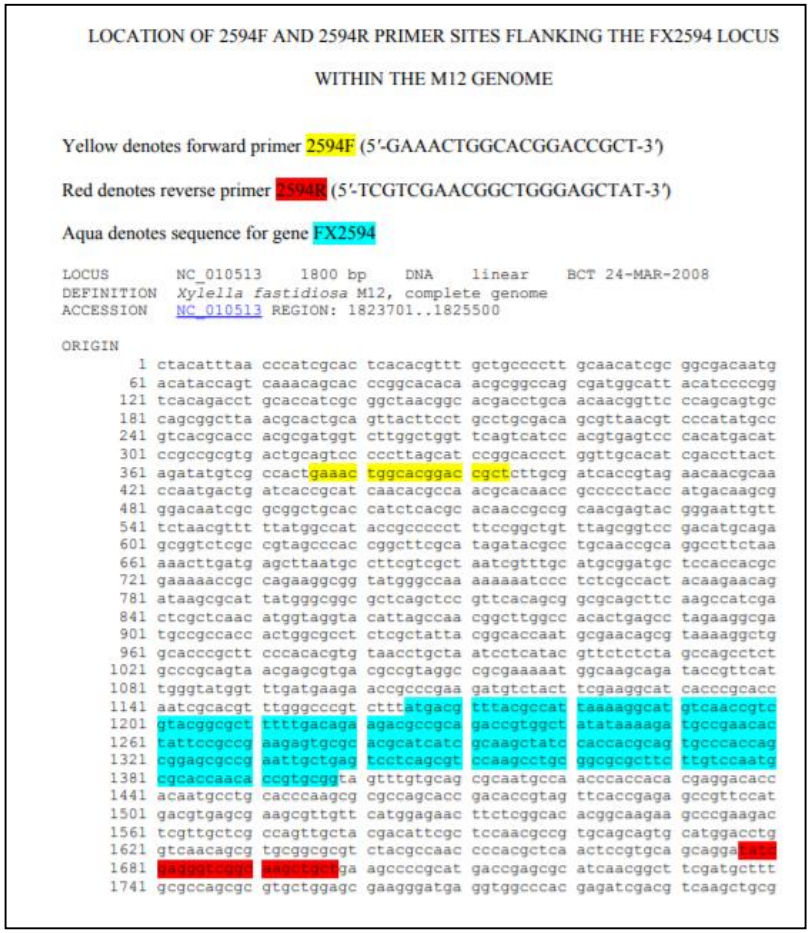
2594F 2594R	GAAACTGGCACGGACCGCT AGCAGCTTGCCGACCCTCGATA	1323	ALS OLS	This study

ALS = almond leaf scorch strains; OLS = oleander leaf scorch

Nissen *et al.*,2010

Program for designing the primer pair of 2594F and 2594R for *Xylella fastidiosa*

OLIGO Primer Analysis Software was used to facilitate the design of 2594F and 2594R, which flank FX2594 in the M12 genome.



LOCATION OF 2594F AND 2594R PRIMER SITES FLANKING THE FX2594 LOCUS WITHIN THE M12 GENOME

Yellow denotes forward primer 2594F (5'-GAAACTGGCACGGACCGCT-3')

Red denotes reverse primer 2594R (5'-TCGTCGAACGGCTGGGAGCTAT-3')

Aqua denotes sequence for gene FX2594

```
LOCUS       NC_010513    1800 bp    DNA    linear    BCT 24-MAR-2008
DEFINITION  Xylella fastidiosa M12, complete genome
ACCESSION   NC_010513 REGION: 1823701..1825500

ORIGIN
        1 ctacatttaa cccatcgcac tcacacgttt gctgcccctt gcaacatcgc ggcgacaatg
       61 acataccagt caaacagcac ccggcacaca acgcggccag cgatggcatt acatccccgg
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     1681 gaggctcggc aagctgctga agccccgcat gaccgagcgc atcaacggct tcgatgcttt
     1741 gcgccagcgc gtgctggagc gaagggatga ggtggcccac gagatcgacg tcaagctgcg
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Nissen *et al.*,2010

Conventional PCR(C-PCR)

Primer pair CVC-1and 272-2-Int

PCR conditions: 94°C for 4 min followed by 30 cycles of (94°C for 1 min, 62°C for 1 min, 72°C for 1 min) and a final step of 72°C for 10 min.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	18.3	N.A.
Taq DNA polymerase buffer (Invitrogen)	×10	2.5	×1
$MgCl_2$	50 mM	0.75	1.5 mM
dNTPs	20 mM	0.25	0.2 mM
Forward primer (CVC-1)	20 µM	0.5	0.4 µM
Reverse primer (272-2-Int)	20 µM	0.5	0.4 µM
Platinum Taq DNA polymerase (Invitrogen)	5 U μL^{-1}	0.2	0.04 U μL^{-1}
Subtotal		23	
Genomic DNA from plant extract (final concentration and its 10- and 100-fold dilutions) or bacterial suspension		2	
Total		25	

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

EPPO,2016

Molecular Diagnosis of *Xylella*

Conventional PCR (C-PCR)

Primer set RST31-RST33

- The test is based on Minsavage *et al.*,1994.
- The target sequence is located in the 3′ end of the gene *rpoD*, coding for an RNA polymerase sigma-70 factor/sigma-70 (gene rpoD).
- The forward primer RST31 sequence is:
- RST31 (forward): 5′-GCGTTAATTTTCGAAGTGATTCGATTGC-3′
- RST33 (reverse): 5′-CACCATTCGTATCCCGGTG-3′
- Amplicon size: 733 bp.
- **PCR conditions**
- 95°C for 1 min followed by 40 cycles of (95°C for 30 s, 55°C for

Primer Name	Sequence (5'-3')	Target Gene	Reference
RST31	GCGTTAATTTTCGAAGTGATTCGA	Unique *E.coli* R 1 fragment	Minesavage *et al.*, 1994
RST33	CACCATTCGTATCCCGGTG		Minesavage *et al.*, 1994

EPPO Standards,2016

Molecular Diagnosis of *Xylella*

PCR Primers useful for detection of *X. fastidiosa*

Conventional PCR(C-PCR)

- To detect *X. fastidiosa*, three specific primers sets can be used:
- RST31/33(target gene: *rpoD*, coding for an RNA polymerase sigma-70 factor). For the PD strain(Pierce's disease of grapevine, the RST primers can be used (Minesavage *et al.*,1994)
- XF1-F/XF6-R (target gene: 16S rDNA);

Primer Name	Sequence (5'-3')	Target Gene	Reference
RST31	GCGTTAATTTTCGAAGTGATTCGA	Unique *E.coli* R 1 fragment	Minesavage *et al.*, 1994
RST33	CACCATTCGTATCCCGGTG		Minesavage *et al.*, 1994
XF1-F	CAGCACATTGGTAGTAATAC	16S rDNA	Firrao and Bazzi, 1994
XF6-R	ACTAGGTATTAACCAATTGC		Firrao and Bazzi, 1994

Janse and Obradovic,2010

Molecular Diagnosis of *Xylella*

PCR Primers useful for detection of *X. fastidiosa*

Conventional PCR (C-PCR)

- To detect *Xylella fastidiosa*, three specific primers sets can be used.
- For the Pierce's disease strain of *Xylella fastidiosa* the RST primers should be used (Minesavage et al, 1994).
- For strains not occurring in grapevine the XF primers can be used.
- It is important to use housekeeping genes such as ribosomal DNA to ensure the DNA template does not contain PCR inhibitors.
- This eliminates the possibility of a false negative result.
- All primers were used at a concentration of 100 ng/µl.

Primer Name	*Sequence (5'-3')*	*Target Gene*	*Reference*
RST31	GCGTTAATTTTCGAAGTGATTCGA	Unknown fragment	Minesavage *et al.*,1994
RST33	CACCATTCGTATCCCGGTG		Minesavage *et al.*,1994
XF1-F	CAGCACATTGGTAGTAATAC	16S rDNA	Firrao & Bazzi, 1994
XF6-R	ACTAGGTATTAACCAATTGC		Firrao & Bazzi, 1994
FD2	AGAGTTTGATCATGGCTCAG	16S rDNA	Weisburg *et al.*, 1991
RP1	ACGGTTACCTTGTTACGACTT		Weisburg *et al.*, 1991

FD2/RP1 primer (Weisburg *et al.*,1991)

The National Diagnostic Protocol (NDP) for *Xylella fastidiosa*,2012

Conventional PCR(C-PCR)

Master mix for the RST31/33 primer set

PCR conditions: 95°C for 1 min followed by 40 cycles of (95°C for 30 s, 55°C for 30 s, 72°C for 45 s) and a final step of 72°C for 5 min.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	18.6	N.A.
Taq DNA polymerase buffer (Invitrogen)	×10	2.5	×1
$MgCl_2$	50 mM	0.75	1.5 mM
dNTPs	20 mM	0.25	0.2 mM
Forward primer (RST31)	20 µM	0.375	0.3 µM
Reverse primer (RST33)	20 µM	0.375	0.3 µM
Platinum Taq DNA polymerase (Invitrogen)	5 U μL^{-1}	0.15	0.03 U μL^{-1}
Subtotal		23	
Genomic DNA from plant tissue extract or bacterial suspension		2	
Total		25	

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

EPPO,2016

Conventional PCR(C-PCR)

Master mix for the RST31/33 primer set

Master mix composition for PCR and cycling conditions for a final reaction volume of 20 µl using the primers of Minsavage *et al.*,1994

The oligonucleotide primers used are:

RST31 (forward): 5′-GCG TTA ATT TTC GAA GTG ATT CGA TTG C-3′

RST33 (reverse): 5′-CAC CAT TCG TAT CCC GGT G-3′

The master mix used for this PCR developed by Minsavage *et al.* (1994) is described in Table 2.

Table 2. Master mix composition, cycling parameters and amplicons for conventional PCR using the primers of Minsavage *et al.* (1994)

Reagents	Final concentration
PCR grade water	–†
PCR buffer (Invitrogen)[1]	1×
dNTPs	200 µM
$MgCl_2$	1.5 mM
Primer RST31 (forward)	0.5 µM
Primer RST33 (reverse)	0.5 µM
Taq DNA polymerase (Invitrogen)[1]	1.25 U
DNA volume	2 µl bacterial suspension or DNA extract
Cycling parameters	
Initial denaturation	95 °C for 1 min
Number of cycles	40
- Denaturation	95 °C for 30 s
- Annealing	55 °C for 30 s
- Elongation	72 °C for 45 s
Final elongation	72 °C for 5 min
Expected amplicons	
Size	733 bp

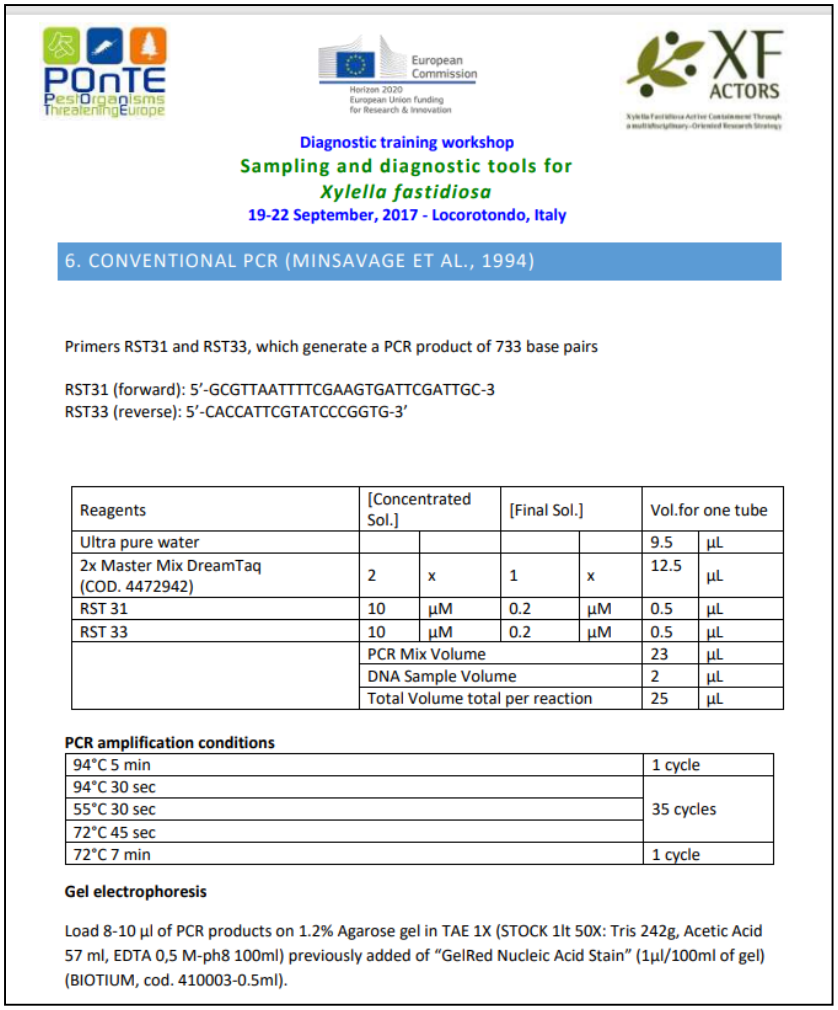
† For a final reaction volume of 20 µl.

bp, base pairs; PCR, polymerase chain reaction.

Conventional PCR(C-PCR)

Master mix for the RST31/33 primer set

2x Master Mix DreamTaq (COD. 4472942) and PCR amplification conditions



Diagnostic training workshop

Sampling and diagnostic tools for

Xylella fastidiosa

19-22 September, 2017 - Locorotondo, Italy

6. CONVENTIONAL PCR (MINSAVAGE ET AL., 1994)

Primers RST31 and RST33, which generate a PCR product of 733 base pairs

RST31 (forward): 5'-GCGTTAATTTTCGAAGTGATTCGATTGC-3
RST33 (reverse): 5'-CACCATTCGTATCCCGGTG-3'

Reagents	[Concentrated Sol.]		[Final Sol.]		Vol.for one tube	
Ultra pure water					9.5	µL
2x Master Mix DreamTaq (COD. 4472942)	2	x	1	x	12.5	µL
RST 31	10	µM	0.2	µM	0.5	µL
RST 33	10	µM	0.2	µM	0.5	µL
	PCR Mix Volume				23	µL
	DNA Sample Volume				2	µL
	Total Volume total per reaction				25	µL

PCR amplification conditions

94°C 5 min	1 cycle
94°C 30 sec	35 cycles
55°C 30 sec	
72°C 45 sec	
72°C 7 min	1 cycle

Gel electrophoresis

Load 8-10 µl of PCR products on 1.2% Agarose gel in TAE 1X (STOCK 1lt 50X: Tris 242g, Acetic Acid 57 ml, EDTA 0,5 M-ph8 100ml) previously added of "GelRed Nucleic Acid Stain" (1µl/100ml of gel) (BIOTIUM, cod. 410003-0.5ml).

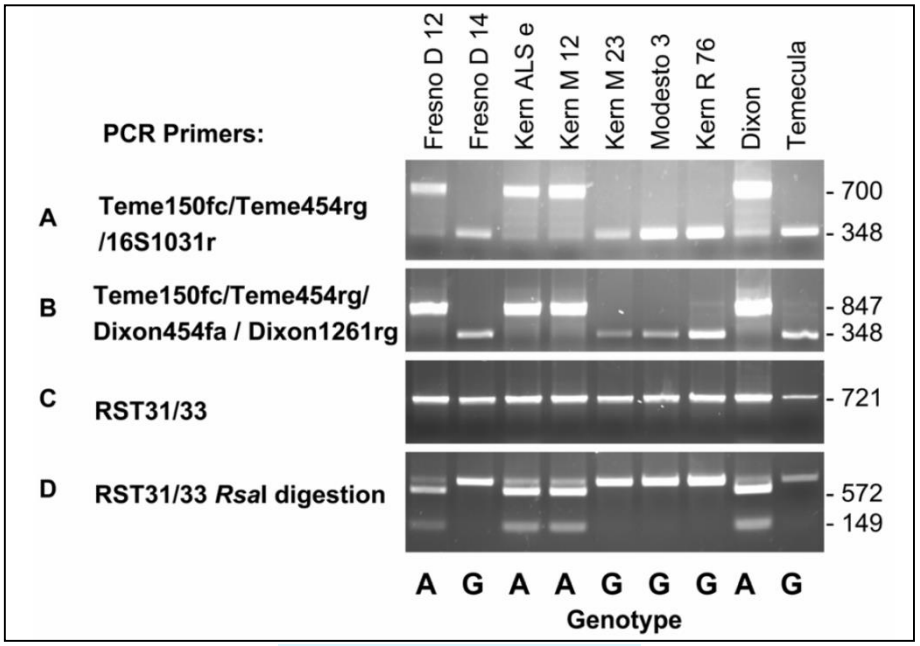
Diagnostics methods
Master mix for the RST31/33 primer set
*Rsa*I digestion of RST31-RST33 amplicons

- Restriction digestion:
- Aliquots (9 µl) of PCR products of the RST31-RST33 primer pair were digested in a 10.5-µl reaction containing 1X NEBuffer #4, 5 U of *RsaI* restriction endonuclease (New England BioLabs Inc., Ipswitch, MA) and 0.5 µl sterile deionized water.
- Reactions were incubated at 37°C for 3 h and then terminated by adding 2 µl of 6X blue gel loading dye (New England BioLabs Inc., Ipswitch, MA).
- Restriction fragment length polymorphisms (RFLP) were identified by electrophoresis in a 1% agarose gel, with all of digested samples being loaded into wells.
- Visualization and determination of size of separated DNA fragments was as previously described.

Nissen *et al.*,2010

Diagnostics methods
Master mix for the RST31/33 primer set
*Rsa*I digestion of RST31-RST33 amplicons

- Polymerase chain reaction (PCR) amplifications of almond leaf scorch isolates from San Joaquin Valley of California using the **A,** three primer format, **B,** four primer format, and **C,** RST31-RST33 primer set.
- Panel **D** is the *Rsa*I digestion of RST31-RST33 amplicons. Numbers on the right are DNA fragment size in base pairs.



Chen *et al.*,2005

Conventional PCR(C-PCR)

Master mix for the 272-1-int/272-2-int primer set

Master mix composition, cycling parameters and amplicons for conventional PCR using the primers of Pooler and Hartung,1995

The oligonucleotide primers used are:

272-1-int (forward): 5′-CTG CAC TTA CCC AAT GCA TCG-3′

272-2-int (reverse): 5′-GCC GCT TCG GAG AGC ATT CCT-3′

The master mix used for this PCR is described in Table 3.

Table 3. Master mix composition, cycling parameters and amplicons for conventional PCR using the primers of Pooler and Hartung (1995)

Reagents	Final concentration
PCR grade water	–†
PCR buffer (Invitrogen)[1]	1×
dNTPs	200 µM
$MgCl_2$	1.5 mM
Primer 272-1-int (forward)	0.4 µM
Primer 272-2-int (reverse)	0.4 µM
Taq DNA polymerase (Invitrogen)[1]	1.0 U
DNA volume	2 µl bacterial suspension or DNA extract
Cycling parameters	
Initial denaturation	94 °C for 1 min
Number of cycles	40
- Denaturation	94 °C for 1 min
- Annealing	67 °C for 1 min
- Elongation	72 °C for 1 min
Final elongation	72 °C for 10 min
Expected amplicons	
Size	500 bp

† For a final reaction volume of 20 µl.

bp, base pairs; PCR, polymerase chain reaction.

Conventional PCR(C-PCR)

Master mix for the S-S-X.fas-0067-a-S-19/S-S-X.fas-1439-a-A-19; S-S-X.fas-0067-a-S-19/S-S-X.fas-0838-a-A-21 and S-S-X.fas-0838-a-S-21/S-S-X.fas-1439-a-A-19 primer sets targeting 16S rRNA gene(Rodrigues *et al.*,2003)

- Set A:
- S-S-X.fas-0067-a-S-19 (forward): 5′-CGG CAG CAC ATT GGT AGT A-3′
- S-S-X.fas-1439-a-A-19 (reverse): 5′-CTC CTC GCG GTT AAG CTA C-3′
- Primer set A amplifies a product of 1348 bp.
- Set B:
- S-S-X.fas-0067-a-S-19 (forward): 5′-CGG CAG CAC ATT GGT AGT A-3′
- S-S-X.fas-0838-a-A-21 (reverse): 5′-CGA TAC TGA GTG CCA ATT TGC-3′
- Primer set B amplifies a product of 745 bp.
- Set C:
- S-S-X.fas-0838-a-S-21 (forward): 5′-GCA AAT TGG CAC TCA GTA TCG-3′
- S-S-X.fas-1439-a-A-19 (reverse): 5′-CTC CTC GCG GTT AAG CTA C-3′
- Primer set C amplifies a product of 603 bp.

Conventional PCR(C-PCR)

Master mix for the S-S-X.fas-0067-a-S-19/S-S-X.fas-1439-a-A-19; S-S-X.fas-0067-a-S-19/S-S-X.fas-0838-a-A-21 and S-S-X.fas-0838-a-S-21/S-S-X.fas-1439-a-A-19 primer sets targeting 16S rRNA gene(Rodrigues *et al.*,2003)

Reagents	Final concentration
PCR grade water	–[†]
PCR buffer	1×
dNTPs	200 µM
$MgCl_2$	1.5 mM
Primer (forward set A, or B or C)	0.2 µM
Primer (reverse set A, or B or C)	0.2 µM
Taq DNA polymerase (Invitrogen)[1]	2.0 U
DNA volume	2 µl bacterial suspension or DNA extract
Cycling parameters	
Initial denaturation	94 °C for 3 min
Number of cycles	30
- Denaturation	94 °C for 1 min
- Annealing	55 °C for 30 s
- Elongation	72 °C for 2 min
Final elongation	72 °C for 7 min
Expected amplicons	
Size	Primer set A: 1348 bp Primer set B: 745 bp Primer set C: 603 bp

[†] For a final reaction volume of 20 µl.

bp, base pairs; PCR, polymerase chain reaction.

ISPM 27, DP 25: *Xylella fastidiosa*,2018

Conventional PCR(C-PCR)

Master mix composition, cycling parameters and amplicons for conventional PCR using the *gyrB* gene targeting primers of Rodrigues *et al.*,2003

- The gyrB primers used are:
- FXYgyr499 (forward): 5′-CAG TTA GGG GTG TCA GCG-3′
- RXYgyr907 (reverse): 5′-CTC AAT GTA ATT ACC CAA GGT-3′
- The gyrB primer set produces an amplicon of 429 bp.

Reagents	Final concentration
PCR grade water	–[†]
PCR buffer	1×
dNTPs	200 µM
$MgCl_2$	1.5 mM
Primer FXYgyr499 (forward)	0.4 µM
Primer RXYgyr907 (reverse)	0.4 µM
Taq DNA polymerase (Invitrogen)[1]	2.5 U
DNA volume	2 µl bacterial suspension or DNA extract
Cycling parameters	
Initial denaturation	94 °C for 3 min
Number of cycles	30
- Denaturation	94 °C for 1 min
- Annealing	60 °C for 1 min
- Elongation	72 °C for 2 min
Final elongation	72 °C for 7 min
Expected amplicons	
Size	429 bp

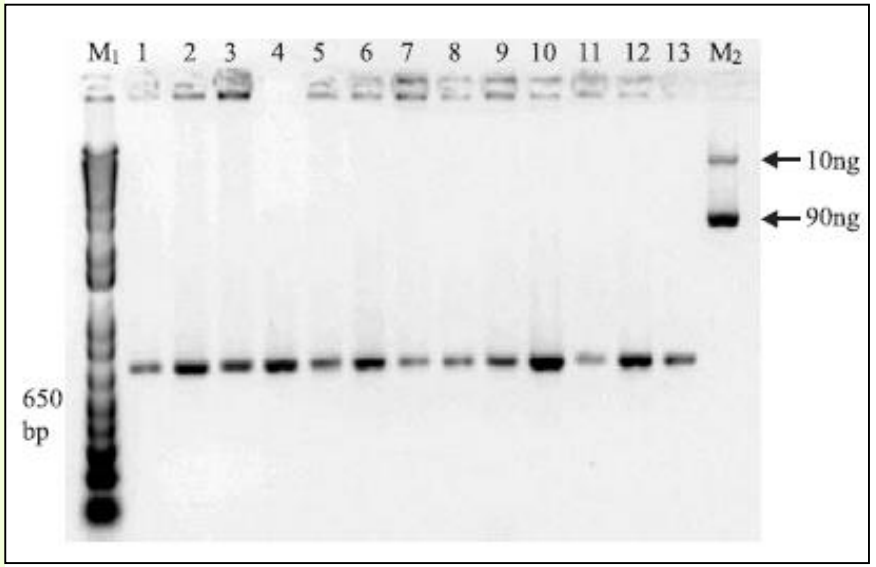
[†] For a final reaction volume of 20 µl.
bp, base pairs; PCR, polymerase chain reaction.

Diagnostics methods

PCR amplify 16S-23S spacer region of rDNA

Conventional PCR(C-PCR)

- Gel electrophoresis of the amplified rDNA internal transcribed spacer (ITS) region of *7 Xylella fastidiosa* strains.
- M1- Molecular marker 1Kb Plus DNA.
- Ladder; 1-13 *Xylella fastidiosa* strains:
- 1-Citrus (X0); 2-4-Coffee (6738, 6740, 6755); 5-7-Grapevine (6068, 6752, 6753); 8-9- Mulberry (6744, 6745); 10-Almond (6746); 11-Elm (6748); 12- Ragweed (6749); 13-Periwinkle (6751);
- M2- Molecular marker pGEM.



A fragment of approximately 510 bases containing the 16S-23S rDNA region of all strains was obtained.

Martinati *et al.*,2007

Diagnostics methods

In citrus plants and sharpshooter leafhoppers

Nested-PCR

- Nested PCR is an effective method for detecting organisms or their products in environmental samples where the presence of low concentrations of the DNA targets and high concentrations of contaminants could inhibit DNA amplification.
- The detection of *X. fastidiosa* in insect samples is difficult as the number of bacterial cells present in insects is less than that present in samples of plant tissues, so the use of nested-PCR could be an efficient alternative assay.

Ciapina *et al.*,2004

Diagnostics methods

In citrus plants and sharpshooter leafhoppers

Nested-PCR

- Samples were analysed using up to 3 different bacterial extraction methods and a 2-step, nested polymerase chain reaction (PCR) amplification using oligonucleotide primers specific to *X. fastidiosa*.
- Eleven of the 27 taxa could not be tested due to failure to amplify *X. fastidiosa* DNA in the positive controls, regardless of the extraction method.
- So, the nested PCR using the RST sets (primers RST3I/RST33)(genes RNA polymerase sigma factor) was highly sensitive and specific for detection of *X. fastidiosa* in potential insect vectors.

McElrone *et al.*,1999; PAIÃO *et al.*,??

Diagnostics methods

In citrus plants and sharpshooter leafhoppers

Nested-PCR

1. DNA extracts from three of the oleander plants with high ELISA absorbance values were tested by nested PCR with primer pair 272-1/272-2 followed by the pair 272-1 int/272-2 int.
2. Two of the samples were positive for the bacterium and one of the PCR products was cloned and sequenced in both directions (GenBank Accession No. EU009615).

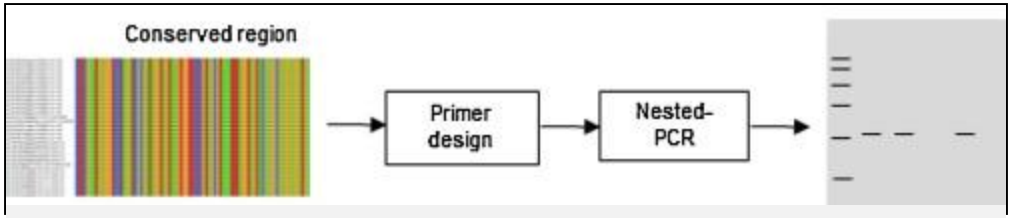


Fig for unrelated parasite from Costa *et al.*,2016

Astúa *et al.*,2008

Diagnostics methods

In citrus plants and sharpshooter leafhoppers

Nested-PCR

- The conditions for DNA amplification were 8 µl of extracted DNA, 1X PCR Buffer (Invitrogen, Carlsbad, CA, USA); 2 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} of each dNTP; 1.0 unit of *Taq* polymerase and 30 ng of each primer: CVC-1/272-2 int or 272-1/272-2.
- The final reaction mixture volume was 20 µl. The first primer set was used for plant and bacterial samples and the second was used for insect samples in the first round of the nested-PCR.
- The PCR cycling conditions were: 94°C for 2min, followed by 35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 90s.
- After a final extension at 72°C for 5 min, reactions were held at 4°C.
- For the nested PCR, 3 µl of the first PCR reaction was used as a template using the CVC-1/272-2 int primer set and it was amplified using the conditions for DNA amplification and PCR cycling programme described above.

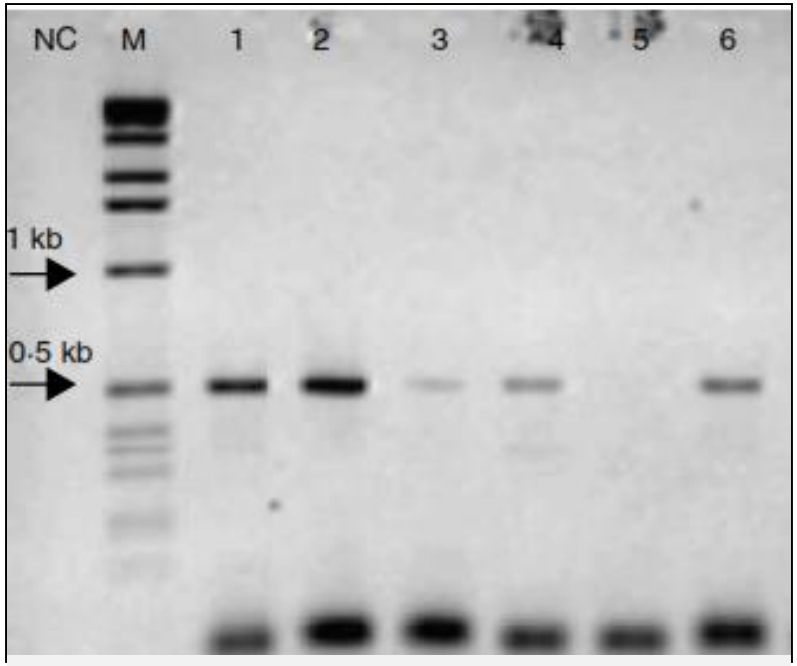
Ciapina *et al.*,2004

Diagnostics methods

In citrus plants and sharpshooter leafhoppers

Nested PCR

- Detection of *Xylella fastidiosa* in fed sharpshooter by nested-PCR using the CVC-1/272-2 int primer.
- Lanes:
- M, Ladder 1kb DNA molecular size;
- NC, Negative Control (macerated head of healthy sharpshooter);
- Positive samples showed an amplified fragment of 500 bp(0.5 kb) indicated by arrow.



The use of Chelex 100 resin allowed a fast and efficient DNA extraction to be used in the detection of *X. fastidiosa* in citrus plants and insect vectors by PCR and nested-PCR assays, respectively.

Ciapina *et al.*,2004

Molecular Diagnosis of *Xylella*
Conventional PCR(C-PCR)
Multiplex PCR

- PCR-based diagnosis using conserved primers for *X. fastidiosa* strains (RST31/33) and also specific to *X. fastidiaosa* subsp. *pauca* (CVC1/272-2 int) were positive for all symptomatic tested olive leaf scorch samples (n= 8 of 9), but no was obtained using twigs from asymptomatic trees (n= 20).
- For DNA extraction and bacterial isolation, we used leaves and twigs collected from non-symptomatic sections of symptomatic branches.

Coletta-Filho *et al.*,2016

Molecular Diagnosis of *Xylella*
Conventional PCR(C-PCR)
Multiplex PCR

- Primers CVC-1 and 272-2-int, which generate a PCR product of 500 base pairs, are used for the detection of *X. fastidiosa* causing Citrus Variegated Chlorosis (CVC).
- DNA fragments are used (Pooler & Hartung,1995):
- CVC-1: 5′-AGATGAAAACAATCATGCAAA-3′
- 272-1-int: 5′-GCCGCTTCGGAGAGCATTCCT-3′.

EPPO,2004; Huang and Sherald,2004

Molecular Diagnosis of *Xylella*

PCR primers for *X. fastidiosa* strains and *X. fastidiosa* subsp. *pauca*

PCR amplifications for both primer sets

- PCR was carried out by using the primers RST31 and RST33 that amplify a 733-bp DNA fragment of several strains of *X. fastidiosa* (Minsavage *et al.*, 1994), and CVC1/272-2-int for specific amplification of a 500-bp product of the citrus (Pooler & Hartung, 1995) and coffee (Coletta Filho & Machado, 2001) strains of the bacterium.
- The two primer pairs were used in the same reaction (multiplex PCR).

Marucci *et al.*,2003; Coletta-Filho *et al.*,2016

Molecular Diagnosis of *Xylella*

PCR primers for *X. fastidiosa* strains and *X. fastidiaosa* subsp. *pauca*

PCR amplifications for both primer sets

- PCR amplification was performed in a 13 µL volume containing 1× master mix PCR (Dream Taq DNA polymerase), 10 pmol of each primer of sets RST31/RST33 (Minsavage *et al.*, 1993) and CVC1/272-2 int (Pooler & Hartung, 1995), and 3 µL of total DNA (100 ng/µL).
- Amplifications for both primer sets were conduced with the following setup:
- Initial denaturation step at 95°C for 5 min, 36 cycles at 95°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min, with a final extension at 72°C for 5 min. The amplicons were separated on a 1% agarose gel.

Coletta-Filho *et al.*,2016

Molecular Diagnosis of *Xylella*
Conventional PCR(C-PCR)
Gel electrophoresis of RST 31/33 primers

- Gel electrophoresis of RST 31/33 primers in olive samples inoculated with different stains of *Xylella fastidiosa* subsp. *pauca*.
- L= (GeneRuler 1Kb ladder marker - Fermentas). P and N represent positive and negative controls.
- Lanes 1 and 2 represent olive plants inoculated with PBS; 3 to 7 represents plants inoculated with *X. fastidiosa* strain 9a5c (ST13); 8 to 10 represent plants inoculated with strain MFG01 (ST16) isolate from olive plants with leaf scorch symptoms; 11 to 15 represent olive plants inoculate with strain PLS8 (ST71) from plum.

Bands represent samples infected by *X. fastidiosa* (positive samples).

Note: CVC1/272-2-int for specific amplification of a 500-bp product of the citrus and coffee strains of the bacterium(Marucci *et al.*,2003).

Coletta-Filho *et al.*,2016

Conventional multiplex PCR

Mainly used for assignment of an isolate to *Xylella fastidiosa* subsp. *fastidiosa*, *multiplex* or *sandyi*

- The target sequences are a gene that encodes a putative methyltransferase of the restriction/methylation system for the XF1968 primers, a gene that encodes a putative fimbrial protein for the XF2542 primers (these were assigned to the CVC *X. fastidiosa* 9a5c strain) and a gene that encodes an intergenic region between the genes coding for a conserved hypothetical protein and a glycine cleavage H protein for the ALM primers (this target area was assigned to the genome of the ALS strain M12).
- Amplicon size:
- 638 bp with subsp. *sandyi*, *multiplex*,
- 521 bp with subsp. *multiplex*, and
- 412 bp with subsp. *fastidiosa*, *multiplex*.

Hernandez-Martinez *et al.*,2006; EPPO,2018

Conventional multiplex PCR

Mainly used for assignment of an isolate to *Xylella fastidiosa* subsp. *fastidiosa*, *multiplex* or *sandyi*

- **Primers:**
- Oligonucleotides for subsp. *multiplex*, *sandyi*:
  - forward primer XF1968-L 5′-GGAGGTTTACCGAAGACAGAT-3′;
  - reverse primer XF1968-R 5′-ATCCACAGTAAAACCACATGC-3′.
- Oligonucleotides for subsp. *multiplex*:
  - forward primer ALM1 5′-CTGCAGAAATTGGAAACTTCAG-3′;
  - reverse primer ALM2 5′-GCCACACGTGATCTATGAA-3′.
- Oligonucleotides for subsp. *fastidiosa*, *multiplex*:
  - forward primer XF2542-L 5′-TTGATCGAGCTGATGATCG-3′;
  - reverse primer XF2542-R 5′-CAGTACAGCCTGCTGGAGTTA-3′.

Hernandez-Martinez *et al.*,2006; EPPO,2018

Conventional multiplex PCR

Mainly used for assignment of an isolate to *Xylella fastidiosa* subsp. *fastidiosa*, *multiplex* or *sandyi*

- **DNA Extraction Method:**
- For pure cultures, a single colony of a fresh pure culture is suspended in 0.9 mL of PCR-grade water; lysis should be performed at 100°C for 5 min.
- Extracts of total nucleic acids can be stored at 4°C for immediate use or at −20°C for further use.

Hernandez-Martinez *et al.*,2006; EPPO,2018

Conventional multiplex PCR

Mainly used for assignment of an isolate to *Xylella fastidiosa* subsp. *fastidiosa*, *multiplex* or *sandyi*

- **Master Mix:**

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	10.25	N.A.
PCR buffer (Promega)	10x	2.5	1x
$MgCl_2$	50 mM	1.25	2.5 mM
dNTPs	20 mM	1	0.8 mM
Forward primers (XF1968-L, ALM1, XF2542-L) for each	20 µM	1.25 for each (total 3.75)	1 µM
Reverse primers (XF1968-R, ALM2, XF2542-R) for each	20 µM	1.25 for each (total 3.75)	1 µM
Promega Taq DNA polymerase	5 U μL^{-1}	0.5	0.1 U μL^{-1}
Subtotal		23	
Genomic DNA extract		2	
Total		25	

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

N.A.: not applicable, not available

EPPO,2018

Conventional multiplex PCR

Mainly used for assignment of an isolate to *Xylella fastidiosa* subsp. *fastidiosa*, *multiplex* or *sandyi*

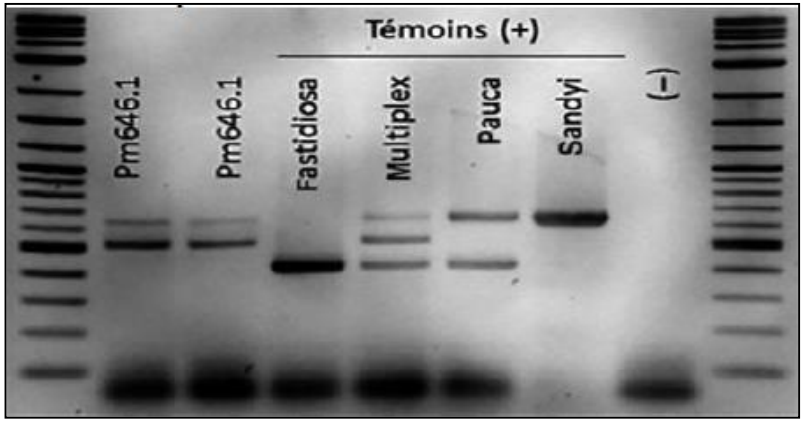
- PCR conditions:
- 95°C for 3 min, 40 cycles (95°C for 30 s, 55°C for 30 s and 72°C for 30 s) and a final step at 72°C for 5 min before cooling at 15°C.
- NIC (negative isolation control) and NAC (negative amplification control) should produce no amplicons.
- PIC (positive isolation control) and PAC (positive amplification control) should produce amplicons of the relevant size.

Hernandez-Martinez *et al.*,2006; EPPO,2018

Conventional multiplex PCR

Mainly used for assignment of an isolate to *Xylella fastidiosa* subsp. *fastidiosa*, *multiplex* or *sandyi*

- Electrophoretic analysis of PCR amplicons obtained from samples of *Polygala myrtifolia* (Pm646.1) by Hernandez-Martinez *et al.*,2006.
- It should be noted that although the test is not recommended for the detection of *X. fastidiosa* subsp. *pauca*, some sequence type of subsp. *pauca* can produce bands with this PCR.



Hernandez-Martinez *et al.*,2006; EPPO,2018

Genotype identification of bacteria
MLST vs MLSA

- Both methods depend on the sequencing of multiple (usually four to eight) housekeeping genes, i.e., genes conferring a basic metabolic function.
- **Multilocus Sequence Typing (MLST):**
- MLST is usually applied to strains that belong to a well-defined species. Here a sequence of multiple (usually 5-7) housekeeping genes(HKGs) is analysed.
- **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.

PAMBD.org;..

Genotype identification of bacteria
MLST vs MLSA

- **MLSA** relies on the comparison of partial DNA sequences of each gene or of concatenated sequences among strains, while
- **MLST** is based on the analysis of the combination of alleles at each locus, defining a sequence type (ST).
- **MLSA** provides a framework for species definition and allows the identification of species by electronic taxonomy, while
- **MLST** usually allows strains to be distinguished below the species level.

Jacques *et al.*,2012

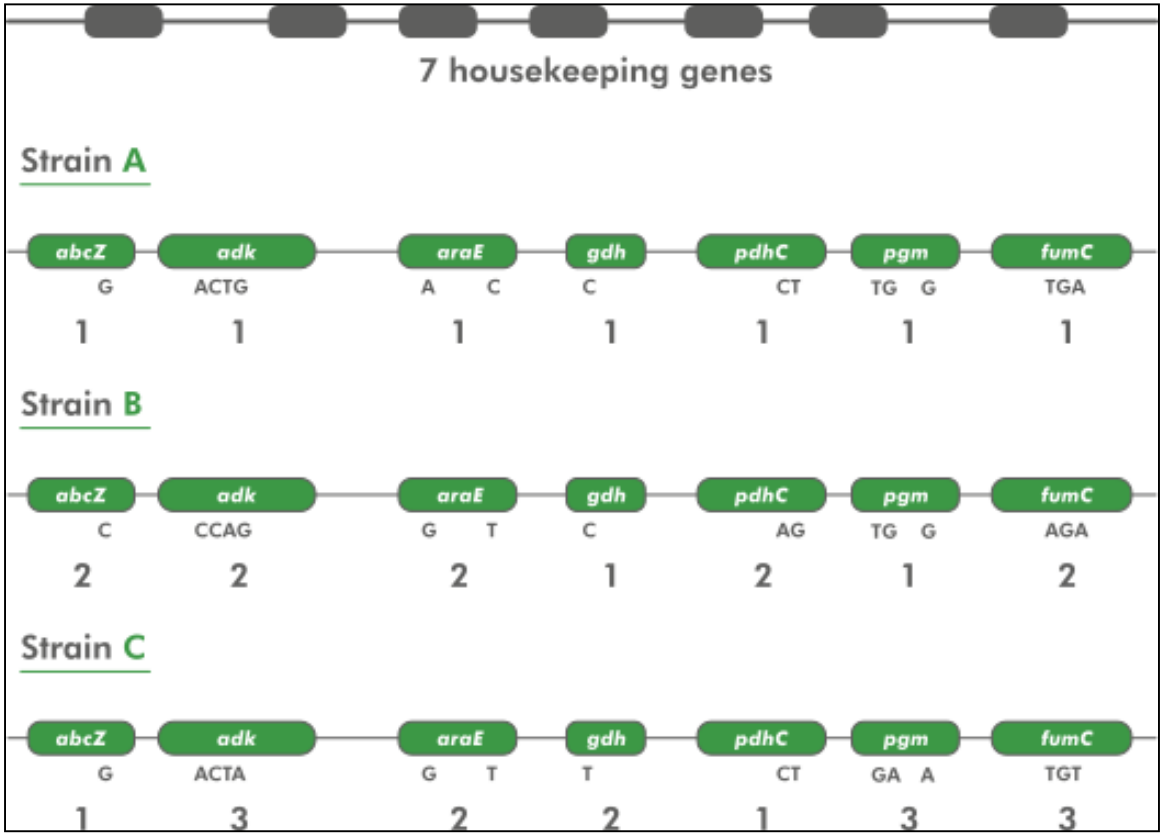
Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme

- Briefly, in MLST each allele of a given gene is assigned a number, so different strains of bacteria can be characterized by a series of numbers, representing one allele for each locus analyzed.
- The combination of the allele numbers at each locus determine the so called sequence type (ST) for each analyzed strain.
- For each house-keeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST).

Genotype identification of bacteria

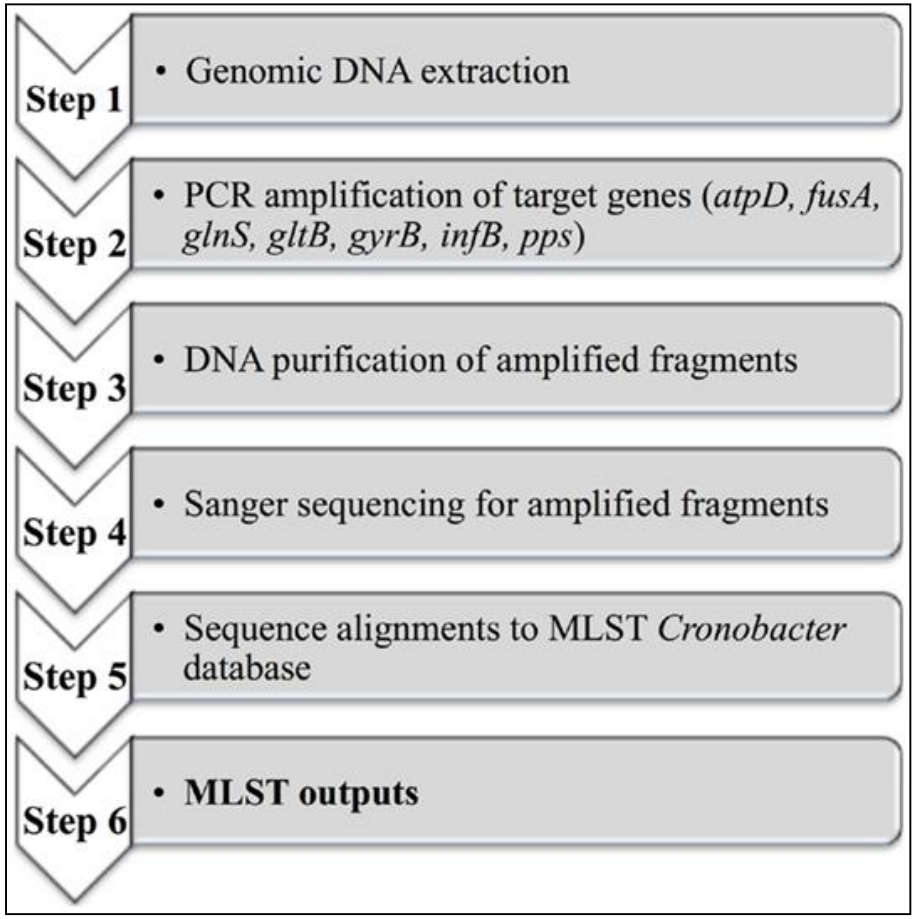
Multilocus Sequence Typing (MLST) Scheme



BIONUMERICS

Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme



Yand and Fanning,2015

Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme

Analysis of MLST sequences in BIONUMERICS

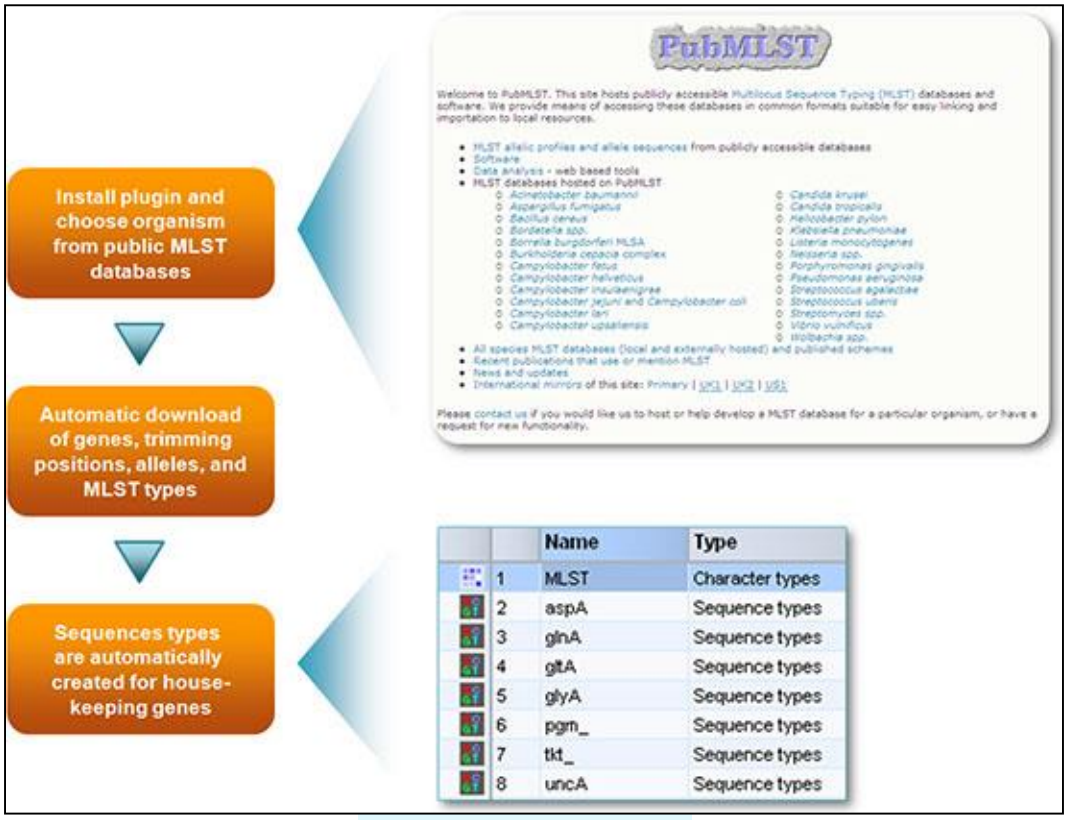
- Download BIONUMERICS software. BIONUMERICS software is widely used for the storage and analysis of MLST sequences.
- BIONUMERICS automatically analyses batches of sequence trace files, connects to online MLST databases, retrieves corresponding allele numbers, sequence types as well as available clonal complex information.
- BIONUMERICS can process hundreds of isolates in only seconds. Results are stored in the database and are available for statistical and population analysis, clustering, partitioning, identification using BIONUMERICS' impressive set of analysis tools.

Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme

Analysis of MLST sequences in BIONUMERICS

- Setup scheme:



BIONUMERICS

Identification of bacteria

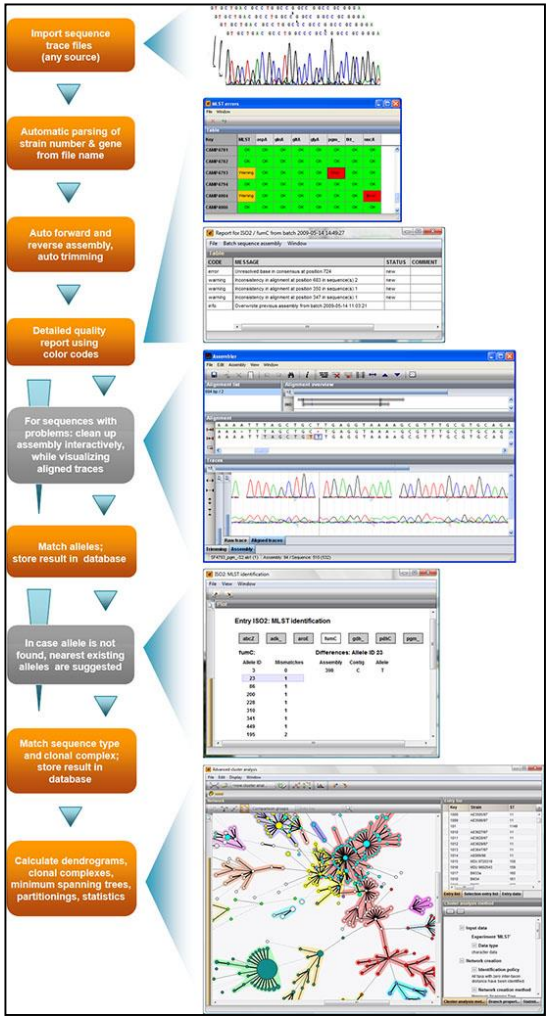
Multilocus Sequence Typing (MLST) Scheme

Analysis of MLST sequences in BIONUMERICS

Workflow scheme:

Fully automatic processing workflow

- Automatic import and assembly of batches of sequencer trace files from various sources (AB, Beckman, Amersham, FASTA); file names are parsed into strain and gene information using a parsing definition.
- Consensus sequences are automatically trimmed using start and stop signatures and placed in the right direction.
- When the batch assembly is finished, an overview report is shown, listing status of each strain/gene combination.
- Double-click on a problem contig to display the detailed information window.
- Double-click on a particular problem to open the Assembler with the problem position selected.
- For each problem position, show nearest existing alleles and suggested bases - easy verification with chromatograms.
- Alleles and MLST types can be identified by real-time connection to MLST server database, or by comparing to locally stored allele database (faster). In the latter case, local database can be updated automatically at startup.
- Allele and MLST type information for own strains is stored in the database and can be updated at any time for a selection of strains. BIONUMERICS will prompt you for any change in allele/MLST type definition that has occurred in the MLST server database.
- Calculate population modelling networks in the finest and most comprehensive cluster analysis application available today, using standard or custom priority rules and with branch significance support indication.
- Calculate and display partitioning for clonal complexes and use BIONUMERICS' rich set of statistics tools.



BIONUMERICS

1. Multilocus sequence typing (MLST)
Conventional/traditional MLST scheme using housekeeping genes
Drawbacks

- This MLST scheme relies on sequence analysis of seven housekeeping genes which are required for basic cellular maintenance.
- Housekeeping genes are usually relatively conserved, making them good candidates for use in evolutionary phylogenetic analyses of distantly related groups.
- The seven concatenated HKGs (series of housekeeping genes) which were used to delineate *fastidiosa* isolates in differnet species are:
  leuA; petC; malF; cysG; holC; nuoL; gltT*X* (PubMLST).
- One drawback to using housekeeping genes is that they may not provide enough variability to differentiate closely related isolates.

Parker *et al.*,2012; PubMLST: MLST allelic profiles and sequences

2. MLSA-E, an alternative method for discriminating between closely related *X. fastidiosa* Using environmentally mediated genes

- An alternative for discriminating between closely related *X. fastidiosa* isolates is to perform MLSA of genes influenced by environmental factors, termed environmentally mediated genes.
- Environmentally mediated genes are involved in adaptation to environmental changes and are usually subject to positive selection pressure, so they should have greater sequence variability than conserved housekeeping genes under stabilizing selection pressure.

Parker *et al.*,2012

1. Multilocus sequence typing (MLST) Methods

Using 7 housekeeping genes

- The MLSA-MLST method is used for the identification of Xylella fastidiosa's sub-species.
- This method involves the analysis of partial sequences of 7 housekeeping genes (cysG, gltT, holC, leuA, malF, nuoL and petC) from the bacterium's genome.
- This multi-loci analysis is a reference method in taxonomy research and allows determining the phylogenetic position of strains and naming them.
- These housekeeping genes are highly maintained within the *X. fastidiosa* species but different strains have allele variants.
- In this method, an allele number is allocated to each housekeeping gene and these 7 numbers define some kind of an identity document called the Sequence Type (ST).

Multilocus sequence typing (MLST)
Methods
Using 7 housekeeping genes

Gene	Gene function	Biochemical function	Temecula (PD0001) gene position	M12 (ALS0299) gene position	9a5c (CVC0018) gene postion
leuA	2-isopropylmalate synthase	amino acid biosynthesis	PD1047	Xfasm_1205	XF1818
petC	ubiquinol cytochrome C oxidoreductase, cystochrome C1 subunit	electron transport	PD1775	Xfasm_1943	XF0910
malF	ABC transporter sugar permease	transport of carbohydrates	PD1465	Xfasm12_1606	XF2447
cysG	siroheme synthase	biosynthesis of heme, porphyrin	PD1840	Xfasm12_2018	XF0832
holC	DNA polymerase III holoenzyme, chi subunit	replication	PD0104	Xfasm12_0112	XF0136
nuoL	NADH-ubiquinone oxidoreductase, NQO12 subunit	aerobic respiration	PD0259	Xfasm12_0280	XF0316
gltT	glutamate symport protein	transport of amino acids	PD1516	Xfasm12_1656	XF0656

Multilocus sequence typing (MLST) is a technique in molecular biology for the typing of multiple loci. Approximately 450-500 bp internal fragments of each gene are used. 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).

Janse *et al.*,2012; Wikipedia,2019

Multilocus sequence typing (MLST)
Methods
Using 7 housekeeping genes

- The sequences for the primers are as follows:
- forward primer leuA-for 5′-GGTGCACGCCAAATCGAATG-3′; reverse primer leuA-rev 5′-GTATCGTTGTGGCGTACACTG-3′;
- forward primer petC-for 5′-GCTGCCATTCGTTGAAGTACCT-3′; reverse primer petC-rev 5′-GCACGTCCTCCCAATAAGCCT-3′;
- forward primer malF-for 5′-TTGCTGGTCCTGCGGTGTTG-3′; reverse primer malF-rev 5′-GACAGCAGAAGCACGTCCCAGAT-3′;
- forward primer cysG-for 5′-GCCGAAGCAGTGCTGGAAG-3′; reverse primer cysG-rev 5′-GCCATTTTCGATCAGTGCAAAAG-3′;
- forward primer holC-for 5′-ATGGCACGCGCCGACTTCT-3′; reverse primer holC-rev 5′-ATGTCGTGTTTGTTCATGTGCAGG-3′;
- forward primer nuoL-for 5′-TAGCGACTTACGGTTACTGGGC-3′; reverse primer nuoL-rev 5′-ACCACCGATCCACAACGCAT-3′;
- forward primer gltT-for 5′-TCATGATCCAAATCACTCGCTT-3′; reverse primer gltT-rev 5′-ACTGGACGCTGCCTCGTAAACC-3′.
- The workflow is described in the PubMLST *Xylella fastidiosa* database (http://pubmlst.org/xfastidiosa).

EPPO,2016

Multilocus sequence typing (MLST)

Some more housekeeping genes and specific primers used for multilocus sequence typing (MLST) in *X. fastidiosa*

Gene	Position	Forward primer	Reverse primer	Gene function
holC[a]	XF0136	GATTTCCAAACCGCGCTTTC	TCATGTGCAGGCCGCGTCTCT	DNA polymerase III holoenzyme, chi subunit
rfbD[a]	XF0257	TTTGGTGATTGAGCCGAGGGT	CCATAAACGGCCGCTTTC	dTDP-4-dehydrorhamnose-3, 5-epimerase
nuoL[a]	XF0316	CATTATTGCCGGATTGTTAGG	GCGGGAAACATTACCAAGC	NADH-ubiquinone oxidoreductase, NQO12 subunit
nuoN[a]	XF0318	GGGTTAAACATTGCCGATCT	CGGGTTCCAAAGGATTCCTAA	NADH-ubiquinone oxidoreductase, NQO14 subunit
gltT[a]	XF0656	TTGGGTGTGGGTACGTTGCTG	CGCTGCCTCGTAAACCGTTGT	Glutamate symport protein
cysG[a]	XF0832	GGCGGCGGTAAGGTTG	GCGTATGTCTGTGCGGTGTGC	cysG Siroheme synthase
petC[a]	XF0910	CTGCCATTCGTTGAAGTACCT	CGTCCTCCCAATAAGCCT	Ubiquinol cytochrome c oxidoreductase
pilU[a]	XF1632	CAATGAAGATTCACGGCAATA	ATAGTTAATGGCTCCGCTATG	Twitching motility protein
leuA[a]	XF18188	GGGCGTAGACATTATCGAGAC	GTATCGTTGTGGCGTACACTG	2-Isopropylmalate synthase
lacF[a]	XF2447	TTGCTGGTCCTGCGGTGTTG	CCTCGGGTCATCACATAAGGC	ABC transporter sugar permease
rfbD[b]	XF0257	TTTGGTGATTGAGCCGAGGGT[a]	TCCATAAACGGCGCCTTC	dTDP-4-dehydrorhamnose-3, 5-epimerase
cysG[b]	XF0832	GGCGGCGGTAAGGTTG[a]	GCATATGTCTGTGCGGTGTGC	cysG Siroheme synthase
holC[c]	XF0136	ATGGCACGCGCCGACTTCT	ATGTCGTGTTTGTTCATGTGCAGG	DNA polymerase III holoenzyme, chi subunit
nuoL[c]	XF0316	TAGCGACTTACGGTTACTGGGC	ACCACCGATCCACAACGCAT	NADH-ubiquinone oxidoreductase, NQO12 subunit
gltT[c]	XF0656	TCATGATCCAAATCACTCGCTT	ACTGGACGCTGCCTCGTAAACC	Glutamate symport protein
cysG[c]	XF0832	GCCGAAGCAGTGCTGGAAG	GCCATTTTCGATCAGTGCAAAAG	cysG Siroheme synthase
petC[c]	XF0910	GCTGCCATTCGTTGAAGTACCT	GCACGTCCTCCCAATAAGCCT	Ubiquinol cytochrome c oxidoreductase
leuA[c]	XF18188	GGTGCACGCCAAATCGAATG	GTATCGTTGTGGCGTACACTG	2-Isopropylmalate synthase
malF[c]	XF2447	TTGCTGGTCCTGCGGTGTTG	GACAGCAGAAGCACGTCCCAGAT	ABC transporter sugar permease
pilU[c]	XF1632	CCGTAATCACAACTCAACAGGACA	CTGCGAATCAGCATGGCGTA	Twitching motility protein
acvB[d]	PD_1902	ACAGTATCGCCGTCGAAGTGATGA	CATGCATACRGCGATGYTTCCGAT	Virulence protein: suggested to regulate pathogenicity
copB[d]	PD_0101	ATGAACACCCGTACCTGGTTCGTA	ATTTAGTCTCCACCATGAGCCGCA	Copper resistance protein B precursor
cvaC[d]	PD_0215	TGCGTGAATTRACATTGACCG	CCTAGTCTGCGGCTTAAGCAGATT	Colicin V precursor
fimA[d]	PD_0062	CCCAGTGCGTCGTTATCGATTATTGGT	TTTYGYACTCTCAAGCATCGCATC	Fimbrial subunit precursor
gaa[d]	PD_0315	TGAGAGCTGCYGATGTTCCAATGA	ACAGCTTCTGGCAAGAACAAGCAC	Glutaryl-7-aminocephalosporanic acid acylase precursor
pglAa[d]	PD_1485	TAGTGCTGGCCTAACGATGTYGGT	CCGTATCAGCAACCACATGGAAGT	Polygalacturonase precursor
pilA[d]	PD_1924	ATCGCKCTGCCYATGTACCAAA	CAGCATTGATCGTRTTGCTGTRTG	Fimbrial protein
rpfF[d]	PD_0407	GCGCTCCATAGTTCGGAGTGATTT	ATGTCCGCTGTACATCCCATTCCT	Regulator of pathogenicity factors
xadAa[d]	PD_0731	TGGGAGGTCAAAGYACTGCCATCA	GCATTGGCAGCAACACTCGAATCA	Outer membrane afimbrial adhesin
gltT[e]	PD1516	TTTTTCAGGGGTGTCGCGC	TTCCAACGTTACTGGACGCT	Glutamate symport protein
cysG[e]	PD1840	CCAAACATAGAAGCACGCCG	CGTATGTCTGTGCGGTGTG	Siroheme synthase
leuA[e]	PD1047	GGCCAGTGCTGTGTTTTGTT	GGGCTACTTGCTGGAGGAAG	2-Isopropylmalate synthase
lacF[e]	PD1465	TTCTTTGGTGGGTTGGGTGT	CACACAGCATCAACGTCGTC	ABC transporter sugar permease

[a] Schuenzel et al. (2005).
[b] Almeida et al. (2008).
[c] Yuan et al. (2010).
[d] Parker et al. (2012).
[e] Harris and Balci (2015).

Baldi and La Porta,2017

Multilocus sequence typing (MLST)
Primers

forward primers	reverse primer
leuA-for 5′-GGTGCACGCCAAATCGAATG-3′	leuA-rev 5′-GTATCGTTGTGGCGTACACTG-3′;
petC-for 5′-GCTGCCATTCGTTGAAGTACCT-3′	petC-rev 5′-GCACGTCCTCCCAATAAGCCT-3′
malF-for 5′-TTGCTGGTCCTGCGGTGTTG-3′	malF-rev 5′-GACAGCAGAAGCACGTCCCAGAT-3′
cysG-for 5′-GCCGAAGCAGTGCTGGAAG-3′	cysG-rev 5′-GCCATTTTCGATCAGTGCAAAAG-3′
holC-for 5′-ATGGCACGCGCCGACTTCT-3′	holC-rev 5′-ATGTCGTGTTTGTTCATGTGCAGG-3′
nuoL-for 5′-TAGCGACTTACGGTTACTGGGC-3′	nuoL-rev 5′-ACCACCGATCCACAACGCAT-3′
gltT-for 5′-TCATGATCCAAATCACTCGCTT-3′	gltT-rev 5′-ACTGGACGCTGCCTCGTAAACC-3′

The workflow is described in the PubMLST *Xylella fastidiosa* database (http://pubmlst.org/xfastidiosa).

Multilocus sequence typing (MLST)

Methods

DNA extraction method

- Nucleic acid source: pure culture plant extract or insects.
- For pure cultures, a single colony of a fresh pure culture is suspended in 0.9 mL of PCR-grade water; lysis should be performed at 100°C for 5 min.
- Extracts of total nucleic acids can be stored at 4°C for immediate use or at −20°C for further use.

EPPO,2018

Multilocus sequence typing (MLST)

Methods

Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	36.2	N.A.
PCR buffer (Invitrogen)	10x	5	1x
$MgCl_2$	50 mM	1.5	1.5 mM
dNTPs	20 mM	0.5	0.2 mM
Forward primers (leuA-for, petC-for, malF-for, cysG-for, holC-for, nuoL-for, gltT-for)	20 µM	0.75	0.3 µM
Reverse primers (leuA-rev, petC-rev, malF-rev, cysG-rev, holC-rev, nuoL-rev, gltT-rev)	20 µM	0.75	0.3 µM
DNA Polymerase Platinum (Invitrogen)	5 U μL^{-1}	0.3	0.03 U μL^{-1}
Subtotal		45	
Genomic DNA		5	
Total		50	

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

N.A.: not applicable, not available

EPPO,2016

Multilocus sequence typing (MLST)
Methods
Master mix

- Each reaction mixture contained 5 to 15 ng/µl of DNA template, 1x buffer solution (Promega), 1 mM deoxynucleotide triphosphates, 1 µM of each primer, and 5U of *Taq* polymerase (Promega) for a total 30-µl reaction volume.
- The thermocycler (Eppendorf Mastercycler) reaction conditions were an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds for primer annealing, and an extension period at 72°C for 1 min.
- The final step was an extension period at 72°C for 5 min.
- For genes over 1,000 bp, the extension time was lengthened from 1 min to 90 seconds.

Schuenzel *et al.*,2005

Multilocus sequence typing (MLST)
Methods
PCR conditions (for pure cultures)

- 95°C for 3 min, 35 cycles of (95°C for 30 s, 65°C for 30 s and 72°C for 60 s) and a final step of 72°C for 10 min If the amplicons are of good quality and at the expected size, a template should be sent for sequencing with reverse and forward primers.
- The results of sequencing should be compared with sequences available on http://pubmlst.org/xfastidiosa/ (Scally *et al.*,2005).
- NIC (negative isolation control) and NAC (negative amplification control) should produce no amplicons.
- PIC (positive isolation control) and PAC (positive amplification control) should produce amplicons of the relevant size.

EPPO,2018

Multilocus sequence typing (MLST)

Methods

Sequencing

- 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 depending on the degree of discrimination desired.

Wikipedia,2020

Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme

- Briefly, in MLST each allele of a given gene is assigned a number, so different strains of bacteria can be characterized by a series of numbers, representing one allele for each locus analyzed.
- The combination of the allele numbers at each locus determine the so called sequence type (ST) for each analyzed strain.
- For each house-keeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST).

Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme

- Most bacterial species have sufficient variation within house-keeping genes to provide many alleles per locus, allowing billions of distinct allelic profiles to be distinguished using seven house-keeping loci.
- For example, an average of 30 alleles per locus allows about 20 billion genotypes to be resolved.
- MLST is based on the well established principles of multilocus enzyme electrophoresis, but differs in that it assigns alleles at multiple house-keeping loci directly by DNA sequencing, rather than indirectly via the electrophoretic mobility of their gene products.

PubMLST

Multilocus sequence typing (MLST)

Interpretation of sequencing results: A table of correspondence between Sequence Types (ST) and sub-species is presented.

ST	*leuA*	*petC*	*malF*	*cysG*	*holC*	*nuoL*	*gltT*	clonal complex	reference
1	1	1	1	1	1	1	1	fastidiosa	Yuan et al. (2010)
2	1	1	4	1	1	1	1	fastidiosa	Yuan et al. (2010)
3	1	1	1	20	1	1	1	fastidiosa	Yuan et al. (2010)
4	1	1	1	4	1	1	1	fastidiosa	Yuan et al. (2010)
5	2	2	2	2	2	2	2	sandyi	Yuan et al. (2010)
6	3	3	3	3	3	3	3	multiplex	Yuan et al. (2010)
7	3	3	3	7	3	3	3	multiplex	Yuan et al. (2010)
8	3	3	5	5	4	3	7	multiplex	Nunney et al. (2013)
9	3	3	5	5	4	3	4	multiplex	Yuan et al. (2010)
10	5	4	3	3	6	3	5	multiplex	Yuan et al. (2010)
11	7	7	7	9	10	8	8	pauca	Nunney et al. (2012)
12	7	7	7	9	13	8	8	pauca	Nunney et al. (2012)
13	7	6	7	9	10	7	8	pauca	Yuan et al. (2010)
14	8	8	8	11	12	9	9	pauca	Yuan et al. (2010)
15	5	3	3	3	4	3	5	multiplex	Nunney et al. (2013)
16	7	6	8	10	11	8	8	pauca	Nunney et al. (2012)
17	1	1	10	12	18	10	1	fastidiosa	Nunney et al. (2014b)
18	9	1	9	13	14	5	10	fastidiosa	Nunney et al. (2012)
19	10	1	10	14	15	11	1	fastidiosa	Nunney et al. (2012)
20	1	1	10	12	17	11	11	fastidiosa	Nunney et al. (2012)
21	10	1	10	14	15	11	12	fastidiosa	Nunney et al. (2014a)
22	3	3	5	12	4	3	3	multiplex	Nunney et al. (2012)
23	3	3	5	3	6	3	3	multiplex	Nunney et al. (2013)
24	3	3	5	3	4	3	7	multiplex	Nunney et al. (2013)
25	3	3	3	17	3	3	3	multiplex	Nunney et al. (2013)
26	5	3	3	3	6	3	5	multiplex	Nunney et al. (2013)
27	6	3	5	6	7	3	7	multiplex	Nunney et al. (2013)
28	6	3	5	18	7	4	7	multiplex	Nunney et al. (2013)
29	4	3	6	18	5	4	3	morus	Nunney et al. (2014b)
30	4	5	6	8	5	4	3	morus	Nunney et al. (2014b)
31	4	3	6	18	8	6	3	morus	Nunney et al. (2014b)
32	4	3	5	12	4	4	3	multiplex	Nunney et al. (2013)
33	11	9	14	15	19	13	10	fastidiosa/sandyi	Nunney et al. (2012)/Jacques et al. (2016)
34	3	3	3	3	3	3	6	multiplex	Nunney et al. (2013)
35	3	10	3	3	3	3	3	multiplex	Nunney et al. (2013)
36	5	3	5	19	6	3	5	multiplex	Nunney et al. (2013)

EPPO,2018

Multilocus sequence typing (MLST)

Interpretation of sequencing results: A table of correspondence between Sequence Types (ST) and sub-species is presented.

ST	*leuA*	*petC*	*malF*	*cysG*	*holC*	*nuoL*	*gltT*	clonal complex	reference
37	3	3	5	21	4	3	3	multiplex	Nunney et al. (2013).
38	3	3	5	16	4	3	7	multiplex	Nunney et al. (2013)
39	3	3	5	19	4	3	7	multiplex	Nunney et al. (2013)
40	6	3	5	18	7	3	7	multiplex	Nunney et al. (2013)
41	3	3	5	18	9	3	3	multiplex	Nunney et al. (2013)
42	6	3	5	12	4	3	3	multiplex	Nunney et al. (2013)
43	3	3	5	18	4	3	7	multiplex	Nunney et al. (2013)
44	3	3	5	5	6	3	4	multiplex	Nunney et al. (2013)
45	3	3	5	3	4	3	3	multiplex	Nunney et al. (2013)
46	5	3	3	3	6	3	3	multiplex	Nunney et al. (2013)
47	13	1	10	23	20	5	1	fastidiosa	Nunney et al. (2012)
48	3	3	12	3	6	3	3	multiplex	Nunney et al. (2013)
49	3	3	5	3	6	3	7	multiplex	Nunney et al. (2013)
50	3	11	13	22	21	14	13	multiplex	Nunney et al. (2013)
51	3	3	5	3	4	15	3	multiplex	Nunney et al. (2013)
52	10	1	10	14	18	10	1	fastidiosa	Nunney et al. (2012)
53	7	6	16	24	10	16	14	pauca	Nunney et al. (2014a)
54	11	9	11	25	19	12	1	fastidiosa/sandyi	Nunney et al. (2012)/Jacques et al. (2016)
55	1	1	10	12	18	10	10	fastidiosa	Nunney et al. (2012)
56	11	9	11	15	17	12	10	fastidiosa/sandyi	Nunney et al. (2012)/Jacques et al. (2016)
57	1	1	10	12	18	11	11	fastidiosa	Nunney et al. (2012)
58	6	3	5	12	4	3	7	multiplex	Nunney et al. (2013)
59	9	1	9	13	14	5	1	fastidiosa	Coletta-Filho et al. (2017)
60	9	1	1	13	14	5	1	fastidiosa	Coletta-Filho et al. (2017)
61	11	9	11	15	16	12	10	fastidiosa/sandyi	Nunney et al. (2014a)/Jacques et al. (2016)
62	4	3	6	18	5	6	3	morus	Nunney et al. (2014a)
63	5	6	3	3	6	3	5	multiplex	Coletta-Filho et al. (2017)
64	7	7	7	9	10	7	8	pauca	Coletta-Filho et al. (2017)
65	7	6	7	9	10	8	8	pauca	Coletta-Filho et al. (2017)
66	7	8	8	10	11	8	8	pauca	Coletta-Filho et al. (2017)
67	5	3	8	3	12	3	5	multiplex	Coletta-Filho et al. (2017)
68	14	8	8	11	12	9	8	pauca	Coletta-Filho et al. (2017)
69	7	6	7	9	23	17	8	pauca	Coletta-Filho et al. (2017)
70	14	7	8	11	22	9	8	pauca	Coletta-Filho et al. (2017)
71	5	8	8	11	12	9	9	pauca	Coletta-Filho et al. (2017)
72	12	12	15	26	24	18	1	sandyi	Denancé et al. (2017) /Loconsole et al. (2016)

EPPO,2018

Multilocus sequence typing (MLST)

Interpretation of sequencing results: A table of correspondence between Sequence Types (ST) and sub-species is presented.

ST	*leuA*	*petC*	*malF*	*cysG*	*holC*	*nuoL*	*gltT*	clonal complex	reference
73	7	6	8	27	10	16	8	pauca	Loconsole et al. (2016)/Coletta-Filho et al. (2017)
74	7	6	8	28	25	16	8	pauca	Jacques et al. (2016)
75	9	1	10	29	1	19	1	fastidiosa	Jacques et al. (2016)
76	12	13	15	26	24	18	1	sandyi	Loconsole et al. (2016)/Denancé et al. (2017)
77	1	1	6	30	26	5	1	fastidiosa	Bergsma-Vlami et al. (2017)
78	7	6	7	9	23	8	8	Pauca	Tolocka et al. (2017)
79	3	3	3	26	3	3	3	multiplex	Denancé et al. (2017)
80	7	6	17	31	10	16	15	pauca	B. Landa pers. comm.
81	3	3	3	32	3	3	3	multiplex	B. Landa pers. comm.

EPPO,2018

Multilocus sequence typing (MLST)

Methods

Using housekeeping and non-housekeeping genes

PCR conditions:

95°C for 3 min, 35 cycles of (95°C for 30 s, 65°C for 30 s and 72°C for 60 s) and a final step of 72°C for 10 min If the amplicons are of good quality and at the expected size, a template should be sent for sequencing with reverse and forward primers.

The results of sequencing should be compared with sequences available on http://pubmlst.org/xfastidiosa/ (Scally *et al.*,2005).

Gene	Function	Biochemical function	Primer sequences (forward/reverse)	Gene length/ MLST fragment used* (bp)
holC	DNA polymerase III holoenzyme, chi subunit	Replication	5′-GATTTCCAAACCGCGCTTTC-3′ 5′-TCATGTGCAGGCCGCGTCTCT-3′	379 / 379
nuoL	NADH-ubiquinone oxidoreductase, NQO12 subunit	Aerobic respiration	5′-CATTATTGCCGGATTGTTAGG-3′ 5′-GCGGGAAACATTACCAAGC-3′	1,821 / 557
gltT	Glutamate symport protein	Transport of amino acids	5′-TTGGGTGTGGGTACGTTGCTG-3′ 5′-CGCTGCCTCGTAAACCGTTGT-3′	951 / 654
cysG	Siroheme synthase	Biosynthesis of heme, porphyrin	5′-GGCGGCGGTAAGGTTG-3′ 5′-GCGTATGTCTGTGCGGTGTGC-3′	1,170 / 600
petC	Ubiquinol cytochrome *c* oxidoreductase, cytochrome *c*1 subunit	Electron transport	5′-CTGCCATTCGTTGAAGTACCT-3′ 5′-CGTCCTCCCAATAAGCCT-3′	533 / 533
leuA	2-Isopropylmalate synthase	Amino acid biosynthesis	5′-GGGCGTAGACATTATCGAGAC-3′ 5′-GTATCGTTGTGGCGTACACTG-3′	1,218 / 708
lacF	ABC transporter sugar permease	Transport of carbohydrates	5′-TTGCTGGTCCTGCGGTGTTG-3′ 5′-CCTCGGGTCATCACATAAGGC-3′	730 / 730
RNA polymerase	RNA polymerase sigma-70 factor	Replication	5′GCGTTAATTTTCGAAGTGATTCGATTGC-3′ 5′-CACCATTCGTATCCCGGTG-3	733
HL	HL protein	Hypothetical protein	5′-AAGGCAATAAACGCGCACTA-3′ 5′-GGTTTTGCTGACTGGCAACA-3′	221
16S rDNA	16S ribosomial DNA	Replication	5′-CAGCACATTGGTAGTAATAC-3′ 5′-ACTAGGTATTAACCAATTGC-3′	404
pilJ	Twitching motility protein	Surface structures	5′-CAATGAAGATTCACGGCAATA-3′ 5′-ATAGTTAATGGCTCCGCTATG-3′	915
rfbD	dTDP-4-dehydrorhamnose-3, 5-epimerase	Surface polysaccharides	5′-TTTGGTGATTGAGCCGAGGGT-3′ 5′-CCATAAACGGCCGCTTTC-3′	429
nuoN	NADH-ubiquinone oxidoreductase, NQO14 subunit	Aerobic respiration	5′-GGGTTAAACATTGCCGATCT-3′ 5′-CGGGTTCCAAAGGATTCCTAA-3′	1,398

*According to Scally *et al.*, 2005.

Primer pairs used in the MLST scheme for typing of *Xylella fastidiosa* subspecies (Source: http://pubmlst.org/xfastidiosa/)

Elbeaino *et al.*,2014

2. Multilocus sequence Analysis (MLSA)
Using Environmentally Mediated Genes (MLSA-E)

- An alternative for discriminating between closely related *X. fastidiosa* isolates is to perform MLSA of genes influenced by environmental factors, termed environmentally mediated genes.
- Here multilocus sequence analysis of environmentally mediated genes (MLSA-E) was applied to identify *X. fastidiosa* isolate relationships.
- MLSA-E was used for genes related to processes important for establishing *X. fastidiosa* infections and colonization of the insect vector, such as surface attachment, biofilm formation, virulence, and nutrient transport and utilization.

Parker *et al.*,2012

Multilocus sequence Analysis (MLSA) Methods

Using Environmentally Mediated Genes (MLSA-E)

Genes selected for final *X. fastidiosa* MLSA-E analysis:

1. *acvB* (virulence);
2. *copB* (copper resistance);
3. *cvaC*[a] (toxin);
4. *fimA* (attachment);
5. *gaa*;
6. *pglA*[a] (cell wall degradation);
7. *pilA* (motility);
8. *rpfF* (pathogenicity);
9. xadA[a] (attachment);
10. etc.

Gene	Locus tag (Temecula)	Gene size (bp)	Category	Description	Forward primer (5′→3′)	Reverse primer (5′→3′)	Amplicon size (bp)	Reference(s)
acvB	PD_1902	900	Virulence	Virulence protein: suggested to regulate pathogenicity and disease symptoms	ACAGTA TCGCCG TC GAAG TGATGA	CATGCA TACRGC GATGYT TCCGAT	743	34
copB	PD_0101	930	Resistance	Copper resistance protein B precursor: encodes a copper resistance cation-translocating ATPase induced by copper exposure	ATGAAC ACCCGT AC CTGG TTCGTA	ATTTAG TCTCCA CCATGA GCCGCA	607–862	68, 82
cvaC[a]	PD_0215	309	Toxin	Colicin V precursor: encodes a bacteriocin precursor proposed to be a defense mechanism	TGCGTG AATTRA CA TTGA CCG	CCTAGT CTGCGG CTTAAG CAGATT	330	20, 62
fimA	PD_0062	555	Attachment	Fimbrial subunit precursor: component of type I pili important for biofilm formation and aggregation	CCCAGT GCGTCG TTATCG ATTATT GGT	TTTYGY ACTCTC AA GCAT CGCATC	557	18–20, 24, 25, 46
gaa	PD_0315	1,992	Toxin	Glutaryl-7-aminocephalosporanic acid acylase precursor: encodes a member of the newly described family of β-lactam antibiotic acylases	TGAGAG CTGCYG ATGTTC CAATGA	ACAGCT TCTGGC AAGAAC AAGCAC	1129	4, 5
pglA[a]	PD_1485	1,635	Cell wall degradation	Polygalacturonase precursor: needed for degrading host plant cell walls to allow colonization	TAGTGC TGGCCT AA CGAT GTYGGT	CCGTAT CAGCAA CCACAT GGAAGT	828/829	63, 71
pilA	PD_1924	447	Motility	Fimbrial protein: major structural protein of type IV pili used for twitching motility and upstream migration	ATCGCK CTGCCY AT GTAC CAAA	CAGCAT TGATCG TRTTGC TGTRTG	405	57
rpfF	PD_0407	873	Pathogenicity	Regulator of pathogenicity factors: involved in biosynthesis of diffusible signal factor (DSF) to mediate cell-to-cell signaling for biofilm formation	GCGCTC CATAGT TC GGAG TGATTT	ATGTCC GCTGTA CATCCC ATTCCT	825	8, 9, 60
xadA[a]	PD_0731	2,994	Attachment	Outer membrane afimbrial adhesin: may contribute to initial cell binding to surfaces	TGGGAG GTCAAA GYACTG CCATCA	GCATTG GCAGCA ACACTC GAATCA	1087/1108	25

[a] Genes selected on the basis of *dN/dS*.

dN/dS ratio: Nucleotide substitutions in genes coding for proteins can be either synonymous (do not change amino acid), alternatively called silent substitutions, or non-synonymous (changes amino acid). dN/dS measured across the whole protein sequence between two divergent species in theory tells you something about selection. If this ratio = 1, then the whole coding sequence evolves neutrally, when 0 < dN/dS < 1, it's under constraint, and when > 1 under positive selection.

Parker *et al.*,2012

Multilocus sequence Analysis (MLSA) Methods

Using Environmentally Mediated Genes (MLSA-E)

Temecula/9a5c *dN/dS* for housekeeping genes, nonhousekeeping genes, and the final MLSA-E gene regions used in this study.

Xylella fastidiosa strain Temecula was isolated from a naturally infected grapevine with Pierce's disease in a wine-grape-growing region of California.

Parker *et al.*,2012

Housekeeping genes		Nonhousekeeping genes		Final MLSA-E gene regions	
Gene	*dN/dS*	Gene	*dN/dS*	Gene	*dN/dS*
cysG	0.19	*acvB*	0.29	*acvB*	0.26
gltT	0.09	*copB*	0.28	*copB*	0.26
gyrB[b]	0.06	*cvaC*	0.34	*cvaC*	0.35
holC	0.11	*exbD1*	0.11		
leuA	0.07	*fimA*	0.40	*fimA*	0.37
malF	0.10	*gaa*	0.23	*gaa*	0.22
nuoL	0.10	*pglA*	0.21	*pglA*	0.21
petC	0.28	*pilA*	0.24	*pilA*	0.30
pyrE[c]	0.08	*pilJ*	0.10		
		rpfF	0.19	*rpfF*	0.19
		xadA	0.30	*xadA*	0.36
Mean	0.12		0.24		0.28
Median	0.10		0.24		0.26

[a]Unless noted, housekeeping genes are from the MLST study. Nonhousekeeping genes are from this study. The dN/dS (synonymous substitution) ratios of the nonhousekeeping genes were significantly higher than the dN/dS ratios of the housekeeping genes (Wilcoxon ranksum test, two-tailed P= 0.0013).
[b]*gyrB* used in other *X. fastidiosa* phylogenetic analyses. [C]*pyrE* used in initial MLSA-E analyses in this study.

Multilocus sequence Analysis (MLSA) Methods
Using Environmentally Mediated Genes (MLSA-E)

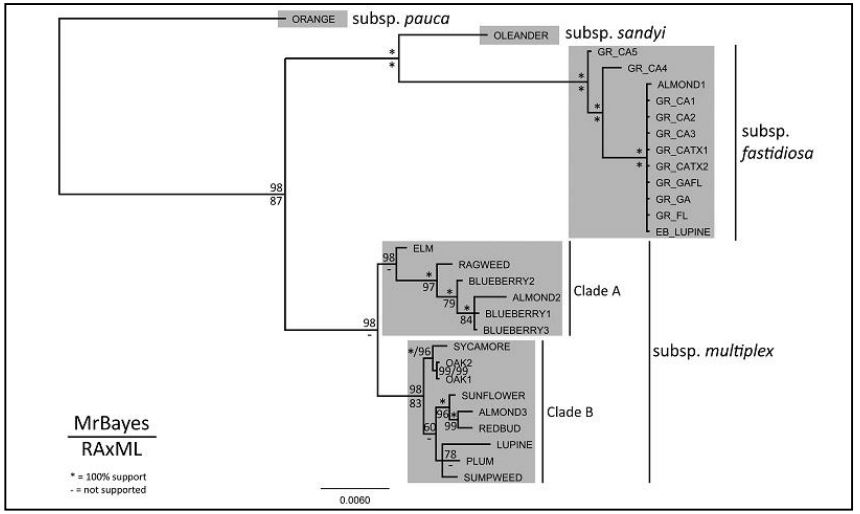
- **Master mix and PCR conditions:**
- Isolate DNA was amplified by PCR on an S1000 thermocycler (Bio-Rad Laboratories, Hercules, CA) in reaction mixtures (50µl) containing the following components: 1.25 U Perfect *Taq* DNA polymerase, 1x Perfect *Taq* PCR buffer, 0.2 mM deoxynucleoside triphosphates, 0.2 µM each primer, and 1 µl DNA template.
- Cycling parameters for all genes were 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 45 s, with a final step of 72°C for 10 min.
- PCR products were verified by gel electrophoresis.
- DNA sequencing was conducted on an ABI 3730xl DNA analyzer.

Parker *et al.*,2012

Multilocus sequence Analysis (MLSA) Methods

Using Environmentally Mediated Genes (MLSA-E)

- Expanded subtree for *X. fastidiosa* subsp. *fastidiosa* showing variation in isolates from grape.
- Branch lengths are transformed to enable viewing of branches and relationships between haplotypes.
- BP (Bayesian posterior) probabilities and maximum likelihood bootstrap values are presented above and below each node, respectively.
- Asterisks indicate 100% support values, while dashes indicate no support on the basis of ML analysis.



MLST is probably the best PCR-based approach to identify and classify *X. fastidiosa*. Also MLST may be more suitable for strain/subspecies studies. (Baldi and La Porta,2017).

Parker *et al.*,2012

Molecular Diagnosis of *Xylella*

Three Real-time PCR tests for detection of *Xylella fastidiosa*

- Three real-time PCR tests are recommended and have been validated.
- Two tests based on Francis *et al.*,2006 including:

1. SYBR green version, and
2. Taqman version.

- The test based on Harper *et al.*,2010; erratum 2013.

EPPO,2016

Real-time PCR

1. SYBR green version

Xylella fastidiosa

- This PCR is suitable for the detection and identification of *Xylella fastidiosa*.
- The test is based on Francis *et al.*,2006.
- The target sequence is a conserved hypothetical protein HL gene.
- Amplicon size: 221 bp.
- Forward primer HL5 sequence: 5′-AAGGCAATAAACGCGCACTA-3′;
- Reverse primer HL6 sequence: 5′-GGTTTTGCTGACTGGCAACA-3′.

EPPO,2016

Real-time PCR

SYBR green version Master mix

PCR conditions: Pre-incubation at 95°C for 5 min, followed by 40 cycles of (95°C for 20 s and 60°C for 40 s); melt-curve analysis is performed immediately after the amplification protocol by collecting data over a temperature range of 65–95°C in 0.5°C increments

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	3.88	N.A.
SYBR Select Master Mix (Applied Biosystems)	2x	5.5	1x
Forward primer (HL5)	10 µM	0.31	0.28 µM
Reverse primer (HL6)	10 µM	0.31	0.28 µM
Subtotal		10	
Bacterial suspension or DNA extract		1	
Total		11	

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

N.A.: not applicable, not available

EPPO,2016

Real-time PCR
2. Taqman version
Xylella fastidiosa

- This PCR is suitable for the detection and identification of *Xylella fastidiosa*.
- The test is based on Francis *et al.*,2006.
- The target sequence is a conserved hypothetical protein HL gene.
- Amplicon size: 221 bp.
- Forward primer HL5 sequence: 5′-AAGGCAATAAACGCGCACTA-3′;
- Reverse primer HL6 sequence: 5′-GGTTTTGCTGACTGGCAACA-3′.
- The probe sequence is: 5′/FAM/-TGGCAGGCAGCAACGATACGGCT-/BHQ1/3′.
- Automatic baseline and manual threshold of 0.1.

EPPO,2016

Real-time PCR

Taqman version Master mix

PCR conditions: Pre-incubation (UNG step) at 50°C for 2 min, initial denaturation at 95°C for 10 min, followed by 45 cycles of (95°C for 15 s and 60°C for 60 s)

Reagent	**Working concentration**	**Volume per reaction (µL)**	**Final concentration**
Molecular-grade water*	**N.A.**	**1**	**N.A.**
Real-time PCR buffer (TaqMan® Universal PCR Master Mix, Thermo Fisher Scientific, 2×)	**2x**	**5**	**1x**
Forward primer (HL5)	**10 µM**	**0.9**	**0.9 µM**
Reverse primer (HL6)	**10 µM**	**0.9**	**0.9 µM**
Probe 1 (probe)	**10 µM**	**0.2**	**0.2 µM**
Subtotal		**8**	
Bacterial suspension or DNA extract		**2**	
Total		**10**	

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

N.A.: not applicable, not available

EPPO,2016

Real-time PCR
Third method
Xylella fastidiosa

- This PCR is suitable for the detection and identification of *Xylella fastidiosa*.
- The test is based on Harper *et al*.,2010, erratum 2013.
- The target sequence is located at the level of the gene coding for the 16S rRNA processing rimM protein.
- Forward primer XF-F sequence:
- 5′-CACGGCTGGTAACGGAAGA-3′;
- Reverse primer XF-R sequence:
- 5′-GGGTTGCGTGGTGAAATCAAG-3′;
- Probe XF-P sequence:
- 5′-6-FAM -TCG CAT CCC GTG GCT CAG TCC-BHQ-1-3′.

EPPO,2016

Real-time PCR

Third method Master mix

PCR conditions: Pre-incubation at 50°C for 2 min followed by 95°C for 10 min, followed by 40 cycles of (94°C for 10 s and 62°C for 40 s). Heating ramp speed: 5°C s^{-1}

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	6.48	N.A.
Taqman Fast Universal Master Mix (Applied Biosystems)	2 x	10	1x
Forward primer (*XF-F*)	10 µM	0.6	0.3 µM
Reverse primer (*XF-R*)	10 µM	0.6	0.3 µM
Probe 1 (*XF-P*)	10 µM	0.2	0.1 µM
BSA	50 µg $µL^{-1}$	0.12	0.3 µg $µL^{-1}$
Subtotal		18	
Bacterial suspension or DNA extract		2	
Total		20	

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

N.A.: not applicable, not available

EPPO,2016

Real-time PCR

Third method Master mix

PCR conditions: Pre-incubation at 50°C for 2 min followed by 95°C for 10 min, followed by 40 cycles of (94°C for 10 s and 62°C for 40 s). Heating ramp speed: 5°C s^{-1}

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	6.48	N.A.
Taqman Fast Universal Master Mix (Applied Biosystems)	2 x	10	1x
Forward primer (*XF-F*)	10 µM	0.6	0.3 µM
Reverse primer (*XF-R*)	10 µM	0.6	0.3 µM
Probe 1 (*XF-P*)	10 µM	0.2	0.1 µM
BSA	50 µg $µL^{-1}$	0.12	0.3 µg $µL^{-1}$
Subtotal		18	
Bacterial suspension or DNA extract		2	
Total		20	

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

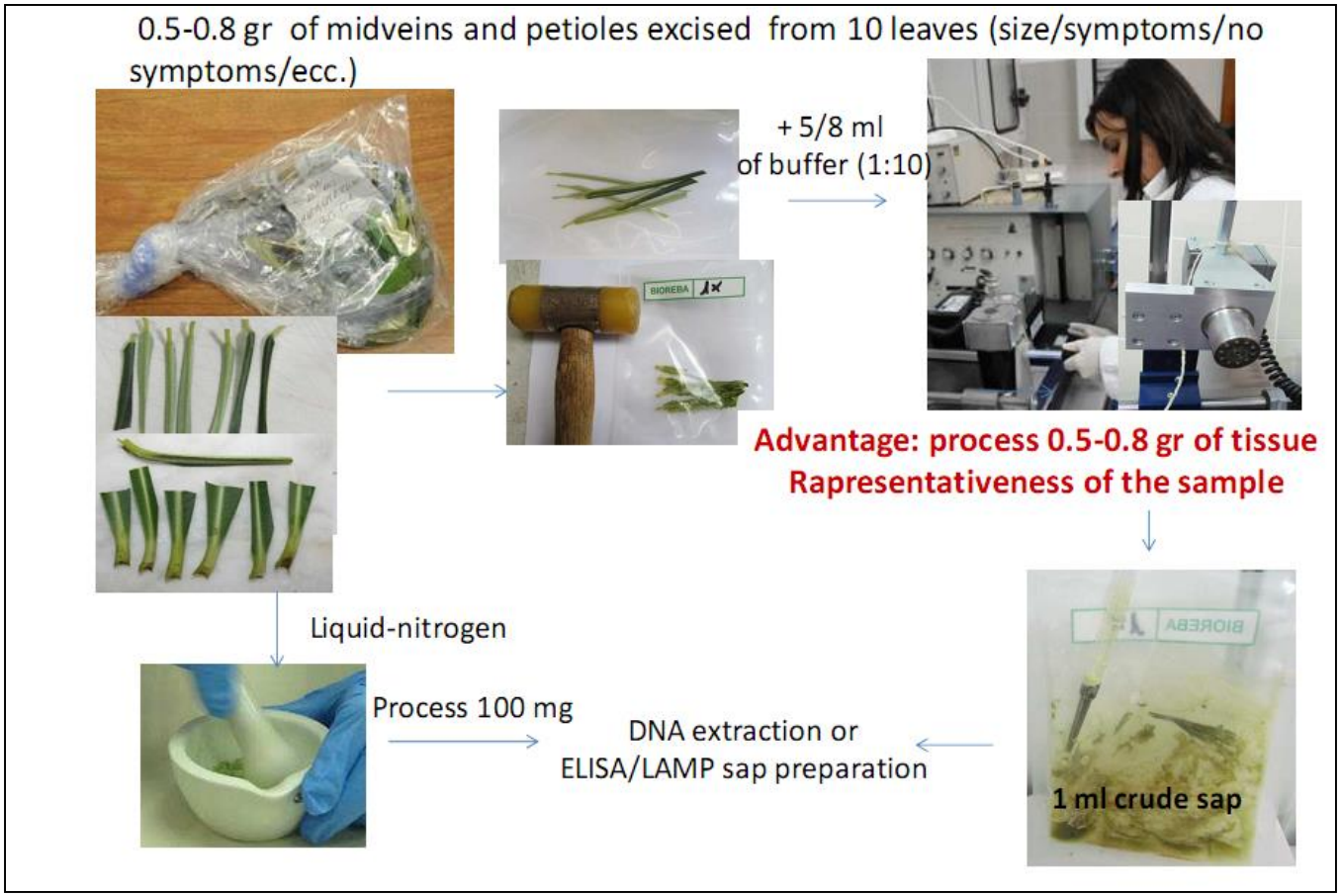
N.A.: not applicable, not available

EPPO,2016

Detection procedure

Sample preparation for DNA extraction or ELISA/LAMP sap preparation

LAMP assay



Loconsole,2016

Detection procedure

Sample preparation for DNA extraction or ELISA/LAMP sap preparation

LAMP assay

A. LAMP ASSAY: based on crude sap preparation for ELISA assay (laboratory tests)

Advantage:

1. crude sap rapresentative of the entire sample to overcome erratic distribution of Xf in plants infected at early stage and in symptomless samples
2. use for testing other susceptible hosts

Loconsole,2016

Molecular Diagnosis of *Xylella*

PCR approaches to *X. fastidiosa* study

Genetic variation

- In order to characterize and differentiate strains and pathotypes of *X. fastidiosa*, several molecular techniques have been used.
- In the late eighties and early nineties, restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) analyses were largely diffused to study strain differentiations of pathogenic bacteria.
- In the following years, RFLP and RAPD were often used in combination with other techniques, such as REP PCR, ERIC-PCR and contour-clamped homogeneous electric field (CHEF), in order to improve sensitivity and reliability of the test.

Baldi and La Porta,2017

Diagnostics methods

Genetic variation

rep-PCR

- Repetitive sequence based-PCR technology (REP-PCR) was used to identify unique PCR products for *X. fastidiosa* causing Pierce's Disease (PD).
- The DNA from *X. fastidiosa* strains were amplified with:
- REP1-R (5'-IIIICGICGIATCCIGGC-3') and
- REP 2 (5'-ICGICTTATCIGGCCTAC-3') primers (Versalovic *et al.*, 1991).
- The overall reactions were adjusted to a final volume of 20 µl, containing:
- 1 X PCR buffer (50 mM KCl, 200 mM Tris-HCl, pH 8,4),
- 2 mM $MgCl_2$,
- 200 µM of each dNTP,
- 1 U of *Taq* DNA polymerase,
- 30 ng of each primer (REP1-R and REP 2) and
- 50 ng of template DNA.

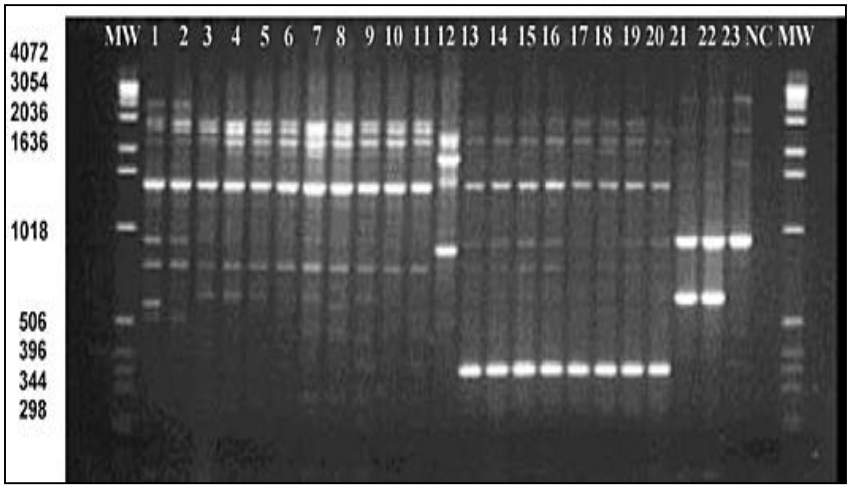
Travensolo *et al.*,2005

Diagnostics methods

Genetic variation

rep-PCR

- The molecular profiles obtained by the REP-PCR amplification showed three distinct groups among the *X. fastidiosa* strains.
- The first group was outlined only by *X. fastidiosa* isolated from citrus (9a5c, 11347, 10348, 10438, 11066, 11067, 11834, 11380, 11775 and 11779), with amplifying fragments ranging from 300 to 4,000 bp.
- The second group was composed of seven strains of *X. fastidiosa* from citrus variegated chlorosis (11037, 11038, B-14, M2-1, 11399, 11400, CVC-5) and one from coffee trees (12288). The distinction of this group was possible due to the presence of a 300 bp amplified fragment.
- The third group, comprised of strains from grapevine (8935 and 9713) and plum (9746), showed a sufficiently distinct profile from the others, with a 630-bp fragment.



Travensolo *et al.*,2005

Molecular Diagnosis of *Xylella*

PCR approaches to *X. fastidiosa* study

Genetic variation

- A more recent technique to study genetic variability among strains exploits the so called short sequence repeats (SSRs) that are located within the prokaryotic genome (Kremer *et al.*, 1999).
- Such short repetitive regions, due to the potentially variable number of tandem repeats (VNTR), can be highly polymorphic among different strains of bacteria and therefore represent a valuable tool for molecular studies.
- A set of 34 SSR markers were developed within the genome of *X. fastidiosa* and used for genotyping studies.

Baldi and La Porta,2017

List of SSR markers specific for *X. fastidiosa*.

SSR markers proved to be powerful tools to distinguish genetically similar isolates, as the average level of polymorphism found among the 34 SSRs was 11.3 alleles per locus.

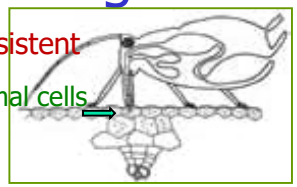
Marker	Forward primer	Reverse primer	Motif	Hosts	Citations WoS - G-Scholar
SSR20[a]	ATGAAGAAGCCAGGATACAT	GCTACACGTGCAACAAC	(ATTGCTG)13	Citrus, coffee, grapevine, plum, Japanese Lantern, periwinkle	8–98
SSR21[a]	AACACGGATCAAGCTCATG	GGAACACGCAATAGTAAGA	(TGTTATC)21	–	
SSR26[a]	CTGTGATCGGTGAATTGA	TCAAGCACACTTCCTACG	(GTGTGTGA)37	–	
SSR29[a]	GCAACGCTGTTATCTCAAT	ATTACGCTTCTTATCGCTGT	(GTGTGCCT)11	–	
SSR30[a]	TACGCTGCACCTGTCTG	CTGTGAACTTCCATCAATCC	(TGATCCTG)15	–	
SSR36[a]	ATGTCACTCAGGTCAGG	CAGAACCACCGACTG	(TGTTGGGG)10	–	
SSR40[a]	ACCTTGACGACGGATG	TAGGAACTGCTGCTACTGAT	(GAAGGCGTA)27	–	
SSR32[a]	AGATGAACCTCGCCAC	GTACTCATCTGCGATGG	(CTGATGTG)9	–	
SSR34[a]	TGATAGAACTGTTTGACGCATTTG	TCGGGAAGTTTGGGGTGAC	(TTGGGTAG)22/(TTGGGTAA)35	–	
OSSR-2[b]	TTGCTTCACCATTAGCCTTATC	GGCCGTACAGGACCGATC	(ATG)9	Grape, citrus, almond, oleander	22–37
OSSR-9[b]	TAGGAATCGTGTTCAAACTG	TTACTATCGGCAGCAGAC	(TTTCCGT)13	–	
OSSR-12[b]	ACAGTCTGTGTCCGCAATTTG	CAGGCGCAGATAGCATTGATC	(AGAGGGTAT)9	–	
OSSR-14[b]	GGCGTAACGGAGGAAACG	ATGAACACCCGTACCTGG	(TGATCCATCCCTGTG)11	–	–
OSSR-16[b]	GCAAATAGCATGTACGAC	GTGTTGTGTATGTGTTGG	(CTGCTA)12	–	
OSSR-17[b]	AGTACAGCGAACAGGCATTG	AGCAACCAGGACGGGAAC	(TGCCTG)10	–	
OSSR-19[b]	GCTGTGAACTTCCATCAATCC	GCAAGTAGGGGTAAATGTGAC	(CAGGATCA)10	–	
OSSR-20[b]	ATCTGTGCGGCGGTTCTG	CACTTGCGGCGTAGATACTTC	(AGGATGCTA)20	–	
CSSR-4[b]	AACCCAATTCTTTTAATATGTG	TTGCAGCATTAGATATTTGAG	(TGCC)7	–	
CSSR-6[b]	CGCACTGTCATCCATTTAATC	GCTGCTTCATCTAGACGTG	(GCTGTA)7	–	
CSSR-7[b]	CACAGCGAACAGGCATTG	AGCAACCAAGACGGGAAC	(CTGTGC)14	–	
CSSR-10[b]	GCAACCACAAAGCCGCAG	AGCACCTCTTAGCATCACTGG	(CAATGA)10	–	
CSSR-12[b]	TAAGTCCATCACCGAGAAG	AAACGGATTTAGGAACACTC	(GAAGGCGTA)27	–	
CSSR-13[b]	CAATGTCACTCAGGTCAG	TTCTGGAATACATCAAATGC	(TGTTGGGG)10	–	
CSSR-16[b]	CGATCAACCCATTCACTG	GCTCCTATTTGCATGATATTG	(GTGGTGGCA)6	–	
CSSR-17[b]	AGAAGTATTCGCTACGCTACG	GGTGATGATTCAGTTGGTGTTG	(CTGATGTG)9	–	
CSSR-18[b]	GTGCTTCCAGAAGTTGTG	GACTGTTCTCTTCGTTCAG	(GCCAA)12	–	
CSSR-19[b]	TGCTGTGATTGGAGTTTTGC	TCAAACGAATCTGTCCATCAAG	(TGGTGAG)7	–	
CSSR-20[b]	GGTATCGCCTTTGGTTCTGG	GACAACCGACATCCTCATGG	(GTAGCA)8	–	
ASSR-9[b]	GGTTGTCGGGCTCATTCC	TTGTCACAGCATCACTATTCTC	(CAAGTAC)11	–	
ASSR-11[b]	AGAGGCAACGCAGGAACAG	GTGAGTTATATCGGTGCAGCAG	(ACGCATC)10	–	
ASSR-12[b]	TGCTCATTGTGGCGAAGG	CGCAACGTGCATTCATCG	(GATTCAG)14	–	
ASSR-14[b]	TTGACTCAAGGAATAAAAC	GAAAAGAGTGTCAATACG	(CTGCGTGC)11	–	
ASSR-16[b]	TTAATCAACAACGCTTATCC	TCGCAGTAGCCAGTATAC	(GCTCCGGTTCTA)26	–	
ASSR-19[b]	CGCCGACTGTCTATGTGAC	TTCCTAGCAATGGCAATGTTG	(ACAACG)10	–	
ASSR-20[b]	TTACTATCGGCAGCAGACG	TGAAGCAATGGTGGATTTAGG	(ACAGAAA)10	–	
GSSR-4[b]	GCGTTACTGGCGACAAAC	GCTCGTTCCTGACCTGTG	(ATCC)7	–	
GSSR-6[b]	TGTTCTCTTCGTTCAGCCAAGC	CGCAGCAGAGCAGCAGTG	(CTTGT)12	–	
GSSR-7[b]	ATCATGTCGTGTCGTTTC	CAATAAAGCACCGAATTAGC	(GGCAAC)24	–	
GSSR-12[b]	TTACGCTGATTGGCTGCATTG	GTCAAACACTGCCTATAGAGCG	(TATCTGT)20	–	
GSSR-14[b]	TTGATGTGCTTTTGCGGTAAG	GACAGGTCCTCTCATTGCG	(TCCCGTA)24	–	
GSSR-15[b]	CCGCAGAGTCCGTTGTAAC	AGCCGACGCACGGTATATC	(AGCCTGC)17	–	
GSSR-19[b]	GCCGATGCAGAACAAGAAC	TCAACTTCGCCACACCTG	(GAAAACAAG)19	–	
GSSR-20[b]	TGGATGGATAGATGATTCAGCC	CGATCAGTGGAGGATGTCTTG	(GAACCACTA)7	–	
COSS1[c]	GAAACAAGATGGCGGTTGC	CATTTAAACGGGCGGCATA	(ATTGCTG)15	Coffee, citrus	0–0
COSSR6[c]	TGCTGCGCGATAACCAAGT	CATCCAATCAGCCCTAACCT	(GTGATGCG)10	–	
CSSR45[d]	ACAGACATCACCGGCATTG	AATGTCGCTGCCAATCCAT	(CACACCGAGATGGAC)8	–	
COSSR4[c]	CAAGGTGACCGCTAGCCTAT	GCTGTCATTGGGTGATGC	(CAATACAC)13	–	
COSSR5[c]	ACACTGACACAACAGCCACCA	AATGGTGGGTGTGATGGTTTC	(CATACAGA)9	–	
COSSR9[c]	AAGTATTCGCTACGCTACGC	GTGTGTTATGTGTGCCATTCGT	(CTGATGTG)10	–	
CSSR42[c]	ATTACGCTGATTGGCTGCAT	GTTTCATTACGCGGAACAC	(TGTTATC)21	–	

[a] Della Coletta-Filho et al. (2001).
[b] Lin et al. (2005).
[c] Francisco et al. (2017).

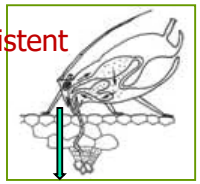
Baldi and La Porta,2017

Vector-borne fastidious bacteria
Persistent and nonpersistent transmission

- Generally, vector acquisition of pathogens increases with time spent feeding on infected plant sources of the pathogen.
- Nonpersistent transmission: Transmission is called nonpersistent if the rate of transmission drops to near zero within a short time(hours).
- Persistent transmission: Describes situations in which the vector can transmit over many days, in some cases, for weeks or months.
- All phytoplasmas are transmitted to plants by phloem-feeding insects in a persistent propagative manner.



Purcell and Almeida,2005;..



Diseases caused by vector-borne fastidious bacterial pathogens

- Some plant parasites transmitted by insect vectors must multiply and circulate throughout the body of the vector to be transmitted.

Type of pathogen	Vector	Type of transmission
Xylella fastidiosa (numerous diseases)	Xylem sap-feeders (Leafhopper known as Glassy-winged sharpshooter)	Noncirculative but persistent, propagative in vector
Ca. Liberibacter Citrus greening	Psyllids	Foregut-borne (non-circulative), persistent
Aster yellows phytoplasma	Leafhoppers (several spp.)	circulative, propagative
Spiroplasma citri Citrus stubborn	Leafhoppers (several spp.)	circulative, persistent manner

Purcell and Almeida,2005;..

Glassy-winged sharpshooter (GWSS)
The main vector of *Xylella fastidiosa*

- The leafhopper insect known as glassy-winged sharpshooter(*Homalodisca coagulata*) (GWSS) is the major vector of:

1. Pierce's disease, and
2. Phony peach disease.

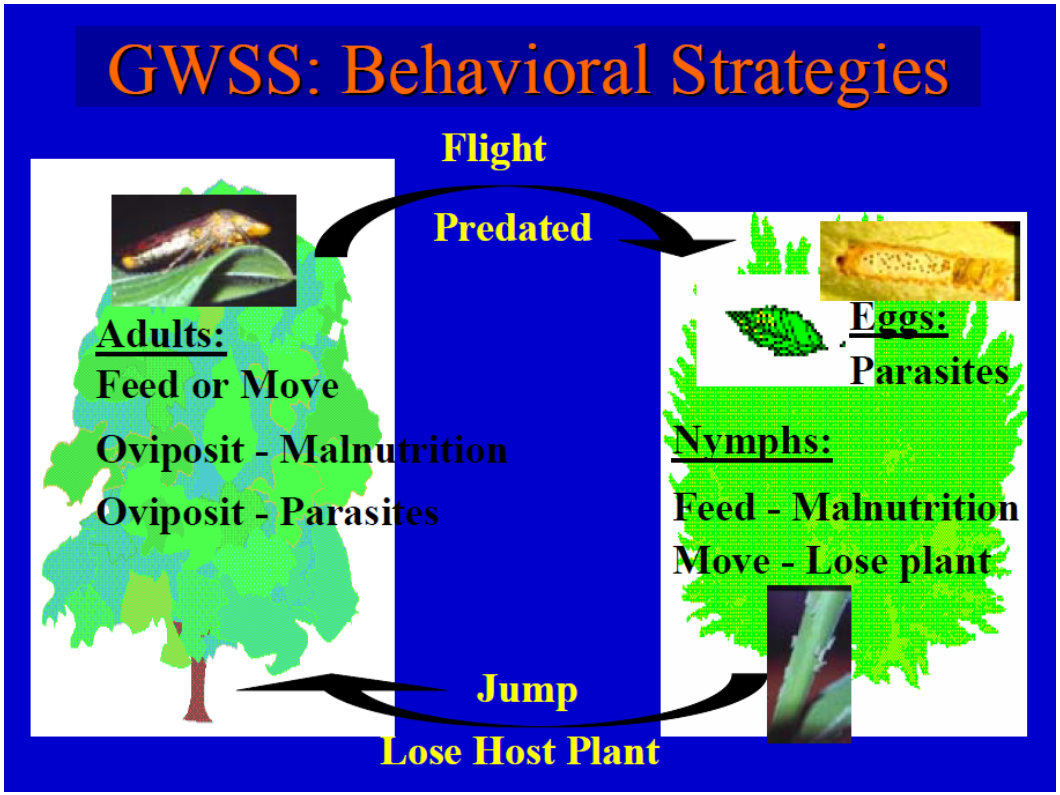
Courtesy Russell et al

Gould & Lashomb,2005

Sharpshooter(GWSS)

A model of the reproductive and behavioral tradeoffs facing glassy winged sharpshooters during adult and immature stages

- These tradeoffs occur because of the low nutritional value of plant xylem, the different requirements of the adults and immatures, and high egg parasitism.

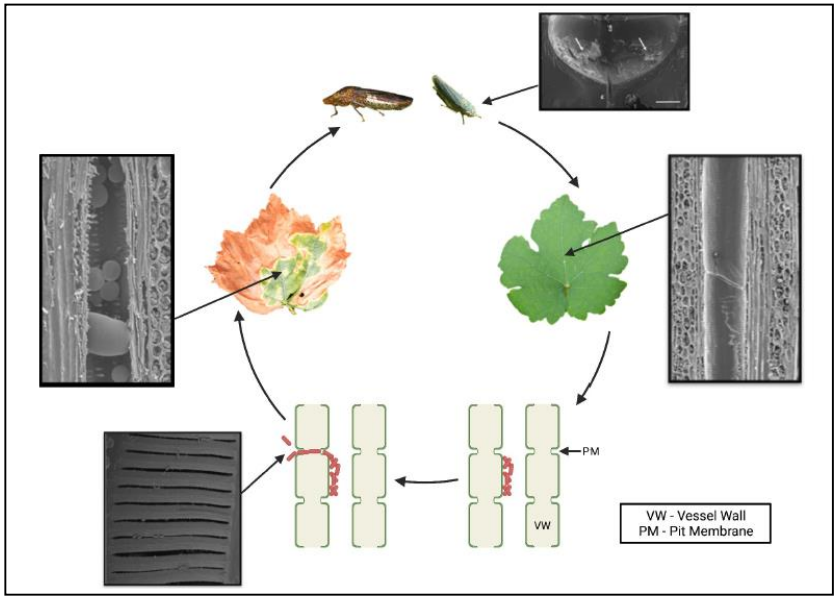


Lashomb *et al.*,2002

PD of grapevine cycle

Role of insect vectors, such as the GWSS and the BGSS and excess tylose production in the xylem lead to PD symptom development

- *Xylella fastidiosa* is acquired by its xylem-feeding insect vectors, such as the GWSS and the BGSS, during the feeding process. Once acquired, it colonizes the insect's foregut and forms robust biofilms (**indicated by white arrows**). *Xylella fastidiosa* is transmitted to a new host plant when the insect vector feeds on a new plant and deposits *Xylella fastidiosa* cells directly into the plant xylem. *Xylella fastidiosa* achieves systemic colonization of the xylem by enzymatic degradation of the xylem pit membranes that connect adjacent xylem vessels.
- *Xylella fastidiosa* colonization induces prolific production of balloon-shaped defense related protrusions called tyloses in the xylem. Systemic colonization and vessel occlusion by bacterial biofilms and excess tylose production lead to PD symptom development.
- GSS, blue-green sharpshooter; GWSS, glassy-winged sharpshooter; PD, Pierce disease; PM, pit membrane; VW, vessel wall.



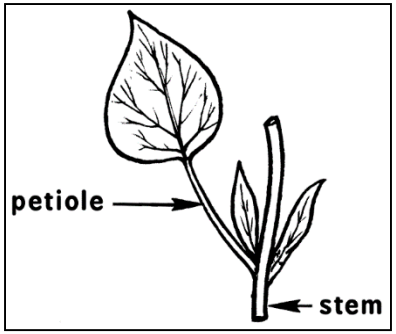
Castro *et al.*,2021

Pathogenicity test

Susceptible cultivars

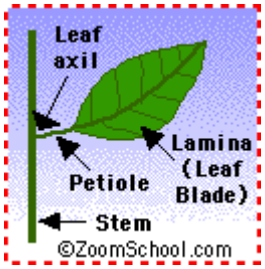
- A general inoculation procedure is described below.
- Pathogenicity tests should use plants of the same host from which the suspect *X. fastidiosa* was isolated.
- Where possible, the most susceptible cultivars should be used.
- Some recommended examples include: for *V. vinifera* (common grape vine), the cultivars 'Chardonnay', 'Cabernet sauvignon', 'Chenin Blanc' and 'Pinot Noir'; for *Camellia sinensis* (*tea*), 'Pera', 'Hamlin', 'Natal' and 'Valencia'; and for *Olea europaea* (olive), 'Cellina di Nardo', 'Frantoio' and 'Leccino' (EPPO, 2018b).
- *Catharanthus roseus* (Madagascar periwinkle) is a herbaceous plant that is easily grown in a greenhouse and is susceptible to *X. fastidiosa* (Monteiro *et al.*,2001).

Pathogenicity test
Needle inoculation



- Pathogenicity testing is recommended when requiring additional information on strain aggressiveness, potential host range, or to fulfil the requirements of Koch's postulates.
- Actively growing, susceptible plants need to be maintained in a greenhouse or growth chamber at 26-28°C.
- Inoculation techniques should deliver inoculum directly into the xylem vessels for development of symptoms.
- The most widely used method for plant inoculation is by needle puncture into the stem at the insertion of the petiole (Hill and Purcell, 1995; Almeida *et al.*, 2001).

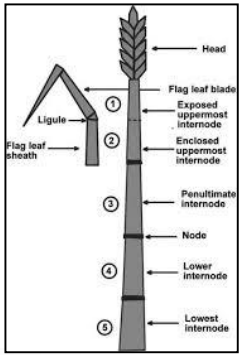
Pathogenicity test
Needle inoculation



- To facilitate the rapid uptake of the inoculum by the transpiration system, inoculated plants should be young and should be grown in pots with dry soil.
- Cultures of bacteria grown for 8-10 days on suitable media should be used for pathogenicity tests.
- Bacteria are removed from solid media and suspended in PBS to produce a turbid suspension of approximately 10^8–10^9 cfu/ml (Abs_{600nm}= 0.2).
- A drop (20-50 µl) of inoculum is placed in a leaf axil and punctured through several times with a fine needle until the liquid is completely absorbed.
- Control plants are treated in the same way except that the suspending medium (PBS) is used instead of bacterial suspension.
- Plants must be maintained in the greenhouse or growing chambers at 26-28°C.

Pathogenicity test

Needle inoculation from axenic cultures



- Deliver the inoculum directly into the xylem vessels through puncturing the stem with a syringe needle or pin.
- The inoculum should be visible turbid suspension(A_{600}=0.2) containing 10^8 to 10^9 CFU/ml in a buffer.
- With pin-prick method, a drop of the inoculum is placed on two of the lower internodes of the host plant.
- Symptoms (typical leaf scorch symptoms) produced by *X. fastidiosa* strains in various hosts require 3 weeks after inoculation (Pierece's disease of grapevine) to 18-24 months (e.g. phony peach) for symptom development.

Schaad *et al.*,2001; EPPO,2016

Pathogenicity test
Needle inoculation

- Inoculation techniques should ensure infiltration directly into the xylem vessels in order for symptoms to develop.
- The most widely used method for plant inoculation is by needle puncture in the stem at the insertion of the petiole.

EPPO,2016

Pathogenicity test

X. fastidiosa pathogenicity assays on grapevines

Needle inoculation

- In brief, 10µL droplets of *X. fastidiosa* bacterial suspensions (OD_{600} = 0.25) in SCP buffer (trisodium citrate, 1 g/L; disodium succinate, 1 g/L; $MgSO_4 \cdot 7H_2O$, 1 g/L; K_2HPO_4, 1.5 g/L; and KH_4PO_4, 1 g/L; pH 7.0) were applied on opposite sides of each of 4-5 internodes of ca. 3 ft high grapevines in pots, starting with the second internode from the base.
- A sterile, tuberculin needle was used to puncture the stems to a depth of 1 to 3 mm through each of the droplets.
- Plants were not watered for at least 36 hours prior to inoculation.
- Inoculated plants were maintained in an air conditioned green house and carefully observed for the appearance of symptoms.
- Observations were recorded from the time the first visible symptoms appeared (ca. 4-6 weeks post inoculation) and continued for another 2 months.
- Disease severity was quantified and expressed as a % of diseased leaves (including bare petioles and bare nodes) on each inoculated plant by measuring the number of symptomatic leaves, the number of bare petioles and the number of bare nodes on each plant, and dividing by the total number of leaves (asymptomatic or symptomatic), bare petioles and bare nodes per plant.

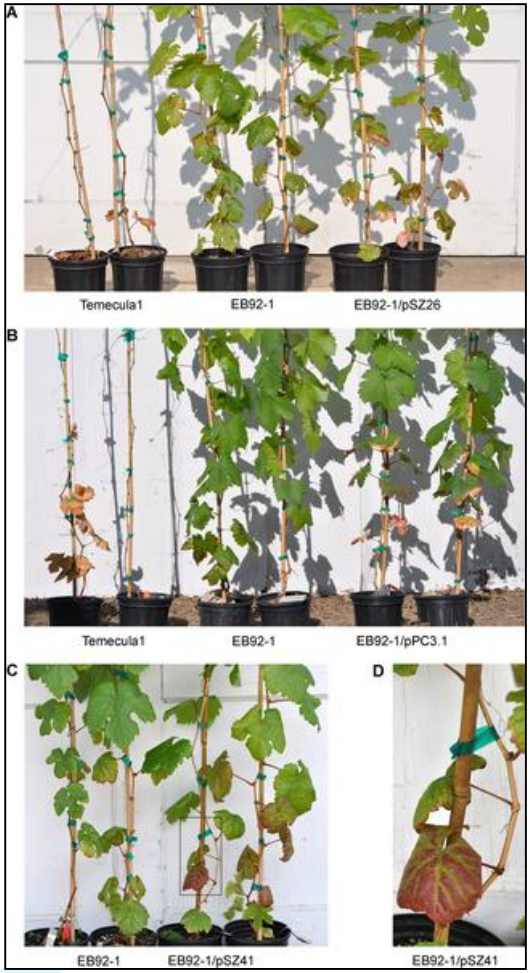
Zhang *et al.*,2015

Pathogenicity test

X. fastidiosa pathogenicity assays on grapevines

Needle inoculation

- EB92-1/pSZ26 elicited typical leaf necrosis delimited with pinkish areas that were identical in appearance to plants inoculated with Temecula1(TM1) strain.
- As the infection progressed the entire lamina underwent necrosis leading to defoliation.
- Enlarged region from boxed area in (C) illustrating unique symptoms(D).



Zhang *et al.*,2015

Pathogenicity test

Recovery of *X. fastidiosa* from grapevine cuttings

- Two grapevine cuttings (*Vitis vinifera*) of the variety "Cabernet sauvignon" were mechanically inoculated with the Temecula strain of *X. fastidiosa* by using the pin-prick method.
- After these vines showed PD symptoms, 23 petioles from symptomatic leaves were collected from these plants, surface sterilized, cut into small (1 mm) pieces, homogenized in buffer, and then dilution-plated on PWG, PD3, and XfD2 media, following published methods.
- After 1-3 weeks, we counted the number of CFUs by using a dissecting microscope.

Almeida *et al.*,2004

Pathogenicity test
Stem tissue inoculation

- An alternative method of inoculation is to raise a flap(a thin and flat piece) of stem tissue by cutting upward with a razor blade to expose the wood.
- A few drops of bacterial suspension are placed under the flap and the flap replaced and wrapped with grafting tape.
- Symptom development usually appears 60-80 days after inoculation; however, this is known to be variable and could be up to 24 months depending on host and strain combination (Hopkins, 2001).
- For both methods of inoculation, if possible the bacterium should be re-isolated to fulfil the requirements for Koch's postulates.

Pathogenicity test

Bioassay on tobacco plants

Xylella fastidiosa

- In addition, a bioassay can be performed on *Nicotiana tabacum* (tobacco) plants by inoculating the petioles with suspensions of *X. fastidiosa* strains associated with almond leaf scorch disease (ALSD) and Pierce's disease (PD) of grapevine (Francis *et al.*,2008).
- Leaf scorch symptoms develop 10-14 days after inoculation.
- All PD strains and the ALSD strain Dixon caused characteristic leaf scorch symptoms, whereas two other ALSD-associated strains (M12 and M23) caused severe leaf chlorosis followed by necrosis, leaf death, and drooping of older leaves.

Pathogenicity test
Bioassay on tobacco plants
Xylella fastidiosa

- 1 month old tobacco plants ('Petite Havana SR1') were inoculated by cutting the top of the stem and removing the lower juvenile leaves so that only three healthy adult leaves in the lower portion of the plant remain (numbered 1–3).
- Bacterial inoculum is prepared from *X. fastidiosa* cultured on solid media at 28°C for about 1 week. Bacteria from two plates are scraped off and resuspended in 1.5 mL succinate-citrate phosphate buffer.
- A 1-mL tuberculin syringe with a 23-gauge needle is used to inject half of the plants with approximately 20 µL of inoculum in each remaining tobacco petiole, near the axils. The other half of the tobacco plants (control plants) are injected in the same manner with buffer only.
- Plants continue growing from the site where the stem was cut. Leaves are classified according to their appearance as control (healthy) or senescent (showing browning symptoms) from buffer-inoculated control plants and asymptomatic (healthy) or symptomatic (marginal leaf scorch) from *X. fastidiosa*-inoculated plants.
- Symptoms start to develop 10–14 days after inoculation (leaf scorch symptoms).
- Francis *et al.*,2008 reports that tobacco inoculated with stains associated with almond leaf scorch and Pierce's disease showed typical symptoms resembling those of grapes and almond infected with *X. fastidiosa*.

EPPO,2016

Pathogenicity test

Bioassay on tobacco plants

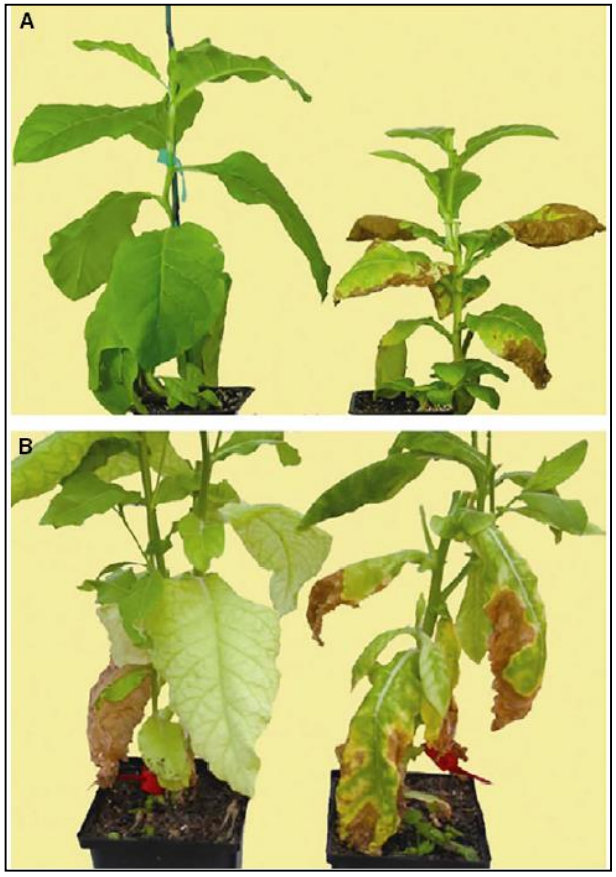
Xylella fastidiosa

- Symptoms were fully developed 6 weeks after inoculation.

A. The control plant mock inoculated with water (left) and plant inoculated with *X. fastidiosa* (right).

B. Advanced symptoms at flowering time (2-3 months after inoculation).

- The water mock-inoculated control plant is showing normal leaf senescence (left) and the *X. fastidiosa* inoculated plant is showing marginal leaf scorching and a chlorotic halo around the edge of the scorch symptoms (right).



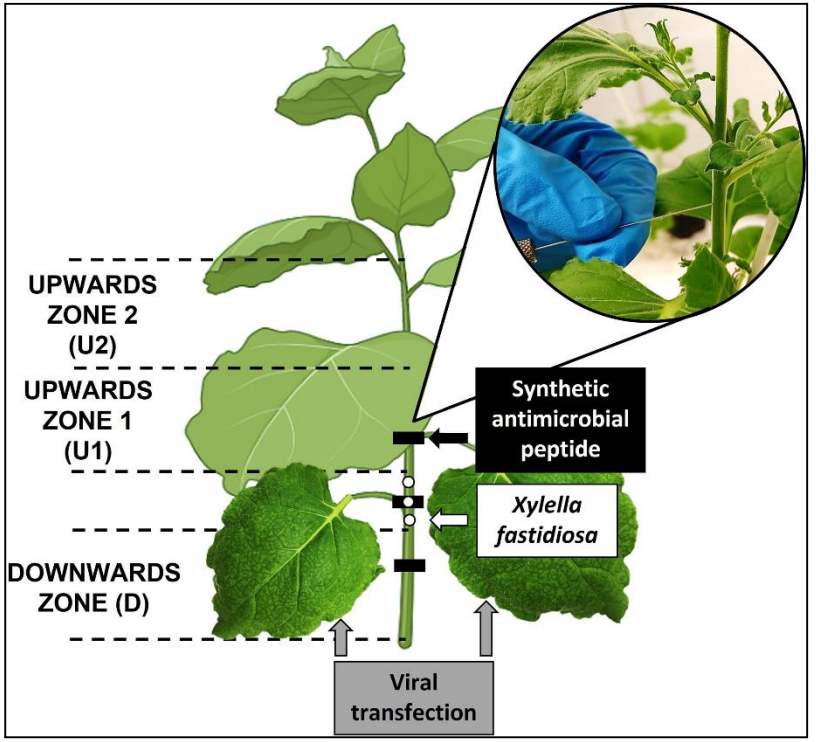
EPPO,2016

Pathogenicity test

Bioassay on tobacco plants

Xylella fastidiosa

- Briefly, a pathogen suspension, at 10^8 CFU/ml ($OD_{600} \cong 0.3$) was injected with a Hamilton 250 µl syringe including a thin needle with bevel tip.
- The needle end was introduced into approximately one half the plant stem diameter to directly access the vascular system.
- Three inoculations of *X. fastidiosa* suspension of 10 µl each (30 µl of total inoculum/plant, $3x10^6$ CFU/plant) were applied at the same side of the stem in a section of 3 cm at around 10 cm above the soil level.



Scheme of pathogen inoculation, peptide application points, and the leaves transfected with PVX constructs, and details of inoculation/delivery process in the stem of the plant.

Baró *et al.*,2022

Maintenance procedures
Storage methods

- Lyophilization and storage on silica gel at 20°C are effective long-term storage methods (Sleesman and Leben,1978; Hopkins,1988b).
- Long-term culturing of *X. fastidiosa* frequently results in the loss of virulence, e.g., after 18 months of weekly culturing on PD2 medium (Hopkins,1984).

Bacterial strains and culture conditions

- *Xylella fastidiosa* subsp. *fastidiosa* strain Temecula (ATCC 700964) was used in this study and was grown in PD2 liquid medium at 28°C.
- Stocks of *X. fastidiosa* cultures were stored in PD2 broth plus 20% glycerol at -80°C.
- Bacteria from these colonies were then transferred to fresh PW and PD3 medium plates for continued growth.
- For long-term storage, bacterial cells were harvested from petri dishes and maintained in PW broth with 20% glycerol at -80°C.

Hernandez-Martinez *et al.*,2006;Cobine *et al.*,2013

Bacterial strains and culture conditions

- Freezer cultures were made by putting 0.75 ml of sterile glycerol and 0.75 ml of Supplemental PW broth culture in cryotubes.
- The tubes were placed in Nalgene's Cryo 1°C Freezing container, put in a -80°C freezer and lowered to the freezer temperature in 1°C increments over four hours.
- After the tubes reached freezer temperature, they were transferred from the freezing container to a freezer box for long-term storage.

Mundell,2005

Identification of the bacterial pathogens
BLOs (bacteria-like organisms)

Phloem-limited prokaryotes
Disease diagnosis and pathogen diagnostics

1. ***candidatus*** **Phlomobacter**
2. ***candidatus*** **Arsenophonus**
3. ***candidatus*** **Liberibacter**

Note that *ca. Liberibacter* spp. are clustered in class *Alphaproteobacteria*, family Rhizobiaceae and *ca.* Phlomobacter and *ca.* Arsenophonus are clustered in class *Gammaproteobacteria*.

Fastidious phloem-limited bacteria

Fastidious Phloem-limited Bacteria

- Small with rippled cell wall, mostly Gram (-)
- No flagella
- Have not been cultivated
- Transmitted by insects

Symptoms: stunting of leaves, shoot proliferation, greening of floral parts

Serratia marcescens: Yellow vine disease of cucurbits

- Watermelon, squash, pumpkin
- Squash bug vector

Candidatus liberobacter asiaticus or *africanus*: Citrus greening

- All citrus…spreads very quickly, worldwide problem
- Psyllid vector

Huanglongbing = yellow dragon

Large "pancake" canker lesion on red grapefruit fruit caused by *X. citri*.

www.apsnet.org

Cuppels Biology 418a

Fastidious phloem-limited bacteria
BLO(bacteria-like organisms)

- Phloem-inhabiting bacteria, which for several years were thought to be rickettsia-like organisms (RLO), were only discovered in 1972 (Agrios,2005).
- More than 20 plant diseases are associated with or known to be caused by these bacteria-like organisms (BLOs), which range in size from 0.25 to 0.5 by 0.8 to 4.0 micrometers (Schubert,2002).

Plant diseases associated with BLO

Disease Association	Plant Host	Geographic Location
Pollen sterility of garlic	*Allium sativum*	Germany
Sugar beet latent rosette	*Beta vulgaris*	Europe
Papaya bunchy top	*Carica papaya*	Caribbean Islands, Cent. Am.
Yellow vine disease of cucurbits	*Citrullus lanatus, Cucumis melo, Cucurbita spp.*	USA
Citrus greening	*Citrus* spp., *Poncirus trifoliata,* and other rutaceous plants	Asia and Africa
Coconut palm decline	*Cocos nucifera*	Tanzania
Carrot yellows	*Daucus carota* subsp. *sativus*	USSR
Strawberries yellows	*Fragaria* X *ananassa*	Australia
Strawberry marginal chlorosis	*Fragaria* X *ananassa*	France and Spain
Brown blast of rubber trees	*Hevea brasiliensis*	China
Hop crinkle	*Humulus lupulus*	Eastern Europe
Larch witches' broom	*Larix decidua*	Germany
Tomato stolbur-like	*Lycopersicon esculentum*	Eastern Europe
Proliferation and stunting	*Melaleuca armilaris*	Israel
Potato leaflet stunt	*Solanum tuberosum*	Israel
Little leaf	*Sida cordifolia*	Puerto Rico
Spinach witches' broom	*Spinacia oleracea*	Italy
Rugose leaf curl of clover	*Trifolium* spp.	Australia
Yellows of clover	*Trifolium repens*	Canada
Clover club leaf	*Trifolium repens*	USA and England
Wheat yellow leaf curl	*Triticum* spp.	China
Yellows disease of grapevine	*Vitis vinifera*	Germany, Greece
Infectious necrosis of grapevine	*Vitis vinifera*	Czechoslovakia
Shoot proliferation	*Wissadula periplocifolia*	Jamaica

Schaad *et al.*,2001

The genus *Candidatus* Phlomobacter

Candidatus Phlomobacter fragariae	Marginal chlorosis of strawberry

- *Candidatus* Phlomobacter can be identified by amplification and sequencing of the 16S rDNA.
- By sequence comparisons, two primers specific for Phlomobacteria have been selected on the 16S rDNA sequence.
- They are efficient and specific for *Candidatus* Phlomobacter detection in plants.
- When used for DNA amplification in insects, these primers are not specific, as *Candidatus* Phlomobacter shares strong homologies with:

1. Insect bacterial symbionts;
2. Parasites;
3. Enterobacteria.

The genus *Ca.* *Arsenophonus*

Emergence and evolution of Arsenophonus bacteria as insect-vectored plant pathogens

- Notably, two species:

1. Phlomobacter fragariae, and
2. Arsenophonus phytopathogenicus, have been characterized as phloem-restricted plant pathogens that are obligatorily transmitted by and hosted in planthoppers.

- Bressan *et al.*, 2012 reviewed the current understanding on the lifestyle transition, evolution, host interaction, and infection cycles of these emerging plant pathogens.

Ca. Arsenophonus phytopathogenicus" is not a sister taxon to "*Ca.* Phlomobacter fragariae", but is a novel species, for the bacterium associated with sugar beet and strawberry diseases.

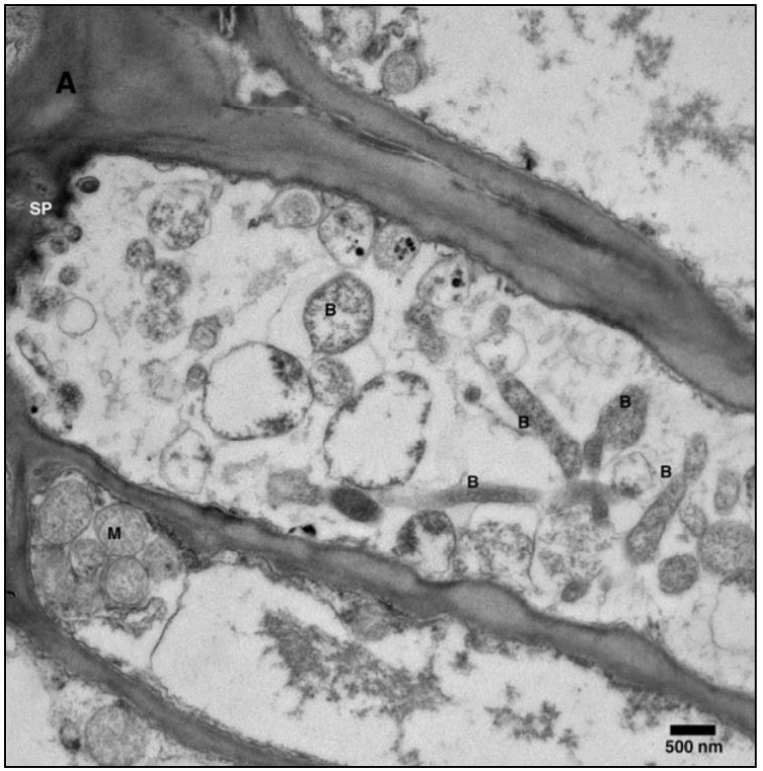
The genus *Candidatus* Liberibacter

- *Candidatus* Liberibacter is a genus of Gram-negative bacteria in the Rhizobiaceae family.
- Liberibacters are restricted to the sieve tubes of the phloem tissue and possess a characteristic double-membrane cell envelope.
- The outer cell wall membrane and the inner cytoplasmic membrane can be observed. The peptidoglycan layer between the inner and outer membrane can be visualized by cytochemical treatments.
- Members of the genus are plant pathogens mostly transmitted by psyllids.

Cell structure

'*Candidatus* Liberibacter asiaticus'(CLas)

- Pleiomorphic round and elongated bacilliform-like shapes characteristic of '*Ca*. L. asiaticus'.
- B, bacterial cells;
- SP, sieve plate;
- M, mitochondria.
- Scale bar is 0.5 μm



Hilf *et al.*,2013

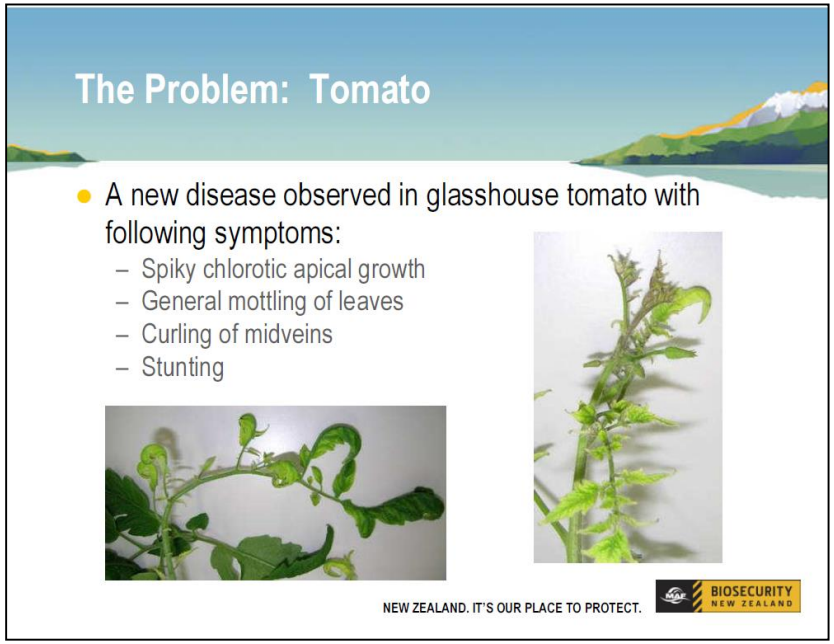
The genus *Candidatus* Liberibacter

Candidatus Liberibacter asiaticus	Huanglongbing (Citrus greening disease)
Candidatus Liberibacter asiaticus subsp. capensis	Present in an ornamental rutaceous (citrus family) tree
Candidatus Liberibacter africanus	Huanglongbing (Citrus greening disease)
Candidatus Liberibacter americanus	Blotchy mottle on sweet orange
Candidatus Liberibacter psyllaurous	Associated with potato and tomato Psyllid yellows
Candidatus Liberibacter solanacearum	Zebra chip disease of carrot, potato and tomato
Candidatus Liberibacter crescens	Isolated from papaya growing in Puerto Rico
Candidatus Liberibacter brunswickensis	Associated with the psyllid Acizzia solanicola on eggplant in Australia
Candidatus Liberibacter europaeus	Found in pear trees, where it seems to cause no symptoms and is vectored by the psyllid, *Cacopsylla pyri*

Liefting; Liefting co-workers; Bull *et al.*,2012; Bressan *et al.*,2012; Wikipedia,2020

'*Candidatus* Liberibacter solanacearum'

Zebra chip disease of potato and spiky chlorotic apical growth, general mottling of leaves, curling of midveins and stunting of tomato

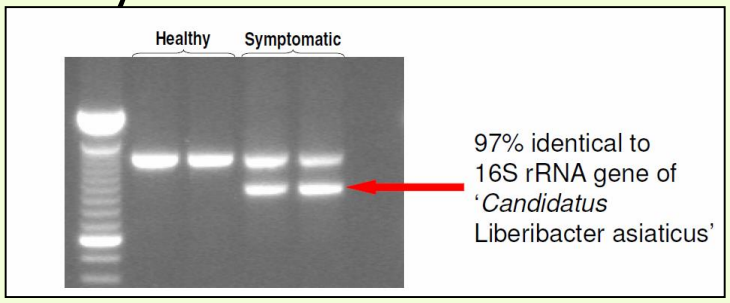


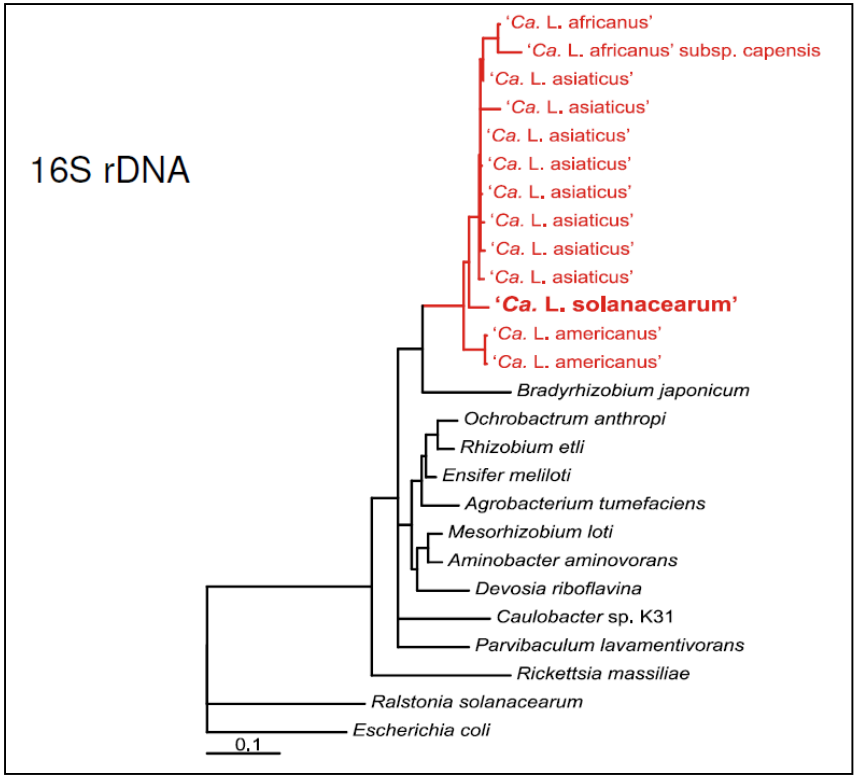
Tomato/potato psyllid (*Bactericera cockerelli*) observed in association with these affected crops.

Phylogenetic analysis of the 16S rRNA gene

'*Candidatus*' Liberibacter including a new Liberibacter species '*Candidatus* Liberibacter solanacearum'

- Range of specific 16S rRNA PCR primers used in different combinations with universal 16S rRNA.
- Primers (fD2/rP1). A unique 1-kb fragment was amplified from symptomatic plants only.





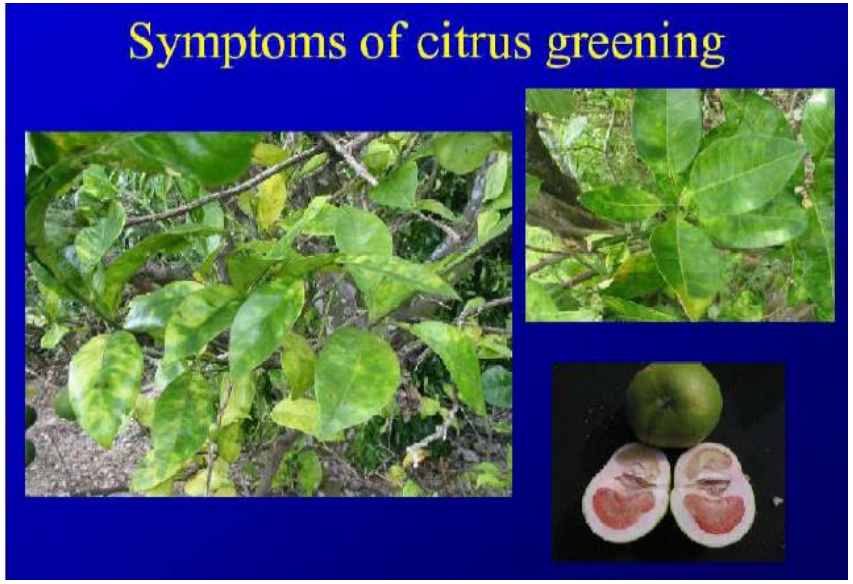
Liefting co-workers

Citrus huanglongbing (greening) disease (citrus greening)

- Huanglongbing also called citrus greening or yellow dragon disease is one of the most serious insect-vectored pathogens of citrus which is thought to have originated in China in the early 1900s.
- Known in China for 100 years where it was called "Huanglongbing" (HLB).
- This name has been translated into English as "yellow dragon disease".
- But Zhao in his 1981 review gives the English name as "yellow shoot disease" and more recently he confirmed that this is the correct meaning in the Chaoshan district of Guangdong Province where the disease was first observed.

HLB or Citrus Greening Disease
candidatus Liberibacter asiaticus, africanus and americanus

- Three species of *Candidatus* Liberibacter cause huanglongbing:
- *Ca. L. asiaticus*,
- *Ca. L. africanus*, and
- *Ca. L. americanus*.
- The Asian form is the most widespread.



Note: **Association of phytoplasma with *Ca.* Liberibacter asiaticus'(CLas) in sweet lime was reported for the first from Iran** (Saberi *et al.*,2017).The HLB-associated phytoplasma was a member of peanut witches' broom (16SrII) phytoplasma group. Recently same type of association (phytoplasma with Huanglongbing (HLB) disease) was reported in pomelo (*Citrus grandis*) from India. Here, the pathogen belongs to 16SrXIV Group of phytoplasma, '*Candidatus* Phytoplasma cynodontis'(Ghosh *et al.*,2019).

Davis; Zhang,2019

HLB or Citrus Greening Disease

Synonyms for citrus greening disease

- South Africa
  - Greening
  - Yellow branch
  - Blotchy mottle
- China
  - Yellow shoot (Huanglongbing)
- Taiwan
  - Decline (Likubin)
- India
  - Die-back
- The Phillippines
  - Leaf mottle
- Indonesia
  - Vein phloem degeneration

Presentation by Dr. Jamba Gyeltshen 4/06/09

Candidatus Liberibacter

Strain differences

- There are three strains of the bacteria:
- ***Candidatus* Liberibacter asiaticum:**
- The Asian form usually produces a more severe disease reaction than the African form. This form is also heat tolerant, producing symptoms under cool to relatively warm conditions (up to 32˚C) (Bové *et al.*,1974).
- ***Candidatus* Liberibacter africanum:**
- The African form of the pathogen is heat sensitive, with symptoms produced under relatively cool conditions (20-24˚C optimum) (Garnier and Bové,1993).
- Extended periods of high temperatures suppress symptom development.
- ***Candidatus* Liberibacter americanus:**
- The American strain discovered in Brazil.

Disease symptoms
BLO damage to hosts

- Within the citrus plant, the bacteria are limited to the phloem. This condition causes the following symptoms:
  - Shoot color yellow
  - Leaves with characteristic blotchy mottling
  - Normally green tissue turns yellow (chlorosis)
  - Total foliage reduced
  - Leaf tips dieback
  - Premature fruit drop
  - Root systems remain poorly developed with relatively few fibrous roots
- Citrus fruits infected with HLB have the following characteristics:
  - Shape lopsided
  - Size small
  - Color remaining green with seeds aborted
  - Taste sour

Floyd and Krass,2006;…

Trees eventually die; but before that, fruit is distorted and bitter, can't be used for juice.

Detection
By symptoms

- Visual inspection is a very important tool for detection but it is not enough because symptoms of HLB can be confused with nutritional disorders (zinc, iron, manganese deficiencies) or with other diseases (*Citrus tristeza virus*, Stubborn, citrus blight).
- Symptoms of HLB develop slowly. Infected trees gradually decline in vigour and yield, and remain stunted or eventually die. The disease develops irregularly so that individual trees may show a mixture of normal and diseased sectors – this in itself being a diagnostic characteristic.
- Symptoms first appear as leaf mottling and chlorosis in one shoot or sector of the tree.
- Later, leaf symptoms resemble nutritional deficiencies (zinc, copper and nitrogen) but may vary depending on the bacterial strain.

www.ippc.int,2007

Detection
By symptoms

- On Leaves: The larger leaves on the lower parts of branches turn yellow along the main and secondary veins that later change to a "blotchy-mottle". As the discoloration spreads away from the veins, the leaves become pale to light yellow with unevenly distributed dark green areas. Leaves on weak terminal twigs are small, up-right and show a variety of chlorotic patterns, suggestive of zinc and iron deficiencies.
- The small leaves are pale at first and develop secondary chlorotic patterns as they mature. Mature leaves show irregular patches between the veins; the veins are often prominent and yellow.
- On Fruits: Fruits are reduced in size, of poor quality, and often fail to develop normal fruit colour.
- The columella is curved causing the fruit to be distorted and lopsided. Infected fruit have a bitter, salty taste, remain small and fall prematurely.
- Seeds in affected fruit are abortive.

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HLB or Citrus Greening Disease

Sectoring of greening in citrus tree canopy

HLB or Citrus Greening Disease

Candidatus Liberibacter asiaticus, africanus and americanus

Fig. 2. Young sweet orange from commercial planting in São Paulo Brazil with "yellow-shoot" symptom of HLB infection.

Fig. 3. Pummelo foliage from south Florida displaying "blotchy-mottle" symptoms.

Fig. 4. Sweet orange foliage from São Paulo, Brazil demonstrating asymetrical "blotchy mottle" symptom relative to the mid vein.

Fig. 5. *Citrus hystrix* tree in residential area of Miami showing severe vein corking symptom of HLB.

Gottwald *et al.*,2004

HLB or Citrus Greening Disease

Candidatus Liberibacter asiaticus, africanus and americanus

Mandarin displaying stylar end lack of coloration, the characteristic leading to the common name for the disease "greening".

Asymetrical "lopsided" and seed death sweet orange.

Gottwald *et al.*,2004; Floyd and Krass,2006

Citrus greening fruit symptoms

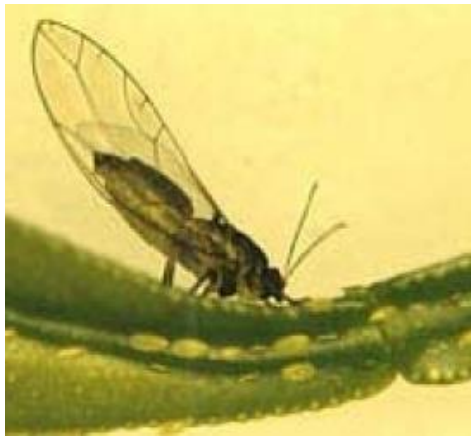
	
HLB-infected Sweet orange fruit from Brazil with blotchy mottle symptom on fruit surface.	HLB-infected Sweet orange fruit from Brazil with diagnostic silver spot that develops when passed firmly with finger.

Gottwald *et al.*,2004

Adult Asiatic or oriental citrus psyllid, *Diaphorina citri* vector of HLB

HLB is vectored by the following two species of psyllids:

1. **Asian citrus psyllid (*Diaphorina citri*);**
2. **African citrus psyllid (*Trioza erytreae*).**

		
Adult Asian citrus psyllid nymphs and characteristic waxy feeding exudate.	**Adult Asian citrus psyllid, *Diaphorina citri*, vector of HLB in Asia.**	**Adult African citrus psyllid. *Tryoza erytreae*, vector of HLB on the African continent.**

Gottwald *et al.*,2004; Floyd and Krass,2006

Diseases caused by vector-borne fastidious bacterial pathogens

- Some plant parasites transmitted by insect vectors must multiply and circulate throughout the body of the vector to be transmitted.

Type of pathogen	Vector	Type of transmission
Xylella fastidiosa (numerous diseases)	Xylem sap-feeders (Leafhopper known as Glassy-winged sharpshooter)	Noncirculative but persistent, propagative in vector
Ca. Liberibacter Citrus greening	Psyllids	Foregut-borne (non-circulative), persistent
Aster yellows phytoplasma	Leafhoppers (several spp.)	circulative, propagative
Spiroplasma citri Citrus stubborn	Leafhoppers (several spp.)	circulative, persistent manner

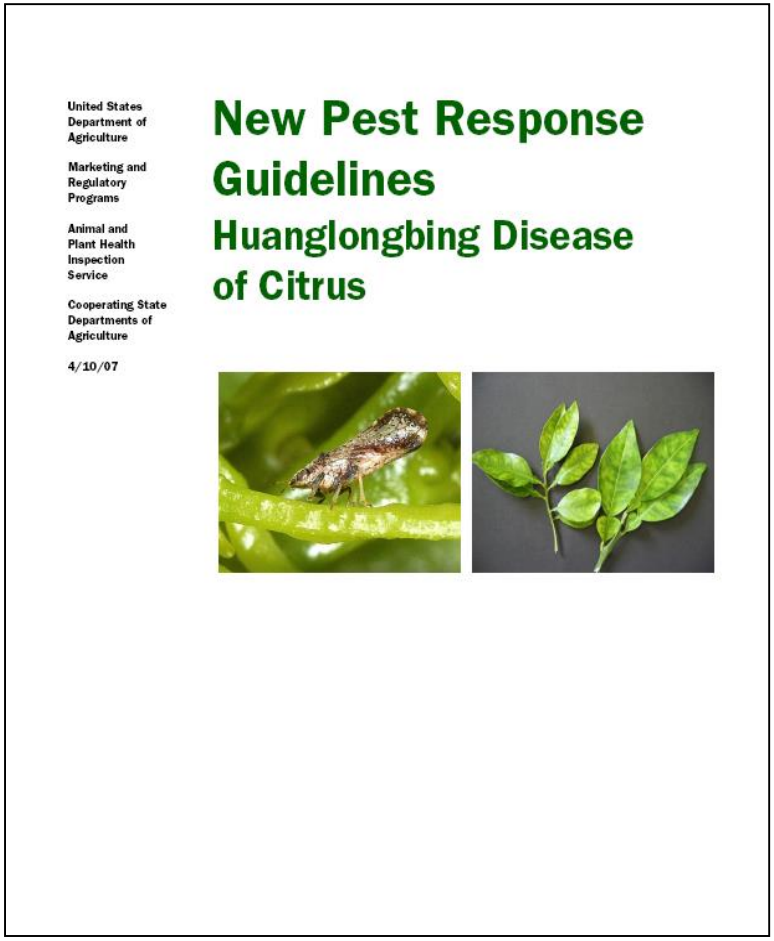
Purcell and Almeida,2005;..

Diaphorina citri
Vector of HLB

A. Wayadande

New Pest Response Guidelines
Citrus Huanglongbing (greening) disease

- Floyd, J. and C. Krass,2006 and 2012.
- USDA/APHIS/PPQ– Emergency and Domestic Programs, Riverdale, Maryland.
- http://www.aphis.usda.gov/import_export/plants/ppq_manuals.shtml



Citrus susceptibility assessment
Plant preference for sampling

- Use the following order of plant preference when sampling:

1. Orange, mandarin, tangelo, and tangerine
2. Pummelo, grapefruit, and sour orange
3. Lemon and lime.

- Sweet oranges and mandarin are more severe to HLB disease.
- In contrast, group of lemon and grapefruits are tolerant and limes (*C. aurantifolia*) and pummelo (*C. grandis*) are the most tolerant.

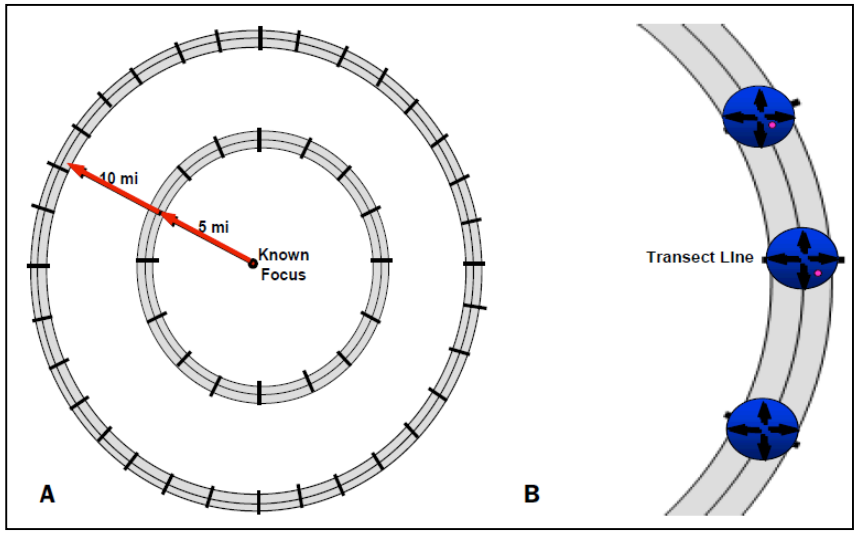
Ahmad *et al.*,2011;..

Sampling method

Examine trees for the presence of yellow shoots, foliar mottling, zinc pattern deficiency, and yellow veins

A. Sampling points along concentric annuli transects at 5-mile increments away from a known positive host tree.

B. Sampling points along an arc transect showing where searching begins to find the nearest host tree for survey.



Floyd and Krass,2006 & 2012

Sampling& Shipping Instructions

- Collect leaves with mid-ribs that are attached to stems.
- Leaf samples
  1. Send a minimum of 20 leaves with stems.
  2. Place leaf and stem samples with paper towels in two resealable plastic bags, one bag inside another larger bag.
  3. Express air from bag.
  4. Seal bag.
  5. Record sample identification number on bag.
  6. Keep samples cool, but **not** frozen (in an ice chest).
- Fruit samples
  1. Place fruit in two paper bags, one bag inside another larger bag.
  2. Record sample identification number on bag.
  3. Keep samples cool but **not** frozen; in an ice chest is good.
  4. Do **not** send fruit samples without accompanying suspect leaf samples from the same tree.
- Pack resealable bags inside a sturdy cardboard box. Include packing material to prevent movement in the box. Omit ice packs.
- Assign and record for each sample a unique ID sample number. Assure that the sample is linked to any survey data collected for that sample by including the Survey ID number on the form.
- Include the completed **PPQ 391 Specimens For Determination on page B-2**—and any relevant tags or barcodes that came with the sample—inside the outer bag.
- Use overnight delivery to ship samples; FedEx® is preferred. Send samples on the same day they are collected, or before noon the following day. Ship samples Monday through Thursday only.

Floyd and Krass,2006

Sample collection

- **Host plant**
- Place leaf samples in plastic bags in cool boxes and refrigerate as soon as possible.
- Leaves can be processed after three weeks if kept in plastic bags at 4°C and if no decay has occurred.
- Alternatively, the midribs can be excised and kept at -80°C.
- Put them into a 1ml of 2 X CTAB buffer (2% CTAB, 1,4 M ClNa, 100 mM Tris- Cl pH 8, 10 mM EDTA) in a 2 ml plastic tube with a screw cap, sealed with parafilm to prevent leakage during transport.

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Sample collection

- **Host vector**
- Preparation of specimens
- Place the adults or nymph in a vial
- Label the vial with the sample number, the date, geographical region.
- Preserve in 70% alcohol and put the vial in a box
- Place the box in a bag.

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Isolation
Squeeze-drop method

- 3- to 4-cm long sections of leaf midveins and petioles were cut from HLB infected citrus and surface sterilized by soaking in a 0.6% sodium hypochlorite solution for 3 min, followed by two separate rinses in sterile water for 2 min each.
- The surface-sterilized tissue was cut into 1-cm sections with a sterile scalpel and, using cooled, flame-sterilized forceps, were squeezed at the fresh cut end of the tissue to express a drop of sap from each section.
- These drops were directly touched onto the surface of duplicate agar plates.
- A sterile loop was then used to streak each drop over the agar surface ("squeeze-drop method").
- The squeezed tissue sections for each petiole and midvein were recombined, minced, and allowed to soak in sterile water (100 to 200 µl) for 10 to 20 min before pipetting 10 to 20 µl of the suspension onto two separate test agar plates and streaking the drop with a sterile loop ("mincesoak method").

Sechler *et al.*,2009

Isolation

Squeeze-drop method

- Each plate was wrapped with Parafilm M, inverted, and incubated at 28°C.
- Half of the plates were placed in a growth chamber under ambient conditions and half in a CO_2 chamber in a 5.0% CO_2 environment.
- At 2 and 4 weeks after plating, all resulting colonies were observed using a binocular dissecting microscope (Nikon SMZ1500) with oblique lighting from below, and portions of several single colonies were streaked onto similar test media.
- Very limited growth of '*Ca*. Liberibacter spp.' occurred on media C3G (selective for spiroplasms) and PD2 (for *Xylella fastidiosa*) when citrus vein extract (CVE) was added, creating the modified media C3G+E and PD2+E, respectively.

Isolation
Liber A medium

- A new medium designated Liber A has been designed and used to successfully cultivate all three '*Candidatus* Liberibacter spp.', the suspect causative agents of huanglongbing (HLB) in citrus.
- The medium containing citrus vein extract and a growth factor sustained growth of '*Ca.* Liberibacter spp.' for four or five single-colony transfers before viability declined.

1. Colonies of '*Ca.* L. asiaticus' were irregular-shaped, convex, and 0.1 to 0.3 mm after 3 to 4 days.
2. The suspect cells of '*Ca.* L. asiaticus' and '*Ca.* L. americanus' under scanning electron microscopy were ovoid to rod shaped, 0.3 to 0.4 by 0.5 to 2.0 µm, often with fimbriae-like appendages.

Sechler *et al.*,2009

Isolation
Liber A medium

- This new medium, named Liber A, contains citrus vein extract and a growth factor, and is able to sustain growth of all strains of bacteria.
- This bacterial growth medium permitted four to five colony transfers before viability was lost, i.e., transfer and growth on new media could not occur.
- All three species are characteristic Gram-negative-like bacteria with the following characteristics:

1. Ovoid to rod-like cells;
2. Micrometer size of 0.2 to 0.3 (width) x 0.5 to 2.0 (length);
3. Fimbriae (thin protein fiber extensions).

Reinhardt,2011

Liber A agar medium
The composition

- The final Liber A agar medium contained:
- 1.5 g of K_2HPO_4,
- 1.0 g of KH_2PO_4, and
- 15.0 g of purified agar per liter.
- After autoclaving and cooling to 55°C in a water bath, followed by the addition of:
- 10 mg of NADP (phosphorylated nicotinamide adenine dinucleotide),
- 20 ml of CVE (citrus vein extract), and
- 5 mg of cycloheximide.
- For liquid Liber A medium, purified agar was omitted.

CVE was prepared from the petiole and midvein of young, fully expanded citrus leaves.

Sechler *et al.*,2009

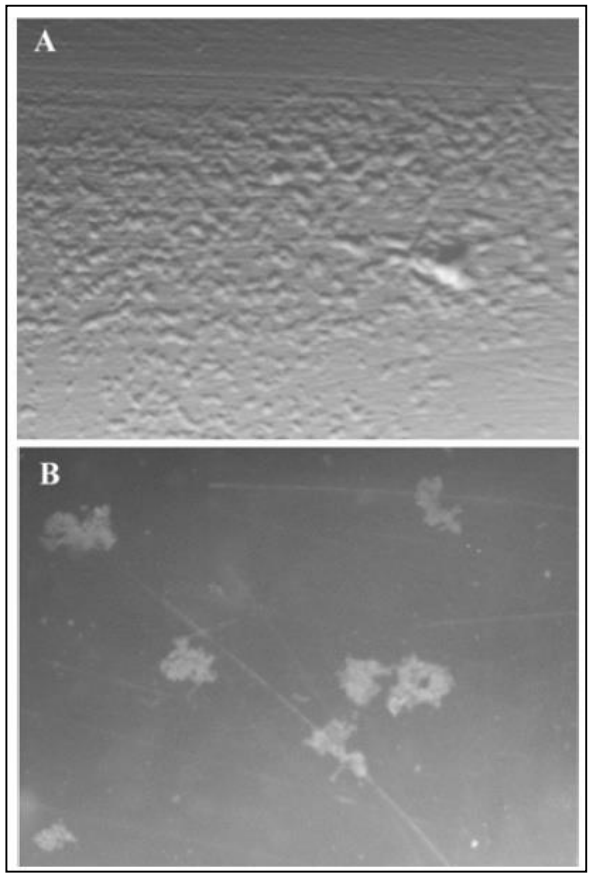
Nicotinamide adenine dinucleotide, abbreviated NAD^+, is a coenzyme found in all living cells and has several essential roles in metabolism. The main role of NAD^+ in metabolism is the transfer of electrons from one molecule to another. NADPH or NADP(nicotinamide adenine dinucleotide phosphate), is a desaturase cofactor significantly increase the percentage of total polyunsaturated fatty acids (essential FAs).

Bacterial colonies

'*Candidatus* Liberibacter americanus'

A. Typical colonies of '*Candidatus* Liberibacter asiaticus' strain China1 in the first streak after 14 days at ×150.

B. '*Ca*. L. americanus' strain Brazil1 in liquid Liber A after 21 days at ×150.



Sechler *et al.*,2009

Liber A agar medium
Drawbacks

- To the best of our knowledge, there were no follow-up or independent repetitions, either within the group or by others.
- In fact, there is still no pure culture of "*Ca*. L. asiaticus" that researchers can access or reproduce.
- These problems suggest the difficulty associated with the long-term reproducibility of culturing methods for "*Ca*. Liberibacter spp."

Ha *et al*.,2019

More media for'*Candidatus* Liberibacter asiaticus'

- Media tested for *in vitro* growth of LAS cells were:

1. K = one-third dilution of King's B medium (K medium);
2. K mdium with 50% juice from the infected fruit (J50 medium),
3. K with 50% commercially-available grapefruit juice (G50 medium), and
4. 100% commercially-available grapefruit juice (G medium).

- Results show that juice-containing media dramatically prolong viability compared to K in experiments reproduced during two years using different juice sources.

King's B, a low-iron medium, was selected for its ability to induce siderophore production in microorganisms, a well-recognized benefit of co-culture.

Parker *et al.*,2012 & 2013

More media for '*Candidatus* Liberibacter asiaticus'

Inoculum preparation from infected seeds

- Pomelo fruit from infected trees were collected (April 2011 to September 2012).
- Entire seed from the fruit were used to create a 'Ca. L. asiaticus' inoculum.
- Fruit were surface sterilized with 1.2% sodium hypochlorite solution followed by 70% ethanol solution.
- Seed were then placed in two to four autoclaved, sterile KLECO canisters with 3 ml of a one-third dilution of King's B media (K) per canister and pulverized(grind) for 2 min in a KLECO 4200 ball mill.
- Seed paste was then scraped into two sterile 50-ml conical tubes, and 35 ml of K was added to each.
- After thorough vortexing, the seed slurries were filtered through a 100-µm nylon net Steriflip Filter Unit (EMD Millipore, Billerica, MA) to remove large seed particles.
- Filtrates from seed from a single fruit were pooled to produce a 'Ca. L. asiaticus' inoculum.

Canister: any container with cylindrical in shape.

Parker *et al.*,2013

Identification and Diagnosis

Thin layer chromatography and serology

'Candidatus Liberibacter'

- Prior to the development of molecular tests, HLB diagnosis was achieved by:

1. Thin layer chromatography for determining the presence of gentisoyl-β-glucoside (Schwarz,1968), although not all HLB-infected citrus species produce it.
2. By serology: The development of monoclonal antibodies held promise for a rapid diagnostic test, but they proved to be too specific for general diagnosis.

Identification and Diagnosis
Thin layer chromatography

- Monoglucose ester gentisic acid (gentisoyl-ß-D-glucose) is a fluorescent substance found in the albedo of fruit and in the bark of the branches of sweet oranges infected with HLB.
- This phenolic marker can be detected by thin layer chromatography (TLC).
- TLC extracts from

1. normal fruit give a yellow -brown fluorescent product, whereas
2. infected fruits give a bright violet fluorescent product which is characteristic for fruit from HLB-infected trees.

- The method is useful for indexing citrus orchards for HLB.

Molecular Diagnosis of BLOs

Primers used for PCR detection

OA1+OI1/OI2c

- Jagoueix *et al*.,1996 recommend the use of three primers (designed based on the 16S rDNA sequences) in the same PCR mixture: OA1+OI1/OI2c.
- Primer sequences:
- OI1: 5´- GCG CGT ATG CAA TAC GAG CGG CA – 3´
- OA1: 5´- GCG CGT ATT TTA TAC GAG CGG CA-3´
- OI2c: 5´- GCC TCG CGA CTT CGC AAC CCA T-3´
- Primer OI2c/OI1 amplify *Ca.* L. asiaticus and *Ca*. L. africanus;
- Primer OI2c/OA1 preferentially amplify *Ca.* L. africanus

www.ippc.int,2007

Molecular Diagnosis of BLOs

Primers used for PCR detection

OA1+OI1/OI2c

- Primer pair OI1 and OI2c is able to amplify the rDNA Las (*Ca.* Liberibacter asiaticus) and Laf species (*Ca.* Liberibacter africanus) (Teixeira et al. 2005a).
- When the 2 *Ca.* Liberibacter spp. are known or supposed to be present in any country, it is desirable to use the 2 forward primers, OI1 + OA1, and the common reverse OI2c primer in the same PCR mixture (Bové 2006).
- Sequence analysis shows that the 16S rDNA amplified from Las has one XbaI restriction site and yields, upon XbaI treatment, 2 fragments of sizes 520 and 640 bp.
- The 16S rDNA amplified from Laf (*Ca.* Liberibacter africanus) has an additional restriction site and yields 3 fragments of 520, 506, and 130 bp.

Valdés *et al.*,2016

Molecular Diagnosis of BLOs

Primers used for PCR detection

OA1+OI1/OI2c

Add 1 µl of the DNA preparation to 39 µl of the following master mix.

Reagents	Initial concentration	Final concentration
Buffer	10X	1X (4 µl)
DNTP s	5 mM	0.2 mM
$MgCl_2$	50 mM	2 mM
Primer OI1	100mM	1 µM
Primer O12c	100 mM	1 µM
Primer OA1	100mM	1 µM
Taq DNA polymerase	5U/µl	0.3 µl (1.5 U/µl)
Water PCR grade		

Molecular Diagnosis of BLOs

DNA amplification program

OA1+OI1/OI2c

- 35 cycles each at 92°C for 40 s (denaturation step) and 72°C for 90 s (annealing and primer extension at the same temperature).
- Analysis by electrophoresis: 8 µl from the PCR reaction are analyzed on 0.7% agarose gel.
- The two Liberibacter spp. yield the same size amplicon of 1160 bp.
- The *Ca*. L. asiaticus amplicon contains one *XbaI* restriction site and yields two fragments (640 bp and 520 bp) upon restriction digestion, whereas the *Ca.* L. africanus amplicon has two restriction sites and yields three fragments (520 bp, 506 bp and 130 bp).

Master mix for *Xbal* : Water (PCR grade): 12.5 µl, Buffer: 2.0 µl, XbaI: 5.0 units, PCR product: 5.0 µl, Total volume: 20 µl.

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Molecular Diagnosis of BLOs

Primers used for PCR detection

Primers A2 and J5

- Hocquellet *et al.*,1996, proposed the use of primers A2 and J5 (designed based on the DNA sequences of the ß- operon) that allow the detection and the direct identification of the two liberibacter species.
- Primer sequences:

  A2: 5´- TAT AAA GGT TGA CCT TTC GAG TTT- 3’

  J5: 5´- ACA AAA GCA GAA ATA GCA CGA ACA A-3´

www.ippc.int,2007

Molecular Diagnosis of BLOs

DNA amplification program

Primers A2 and J5

Add 2 µl of the DNA preparation to 48 µl of the following master mix.

Regeant	Initial concentration	Final concentration
DNTPs	5mM	0,2 MmM
$MgCl_2$	50 mM	2 mM
PrimerA2	100mM	1µM
Primer J5	100mM	1µM
Tris-HCl pH 8.8		78 mM
BSA		200 µg/ml
$(NH_4)_2$ SO_4		17 mM
ß-mercaptoethanol		10 mM
Taq polymerase	5U/µl	2.5 U/µl
Water (PCR grade)		

DNA amplification program: 35 cycles, each at 92ºC for 20 s, 62ºC for 20s and 72ºC for 45 s. Analysis by electrophoresis: 8 µl from the PCR reaction are analyzed on 0.7% agarose gel. These primers direct the amplification of a 669 bp fragment from *Ca.* L. africanus and a 703 bp fragment from *Ca*. L. asiaticus.

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Molecular Diagnosis of BLOs

Primers used for multiplex PCR detection

Primers GB1/GB3 and A2/J5

- Multiplex PCR was developed for routine detection of the three liberibacters in a single reaction mixture with the following two sets of primers: GB1/GB3 and A2/J5.
- A2: 5′- TAT AAA GGT TGA CCT TTC GAG TTT-3′
- J5: 5′- ACA AAA GCA GAA ATA GCA CGA ACA A –3′
- GB1: 5´- AAG TCG AGC GAG TAC GCA AGT ACT – 3′
- GB3: 5′- CCA ACT TAA TGA TGG CAA ATA TAG –3′

Primers GB1/GB3 amplify *Ca*. L. americanus.
Primers OA1+OI1/OI2c amplify *Ca*. L. asiaticus/*Ca*. L. africanus.

www.ippc.int,2007

Molecular Diagnosis of BLOs

Primers used for multiplex PCR detection

Primers GB1/GB3 and A2/J5

Add 1 µl of the DNA preparation to 39 µl of the following master mix.

Regeants	Initial concentration	Final volume
Buffer	10X	4 µl
MgCl 50 mM	50 mM	1.6 µl
DNTP	5 mM	1.6 µl
Primer A2	100 µM	0.4 µl
Primer J5	100 µM	0.4 µl
Primer GB1	100 µM	0.4 µl
Primer GB3	100 µM	0.4 µl
Water		31.2 µl
Taq DNA polimerase	5U/µl	0.3 µl

DNA amplification program: 35 cycles each at 94ºC for 30 s, 62ºC for 30 s and 72ºC for 60 s. Analysis by electrophoresis: 20 µl from the PCR reaction are analyzed on 1,2 % agarose gel.The amplicons obtained are: 1027 bp fragment for *Ca*. L. americanus and 703 bp fragment for *Ca*. L. asiaticus.

www.ippc.int,2007

Molecular Diagnosis of BLOs

Primers used for duplex PCR detection

Primers LSg2f/LSg2r and LAs/RA-s

- Duplex PCR Coletta-Filho, 2005 designed two sets of primers:
- To detect *Ca.* L. americanus:
- LSg2f - TTAAGTTAGAGGTGAAATCC/LSg2r – CAACTTAATGATGGCAAATA and
- To detect *Ca.* L. asiaticus:
- LAs-TGGTGATAGGGTGGATTTAG/RA-s CAACCTCGAAGAAAACAGGAC.

www.ippc.int,2007

Molecular Diagnosis of BLOs

Primers used for Real Time PCR detection

Four primers and one TaqMan probe

- The Real Time PCR performs with four primers and one TaqMan probe combination based on the 16 rDNA sequences.
- HLBas: Specific to *Ca* Liberibacter asiaticus: 5′ - TCG AGC GCG TAT GCA ATA CG A - 3′
- HLB am: Specific to *Ca* Liberibacter americanus: 5′- GAG CGA GTA CGC AAG TAC TAG - 3′
- HLB af: Specific to *Ca.* Liberibacter africanus: 5′- CGA GCG CGT ATT TTA –3′
- HLBr: reverse primer, recognize the three Ca. spp.: 5′ – CTA CCT TTT TCT ACG GGA TAACGC-3′
- Hlbp: the common probe for the pimers, labed with FAM (6-carboxy-fluorescein) and with BHQ (Black Hole Quencher): AGA CGG GTG AGT AAC GCG
- COX: Positive internal control (cytochrome oxidase gene): 5′-GTA TGC CAC GTC GCA TTC CAG A-3 A -3′
- COXr: reverse: 5′- GGA TGCCCT TAG CAG TTT TGG C- 3′
- COXp: probe labeled with TET (tetrachloro-6-carboxy-fluorescein) and BHQ as quencher: ATC CAG ATG CTT ACG CTG

www.ippc.int,2007

Molecular Diagnosis of BLOs

Primers used for Real Time PCR detection

Four primers and one TaqMan probe

- Specificity:
- HLBaspr (HLBas/HLBp/HLBr) and HLBafpr (HLBaf/HLBp/HLBr) detect the Asian and African strains, respectively.
- HLB aspr can detect African strains and the HLBafpr can detect Asian strains but with higher Ct values.
- HLBampr (HLBam/HLBp/HLBr) detect the Americans strains but can not the Asian and African strains.

Molecular Diagnosis of BLOs

Primers used for Real Time PCR detection

Four primers and one TaqMan probe

Add 2 µl of the DNA preparation to 23 µl of the following master mix.

Reagent	Initial concentration	Volume for one reaction	Final concentration
Buffer	10X	2.5 µl	1 X
$MgCl_2$	50 mM	3 µl	6.0 mM
DNTPs	10 mM each	0.6 µl	0,24 mM
Taq polymerase (5 U/ µl)	5 U/µ	0.2 µl	1 unit
Primer mix HLBas/HLBr or HLBam/HLBr	2 µM each	3 µl	240 nM
HLB probe µM	1 µM	3 µl	120 nM
COX primer mix	2 µM each	3 µl	240 nM
COXp probe	1 µM	3 µl	120 nM
Molecular Grade Water		4.7	

DNA amplification program:

1)95°C for 20 seconds

2) 40 or 50 cycles at 95°C for 1 s. and 58°C for 40 s.

www.ippc.int,2007

Molecular Diagnosis of BLOs

Primers used for PCR detection

Specificity	Primer designation	Sequence	Size (bp)
Papaya bunchy top bacterium	PBTF1	(5'AAAGGTTCTGATTGGTTAGGTG3')	
	PBTR1	(5'ATCTTTATGCTCTCCAACTCCTC 3')	705
Yellow vine disease bacterium	YV1	(5'GGGAGCTTGCTCCCCGG3')	
	YV2	(5'CGCTACACCTGGAATTCTAC3')	643
	YV3	(5'GGTTACCTTTGTTACGACTTCA3')	1433
Citrus greening bacteria	OI1	(5'GCGCGTATGCAATACGAGCGGCA3')	
	OI2c	(5'GCCTCGCGACTTCGCAACCCAT3')	1160

Schaad *et al.*,2001

Molecular Diagnosis of BLOs

Nucleotide sequence of primers used for the amplification of Candidatus (Ca.) Liberibacter species by end-point PCR and qPCR

HLB bacterium	Primer sequence (5′–3′)*	DNA region amplified	Type of PCR	Reference
Ca. Liberibacter americanus	F: AGTCGAGCGAGTACGCAAGTACT R: CAACTTAATGATGGCAAATATAG	16S rDNA	Conventional PCR	Nageswara-Rao et al. 2013; Teixeira et al. 2005*a*
	F: GAGCAGTACGCAAGTACTAG Tp: AGACGGGTGAGTAACGCG R: GCGTTATCCCGTAGAAAAAGGTAG	16S rDNA	qPCR	Kim and Wang 2009; W. Li et al. 2006; Li et al. 2009
Ca. Liberibacter asiaticus	F: CGCGTATGCAATACGAGCGGCA R: GCCTCGCGACTTCGCAACCCAT	16S rDNA	Conventional PCR	Nageswara-Rao et al. 2013; Teixeira et al. 2005*a*
	F: TCGAGCGCGTATGCAATACG Tp: AGACGGGTGAGTAACGCG R: GCGTTATCCCGTAGAAAAAGGTAG	16S rDNA	qPCR	Kim and Wang 2009; W. Li et al. 2006; Li et al. 2009
	F: GCCGTTTTAACACAAAAGATGAATATC Tp: ATAAATCAATTTGTTCTAGTTTACGAC R: ACATCTTTCGTTTGAGTAGCTAGATCATTGA	hyv_I and hyv_{II}†	qPCR	Morgan et al. 2012
Ca. Liberibacter africanus	F: GCGCGTATTTTATACGAGCGGCA R: GCCTCGCGACTTCGCAACCCAT	16S rDNA	Conventional PCR	Nageswara-Rao et al. 2013; Teixeira et al. 2005*a*
	F: CGAGCGCGTATTTTATACGAGCG Tp: AGACGGGTGAGTAACGCG R: GCGTTATCCCGTAGAAAAAGGTAG	16S rDNA	qPCR	Kim and Wang 2009; W. Li et al. 2006; Li et al. 2009

Note: Two PCR systems have been used in HLB disease. The first is based on 16S rDNA sequence, using many sequences of primers and probes (Fujikawa and Iwanami 2012). Primer pair OI1 and OI2c is able to amplify the rDNA Las and Laf species. PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; HLB, Huanglongbing. *F, Forward primer; R, Reverse primer; Tp, Taqman probe. †Intragenic tandem-repeats sequence.

Valdés *et al.*,2016

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 *Ca.* Liberibacter

Genus "*Candidatus* Liberibacter"					
Species/subspecies	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
"*Ca.* L. africanus subsp. capensis"	**OI1/OI2c** 16S rRNA gene **A2/J5** Ribosomal protein genes _-operon **CAL1/J5** 16S rRNA gene	Conventional	Plant (DNA extraction)	Garnier *et al.*, 2000	Amplification from *Calodendrum capense* but not from citrus hosts of huanglongbing disease.
"*Ca.* L. americanus"	**OI1/OI2c** 16S rRNA gene **LSg2f/LSg2r** 16S rRNA gene **A2/J5** Ribosomal protein genes β-operon	Conventional	Plant (DNA extraction)	Coletta-Filho *et al.*, 2005	"*Candidatus* Liberibacter americanus" was proposed in 2005 (Teixera *et al.*, 2005) and thus is not included in the ISPP list (updated to 2004).
"*Ca.* L. americanus"	**GB1/GB3** 16S rRNA gene4	Conventional	Plant (DNA extraction)	Teixera *et al.*, 2005	
"*Ca.* L. asiaticus"	**226-primer pair** Specific DNA fragment (unknown)	Conventional	Plant (DNA extraction)	Hung *et al.*, 1999	
"*Ca.* L. asiaticus"	**Rpl-FIP, Rpl-BIP, Rpl-F3, Rpl-B3** *nus*G-*rpl*KAJL-*rpo*B gene cluster	LAMP assay	Plant (DNA extraction)	Okuda *et al.*, 2005	
"*Ca.* L. africanus" "*Ca.* L. asiaticus"	**fD2/rD1** Universal 16S rRNA gene	Conventional	Plant (DNA extraction) (Immunocapture)	Jagoueix *et al.*, 1994	
"*Ca.* L. africanus" "*Ca.* L. asiaticus"	**fD1/rP1** Universal 16S rRNA gene **OI1/OI2c** **O12c/OA1** **O12c/OI1/OA1** 16S rRNA gene	Conventional	Plant (DNA extraction)	Jagoueix *et al.*, 1996	PrimersOI1/OI2c and O12c/OI1/OA1 amplify both *Ca.* L. species, whereas O12c/OA1primers amplify prefentially "*Ca.* L. africanus". Distinction of the two species requires restriction analysis.

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 *Ca.* Liberibacter

"*Ca.* L. africanus" "*Ca.* L. asiaticus"	**OI2/23S1** 16S-23S rDNA spacer region **TRN1/OI4** Isoleucine genes/ 16S rRNA gene	Conventional	Plant (DNA extraction)	Jagoueix *et al.*, 1997	
"*Ca.* L. africanus" "*Ca.* L. asiaticus"	**A2/J5** Ribosomal protein genes β -operon	Conventional	Plant (DNA extraction)	Hocquellet *et al.*, 1999	Direct distinction of the two species.
"*Ca.* L. africanus" "*Ca.* L. americanus" "*Ca.* L. asiaticus"	**HLBr (reverse)** (common) **HLBaf, HLBam, HLBas (forward)** (specific to each of the three species) 16S rRNA gene **COXf, COXr** Cytochrome oxidase gene **Probe** **COXfp** Cytochrome oxidase gene	Single Multiplex real-time (TaqMan)	Plant (DNA extraction)	Li *et al.*, 2006b	

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

Other Phloem-limited bacteria(*Ca* Phlombacter, PBT,YVD& BDB)

Other bacteria "Bacteria-like Organisms" (BLOs)					
"*Candidatus* Phlomobacter fragariae"					
Species	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
Ca. Phlomobacter fragariae	**Fra4/Fra5** 16S rRNA gene	Conventional	Plant (DNA extraction)	Zreik *et al.*, 1998	Bacterium within group 3 of the gamma subclass of *Proteobacteria.*
Papaya bunchy top disease of Cucurbita (PBT)					
Organism	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/observations
PBT (Gamma-3 proteobacterium associated with BLO disease)	**YV1/YV2** **YV1/YV3** 16S rRNA gene	Conventional	Phloem tissue (DNA extraction)	Davis *et al.*, 1998	
Yellow vine disease (YVD)					
Organism	Primer name Target DNA	Variant of PCR protocol	Sample (treatment)	Reference	Synonyms/bbservations
YVD (Gamma-3 proteobacterium associated with BLO disease)	**YV1/YV2** **YV1/Yv3** 16S rRNA gene	Conventional	Phloem tissue (DNA extraction)	Avila *et al.*, 1998	
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	

Palacio-Bielsa *et al.*,2009

DNA electrophoresis profile of *candidatus* Liberibacter asiaticus/africanus

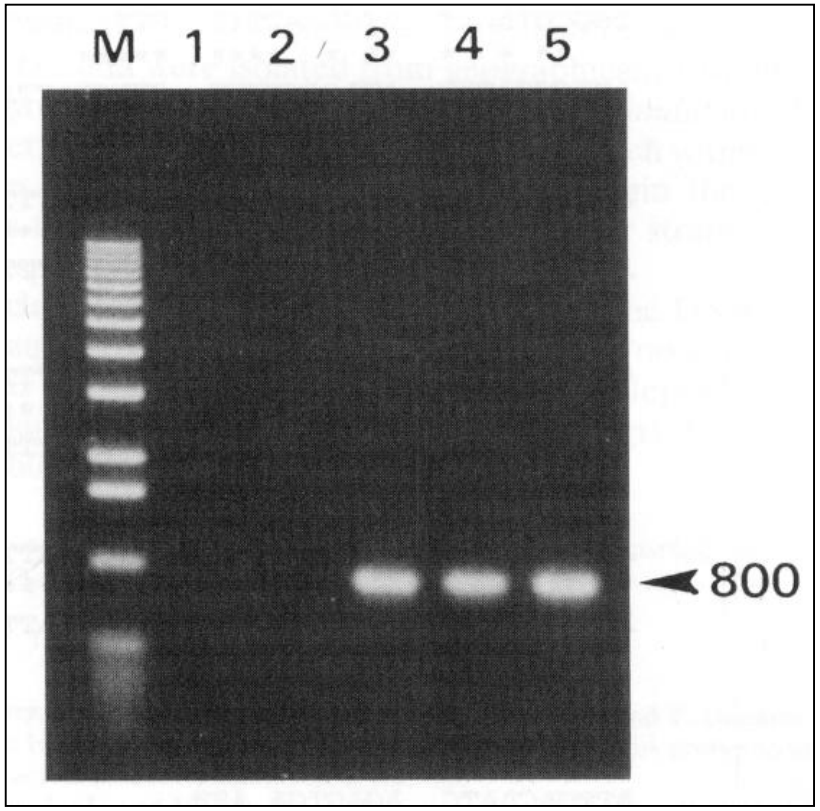


Image courtesy of J.M. Bové & Garnier

Real-time PCR
Primers and probes

Table 2. TaqMan primers

Primer Name	Primer Mix Name	Sequence 5' - 3' Synthesized by Integrated DNA Technologies, Inc.; Primer Purification = Standard Desalting	Target gene	Specific to
HLBas primer	**HLBas primer Mix**	TCG AGC GCG TAT GCA ATA CG	16S rDNA	Las
HLBr primer[1]		GCG TTA TCC CGT AGA AAA AGG TAG		
HLBam primer	**HLBam primer mix**	GAG CGA GTA CGC AAG TAC TAG	16S rDNA	Lam
HLBr primer[1]		GCG TTA TCC CGT AGA AAA AGG TAG		
WG primer	**WG[2] primer Mix**	GCT CTC AAA GAT CGG TTT GAC GG	WG* gene	Psyllids
WGr primer		GCT GCC ACG AAC GTT ACC TTC		

[1]The HLBas and HLBam primer mixes share one common primer, HLBr.
[2] WG refers to glycoprotein.
Note: the "r" in the primer name HLBr and WGr denotes the reverse primer.

Table 3. Probes for Real-time PCR (Synthesized by Integrated DNA Technologies, Inc.)

HLBp probe	56-FAM/AGA CGG GTG AGT AAC GCG/3BHQ-1
WGp probe	5-TET/TTA CTG ACC ATC ACT CTG GAC GC/3BHQ-2

Note: Probes take 7 to 14 business days to arrive once ordered.

Floyd and Krass,2006 & 2012

Pathogenicity test

'*Candidatus* Liberibacter americanus'

- Mottling and stunted growth of *Citrus sinensis* cv. Madame Vinous 3 months after inoculation with '*Candidatus* Liberibacter americanus' strain Brazil1(left) and a water inoculated control (right).

Sechler *et al.*,2009

Pathogenicity test

'*Candidatus* Liberibacter americanus'

Graft transmission

- Buds and growing points from zebra chip(ZC) disease of potato stems were used for grafting to potato cvs. Atlantic, Shepody, Russet Norkotah, and Frontier Russet and tomato cv. Roma in the greenhouse to confirm the presence of the ZC agent.
- Severe symptoms expressed in:

A. Atlantic potato plant, and

B. Roma tomato plant following graft inoculation with a scion from a field-grown potato plant with zebra chip from Texas.

Secor *et al.*,2009;..

Cryogenic storage
'*Ca*. L. asiaticus'(CLas)

- Cells of '*Ca*. L. asiaticus' washed from the surface of Liber A agar medium were stored at -80°C in either:

1. Sterile 50% glycerol in water, or
2. sterile 50% glycerol in saline (0.85% NaCl).

- After 6 months, the cultures were removed from the freezer and streaked onto Liber A agar medium to assess viability.

Sechler *et al.*,2009

Identification of the bacterial pathogens
Mollicutes

Phloem-limited prokaryotes
Disease diagnosis and pathogen diagnostics

1. *Spiroplasma*
2. Phytoplasma

Tenericutes
Plant Pathogenic Mollicutes

- **Domain Bacteria**

  Phylum **"Tenericutes"**
- It now appears the Mollicutes developed from the Gram-positive Firmicutes, and they are now classified with them.
- The Mollicutes are now classified within phylum/division Tenericutes.
- Only, in the 2008 version of MeSH, the Mollicutes is classified as Gram-negative.

Medical Subject Headings (MeSH) is a huge controlled vocabulary or metadata system for the purpose of indexing journal articles and books in the life sciences.

Wikipedia,2008

The class Mollicutes
Phytoplasmas & Spiroplasmas

- Together with acholeplasmas, mycoplasmas, spiroplasmas, and other cell wall less bacteria, phytoplasmas are classified in class *Mollicutes*.
- Mollicutes are distinguished phenotypically from other bacteria by:

1. Their minute size, and
2. Total lack of a cell wall.

- The class of Mollicutes include phytoplasmas and spiroplasma which are bacterial plant pathogens associated with insect vectors.
- Many are able to move about through gliding.

The Mollicutes

Among the first organisms targeted for whole genome sequencing

- The *Mollicutes* were among the first organisms targeted for whole genome sequencing because they have relatively small genomes and many are pathogenic to:

1. plants,
2. animals, or
3. humans.

Modern mollicute systematics
Polyphasic taxonomy

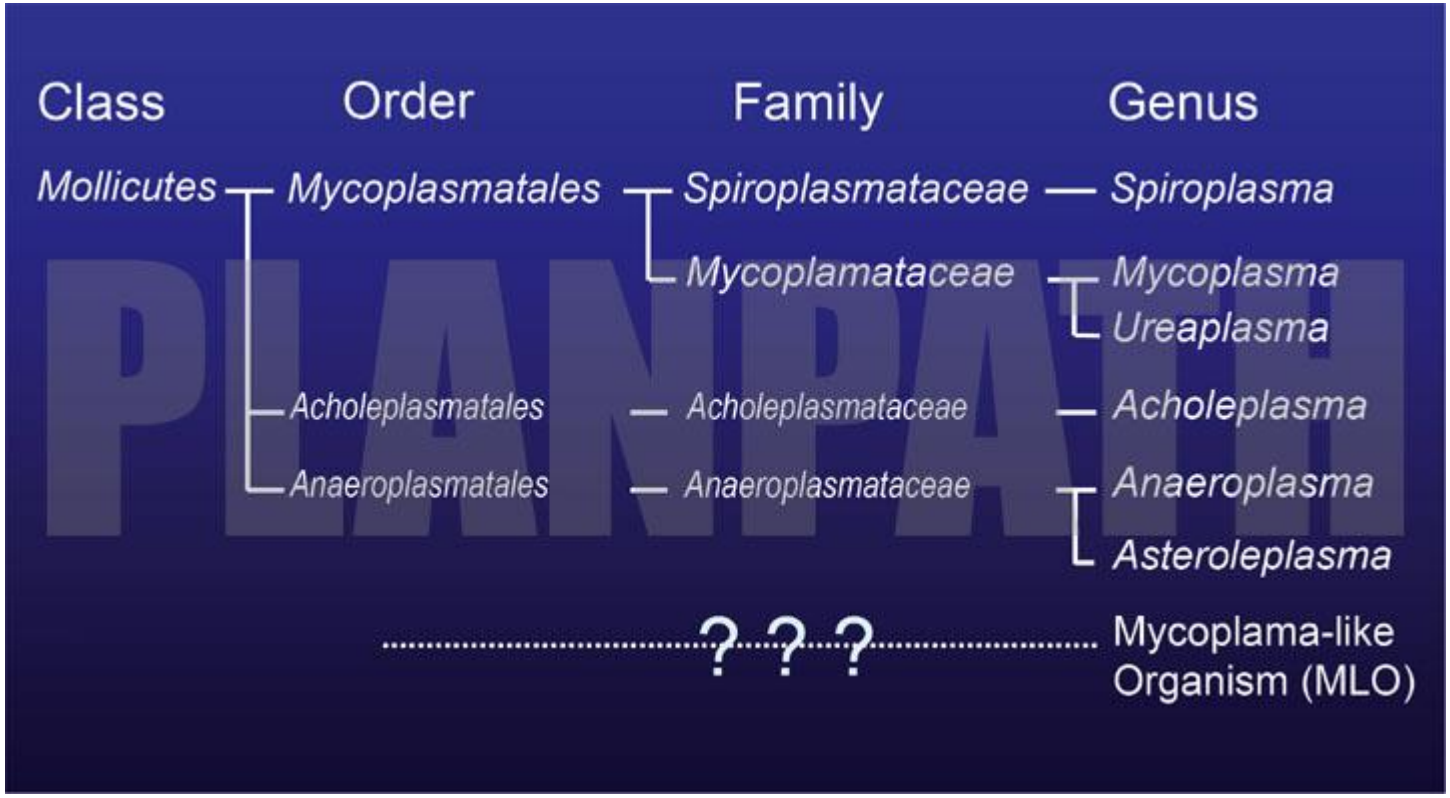
- Modern mollicute systematics has adopted a polyphasic taxonomy system, developed over the last two decades, which is based on:

1. Phenotypic,
2. Genotypic, and
3. Phylogenetic criteria for classification of members of the *Mollicutes*.

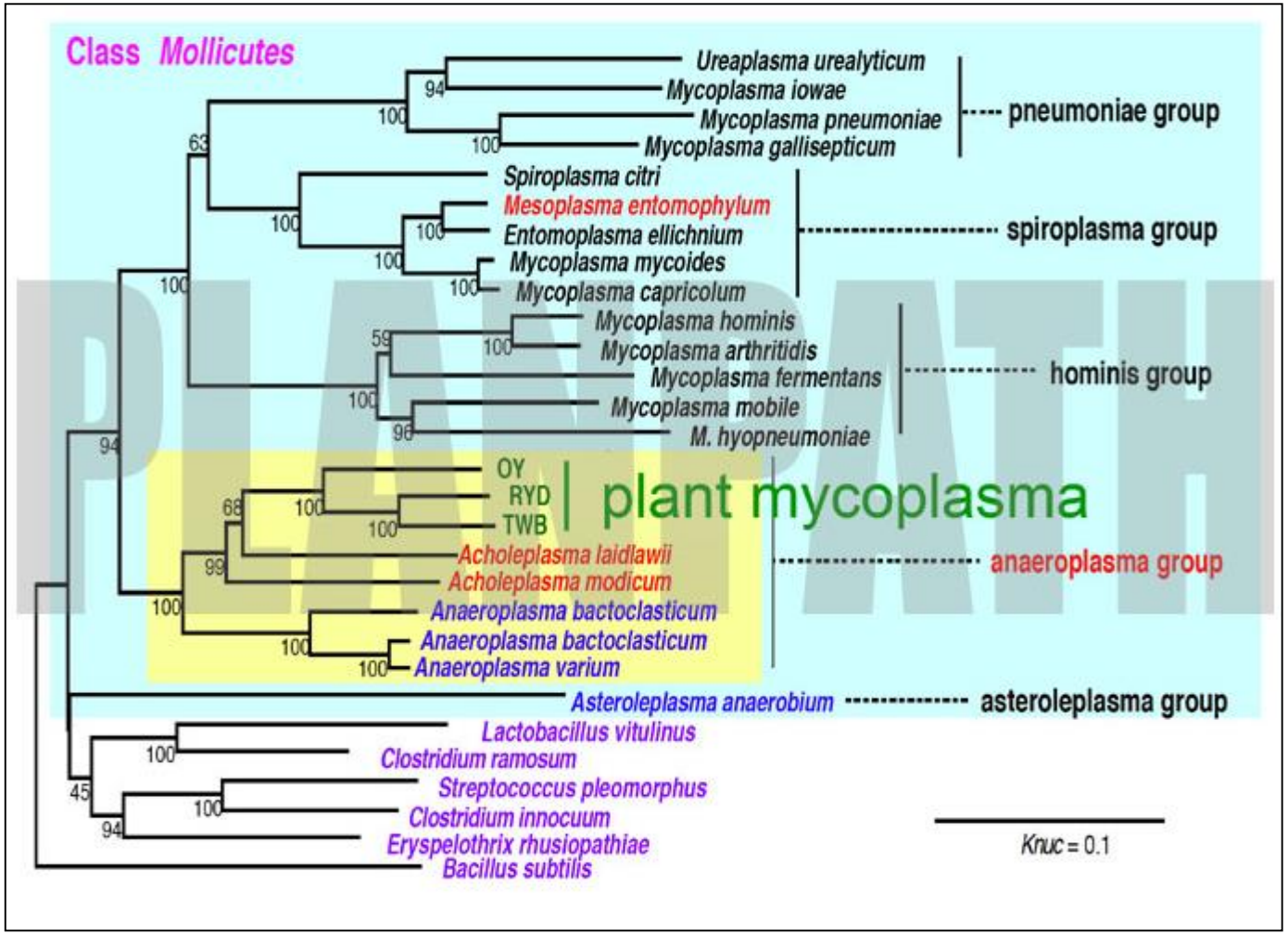
- Comprehensive phylogenetic studies based on 16S rRNA and other housekeeping genes have readily placed phytoplasmas in the class of *Mollicutes*.

Weintraub and Jones,2010

Phylogenetic relationships among *Mollicutes* before molecular phylogenetical analyses



Phylogeny tree
Mollicutes



Characteristics of Mollicutes

- Mollicutes have developed from Gram-positive bacteria by a process of reductive evolution. They cluster within Gram-positive bacteria of the phylum Bacillota.
- These very small pleomorphic microorganisms, typically only 0.2-0.3 μm (200-300 nm) in size can pass through filters with 450-nm pore diameter.
- Cells with a thick three-layer unit membrane.
- Ribosomes similar to those in bacteria.
- Contain DNA and RNA.
- They also have the smallest genome of all bacteria.
- Multiply by binary division.
- Some species (spiroplasmas) can be cultured in artificial media.

Characteristics of Mollicutes

- In agar, spiroplasmas and mycoplasmas are often produce colonies that have a 'fried egg‘ appearance.
- Penicillin resistant and tetracycline sensitive.
- Unlike L-shaped bacteria, they do not revert to bacterial shape when grown in penicillin-deficient medium.
- Susceptible to infection by viruses.
- They do not kill their hosts but they produce predominantly chronic infections.

Mollicutes

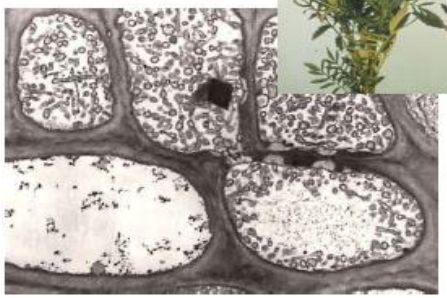
Phytoplasmas & Spiroplasmas

MOLLICUTES

Phytoplasma

- Cannot be cultivated
- Small genome (500-1000 kb)
- Greater than 200 diseases
- Pleomorphic
- Found in phloem sap, insect organs
- Leafhopper-transmitted

Aster yellows

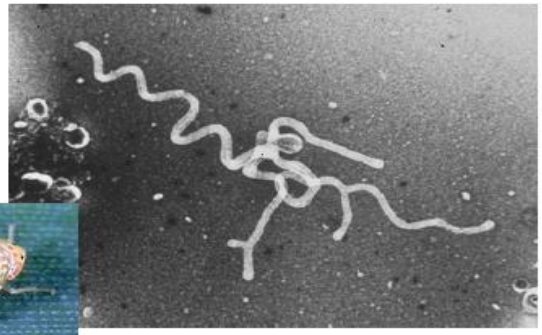


www.apsnet.org

Spiroplasma

- Can be cultivated
- Small genome (500-1000 kb)
- Relatively few diseases
- Helical to branched filaments
- Motile but no flagella
- In phloem sap
- Leafhopper-transmitted
- Can live saprophytically

Corn stunt



www.zarzy.com

www.apsnet.org

Cuppels Biology 418a

Mollicutes
Phytoplasmas & Spiroplasmas

- Plant-infecting phytoplasmas and spiroplasmas are both found in the phloem tissue.
- Neither phytoplasmas nor spiroplasmas are transmissible by mechanical contact.
- They can only be transmitted by vectors, particularly leafhoppers.
- A few can be transmitted by psyllids.
- Transmissible pathogens which can induce systemic infection in their host plants.

Major characteristics
Mollicutes

Table 1. Major characteristics and taxonomy of the class Mollicutes.

Classification	No. of recognized species	Genome size (kb)	Genome G+C (mol%)	Cholesterol requirement	Distinctive properties	Habitat
Mycoplasmataceae						
Genus I: *Mycoplasma*	107 (11)[a]	580–1350	23–40	Yes	Optimum growth 37°C	Humans and animals
Genus II: *Ureaplasma*	7	760–1170	27–30	Yes	Urease positive	Humans and animals
Entomoplasmataceae						
Genus I: *Entomoplasma*	6	790–1140	27–29	Yes	Optimum growth 30°C	Insects and plants
Genus II: Mesoplasma	12	870–1100	27–30	No	Optimum growth 30°C	Insects and plants
Spiroplasmataceae						
Genus I: *Spiroplasma*	34	780–2220	24–31	Yes	Helical filaments	Insects and plants
Acholeplasmataceae						
Genus I: *Acholeplasma*	14	1500–1650	26–36	No	Optimum growth 30–37°C	Animals and plant surfaces
*Anaeroplasma*taceae						
Genus I: Anaeroplasma	4	1500–1600	29–34	Yes	Obligate anaerobes, oxygen sensitive	Bovine-ovine rumen
Genus II: Asteroleplasma	1	1500	40	No		
Undefined taxonomic status						
Phytoplasma	ND[b]	530–1185	23–29	ND	Uncultured in vitro	Insects and plants

Abbreviation: ND, not determined.
[a]The number of Candidatus species is given in parentheses and includes the hemoplasmas (*Eperythrozoon* and *Haemobartonella*) recently transferred to the genus *Mycoplasma* (Neimark et al., 2001).
[b]The taxonomic status of the uncultured phytoplasmas has not been finally defined; seven Candidatus *Phytoplasma* spp. have so far been published.
Updated and modified from Razin et al. (1998).

The Prokaryotes (chapter 1.2.29),2006

Differentiation characteristics of Mollicutes

Property	*Spiroplasma*	Phytoplasma
Morphology	Helical	Non-helical
Cultured	Yes	No
UGA codon	Trp	Stop
Functional sugar PTS	Yes	Probably not
Evolutionary relationship	*Spiroplasma* branch	*Acholeplasma* branch
Spiroplasma species(named)	31	-
Phytopathogenic *Spiroplasma* spp.	3	-
Characterized phytoplasmas	-	51
Phytoplasma groups	-	14
Plant diseases	*S. citri*: Citrus stubborn, many others *S. kunkelii*: Corn stunt *S. phoeniceum*: Periwinkle yellows	Over 300 in 98 families
Location	Sieve tubes	Sieve tubes
Insect vector	Leaf hoppers	Leaf hoppers, psyllids

Phylogenetic studies suggest that the common ancestor for phytoplasmas is Acholeplasma laidlawii, in which the triplet coding for tryptophan (trp) is UGG. In the other prokaryotes, enclosing mycoplasmas and spiroplasmas, trp is coded by UGA, while phytoplasmas use UGA as a stop codon.

Bové and Garnie,1998

Mode of actions in Mollicutes

- Disease symptoms are consistent with the disturbance of plant hormone balance.
- For example, infected plants may be sterile or stunted or develop witches' brooms owing to the release of axillary buds from apical dominance.
- Internally, infected plants often have extensive phloem necrosis.

Identification of the bacterial pathogens *Spiroplasma*

Disease diagnosis and pathogen diagnostics

Distinguishing Properties

Property	Mollicutes (*S. citri*)	Eubacteria	Archae-bacteria
Cell wall	Absent	Present	Present*

Characteristics of the *Spiroplasma*

Spiroplasma shares the simple metabolism, parasitic lifestyle, fried-egg colony morphology

- A genus of Mollicutes, without cell walls.
- Shares the simple metabolism, parasitic lifestyle, fried-egg colony morphology and small genome of other Mollicutes.
- A distinctive helical morphology, unlike Mycoplasma.
- Most spiroplasmas are found either:
- In the gut or hemolymph of insects, or
- In the phloem of plants.
- *Spiroplasmas* are fastidious organisms, which require a rich culture medium.
- Typically they grow well at 30°C, but not at 37°C.
- A few species, notably *Spiroplasma mirum*, grow well at 37°C (human body temperature), and cause cataracts and neurological damage in suckling mice.

Phylogenetic reconstructions
Based on 16S rDNA sequence

- Phylogenetic reconstructions based on 16S rDNA sequence strongly support the closely related serogroups.
- If an isolate cannot be cultivated, then it can only be considered for *Candidatus* status.
- The ICSP Subcommittee on Taxonomy of *Mollicutes* recently recommended that 16S rDNA sequence information be published for all newly described species (ICSP Subcommittee, 11 July 2004 meeting).

Redgassa and Gasparich,2006

Evolutionary relationship of spiroplasmas to other Eubacteria

- Phylogenetic analyses indicate that the class *Mollicutes* is a terminus in the evolution of Gram-positive bacteria, arising from a gram-positive, low G+C content Clostridial lineage of the Eubacteria.
- Recent phylogenetic analyses using the amino acid sequence of phosphoglycerate kinase (Pgk) as a molecular marker indicate a similar derivation, but also suggest that modern mollicutes may be more closely related to the *Streptococcus/Lactobacillus* spp. than to *Clostridium* and *Bacillus* spp.

Redgassa and Gasparich,2006

Evolutionary relationship of spiroplasmas to other Eubacteria

- This evolutionary pathway clearly shows that the *Spiroplasma* genus arose due to reductive evolution, rather than from a primitive organism that evolved prior to the development of the cell wall.
- Recent divergence within *Spiroplasma* can probably be explained by co-evolution with a range of hosts.

Redgassa and Gasparich,2006

The genus *Spiroplasma*

The three characterized phytopathogenic spiroplasmas

- Most spiroplasmas appear to be commensals, but there are a few cases of mutualism or pathogenic.
- Spiroplasmas are most often found in association with insects and ticks.
- Description of the genus *Spiroplasma* began with:

1. The isolation and characterization of the causative agent of citrus stubborn disease *S. citri* .
2. The second described member of the genus *Spiroplasma*, *S. kunkelii*, causes corn stunt disease.
3. The third described member of the genus *Spiroplasma* is *S. phoeniceum*, causes aster yellow (or Periwinkle) disease.

Redgassa and Gasparich,2006

Diseases caused by spiroplasmas

The three characterized phytopathogenic spiroplasmas

Spiroplasma citri	**Citrus stubborn disease**
Spiroplasma kunkelii	**Corn stunt disease**
Spiroplasma phoeniceum	**Periwinkle yellows**

Spiroplasma citri causes stubborn disease(citrus little leaf disease) in Citrus spp. and diseases in other plants (non-rutaceous plant species such as periwinkles).

***Ca.* Phytoplasma phoenicium'**
(Associated with almond lethal disease)

The genus *Spiroplasma*

Differential criteria used to characterize the *S. citri* from *S. kunkelii*

- Common features with *Spiroplasma citri*: Chemoorganotrophic, facultatively anaerobic metabolism, pleomorphic cells lacking cell walls and periplasmic fibrils, and bounded by a single membrane, motile, with helical morphology, and able to pass through a membrane filter of 220 nm pore size but not through 100 nm pores.
- Distinguished features of *Spiroplasma kunkelii*:
- Cells vary from helical filaments 100 to 150 nm diam. and 3 to 10 µm long, to non-helical filaments or spherical cells 0.3 to 0.8 µm diam.

Bradbury,1991

The genus *Spiroplasma*

Common features of *S. kunkelii* with *Spiroplasma citri*

- Like *S. citri*, sterols are required for growth of *S. kunkelii*, thus cholesterol in amounts of 1 to 20 µg/ml is stimulatory.
- Glucose, fructose and lactose are fermented, and arginine is hydrolysed whether or not glucose is present in the medium. In the presence of glucose the reaction is masked at first by acid production. The alkali (red) reaction of the indicator is seen later.
- Urease and phosphatase are negative; coagulated serum not liquefied; film and spot reaction positive; guinea pig erythrocytes not haemadsorbed.
- Temperature range for growth 20° to 32°C; opt. temp. ca. 30°C. The G + C content of the DNA is 26 mol%+1.
- Genome mol. wt. 109. Type strain ATCC 29320.

Bradbury,1991

The genus *Spiroplasma*

Differential characteristics between *S. kunkelii* and *S. citri*

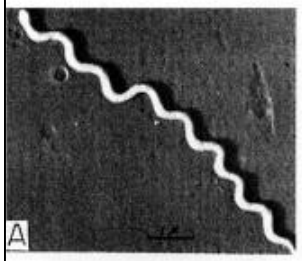
- *Spiroplasma kunkelii* differs from *S. citri* in its:
- negative phosphatase reaction, its narrower temperature range and lower optimum, as well as in serological characters and polyacrylamide gel electrophoresis patterns of whole cell proteins (Whitcomb *et al.*,1986).
- This species forms subgroup 3 of group I of the genus *Spiroplasma* as tabulated in Whitcomb & Tully (1984).
- See also IMI Description 1046.

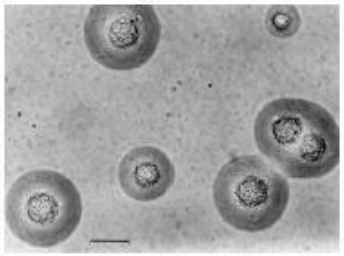
Bradbury,1991

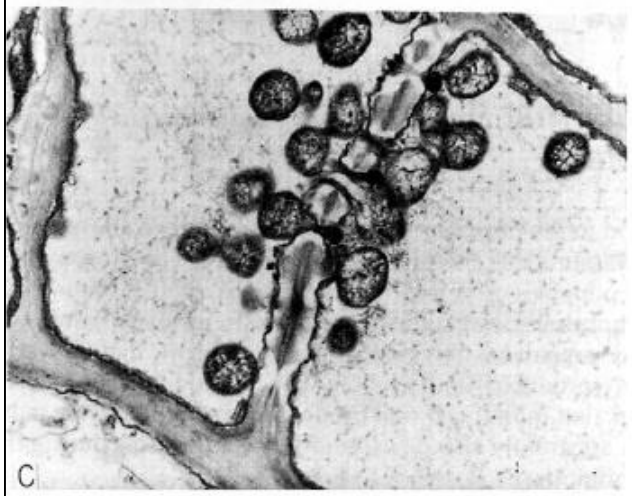
Disease symptoms

Spiroplasma Diseases

"fried egg" colonies







Corn Stunt
S. kunkelii

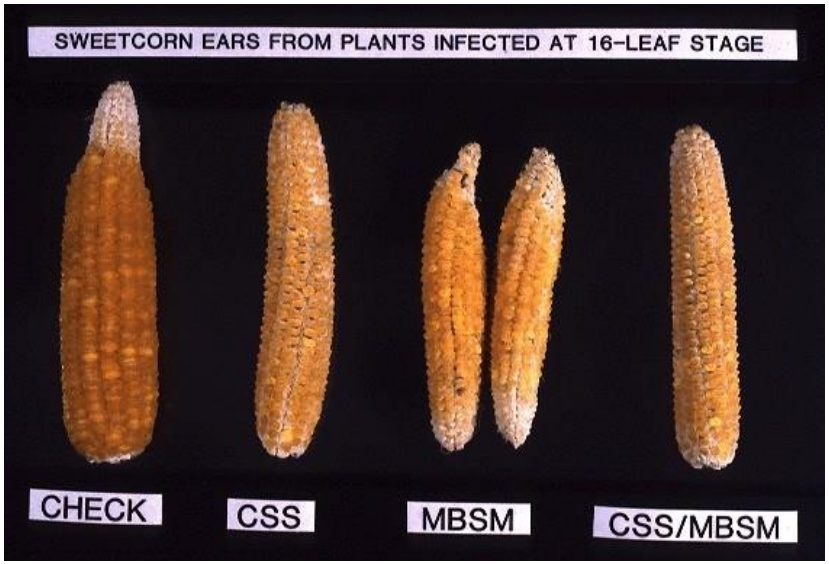
Citrus Stubborn Disease
S. citri

Corn stunt disease symptoms

Spiroplasma kunkelii

- Symptoms on sweet corn ears.
- left to right:

1. control;
2. infected with *S. kunkelii* (CSS);
3. with maize bushy stunt mycoplasma (MBSM); and
4. with both diseases (transmitted by leafhopper *Dalbulus maidis*).



CABI,2014

Phloem discoloration and necrosis caused by *Spiroplasma* infection

Vectors

At least six species of leafhoppers can transmit *Spiroplasma citri*.

Dalbulus maidis

Leafhopper vector of corn stunt *spiroplasma*

Spiroplasma species and their hosts and disease manifestations

Species	Principal host	Disease manifestations
S. alleghenense	*Panorpa helena* scorpion fly	U
S. apis	Bees and flowers	"May disease" of bees
S. cantharicola	*Cantharis* beetle	U
S. chinense	*Calystegia hederaceae* flowers	U
S. chrysopicola	*Chrysops* sp. deerfly	U
S. citri	Dicots and *Circulifer* leafhoppers	Citrus stubborn
S. clarkii	*Cotinus* beetle	U
S. corrscae	*Ellichnia corrusca* firefly	U
S. culicicola	*Aedes* mosquito	U
S. diabroticae	*Diabrotica undecimpunctata* corn rootworm beetle	U
S. diminutum	*Culex annulus* mosquito	U
S. floricola	Insects and flowers	U
S. gladiatoris	*Tabanus gladiator* horsefly	U
S. helicoides	*Tabanus abactor* horsefly	U
S. insolitum	*Eristalis* fly and flowers	U
S. ixodetis	*Ixodes pacificus* ticks	U
S. kunkelii	Maize and leafhoppers	Corn stunt
S. lampyridicola	*Photuris pennsylvanicus* firefly beetle	U
S. leptinotarsae	*Leptinotarsa decemlineata* (Colorado potato beetle)	U
S. lineolae	*Tabanus lineola* horsefly	U
S. litorale	*Tabanus nigrovittatus*	U
S. melliferum	Bees	Honey bee spiroplasmosis
S. mirum	*Haemaphysalis* rabbit ticks	Suckling mouse cataract disease[a]
S. monobiae	*Monobia* wasp	U
S. montanense	*Hybomitra opaca* horsefly	U
S. phoeniceum	*Catharanthus roseus*	Periwinkle disease
S. platyhelix	*Pachydiplax longipennis* dragonfly	U
S. poulsonii	*Drosophila willstoni*	Male lethality[b]
S. sabaudiense	*Aedes* mosquito	U
S. syrphidicola	*Eristalis arbustorum* syrphid fly	U
S. tabanidicola	*Tabanus abdominalis limbatinervis* horsefly	U
S. taiwanense	*Culex tritaeniorhynchus* mosquito	U
S. turonicum	*Haematopta* horsefly	U
S. velocicresens	*Monobia* wasp	U

Abbreviation: U, not known.
[a]Experimental.
[b]Sex-ratio trait.
Data from Williamson et al. (1998).

The Prokaryotes (chapter 1.2.30),2006

Isolation and Culturing

1. Isolation of *Spiroplasma citri* from fruit aspetically without filtration

- *S. citri* can be isolated from seeds or fleshy tissue in small fruits from stubborn suspect trees. The procedure is as follows:
- Wash the fruit in a 1% sodium hypochlorite solution or in soap and water, and dry in the open air or with a sterile towel.
- First flame the equator of the fruit, then cut into the fruit to an approximate depth of 1.25 cm completely around the equator.
- Twist the two halves of the fruit apart.
- Pick out visible aborted seeds with sterile tweezers and place them in a tube of 10 ml of media (use one seed per tube to avoid excessive acid).
- Plug the tubes and incubate at 30-32°C.
- Look for colour change as shown in next Fig. within 7-14 days.

Isolation of *Spiroplasma citri* from fruit aseptically without filtration

- Diagnosis for stubborn disease in culture medium.
- The presence of *Spiroplasma citri* causes a pH change and turns the phenol red indicator dye from reddish-orange (right) to amber-yellow (left).
- Confirmation should be made by taking a drop of liquid from the tube on the left, and observing it under a dark-field microscope.

Isolation of *Spiroplasma citri* from fruit aseptically without filtration

- Examine a drop from each tube showing a colour change under a dark-field microscope for the presence of spiroplasma.
- In an actively growing culture the number of small helical organisms is high.
- Similar aseptic techniques are possible throughout the year using albedo tissue (preferably from immature fruits) and other phloem-containing tissues such as the columella, provided adequate surface sterilization is possible.
- Ten to 20 ml of media may be needed to reduce the effect of inhibitors.

Isolation and Culturing

2. Isolation of *Spiroplasma citri* from plant tissues using filtration

- This is the method of choice for most young leaves, stems and other specimens:
- If the sample is dirty, wash away as much dirt as possible in running water.
- Soak the specimen in 1% sodium hypochlorite for five minutes.
- Rinse in sterile distilled water.
- Place specimen in about 5 ml of medium in a sterile flat dish (such as one-half of a Petri dish) and chop with a sterile razor-blade. This step can also be applied to aborted seed or columella tissue.
- After five minutes or more, filter the medium containing the chopped tissue through a 0.45 µm filter, using slight suction. If penicillin-resistant bacteria are present, finer filters of 0.2 or 0.22 µm may be needed.

Isolation and Culturing

Isolation of *Spiroplasma citri* from plant tissues using filtration

- Transfer aseptically 1 ml of the filtrate to about 10 ml of medium; repeat to make duplicate tubes. If filtrate is limited, use 0.3-3 ml of medium. It is best to make more than one dilution because of inhibitors.
- Incubate tubes at 30-32°C until a colour change is noted. If the organism is present, colour will change in seven to 14 days, and tubes should be held for three to four weeks. Always have known positive controls for comparison.
- Whenever colour change is noted, examine a drop from each tube under a dark-field microscope for the presence of spiroplasmas.
- Transfer cultures promptly to avoid deterioration and store or lyophilize as desired.

Media for spiroplasmas
Serum and serum-free media

- Serum is probably the most important supplement of the culture medium.
- Fetal bovine serum (FBS) has proved superior for most spiroplasmas.
- Serum-free media were also formulated for the culture of some fastidious strains of corn stunt spiroplasma, *Spiroplasma kunkelii*.
- Serum-free media were contained bovine serum albumin and lipids (fatty acids, cholesterol, phosphatidic acid, and phospholipids).
- Davis *et al.*,1988 recommend medium LD59 for *S. kunkelii* because some strains make poor growth in media containing serum.

Lee and Davis,1984 and 1989; Bradbury,1991

A modified medium for *Spiroplasma citri*

- Horse serum or cholesterol was required for growth.
- The medium is a modification of that of Saglio *et al*.,1971 and Fudl-Allah, Calavan and Igwegbe,1972.
- The yeast extract is omitted (Igwegbe,1978).
- The foetal bovine serum may give better results than horse serum, and the serum should be mycoplasma screened by the manufacturer.

Distilled water	780 ml
PPLO broth	21g
Fructose	1g
Glucose	1g
Sucrose	10g
Sorbitol	50g
Tryptone	1g
Phenol red (1 mg/ml)	10 ml
Foetal bovine (or horse) serum	100 ml
Penicillin G (25mg/ml)	25 ml

Media for Rapid Growth of *Spiroplasma citri* and *Spiroplasma kunkelii*
Media LD8, LD8A and LD59

- Several media (LD series) were developed for cultivation of *Spiroplasma citri* and *Spiroplasma kunkelii*.
- The maximum titer of *S. citri* grown in one of the new media, LD8, reached over 6×10^9 colony-forming units per milliliter, with an estimated doubling time of about 4 hr at 31°C.
- In another medium, LD8A, *Spiroplasma kunkelii* grew to a titer of about 2×10^9 CFU/ml, with a doubling time of 11-12 hr at 31°C.
- Media LD8 and LD8A were highly suitable for primary isolation of *S. citri* and *S. kunkelii* , respectively, from diseased plants.

Lee and Davis,1983; Bradbury,1991

C-3G and modified C-3GH(HEPES buffer added) media

Spiroplasma kunkelii

- The C-3G medium of Liao & Chen may be used for isolation and growth of *Spiroplasma kunkelii* or this may be modified by addition of HEPES(N'-2-hydroxyethyl piperazine -N-2-ethanesulfonic acid) buffer, giving C-3GH (M.J. Davis, 1990).
- Gamma globulin-free horse serum is heat inactivated at 56°C for 40 min, filter sterilized and added to the other ingredients after they have been mixed, adjusted to pH 7.4, autoclaved and cooled.

Distilled water	75 ml
Difco PPLO broth base	1.5g
HEPES buffer	1.4 g
Sucrose	12g
Phenol red (0.1% aqueous sol.)	1 ml
gamma globulin-free horse serum	20 ml

Solidified with 0.8% agar. If desired, 80,000 units of penicillin-G may be added to inhibit unwanted bacteria.

Bradbury,1991

LD59 for *S. kunkelii*

- LD59 contains:
- 15 g Difco PPLO broth base, 1 g fructose, 120 g sucrose, 0.6 g L-arginine, 0.6 g L-asparagine, 0.4 g L-methionine, 0.4g α-ketoglutarate, 0.4 g pyruvate, 2 g lactalbumin hydrolysate, 7 g HEPES buffer and 200 ml BSA-lipid solution.
- The BSA-lipid solution is prepared from:
- 0.66 mls Lipid stock I (0.6 ml Tween 80, 20 mg phosphatidic acid, 0·075 ml linoleic acid, 60 mg palmitic acid, and 40 mg cholesterol dissolved in 2 ml absolute alcohol), and
- 3.5 ml Lipid stock II (12 mg lysophosphatidyl choline from egg yolk, 12 mg lysophosphatidyl choline from soybean, and 20 mg phosphatidyl choline from egg yolk dissolved in 10 ml absolute alcohol), which are dissolved in a solution of 9 g bovine serum albumin in 200 ml distilled water at 37°C.

Bradbury,1991

LD59 for *S. kunkelii*

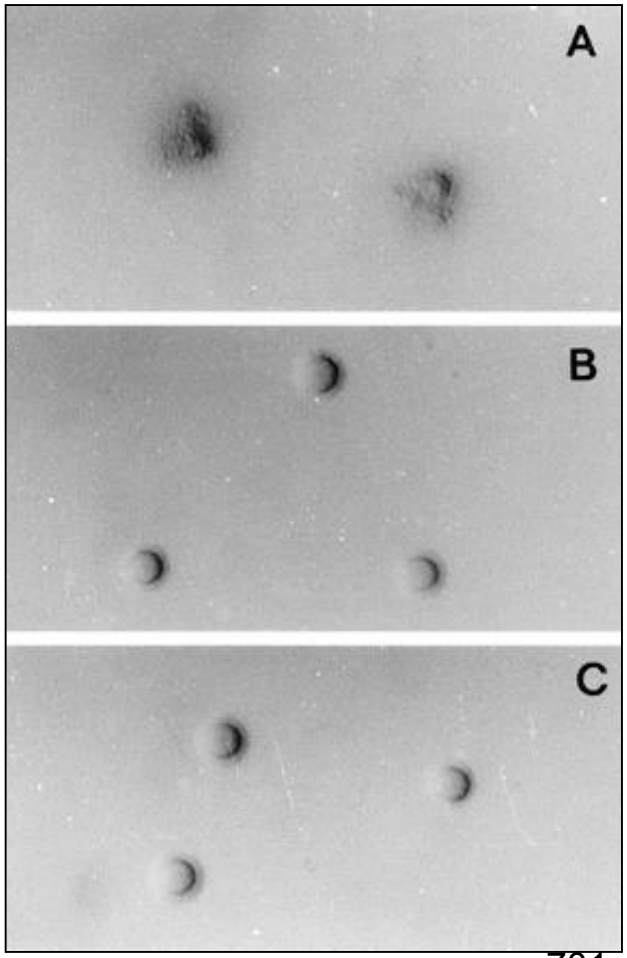
- Growth is better in broth media, but for examination of colonies and selection for purity, semi-solid media can be prepared by adding agar at 0.8-2.2%.
- Colonies on the lower concentrations of agar are diffuse and granular in appearance, while on media with 2.25% agar they are of the classical 'fried egg' type with a denser central zone.
- Maintenance in culture in any of the known media leads after a time to loss of natural pathogenicity to plants, which may be accompanied by an increase in the deleterious effects on leafhoppers experimentally inoculated with the cultured organism.
- Growth is inhibited by homologous antiserum and by tetracycline antibiotics but not by penicillin.

Bradbury,1991

Colony morphology
Spiroplasma kunkelii

- On serum-free medium LD59,

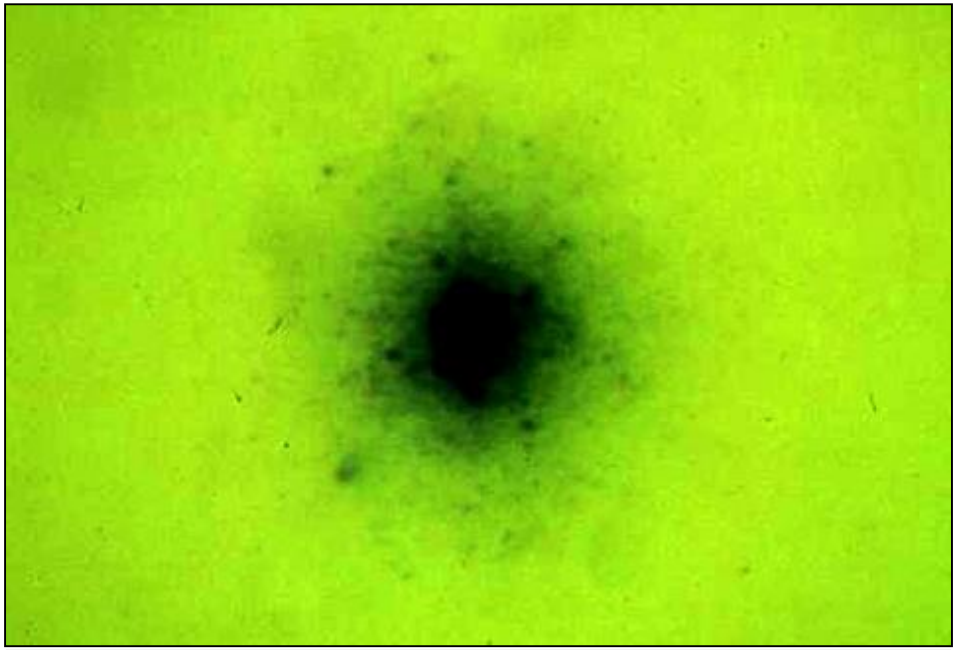
1. non-helical strains of *S. kunkelii* produce minute 'fried egg' colonies (approximately 0.2 mm in diameter after 20 days' incubation),
2. while partially helical strains produce small colonies with granular centres surrounded by satellite colonies.



Lee & Davis,1984;1989

Spiroplasma colony

Spiroplasma shares fried-egg colony morphology



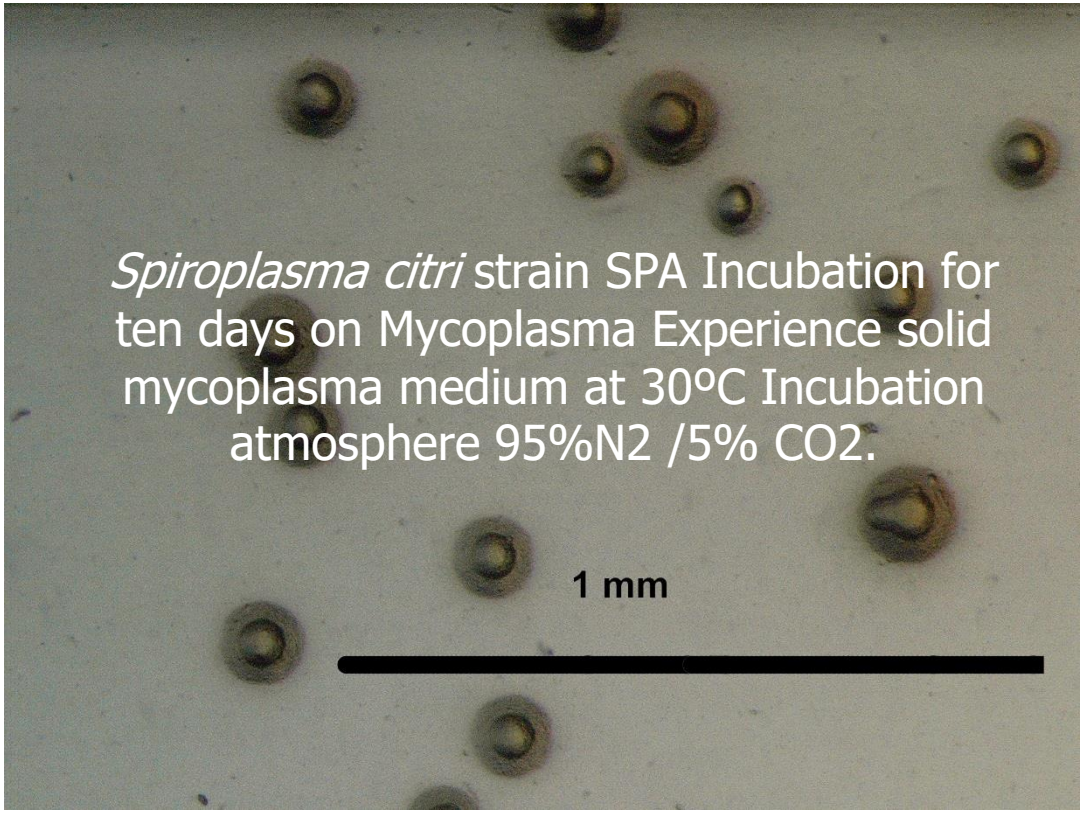
Spiroplasma colonies grown on agar - solidified medium. Note satellite colonies.

Photo: J. Fletcher

The Prokaryotes (chapter 1.2.30),2006;..

Spiroplasma colony

Spiroplasma shares fried-egg colony morphology



Yolk media for cultivation of spiroplasmas

S. citri and *S. kunkelii*

- Citrus stubborn spiroplasma (*S. citri*) and honeybee spiroplasma grew favorably in C-3G basic medium containing 5% yolk fluid or 10% amniotic fluid.
- The serum constituent in serum media could be substituted by yolk, but yolk could not substitute for PPLO broth or sucrose.
- Yolk solution clarified by heating at 56°C graus for 30 min, centrifuging at 10,000 rpm for 30 min, and membrane (0.45 micron) filtering, was slightly better for growth than unclarified yolk solution which hindered microscopic observation.
- *S. citri* grew better in a media with low yolk content (1.25-0.65%), while *S. kunkelii* grew better in 2.5-5.0% yolk medium formulated with phosphate buffer solution (0.01M) at pH 7.2-7.6. SR-3 grew equally well in any medium containing over 0.65% yolk.
- The yolk media were much cheaper than serum media.

Su and Lin,1993

Isolation and culture of *S. citri*
In brief

- Petiole leaf midribs or fruit columella were excised, surface disinfested and diced with a sterile razor blade in 5 ml of LD8 broth medium, passed through a 0.45-µm filter, and incubated at 30°C.
- The presence of *S. citri* was confirmed after 3 to 14 days by examining 10 µl of culture medium by dark-field microscopy at ×400 to 1000 for the presence of motile, helical *spiroplasma*.
- Fried-egg shape colonies were obtained when *S. citri* was subcultured on C-3G solid medium.

Mannaa *et al.*,2013;Ayman *et al.*,2006

Purification
Spiroplasma spp.

- A pure culture is obtained by initial filtration through a 220 nm filter, which will block most microbes except mollicutes.
- The filtrate is then used in a dilution cloning technique to isolate a pure culture.
- This procedure is repeated three times.
- The emerging strain is then designated as the representative strain, which, if the taxon is named, will become the type strain.

Redgassa and Gasparich,2006

Cell characteristics

- Like phytoplasmas and mycoplasmas, spiroplasmas are:
- Pleomorphic;
- Lack a cell wall;
- Sensitive to tetracycline, and
- Resistant to penicillin.

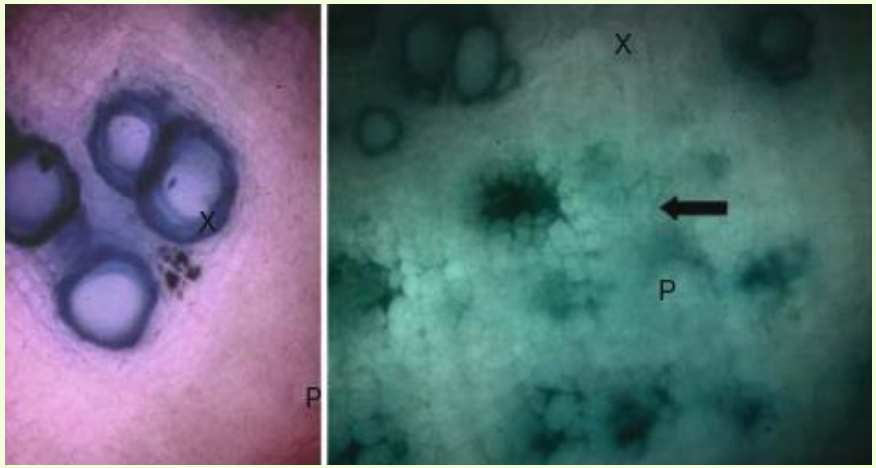
Detection of *S. citri*

Dark-field or phase contrast microscopy
Fluorescent microscopy using Dienes', DAPI, or other fluorescing stains
Electron microscopy
Serology
Ribosomal-sequence PCR
DNA-DNA hybridization

DAPI 4´,6-diamidino-2-phenylidine; a fluorescent DNA binding dye; used to label chromosomes.

Detection of Spiroplasmas
Dienes' stain

- Horseradish root cross section, stained with Dienes' stain.
- Large, thick-walled xylem vessels of both healthy (Panel A) and diseased (Panel B) plants retain the blue dye.
- Blue stained cell patches (arrow) occur in the phloem region in diseased plants only.



The Prokaryotes (chapter 1.2.30),2006

Detection of Spiroplasmas

The fluorescent dye DAPI

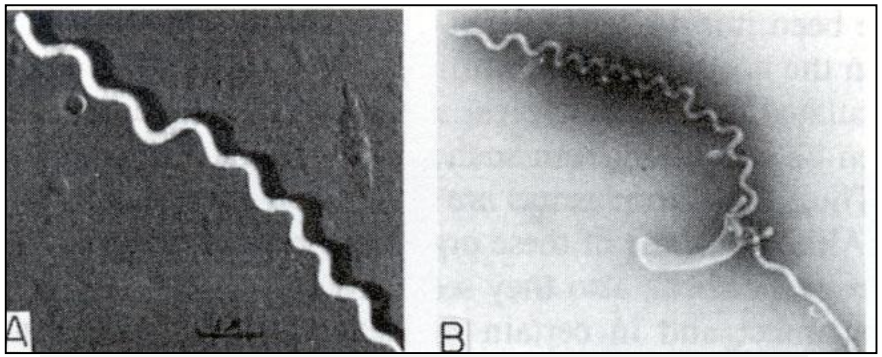
- The fluorescent dye DAPI (4′,6-diamidino-2-phenylindole) binds to DNA in a nonspecific manner.
- Although the binding is nonspecific, labeling host DNA in the nucleus and organelles as well, the dye has been used widely for detection of mollicutes within plant tissues.

Seemuller and Kirkpatrick,1996; Lee *et al.*,2001

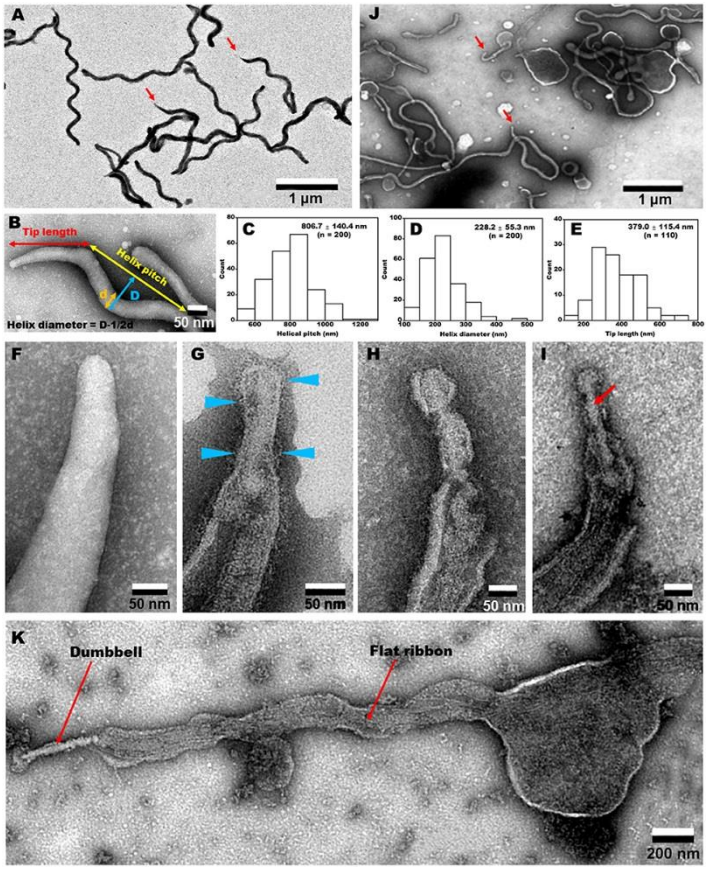
Cell characteristics

Negative-staining EM images of the cells

Spiroplasma cells



The EM images of negatively-stained intact cells revealed helical cell shapes.

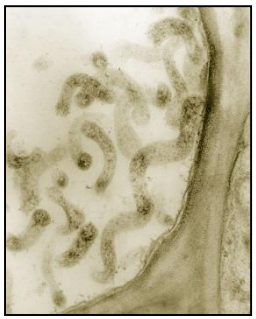


A dumbbell-like structure is connected by a flat ribbon.

Liu *et al.*,2017;....

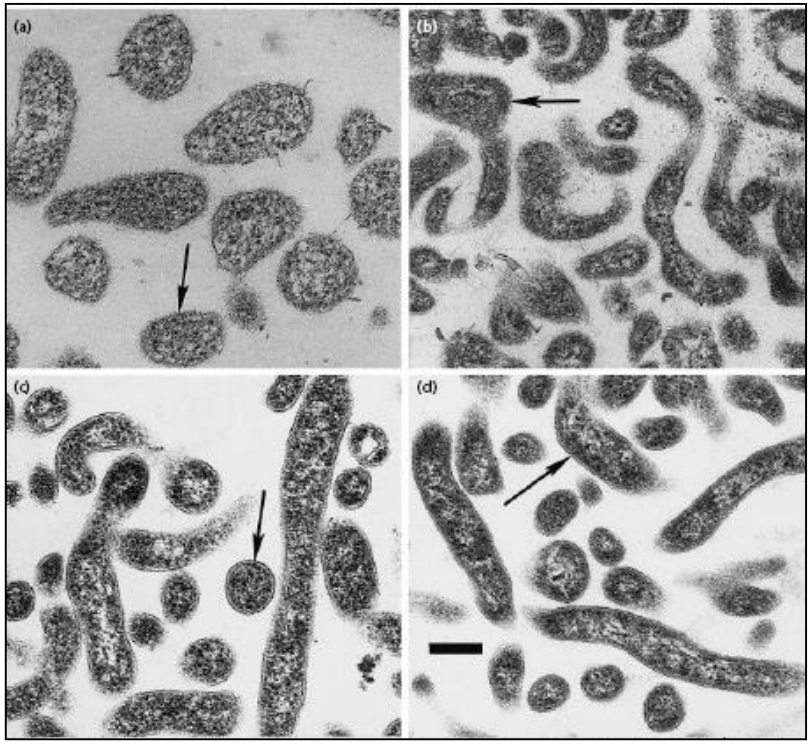
Electron micrograph of some *Spiroplasma* cells

Some unusual strains



Corn Stunt Spiroplasma in phloem cells.

- Some strains were unusual in that they often appeared to grow in a non-helical form when examined by conventional dark-field techniques.
- Electron microscopic observations revealed (Fig. 1a, b) that the cells were bound by a single cytoplasmic membrane.



Arrows indicate unit membrane. Bar, 200 nm.

Williamson *et al.*,1998

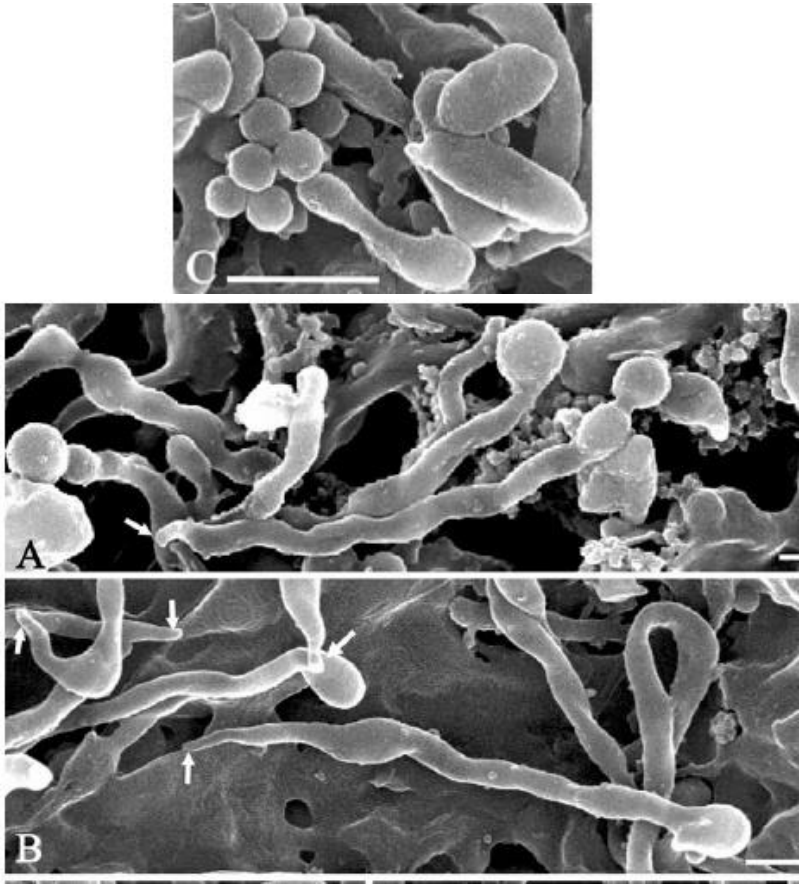
Pili-like structures in plant-pathogenic spiroplasmas

- Scanning electron microscopy (SEM) preparations of cultured.

A. *Spiroplasma kunkelii*,
B. *S. melliferum*,
C. *S. citri*.

- Note:

1. the tip structures (arrows),
2. globular parts of the helix (A, B),
3. a bud-cluster (C).



Ammar *et al.*,2004

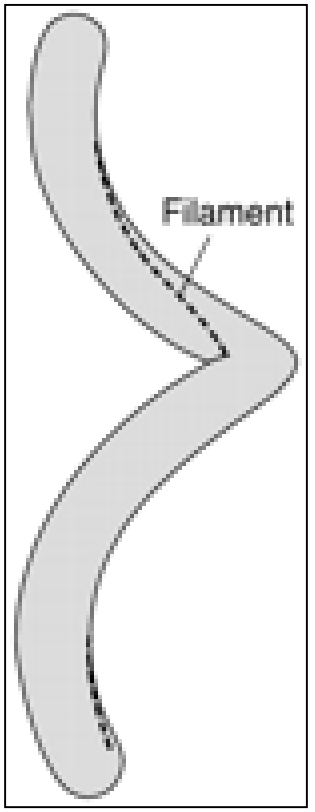
Motility

Spiroplasma : a swimming helix

- Spiroplasmas swim by dynamically changing the conformation of their helical body, sometimes propagating contractile waves that travel the length of the cell.
- *Spiroplasma* lacks periplasm providing rigidity for the cell.
- Instead, the cell's helical shape is defined by a protein ribbon lying just under the membrane, and probably anchored to it.
- This ribbon is composed of seven protofilaments connected side by side.
- Each protofilament is comprised of a linear chain of subunits that can take on multiple conformations.

The motility of *Spiroplasma*

- *In Spiroplasma*, the cell morphology can be viewed as a two-component system:
1. The cholesterol-containing unit membrane defines the cell body
2. The protein ribbon.
- The ribbon in *Spiroplasma* is the main source of the cell's rigidity and defines the cell's morphology.
- As such, the membrane shape will conform in some manner to the shape of the protein ribbon.

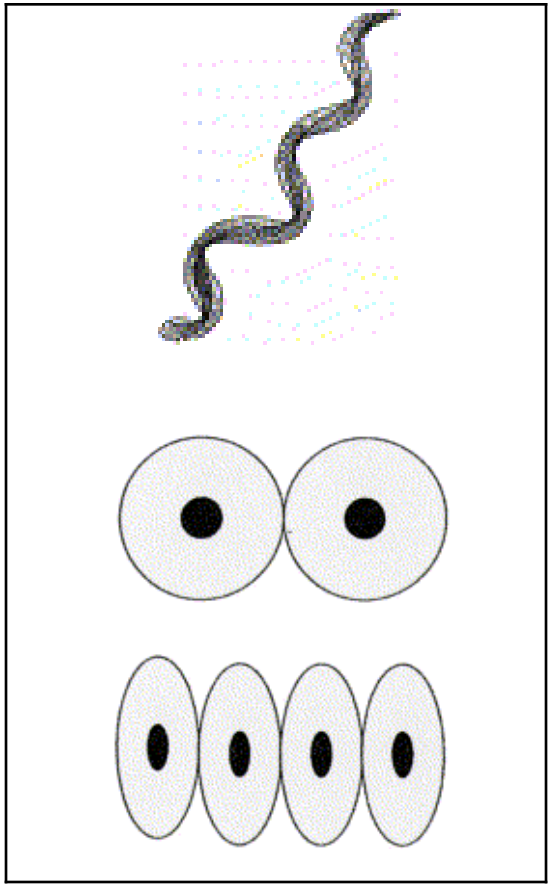


Wolgemuth *et al.*,2003

Motility and helicity:

Role of the cytoskeleton and associated proteins

- Spiroplasma cytoskeleton: Membrane-bound ribbon following the shortest helical line on the cellular coil, and composed of several well-ordered fibrils.
- The 59 kDa fibril protein forms tetramer subunits which assemble into flat fibrils.
- The subunits in the fibrils undergo conformational changes from circular to elliptical, resulting in length changes of the fibrils.



Bové and Garnier,2003

Spiralins

- Spiralins are chemically bound to Spiroplasma-associated fibrils (SpFs) and are separated with difficulty.
- SpFs are unique internal fibrils of spiroplasmas with a molecular weight of 55 kDa.

Biological indexing methods

Suitable conditions for pathogen transmission

- Three different methods of biological indexing:

1. traditional method,
2. inoculated indicator, and
3. inverse inoculation

- were conducted and compared in order to evaluate the most efficient method and the most suitable conditions for transmission of the *S. citri* in the greenhouse.

Mannaa *et al.*,2013

Biological indexing methods

Suitable conditions for pathogen transmission

- For that, young shoots with small emerging leaves from symptomatic trees were collected to be used in these trials.
- Positive control trees, as well as negative control from healthy trees, were also included in the tests.
- Different aspects were evaluated, including:

1. the successful transmission rate, and
2. the time required for the appearance of stubborn symptoms on the indicator plant.

Mannaa *et al.*,2013

Biological indexing methods

Suitable conditions for pathogen transmission

1. Traditional Method

- Side grafting and leaf batch grafting were performed;
- Collected budwood (stem pieces that were 5-7 mm in diameter from Stubborn infected trees) were side-grafted onto the one-year-old Madame Vinous seedlings (stubborn indicator plant).
- After sealing the graft with parafilm, and then labeling and enclosing it inside a plastic bag, the inoculated plants and the negative controls were maintained in a warm, conditioned greenhouse.
- After 1-2 weeks, the plastic bags were opened at the top to reduce humidity and observations of the symptoms were carried out weekly.

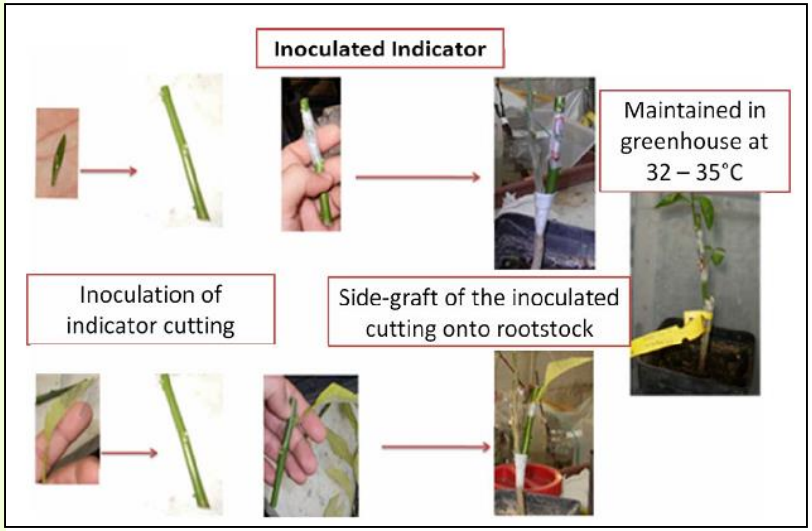
Mannaa *et al.*,2013

Biological indexing methods

Suitable conditions for pathogen transmission

2. Inoculated Indicator

- Wood cuttings containing 4-6 nodes of the indicator "Madame Vinous" were firstly inoculated by chip budding or by leaf inoculation using the bark tissue collected from the sample sources; then, the inoculated indicator cuttings were grafted onto Sour orange rootstock and enclosed inside a plastic bag in order to keep high humidity inside.
- The inoculated plants were maintained in a warm conditioned greenhouse.
- After ten days, the plastic bags were opened at the top in order to reduce the inside humidity, grafting success was evaluated and symptoms observations were carried out weekly.



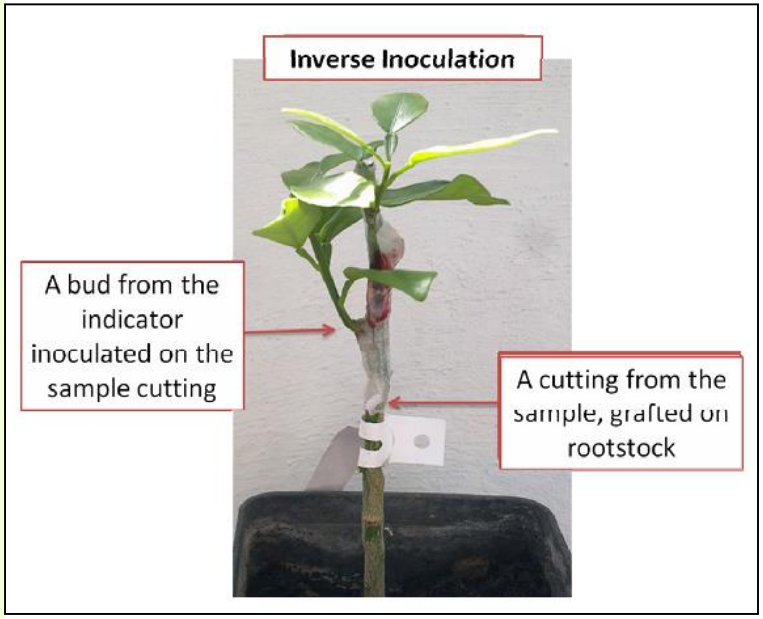
Mannaa *et al.*,2013

Biological indexing methods

Suitable conditions for pathogen transmission

3. Inverse Inoculation

- This new technique was set up in order to overcome the limitations of the traditional indexing related to the low concentration of *S. citri* in the stubborn infected sample and the long time needed for the diffusion of the pathogen in the plants.
- This method is the reverse of the inoculated indicator method, as it consists of the inoculation of a cutting from the sample to be tested with a bud from Madame Vinous sweet orange indicator, and then the obtained inoculated cutting was grafted onto sour orange rootstock.
- Symptoms observations were carried out weekly on leaves emerging from the indicator bud.



Mannaa *et al.*,2013

Spiroplasma Systematics
1. Serological classification system

- The serological classification scheme places new isolates into serological groups or subgroups based on the degree of surface antigen cross reactivity.
- Originally, members of a single serogroup demonstrated:

1. Strong serological relatedness, and had
2. Genomes that exhibited more than 70% DNA-DNA homology.

- Serological relatedness was accepted as a surrogate for DNA-DNA reassociation analyses and formed the basis for the *Spiroplasma* species concept.

Redgassa and Gasparich,2006

Spiroplasma Systematics
Serological classification system

- Species of the genus *Spiroplasma* have been traditionally classified into 34 groups based on cross-reactivity of surface antigens.
- Three of the serogroups contain closely related strain complexes that are further divided into subgroups:

1. Group I has nine subgroups, and
2. Groups VIII and XVI have three subgroups each.

- Most groups and subgroups have been given binomial names.

Redgassa and Gasparich,2006

Spiroplasma Systematics
Serological classification system

- Phylogenetic reconstructions based on 16S rDNA sequence strongly support the closely related serogroups.
- To date, 36 *Spiroplasma* species have been fully characterized and given binomial names.
- Hundreds of other isolates have been partially described, and some of these undoubtedly represent new *Spiroplasma* species.

Redgassa and Gasparich,2006

Revised classification of Spiroplasmas

Strains were examined by standard methods for their ability to utilize glucose, arginine and urea.

Williamson *et al.*,1998

Group	Species	Representative strain (ATCC)	G+C (±1 mol%)	Glucose/ arginine	Host/habitat
I-1	*S. citri*	Maroc-R8A2^T (27556)	26	+/+	Phloem/leafhopper
I-2	*S. melliferum*	BC-3^T (33219)	26	+/+	Honey bee
I-3	*S. kunkelii*	E275^T (29320)	26	+/+	Phloem/leafhopper
I-4		277F (29761)	26	+/+	Rabbit tick
I-5		LB-12 (33649)	26	+/−	Plant bug
I-6	*S. insolitum*	M55^T (33502)	26	+/−	Flower surface
I-7		N525 (33287)	26	+/+	Plant surface
I-8	*S. phoeniceum*	P40^T (43115)	26	+/+	Phloem/vector
II		DW-1 (43153)	26	ND	*Drosophila*
III	*S. floricola*	23-6^T (29989)	26	+/−	Plant surface
IV	*S. apis*	B31^T (33834)	30	+/−	Honey bee
V	*S. mirum*	SMCA^T (29335)	30	+/+	Rabbit tick
VI	*S. ixodetis*	Y32^T (33835)	25	+/−	Ixodid tick
VII	*S. monobiae*	MQ-1^T (33825)	28	+/−	*Monobia* wasp
VIII-1	*S. syrphidicola*	EA-1^T (33826)	30	+/+	Syrphid fly
VIII-2	*S. chrysopicola*	DF-1^T (43209)	29	+/−	Deer fly
VIII-3		TAAS-1 (51123)	31	+/+	Horse fly
IX	*S. clarkii*	CN-5^T (33827)	29	+/+	Green June beetle
X	*S. culicicola*	AES-1^T (35112)	26	+/−	Mosquito
XI	*S. velocicrescens*	MQ-4^T (35262)	26	+/−	*Monobia* wasp
XII	*S. diabroticae*	DU-1^T (43210)	25	+/−	Beetle
XIII	*S. sabaudiense*	Ar-1343^T (43303)	29	+/−	Mosquito
XIV	*S. corruscae*	EC-1^T (43212)	26	+/−	Horse fly/beetle
XV		I-25 (43262)	26	+/−	Leafhopper
XVI-1	*S. cantharicola*	CC-1^T (43207)	26	+/−	Cantharid beetle
XVI-2		CB-1 (43208)	26	+/−	Cantharid beetle
XVI-3		Ar-1357 (51126)	26	+/−	Mosquito
XVII		Tab 4c (700271)	25	+/−	Horse fly
XVIII	*S. litorale*	TN-1^T (43211)	25	+/−	Horse fly
XIX	*S. lampyridicola*	PUP-1^T (43206)	25	+/−	Firefly
XX	*S. leptinotarsae*	LD-1^T (43213)	25	+/+	Colorado potato beetle
XXI		W115 (43260)	24	+/−	Flower surface
XXII	*S. taiwanense*	CT-1^T (43302)	26	+/−	*Culex* mosquito
XXIII	*S. gladiatoris*	TG-1^T (43225)	26	+/−	Horse fly
XXIV	*S. chinense*	CCH^T (43960)	29	+/−	Flower surface
XXV	*S. diminutum*	CUAS-1^T (49235)	26	+/−	*Culex* mosquito
XXVI	*S. alleghenense*	PLHS-1^T (51752)	31	+/+	Scorpionfly
XXVII	*S. lineolae*	TALS-2^T (51749)	25	+/−	Horse fly
XXVIII	*S. platyhelix*	PALS-1^T (51748)	29	+/+	Dragonfly
XXIX		TIUS-1 (51751)	28	+/−	Tiphiid wasp
XXX		BIUS-1 (51750)	28	+/−	Flower surface
XXXI	*S. montanense*	HYOS-1^T (51745)	28	+/+	Horse fly
XXXII	*S. helicoides*	TABS-2^T (51746)	27	+/−	Horse fly
XXXIII	*S. tabanidicola*	TAUS-1^T (51747)	26	+/−	Horse fly
XXXIV		BARC 1901 (700283)	25	+/−	Horse fly
		BARC 2649 (700284)	28	+/+	Horse fly

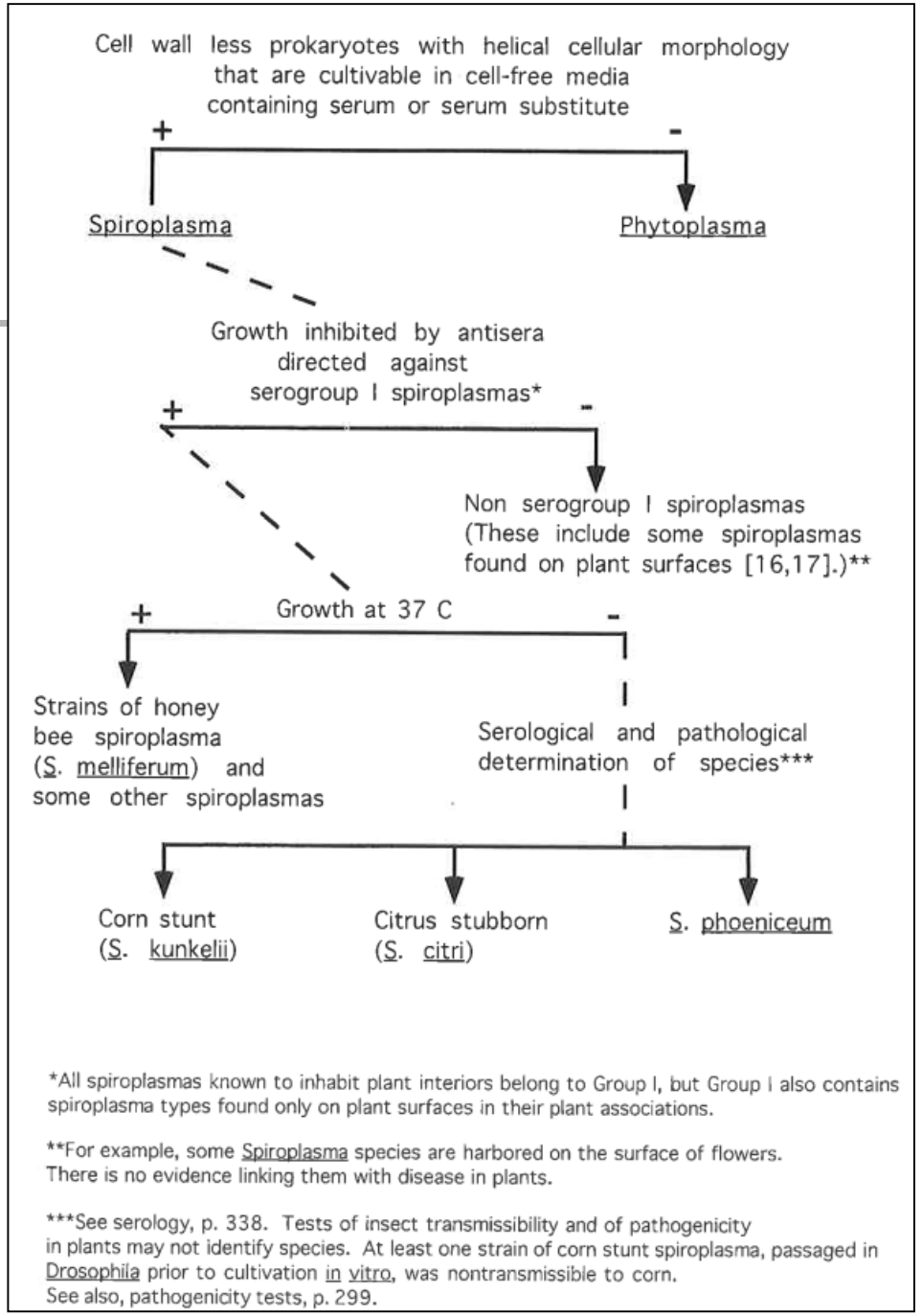
Spiroplasma species and/or representative strains by serogroup[1]

Serogroup[2]	Binomial Name	Host(s)	Disease
I-1	*Spiroplasma citri*	Dicots, leafhoppers	Citrus stubborn
I-2	*S. melliferum*	Honey bees	Honeybee spiroplasmosis
I-3	*S. kunkelii*	Maize, leafhoppers	Corn stunt
I-4	*S.* sp.	Rabbit ticks	NK[4]
I-5	*S.* sp.	Green leaf bugs	NK
I-6	*S. insolitum*	*Eristalis* flies, flowers	NK
I-7	*S.* sp.	Coconut palms	NK
I-8	*S. phoeniceum*	*Catharanthus roseus*	Periwinkle disease
I-9	*S. penaei*	*Penaeus vannamei* shrimp	Shrimp disease
II	*S. poulsonii*	*Drosophila*	Sex ratio trait
III	*S. floricola*	Insects, flowers	Beetle "lethargy"
IV	*S. apis*	Bees, flowers	May disease
V	*S. mirum*	Rabbit ticks	Suckling mouse cataract disease
VI	*S. ixodetis*	*Ixodes pacificus* ticks	NK
VII	*S. monobiae*	*Monobia* wasps	NK
VIII-1	*S. syrphidicola*	*Eristalis arbustorum* flies	NK
VIII-2	*S. chrysopicola*	*Crysops* sp. Flies	NK
VIII-3	*S.* sp.	Horse fly	NK
IX	*S. clarkii*	*Cotinus* beetles	NK
X	*S. culicicola*	*Aedes* mosquitoes	NK
XI	*S. velocicrescens*	*Monobia* wasps	NK
XII	*S. diabroticae*	*Diabrotica undecimpunctata* beetles	NK
XIII	*S. sabaudiense*	*Aedes* mosquitoes	NK
XIV	*S. corruscae*	*Ellychnia corrusca* beetles, horse flies	NK
XV	*S.* sp.	Leafhopper	NK
XVI-1	*S. cantharicola*	Cantharid beetle	NK
XVI-2	*S.* sp.	Cantharid beetle	NK
XVI-3	*S.* sp.	Mosquito	NK
XVII	*S. turonicum*	Horse fly	NK
XVIII	*S. litorale*	*Tabanus nigrovittatus*	NK
XIX	*S. lampyridicola*	*Photuris pennsylvanicus* beetles	NK
XX	*S. leptinotarsae*	*Leptinotarsa decemlineata*	NK
XXI	*S.* sp.	*Prunus sp.* flowers	NK
XXII	*S. taiwanense*	*Culex tritaeniorhynchus*	NK
XXIII	*S. gladiatoris*	*Tabanus gladiator*	NK
XXIV	*S. chinense*	*Calystegia hederaceae*	NK
XXV	*S. diminutum*	*Culex* mosquito	NK
XXVI	*S. alleghenense*	Scorpionfly	NK
XXVII	*S. lineolae*	Horse fly	NK
XXVIII	*S. platyhelix*	Dragonfly	NK
XXIX	*S.* sp.	Tiphiid wasp	NK
XXX	*S.* sp.	Flower surface	NK
XXXI	*S. montanense*	Horse fly	NK
XXXII	*S. helicoides*	Horse fly	NK
XXXIII	*S. tabanidicola*	Horse fly	NK
XXXIV	*S.* sp.	Horse fly	NK
Ungrouped[5]	*S. atrichopogonis*	Biting midge	NK

[1] Table modified from Williamson *et al.* (1998) and Gasparich (2002), [2] Serogroups are designated by Roman numerals; subgroups are indicated by hyphenated numbers,
[4] NK, none known, [5] No group number yet assigned to this serologically distinct species.

Redgassa and Gasparich,2006

Key to differentiate *Spiroplasma* from Phytoplasma



Schaad *et al.*,2001

Differentiation Tests

Species-level tests

- **At group level:**
- **Growth inhibition test (GI)**
- **At subgroup level:**
- **Deformation test (DF)**
- **Metabolic inhibition test (MIT)**

Growth inhibition test (GI)

With polyclonal and monoclonal antibodies

- In order to calibrate the dilutions of antibodies and *S. citri* cultures necessary to prevent spiroplasma growth, inhibition tests were first performed on solid media.
- A large growth inhibition zone was observed with polyclonal serum for dilutions up to 1:64 (Next Table).
- No inhibition was observed with the two monoclonal antibodies even at the highest concentration tested, i.e., 4 mg/ml.
- Growth inhibition by the polyclonal serum was then evaluated with various titers of *S. citri* cultures (Next Table).
- The inhibition was optimum for low *S. citri* titers and high concentrations of antibodies.
- However, a growth inhibition zone of 2.5 mm was still obtained when a disk with a tenfold dilution of serum was placed on a culture plate containing 10^8 *spiroplasmas* per ml.

Malembic *et al.*,2002

Growth inhibition(GI) induced by polyclonal antibodies

Antiserum dilution	Inhibition zone (mm)	*S. citri* titer (CFU/ml)	Inhibition zone (mm) at antiserum dilution	
			0	1:10
—	11	10^2	14	10
1:4	9	10^3	12.5	8
1:8	8.2	10^4	12	7
1:16	7	10^5	11	6
1:32	5.5	10^6	10	5.5
1:64	4.5	10^7	8	3.5
1:128	0	10^8	5.5	2.5
1:256	0			
1:512	0			
PBS control	0			

PBS= phosphate buffer saline, pH 7.2

[a] *S. citri* was grown on solid medium, and growth inhibition was determined as a function of antiserum dilution with *S. citri* at 10^5 CFU/ml and as a function of *S. citri* titer with undiluted and 1:10 diluted antiserum.

Malembic *et al.*,2002

Growth inhibition test (GI)

- Fifty microliters of a *spiroplasma* culture at 10^5 CFU/ml were spread onto SP4 medium solidified with 1% agar in 60-mm-diameter petri dishes.
- Twofold dilution series of crude antiserum, monoclonal antibodies (4 mg/ml), or recombinant antibodies (300 µg/ml) in a final SP4 volume of 25 µl were blotted onto sterile 5-mm-diameter paper disks laid on agar in the center of the dishes.
- Negative controls were PBS and the periplasmic fraction of non transformed *E. coli* cells in place of antibodies.
- After 5 to 7 days at 32°C, the growth inhibition areas were measured from the edges of the disks.

Malembic *et al.*,2002

Spiroplasm SP4 medium

- PPLO Broth w/o Crystal Violet (BD 255420) 11.0 g
- Tryptone (BD 211705) 10.0 g
- Noble Agar (for solid medium) 8.0 g
- DI Water 525.0 mL
- Adjust pH to 7.4 to 7.5.
- Gently boil to dissolve agar, if necessary.
- Autoclave for 15 minutes at 121°C.
- Cool:

1. agar medium to 50 to 55°C,
2. broth to 55°C or cooler.

ATCC,2013

Deformation test (DF)

- The deformation test (DF) is widely accepted for *Spiroplasma* characterization.
- The DF test screens new isolates against antisera to representatives of existing serogroup strains.
- During the initial screening, the new isolate is generally tested against pools of representative antisera and then examined for deformation.
- Tentative serological relatedness is determined by observation of 50% or greater cells deformed (e.g. clumping, blebs).

Redgassa and Gasparich,2006

Deformation test (DF)

- This initial screening is followed by reciprocal DF tests against antisera to individual strains as indicated by the preliminary screen.
- Reciprocal, terminal titers of 320 or greater indicate strong serological relatedness, and would allow placement of a novel isolate in a pre-existing serogroup.

Redgassa and Gasparich,2006

Deformation test (DF)

- The *S. citri* deformation test was performed in 96-well microtiter plates.
- Two fold dilution series of crude antiserum, monoclonal antibodies (initial concentration, 4 mg/ml), or recombinant antibodies (initial concentration, 300 µg/ml) were done in 25 µl of SP4 medium.
- Then, 25 µl of SP4 medium containing 5×10^8 CFU of spiroplasmas per ml was added to each dilution.
- As a negative control, the same procedure was carried out with either PBS or periplasmic fraction of nontransformed *E. coli* cells in place of antibodies.
- The microtiter plate was incubated at room temperature for 30 min, and 6-µl samples from each well were observed under a dark-field microscope to determine *spiroplasma* morphology and motility.

Malembic *et al.*,2002

Metabolism inhibition test (MIT)

- The *S. citri* metabolism inhibition test was performed in 96-well microtiter plates.
- Horizontally, a two fold dilution series of crude antiserum, monoclonal antibody (800 µg/ml), or recombinant antibody (80 µg/ml) suspensions were distributed in a final volume of 50 µl of SP4 medium.
- Vertically, 200 µl of 1:10 dilution series of a 10^6 CFU/ml *spiroplasma* culture in SP4 medium was added to each well.
- Negative controls were done with PBS in place of antibodies.
- Glucose fermentation by *S. citri* leads to the production of lactic acid in SP4 medium, resulting in color change of the phenol red pH indicator from red to yellow.
- After incubation for 5 days at 32°C, color changes were recorded for each well.

Malembic *et al.*,2002

Spiroplasma Systematics

2. Current requirements for classification of *Spiroplasma* species

- The current minimal standards for spiroplasma species designation rely upon:

1. Phenotypic, and
2. Genotypic information.

- Numerous biological characteristics must be determined; however, none of the traits tested are genus-specific.
- Microscopic morphology is determined using dark field and electron microscopy.
- Filterability through a 220 nm membrane and resistance to penicillin (500U/ml).
- Other characters are based on cell biochemistry such as glucose fermentation, arginine hydrolysis and urea hydrolysis.

Redgassa and Gasparich,2006

Current requirements for classification of *Spiroplasma* species

- The original requirement to determine cholesterol utilization has been questioned, since some spiroplasmas do not have a sterol requirement (e.g. *S. floricola* and *S. gladiatoris*).
- All known spiroplasmas are filterable through a 220 nm membrane, resistant to penicillin, ferment glucose and are unable to hydrolyze urea.
- G+C base composition (mol%) is determined using a melting temperature method, with reported compositions falling within the range of 24-31%.
- Although not required, determination of genome size and 16S rDNA sequence was recommended.
- Genome sizes for spiroplasmas range from 780-2,220 kbp and are determined using pulsed-field gel electrophoresis.

Redgassa and Gasparich,2006

Polyphasic taxonomy
Phenotypic, genotypic and phylogenetic information

- Polyphasic taxonomy calls for the inclusion of phenotypic, genotypic and phylogenetic information in classification.
- At the species level, 16S rDNA sequence can distinguish most species unless they are part of closely related strain clusters, such as serologically related subgroups .
- Additional DNA sequences used to enhance molecular phylogenies *Spiroplasma* phylogenetics, including:

1. The 16S-23S rDNA intergenic region,
2. The *gyrB* gene, and
3. The translated sequence of the metabolic gene *pgk*.

Redgassa and Gasparich,2006

Polyphasic taxonomy
Multilocus approach

- There is no single molecule that represents all organismal relationships adequately.
- Different molecules carry different types of information.
- Ultimately, a multilocus approach that is representative of the whole genome may provide the best evidence for evolutionary relationships.
- It has been suggested that 16S rDNA phylogenies could be used in concert with serology to serve as primary characters for taxonomic classification.

Redgassa and Gasparich,2006

Molecular Diagnosis of *Spiroplasma*

Resolving power of 16S rRNA sequence analysis, DNA-DNA homology, and serology at various hierarchical levels

Test	Family	Genus	Inter-group	Group*	Subgroup**	Serovar
16S rDNA	+	+	+	+	±	?
DNA-DNA	-	-	-	+	+	±
Serology	-	-	-	+	+	?

*The Spiroplasma group is a putative species.
**The Spiroplasma subgroup may be eligible for species designation.

Courtesy Whitcomb et al

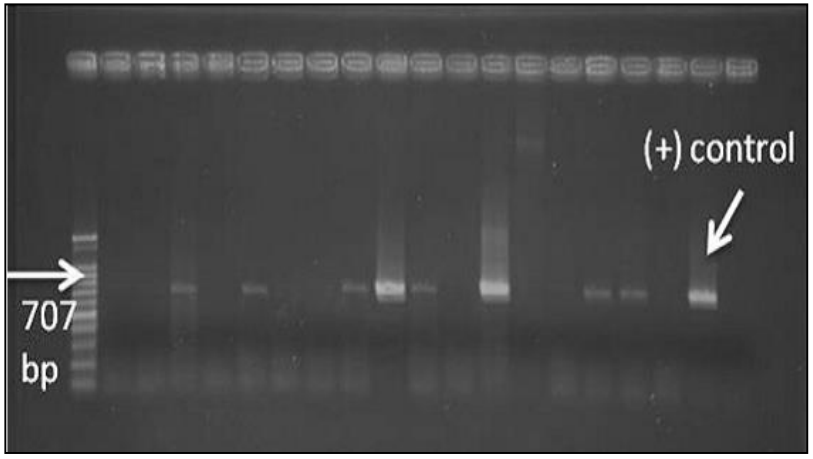
Molecular Diagnosis of *Spiroplasma*
The sequence of the used primer pair

Primer	Primer sequence (5′ to 3′)	Target gene	Reference
Spiralin-f	GTCGGAACAACATCAGTGGT	spiralin gene	(Foissac *et al.*, [9])
Spiralin-r	TGCTTTTGGTGGTGCTAATG		
P89-f	ATTGACTCAACAAACGGGATAA	putative adhesion gene	(Yokomi *et al.*, [10])
P89-r	CGGCGTTTGTTTGTTAATTTTTGGTA		
P58-6f	GCGGACAAATTAAGTAATAAAAGAGC	putative adhesion like multigene	
P58-4r	GCACAGCATTTGCCAACTACA		

Mannaa *et al.*,2013

Molecular Diagnosis of *Spiroplasma*
Molecular Detection by PCR

- Out of the 130 collected suspected symptomatic samples, 96 reacted positively to the PCR assay using the P89 primer pair, and the amplicons of the expected size were produced.
- Agarose gel analysis showing PCR amplification (707 bp) of *S. citri*-infected Egyptian samples.



Mannaa *et al.*,2013

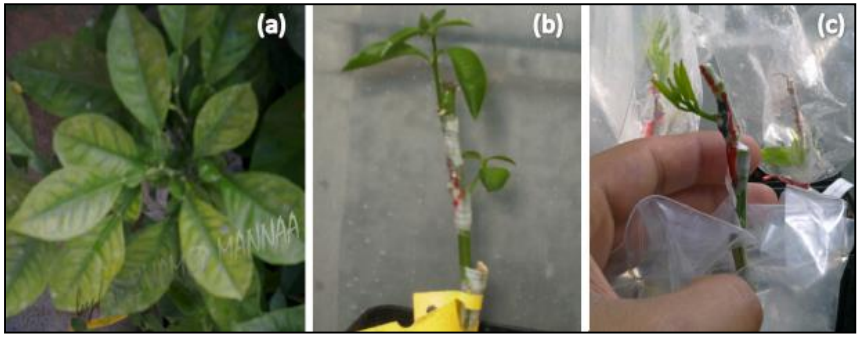
Pathogenicity tests
Biological Indexing
S. citri transmission rate

- *S. citri* transmission rate which was evaluated through the observation of typical symptoms on the leaves of the indicator plants that include (chlorosis, cup shaped leaves with short internodes and small sized).
- using:

a) traditional method;

b) inverse inoculation method;

c) inoculated cutting method.



Mannaa *et al.*,2013

Pathogenicity tests

Biological Indexing

S. citri transmission rate

- Results obtained by comparing the three methods of biological indexing.
- using:

a) traditional method;

b) inverse inoculation method;

c) inoculated cutting method.

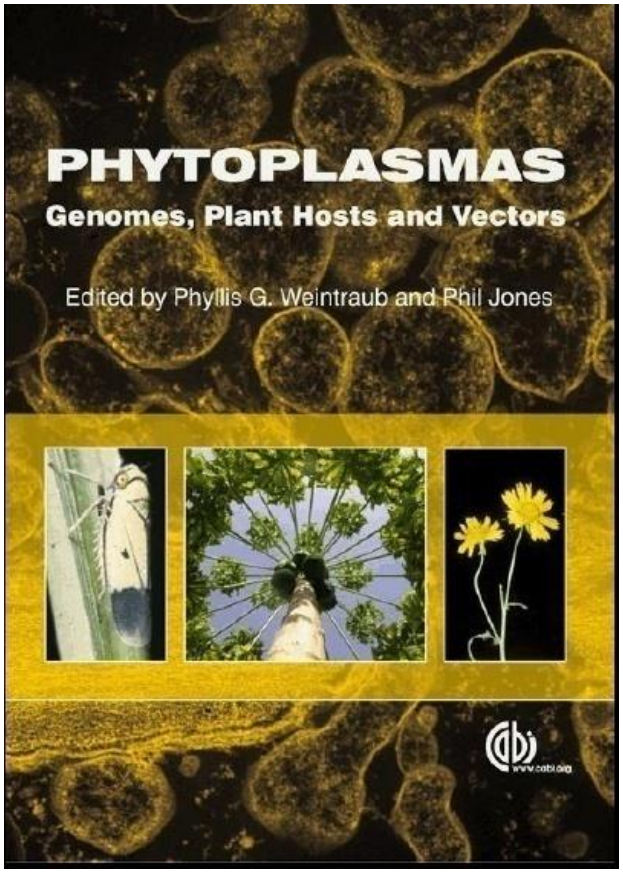
Method	Time needed for observing the first symptoms	No. of symptomatic plants/No. of tested	% successful transmission
Traditional method	3 months	6/20	30%
Inoculated cutting	5 weeks	5/20	25%
Inverse inoculation	4 weeks	17/20	85%

Mannaa *et al.*,2013

Identification of the bacterial pathogens
Phytoplasma

Disease diagnosis and pathogen diagnostics

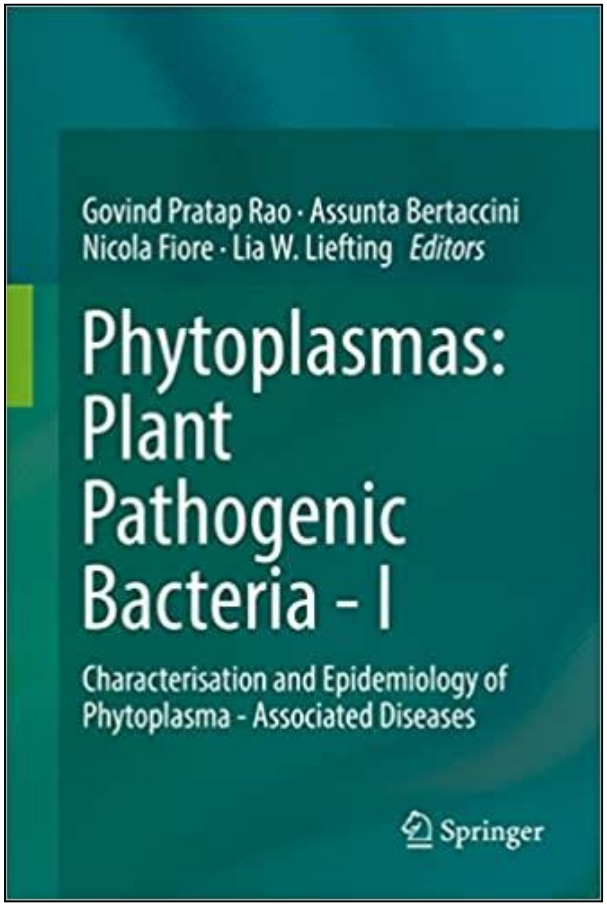
Phytoplasmas:
Genomes, plant hosts, and vectors



Product Details

- Phytoplasmas: genomes, plant hosts, and vectors.
- Editors: Weintraub, P. G. and P. Jones.
- Publisher: CABI, 2010,
- 331 pages.

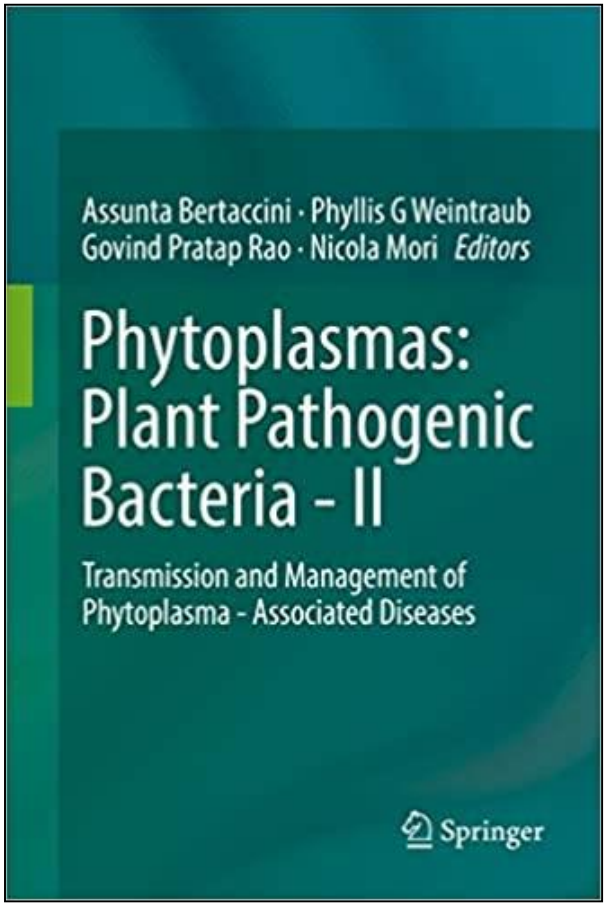
Phytoplasmas: Plant Pathogenic Bacteria-I: Characterisation and Epidemiology of Phytoplasma-Associated Diseases



Product Details

- **Phytoplasmas: Plant Pathogenic Bacteria - I: Characterisation and Epidemiology of Phytoplasma - Associated Diseases.**
- Editors: Govind Pratap Rao, Assunta Bertaccini, Nicola Fiore, Lia W. Liefting.
- Publisher: Springer; 1st ed. 2018 edition.
- 358 pages.

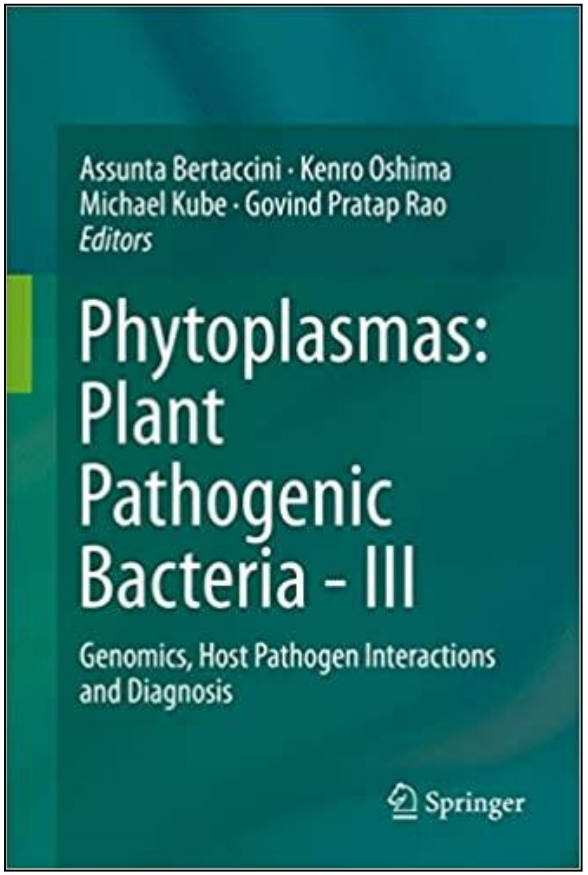
Phytoplasmas: Plant Pathogenic Bacteria- II: Transmission and Management of Phytoplasma-Associated Diseases



Product Details

- **Phytoplasmas: Plant Pathogenic Bacteria- II: Transmission and Management of Phytoplasma-Associated Diseases.**
- Editors: Assunta Bertaccini, Kenro Oshima, Michael Kube and Govind Pratap Rao.
- Publisher: Springer; 1st ed. 2019 edition.
- 415 pages.

Phytoplasmas: Plant Pathogenic Bacteria - III: Genomics, Host Pathogen Interactions and Diagnosis

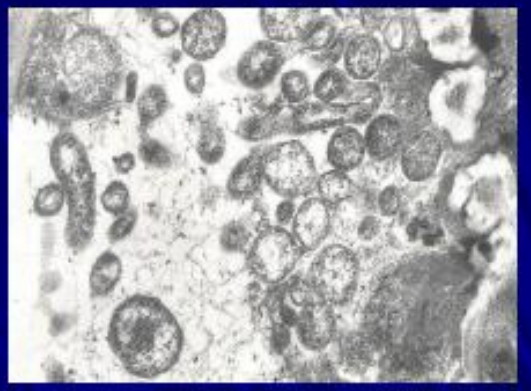


Product Details

- **Phytoplasmas: Plant Pathogenic Bacteria - III: Genomics, Host Pathogen Interactions and Diagnosis.**
- Editors: Assunta Bertaccini, Kenro Oshima, Michael Kube and Govind Pratap Rao.
- Publisher: Springer; 1st ed. 2019 edition.
- 236 pages.

1. What are Phytoplasmas?

- Phloem-limited insect-transmitted plant pathogenic bacteria that lack a cell wall
- Related to Gram positive bacteria (*Bacillus*) do not encode typical genes related to pathogenicity present in other plant pathogenic bacteria
- Can not be grown in axenic culture
- Genomes of some have been sequenced (sizes range from 530 – 1200 kb) they are the smallest known self-replicating life form
- Symptoms include yellowing, phyllody, proliferation, stunting, general decline, witches' broom



Bonants,2008

Phytoplasmas
History

- Since their discovery in 1967 as 'mycoplasma-like organisms', the phytoplasmas have quickly become established as a unique group of plant pathogens.
- Diseases, frequently called 'yellows', have been known since the late 1800s; originally thought to be associated with viruses, many are now known to be caused by phytoplasmas.
- During the 1970s, research centred on diagnosis using symptoms and electron microscopy to visualize the phytoplasmas in the phloem sieve cells of their hosts.
- In the 1990s the term phytoplasma had been proposed, and by 2004 a distinct taxonomic group, '*Candidatus* Phytoplasma', was defined.

Phytoplasmas

Increased number of phytoplasma publications and sequenced records

- A Google Scholar search (using phytoplasma OR 'mycoplasmalike organism' as a search term) revealed that phytoplasma has been a subject of over 5600 scientific articles, of which 4590 appear in professional media with recorded publication dates.
- More than 2100 of the 4590 articles were published since 2004.

Weintraub and Jones,2010

Phytoplasmas

Increased number of phytoplasma publications and sequenced records

- At the time of writing, 1546 phytoplasma 16S rRNA gene sequences have been registered in and released by the GenBank including sequences submitted through the:

1. European Molecular Biology Laboratory (EMBL),
2. The DNA DataBank of Japan (DDBJ), and
3. The National Center for Biotechnology Information (NCBI), USA).

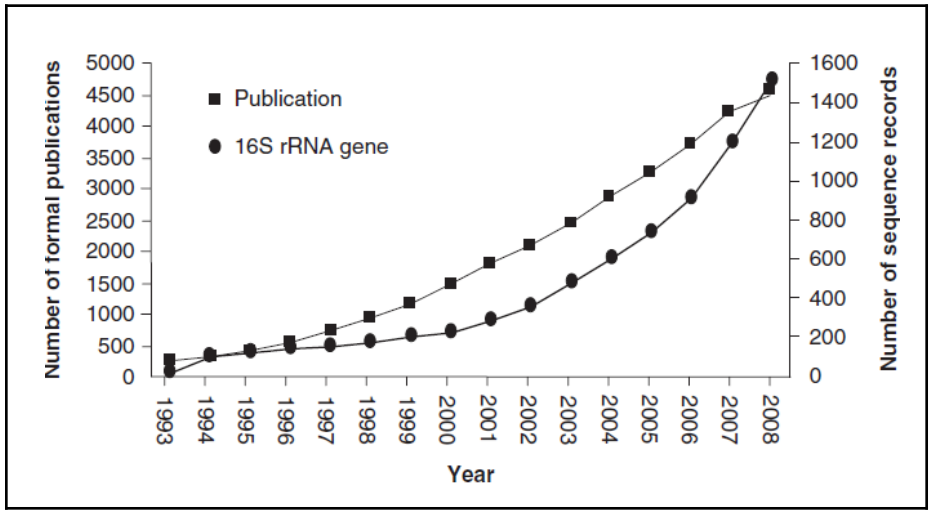
- This number has more than doubled in less than 3 years.

Weintraub and Jones,2010

Phytoplasmas

Increased number of phytoplasma publications and sequenced records

- Increase in the number of phytoplasma research publications and the number of phytoplasmal 16S rRNA gene sequence records in the GenBank from 1993 to 2008.



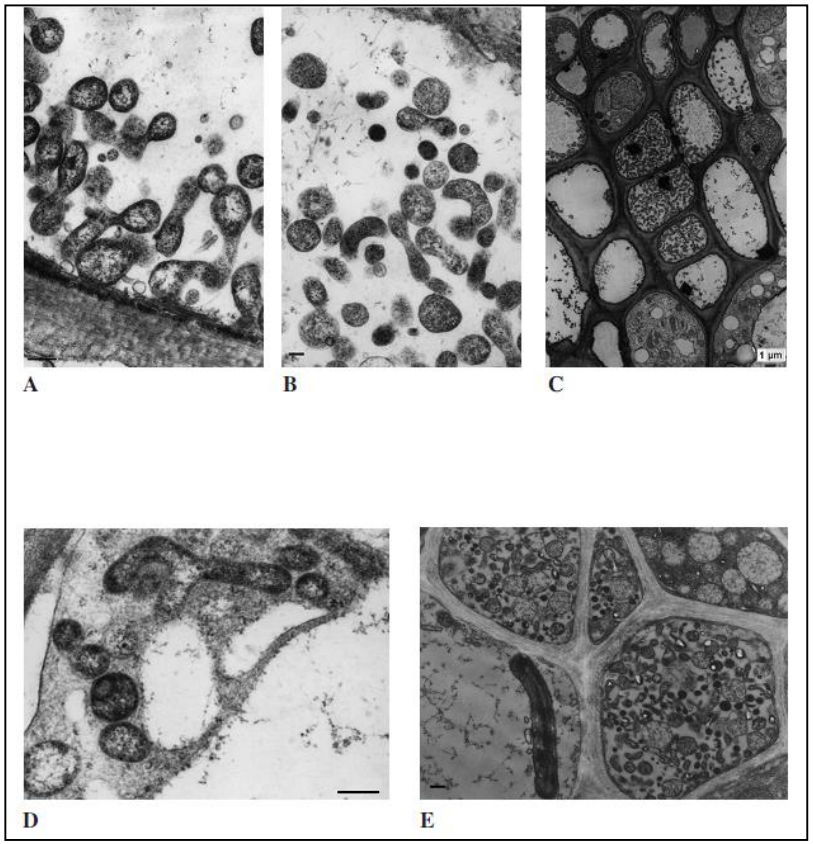
Weintraub and Jones,2010

Morphology
Cell characteristics

- Mollicutes belong to the Gram-positive bacteria with genomes of low GC.
- Phytoplasmas (Phytos: Greek for plant, Plasma, Greek for thing moulded) have a triple-layered plasma membrane.
- Lack cell wall and cells rigidity, and appear as pleomophic bodies and in transmission electron microscopy (EM) with a mean diameter of 200-800 nm.
- Their shape is normally pleiomorphic or filamentous and normally have a diameter of less than 1 micrometer.
- Like other prokaryotes, DNA is free in the cytoplasm.
- They are believed to reproduce through binary fission. Also reproduce asexually by budding.

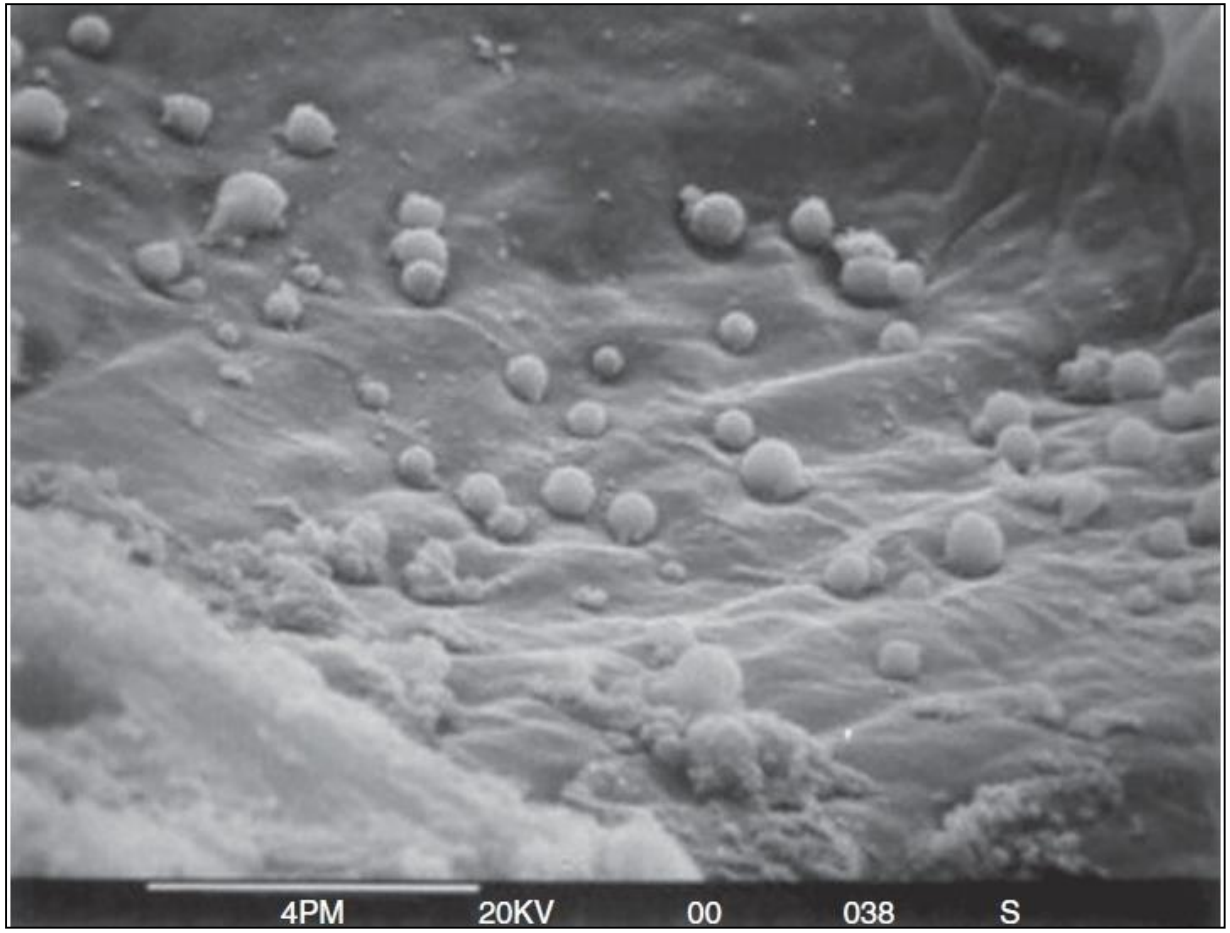
Electron micrograph of phytoplasma bodies accumulated on sieve tubes of phloem cells

- A-E, Electron micrographs of cross sections of sieve tubes showing variations in the size and shape of phytoplasmas infecting plants.
- Cell size: 0.1-0.8 µm in diameter.
- Bar=200 nm unless specified.



Bertaccini and Duduk,2009;..

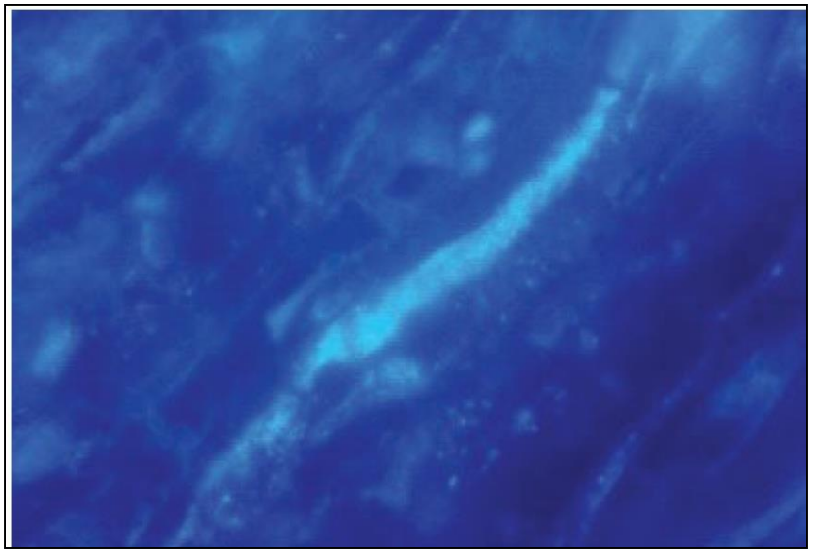
Scanning electron micrograph of a phloem sieve tube of phytoplasma infected plants, showing phytoplasmas attached to the sieve tube plasma membrane



Weintraub and Jones,2010

Fluorescence microscopy
DAPI fluorescence test

- Phytoplasma colonization in petioles of alder viewed by fluorescence microscopy using the DNA dye 4'-6-diamidino-2-phenylindole.
- Several sieve tubes show phytoplasmal infections as single particles, while others show them as small aggregates or larger fluorescent areas.



Phytoplasma colonization in petioles of alder yellows-affected *Alnus glutinosa* (alder).

Weintraub and Jones,2010

Antibiotic sensitivity

- Phytoplasmas differ from bacteria in that they lack a cell wall or penicillin-binding sites.
- They are, therefore, resistant to penicillin, to which bacteria are sensitive.
- However, phytoplasmas are sensitive to tetracycline antibiotics.

Non-sterol-requiring mollicutes

No sterols in cellular membrane

- '*Ca*. Phytoplasma' membranes are:

1. Resistant to digitonin (a glycoside used as a detergent, it effectively water-solubilizes lipids), and
2. Sensitive to hypotonic salt solutions, and are therefore similar to those of non-sterol-requiring mollicutes (Lim *et al*.,1992).

Phytoplasmas

Genome size

- Diversity was found in:
  1. genome size, composition,
  2. metabolic pathways, and
  3. number of repeats (ORFs).
- They possess DNA and RNA and thought to reproduce by budding.

Genome size

Have very small genomes (680-1,600 kb)

1. Base composition of DNA:

- All phytoplasmas have AT-rich genomes.
- They have a genome with a low G+C content.
- GC content ranges from 21.4% for '*Ca.* Phytoplasma mali' to 27.7% for '*Ca.* Phytoplasma asteris' OY-M.
- According to results from buoyant density centrifugation, the G+C content of '*Ca.* Phytoplasma' DNA is 23-29 mol%.
- They have similar numbers of tRNA genes and two copies of the rRNA operon.

Genome size

Have very small genomes (680-1,600 kb)

2. Chromosome size:

- Differences in the chromosome size between phytoplasma species have been reported.
- It ranges from 530 kbp to 1,350 bp depending on taxonomic group and containing unknown amount of plasmids.
- Short circular extrachromosomal DNAs (1.7-7.4 kb) or plasmids were found in all members of the aster yellows group (16SrI) and stolbur groups (16SrXII) and in some members of X-disease (16SrIII) and clover proliferation (16SrVI) groups.
- Genes encoded in extrachromosomal DNAs such as plasmids are known to play important roles in the pathogenicity and virulence of many plant pathogenic bacteria including phytoplasmas.

Bertaccini and Duduk,2009

Genome evolution

- Phytoplasmas phylogenetically belong to Gram-positive bacteria.
- Phytoplasmas suffer extreme genome reductions (reductive, regressive or degenerative evolution) compared with their Gram-positive relatives.
- It has now become clear that phytoplasmas undergo rapid genome evolution, which may be a consequence of their life cycle.
- Phytoplasmas continuously cycle between plants and insects and, in nature, require both organisms for survival and dispersal.

Weintraub and Jones,2010

Genome evolution

- They probably diverged from Gram-positive bacteria, and belong to the '*Candidatus* Phytoplasma' genus.
- The genomes of phytoplasmas are very small when compared with those of their ancestors (walled bacteria in the *Bacillus/Clostridium* group) because they lack several pathways for the synthesis of compounds necessary for their survival.
- These substances must be obtained from host plants and insects (Bai *et al.*,2006).

Bertaccini and Duduk,2009

General features of four sequenced phytoplasma genomes

Strains: Onion Yellows M (OY-M), Aster yellows witches'-broom, *'Ca.* Phytoplasma australiense´and strain AT of *'Ca.* Phytoplasma mali'

Strain	OY-M	AY-WB	*tuf*-Australia; *rp*-A	AT
'Ca. Phytoplasma' species	asteris	asteris	australiense	mali
16S rDNA group	IB	IA	XIIB	X
Cluster	I	I	I	II
Chromosome size (bp)	860,631	706,569	879,324	601,943
Chromosome composition	Circular	Circular	Circular	Linear
G+C content (%)	27.7	26.9	27	21.4
Protein-coding regions (%)	73	72	74	79
Coding sequences	793	708	839	536
Genes encoding proteins	754	671	684	497
Protein-coding genes with assigned functions	446	450	414	338
(Conserved) hypothetical proteins[a]	308	221	270	159
Single-copy proteins	486	482	482	408
Multiple-copy proteins	268	191	202	89
Multiple-copy proteins in PMUs	175	134	143	4
Transposase similar to tra5[b]	7 (6)	6* (20)	5 (1)	1
Fragmented genes	46	102	159	16
Avg ORF size (bp)	833	776	778	955
tRNA genes	32	31	35	32
rRNA operons	2	2	2	2
Extrachromosomal DNAs (plasmids)	2	4	1	0

In general, a phytoplasma genome consists of one chromosome and several small plasmids with a unique replication gene , although 'Ca. P. mali' harbors no plasmids.

(subgroup *tuf-A*ustralia;*rp-A*)

Weintraub and Jones,2010

Habitat

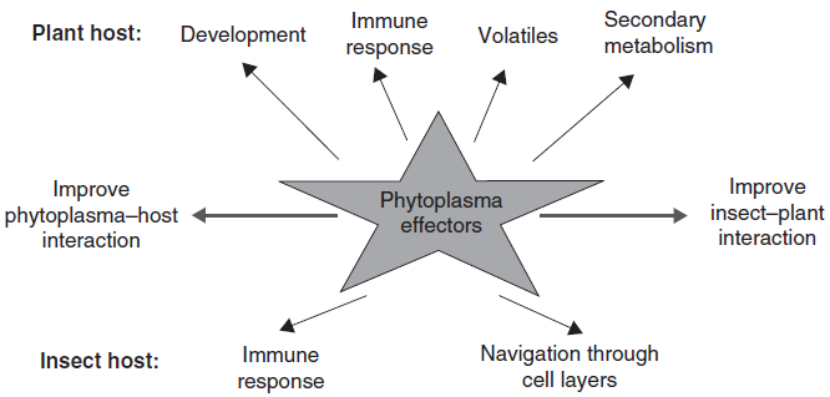
Insect and plant hosts

- Phytoplasmas are obligate parasites occurring in plant phloem tissue and in several insects.
- **In insect hosts:**
- They may cause premature mortality.
- **In plant hosts**
- Phytoplasmas are plant-pathogenic prokaryotes. They may cause complex syndromes with specific symptoms, such as virescence, phyllody, sterility of flowers, proliferation of auxiliary or axillary shoots, abnormal elongation of internodes and many other, often less specific symptoms.
- An array of symptoms may be produced due to profound disturbances in the normal balance of plant hormones or growth regulators.

Phytoplasma candidate virulence factors

Schematic illustration of the possible functions of phytoplasma effectors

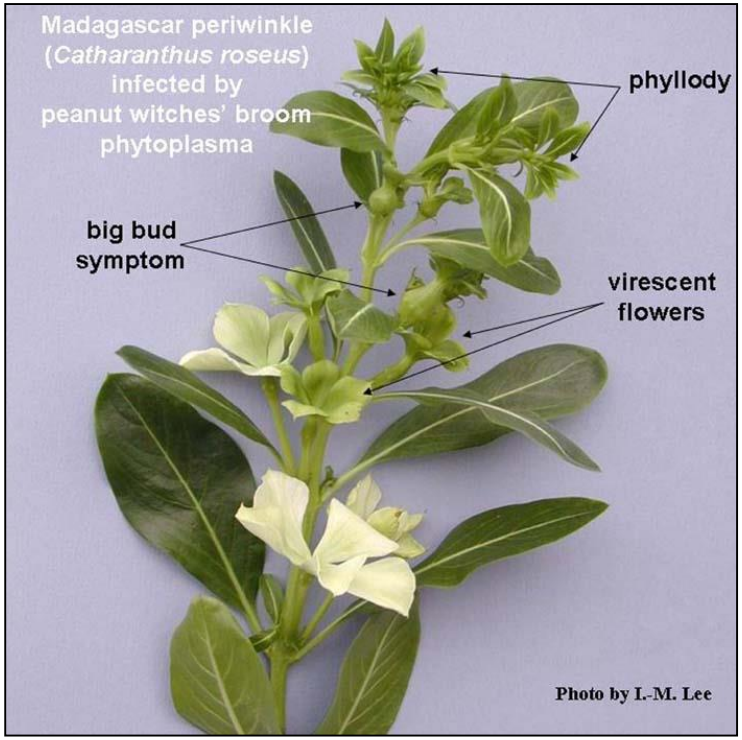
- Effectors perturb the development, immune response, volatile production and secondary metabolism of the plant host and the immune response of insects.
- They can also aid phytoplasma navigation through the various cell layers of insect hosts.
- The overall effect is that phytoplasma fitness is enhanced through manipulation of plant and insect hosts and insect–plant interaction.



Weintraub and Jones,2010

Symptoms
Common symptoms

- Phytoplasmas are unculturable plant-pathogenic, wall-less bacteria (mollicutes) that cause diseases in several hundred plant species worldwide (McCoy *et al.*,1989).
- **Some of these symptoms includes:**
- Yellowing,
- Phyllody (flowers that become leafy),
- Virescence (green coloration of plant parts that are not normally green),
- Witches'-broom (growth of a dense mass of shoots from a single point),
- Stunting,
- Big bud,
- Proliferation, and
- General decline.



Phyllody: production of leaf like structures in place of flowers. Virescence: loss of normal flower color, green flowers.

Virescence

Green coloration of plant parts that are not normally green

- Symptoms of strawberry green petal I-C subgroup phytoplasma.

Phytoplasma infected periwinkle exhibiting virescence and phyllody symptoms

Courtesy Ing-Ming Lee

Witches' brooming in lime

Bové and Garnier

Stunting

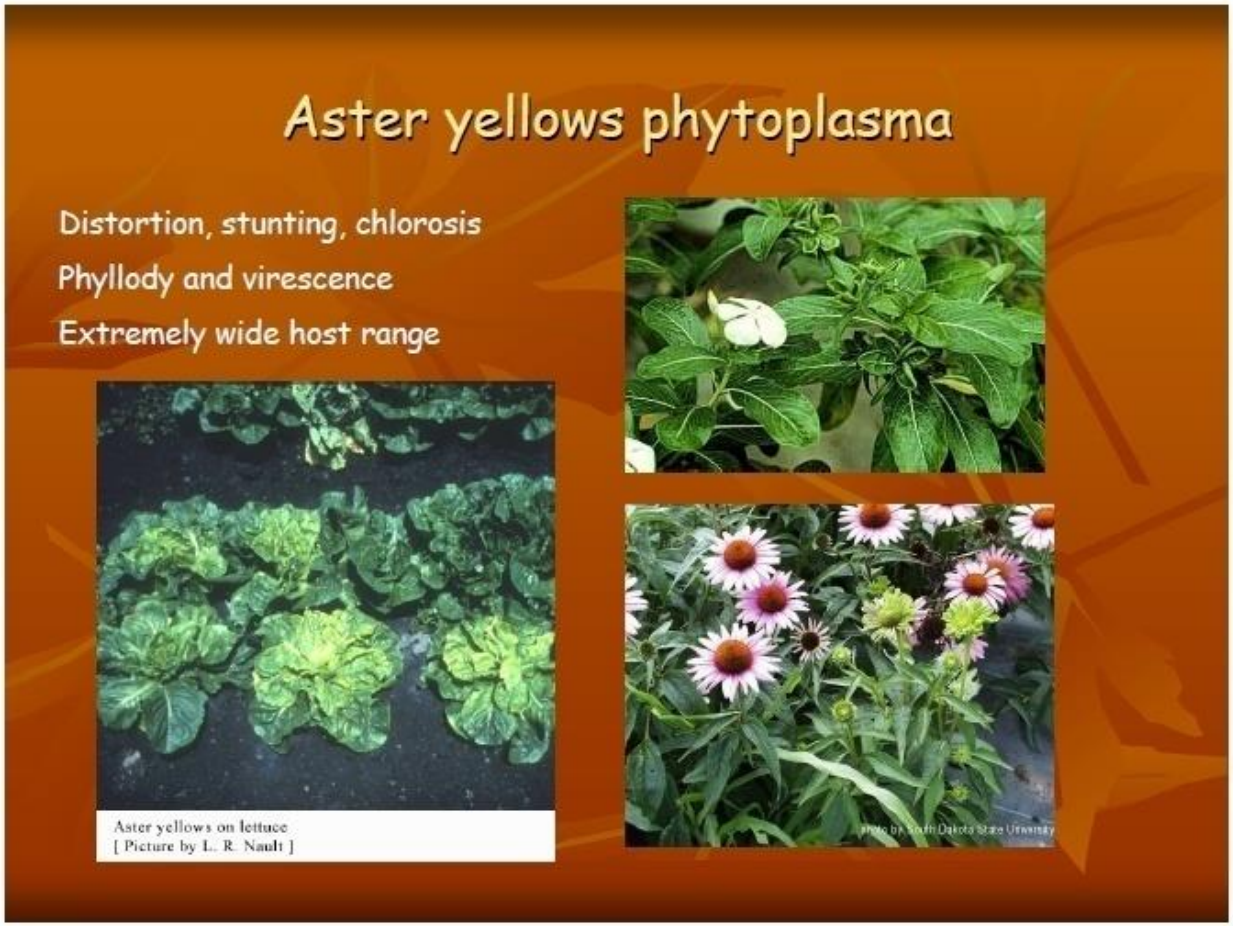
Scarlet Plume(*Euphorbia fulgens*) with and without phytoplasma

Nicolaisen,2001

Yellows

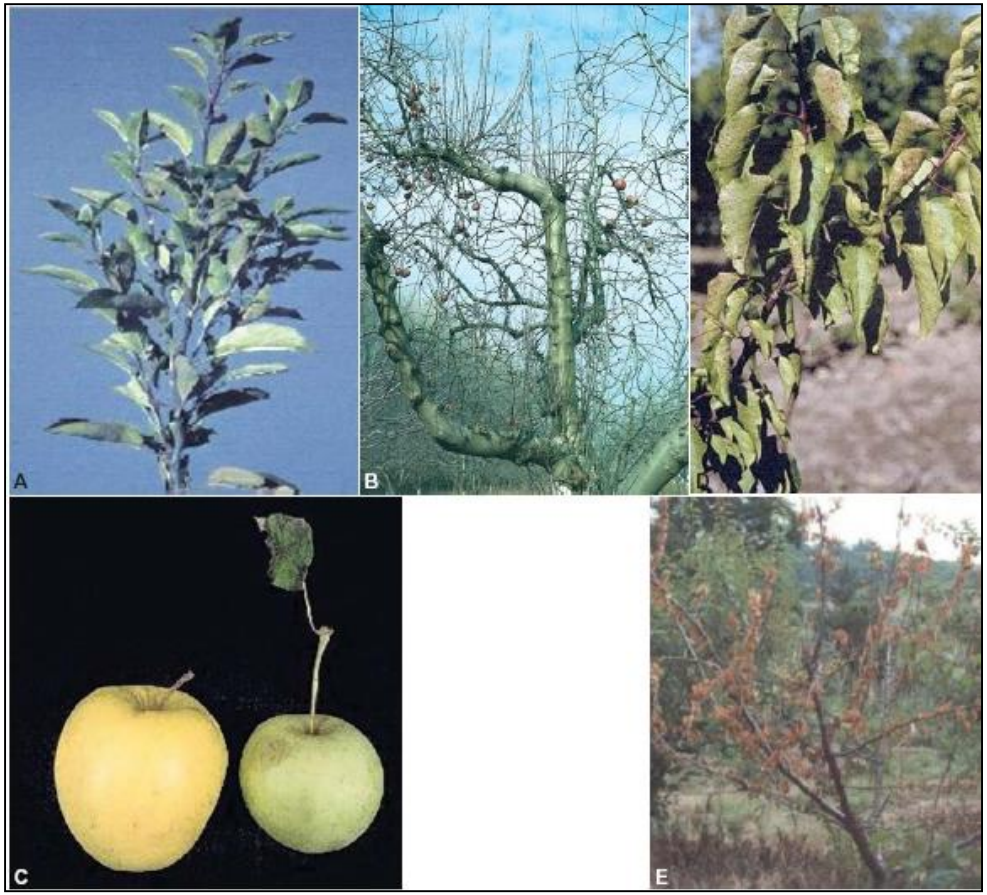
Aster and lethal yellows

Aster yellows phytoplasma



A. Wayadande

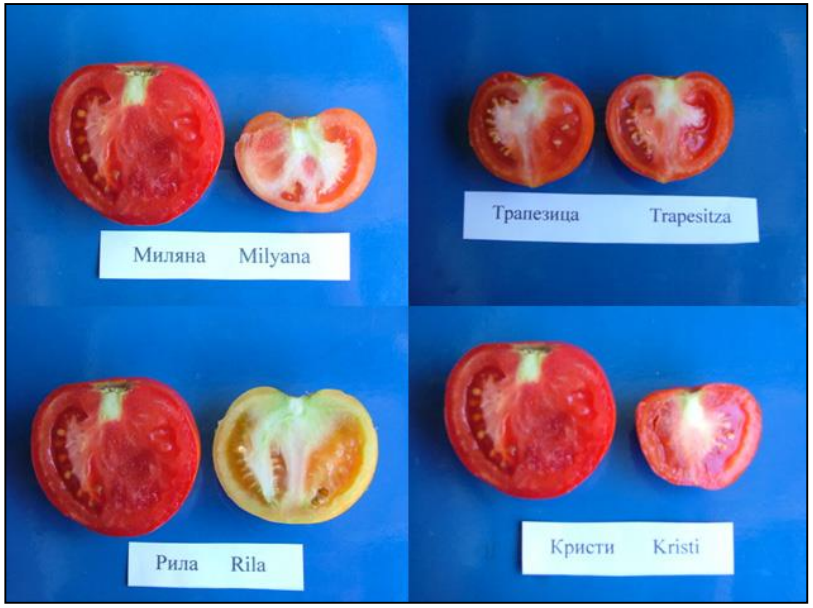
Apple proliferation symptoms on young twig (A), mature apple tree (B), and on reduced fruit size (C, right). (D) European stone fruit yellows symptoms on apricot, followed by death of the tree (E) within a short time



[Photographs courtesy of (A) E. Seemuller, Heidelberg, Germany, and (C–E) L. Giunchedi, University of Bologna, Italy.]

Potato stolbur
Symptoms

Symptoms of potato stolbur phytoplasma. Aerial tubers, yellowing and upward rolling of the top leaves in stolbur-infected potato plant.



In each picture: healthy (left) and stolbur infected (right) tomato from which seeds were used.

Jovic *et al.*,2011; Calari *et al.*,2011

Phytoplasmas life cycle

Phytoplasma vectors

Persistent and nonpersistent transmission

- Generally, vector acquisition of pathogens increases with time spent feeding on infected plant sources of the pathogen.
- Nonpersistent transmission: Transmission is called nonpersistent if the rate of transmission drops to near zero within a short time(hours).
- Persistent transmission: Describes situations in which the vector can transmit over many days, in some cases, for weeks or months.
- All phytoplasmas are transmitted to plants by phloem-feeding insects in a persistent propagative manner.

Purcell and Almeida,2005;..

Diseases caused by vector-borne fastidious bacterial pathogens

- Some plant parasites transmitted by insect vectors must multiply and circulate throughout the body of the vector to be transmitted.

Type of pathogen	Vector	Type of transmission
Xylella fastidiosa (numerous diseases)	Xylem sap-feeders (Leafhopper known as Glassy-winged sharpshooter)	Noncirculative but persistent, propagative in vector
Ca. Liberibacter Citrus greening	Psyllids	Foregut-borne (non-circulative), persistent
Aster yellows phytoplasma	Leafhoppers (several spp.)	circulative, propagative
Spiroplasma citri Citrus stubborn	Leafhoppers (several spp.)	circulative, persistent manner

Purcell and Almeida,2005;..

Phytoplasmas life cycle
Phytoplasma vectors

- Phytoplasmas are mainly spread by insects in the families:

1. Cicadellidae (leafhoppers),
2. Fulgoroidea (planthoppers) and
3. Psyllidae (jumping plant lice).

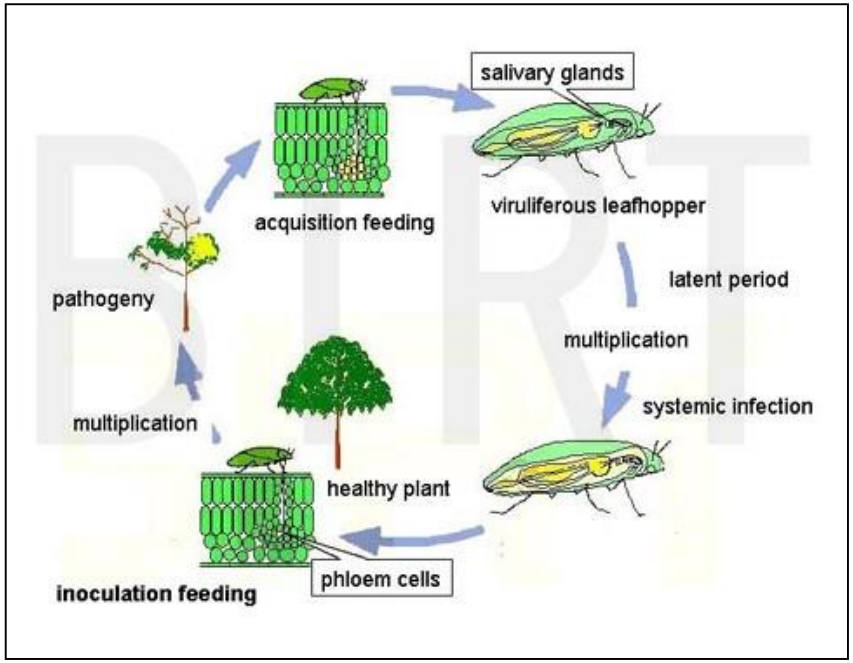
- The sapsucking insects feed on the phloem tissues of infected plants, picking up the phytoplasmas and transmitting them to the next plant they feed on.
- For this reason the host range of phytoplasmas is strongly dependent upon its insect vector.

Phytoplasmas life cycle

Phytoplasma vectors

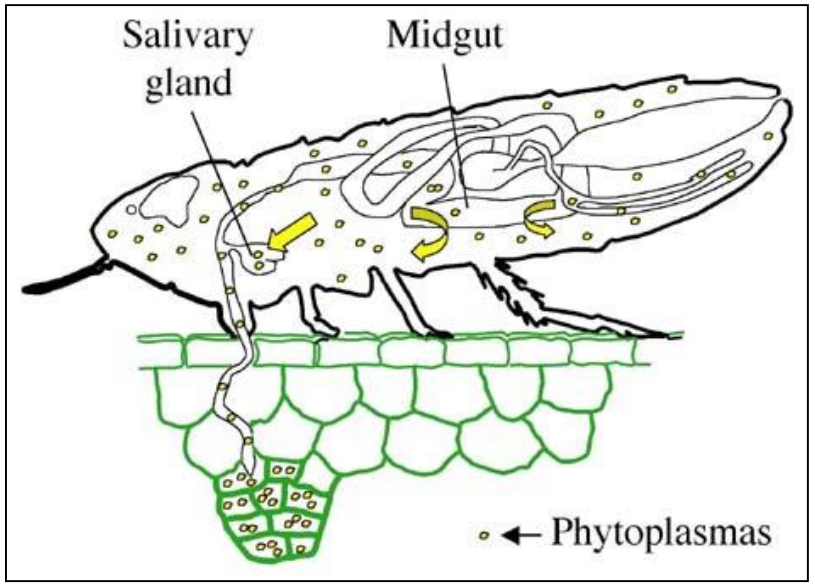
- Organisms of the genus *Ca. Phytoplasmas* inhabit:

1. The phloem sieve elements of vascular plants and,
2. The gut, haemolymph, salivary gland, and other organs of sap-sucking insects.



Phytoplasma vectors
Persistent transmission

- Leafhoppers acquire phytoplasmas while feeding.
- Phytoplasmas move from the gut lumen across the gut epithelial cell layer into the hemolymph.
- They replicate frequently at high levels in various insect tissues, including cells of muscle and salivary glands.
- They enter the saliva via the salivary gland cells.
- Phytoplasmas are introduced into plant hosts with insect saliva during feeding.
- The latent period in the insect is at least two weeks.

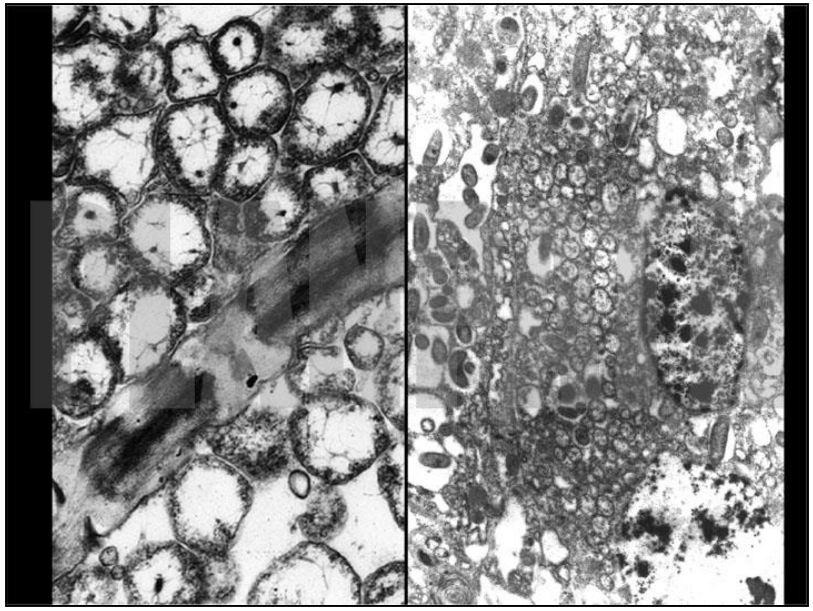


A schematic illustration of the phytoplasma infection route in a leafhopper

Saskia's phytoplasma website

Phytoplasma vectors
Persistent transmission

- Phytoplasmas in:
- Plant cell (left), and
- Insect cell (right).



Phytoplasma vectors

The citrus witches broom vector

Persistent transmission

Hishimonus phycitis

The citrus witches broom vector

Diseases caused by '*Candidatus* Phytoplasma'

- As of today there are hundreds of diseases caused by phytoplasmas and about 100 known insect vectors.

Weintraub and Jones,2010

Diseases caused by formal '*Candidatus* (*Ca.*) Phytoplasma'

'*Ca.* Phytoplasma allocasuarinae'	Associated with allocasuarina yellows
'*Ca.* Phytoplasma americanum'	Associated with potato purple top wilt disease
'*Ca.* Phytoplasma asteris'	Associated with aster yellows
'*Ca.* Phytoplasma aurantifolia'	Associated with witches'-broom disease of small-fruited acid lime
'*Ca.* Phytoplasma australiense'	Associated with Australian grapevine yellows
'*Ca.* Phytoplasma brasiliense'	Associated with hibiscus witches'-broom in Brazil
'*Ca.* Phytoplasma *caricae*'	Associated with papaya bunchy top
'*Ca.* Phytoplasma castaneae'	Associated with chestnut witches'-broom in Korea
'*Ca.* Phytoplasma costaricanum'	Associated with an emerging disease in soybean
'*Ca.* Phytoplasma cynodontis'	Associated with Bermuda grass white leaf
'*Ca.* Phytoplasma convolvuli'	Associated with bindweed yellows
'*Ca.* Phytoplasma fraxini'	Associated with ash yellows
'*Ca.* Phytoplasma fragariae'	Yellow disease of strawberry
'*Ca.* Phytoplasma japonicum'	Associated with Japanese hydrangea phyllody
'*Ca.* Phytoplasma lycopersici'	Parsley leaf of tomato

These species share <97.5% similarity among their 16S rRNA gene sequences.

Diseases caused by informal '*Candidatus* (*Ca.*) Phytoplasma'

'*Candidatus* Phytoplasma mali'	Associated with apple proliferation
'*Ca.* Phytoplasma omanense'	Witches broom of Cassia
'*Ca.* Phytoplasma oryzae'	Associated with rice yellow dwarf
'*Ca.* Phytoplasma phoenicium'	Associated with almond lethal disease
'*Ca.* Phytoplasma pini'	Yellowing and shoot-proliferation of pine trees(*Pinus* spp.)
'*Ca.* Phytoplasma prunorum'	Associated with European stone fruit yellows
'*Ca.* Phytoplasma pyri'	Associated with pear decline
'*Ca.* Phytoplasma rhamni'	Associated with buckthorn witches'-broom
'*Ca.* Phytoplasma rubi'	Associated with *Rubus* stunt
'*Ca.* Phytoplasma spartii'	Associated with spartium witches'-broom
'*Ca.* Phytoplasma sudamericanum'	Associated with *Passiflora* witches' broom
'*Ca.* Phytoplasma tamaricis	Witches -broom -diseased salt cedar
'*Ca.* Phytoplasma trifolii'	Associated with clover proliferation
'*Ca.* Phytoplasma ulmi'	Associated with elm yellows
'*Ca.* Phytoplasma ziziphi'	Associated with jujube witches'-broom

IRPCM Phytoplasma taxonomy group,2004; Bull *et al.*,2010 & 2012

Candidatus species designation

Not yet been formally proposed

- Six species-level taxa for which the *Candidatus* species designation has not yet been formally proposed.
- Phytoplasmas associated with:
- X-disease of peach,
- Grapevine flavescence dore´e,
- Central American coconut lethal yellows,
- Tanzanian lethal decline of coconut,
- Nigerian lethal decline of coconut
- Loofah witches'-broom.

Recommended detection methods

Light & Electron microscopy (Dienes and DAPI staining; immunogold technique), ELISA and PCR (PCR amplification, and RFLP analysis)

Comparison of different phytoplasma detection methods: The cost of equipment is based on a rough estimate.

	Microscope DAPI/ Dienes	electron microscope	ELISA	PCR
Specificity	universal	universal	universal method not developed	can be both universal or directed towards e.g. one group of phytoplasmas
Possibility of distinguishing phytoplasma from plant material	can be difficult	can be difficult	possible with a good antiserum	easy with good primer combinations
Sensitivity	moderate	moderate	moderate	very high
Robustness	high	moderately high	very high	moderately high
Suitable for high throughput	labour intensive	labour intensive	not labour intensive	not labour intensive
Equipment costs (main equipment in brackets)	~25.000 EURO (flourescence microscope)	~250.000 EURO (electron microscope, ultramicrotome)	~15.000 EURO (ELISA reader)	~15.000 EURO (PCR cycler, electrophoresis)
Comments	Demands very skilled technicians.	Demands very skilled technicians.	Difficult to develop new test, for example for poinsettia.	Danger of cross contamination

Mogens Nicolaisen,2001

Recommended detection methods
MLSA markers

- In addition to the 16S rRNA gene marker, multi-locus sequence analysis (MLSA) using other genetic markers has also been widely used for finer differentiation of closely related phytoplasmas.
- These markers include genes encoding:

1. ribosomal proteins (*rp*),
2. protein translocase subunit *SecY*, and
3. translation elongation factor Tu-EF.

Wei *et al.*,2021

Recommended detection methods
MLSA markers

- Unfortunately, selection of suitable markers for MLSA is not straightforward.
- Due to the highly variable evolutionary rates among different bacteria and among different genes (Kuo and Ochman, 2009), the markers that work well for one group do not necessary work well for others.
- Despite these, extensive efforts have been devoted to the development of MLSA markers for phytoplasmas (Martini *et al.*,2019).

Cho *et al.*,2020

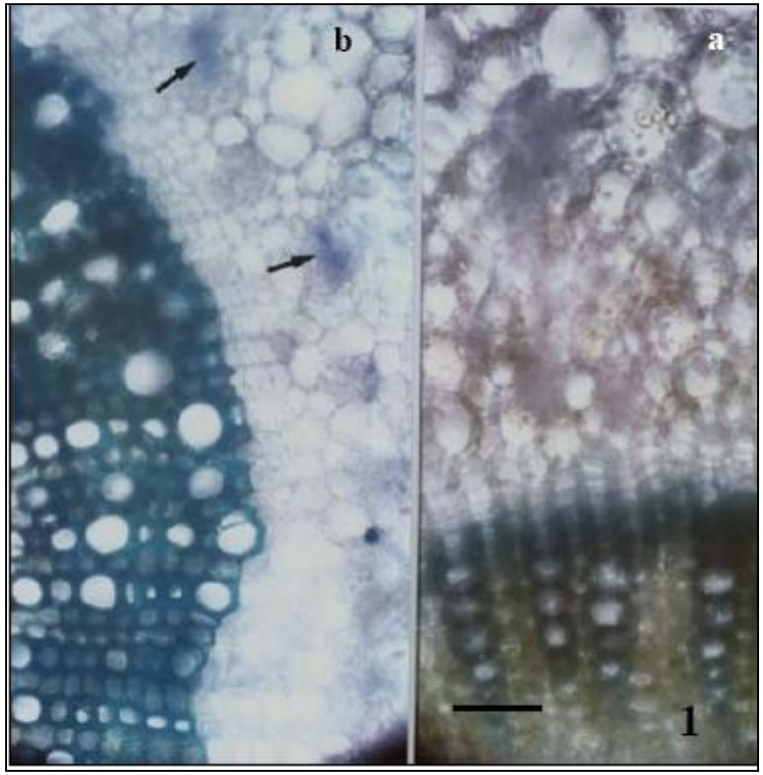
Phytoplasma detection methods
Light & Electron microscopy
Diene's stain

- Hand cut sections of:

a) Healthy and

b) Phytoplasma-infected *Catharanthus roseus*.

- Arrows show the blue spots indicating phytoplasma presence (bars=125 μ).



Evidence of phytoplasmas will be the presence of dark-blue granular structures in the phloem.

Musetti *et al.*,2004

Light microscopy
Diene's stain

- Samples are obtained from the main vein, petioles, or stems of infected plants.
- The samples are cut into 2-3 mm pieces and soaked in Karnowsky fixing solution** (or in glutaraledhyde solution 2% in 0.01 M phosphate buffer, pH 7.0).

***Diene's Stain:**

Methylone Blue	2.5 g
Azure II	1.25 g
Maltose	10 g
Sodium Carbonate	0.25 g

Complete to 100 ml with distilled water

**** Karnowsky Fixing Solution:**

Paraphormaldehyde Solution 8% (12 ml)
Cacodylate buffer 0.1 M, containing
$Ca\ Cl_2\ 2H_2O$ 0.1% (33 ml)
Glutaraldhyde Solution 70% (2 ml

Fluorescence Microscopy
DAPI staining

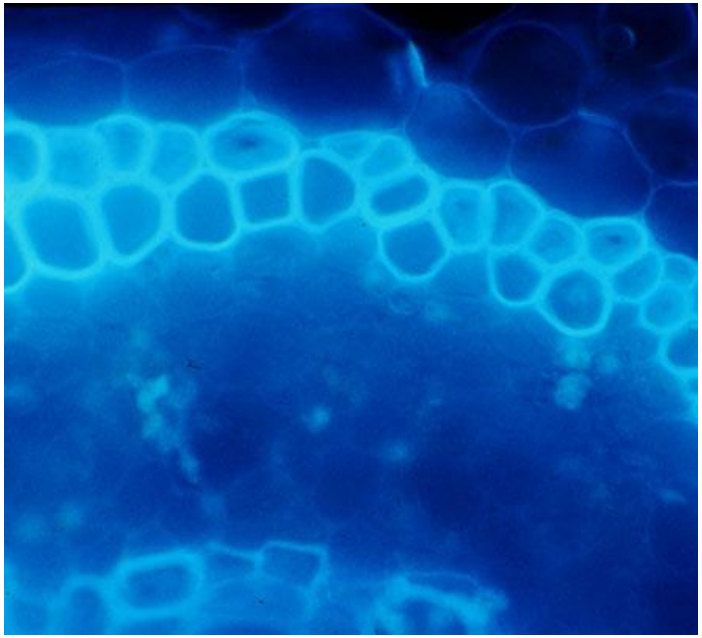
- A rapid and cheap, but not specific technique, which sometimes yields results difficult to interpret.
- Pieces of young tissues (petioles of young leaves, phloem tissue of shoots, branches, and roots) are fixed overnight at 4°C in 4% paraformaldehyde in PBS.
- Longitudinal sections of 20-µm thickness are stained with 1 µg/ml DAPI (4,6 diamidino-2-phenylindole and observed under an epifluorescence microscope.

Burns,2009

Fluorescence Microscopy

The DAPI technique

- Circular, yellow spots in the phloem are often nuclei, and some cell wall junk often fluoresces and can be mistaken for phytoplasmas.
- It may take a while to be sure of what you are seeing.
- Xylem and fibers fluoresce.



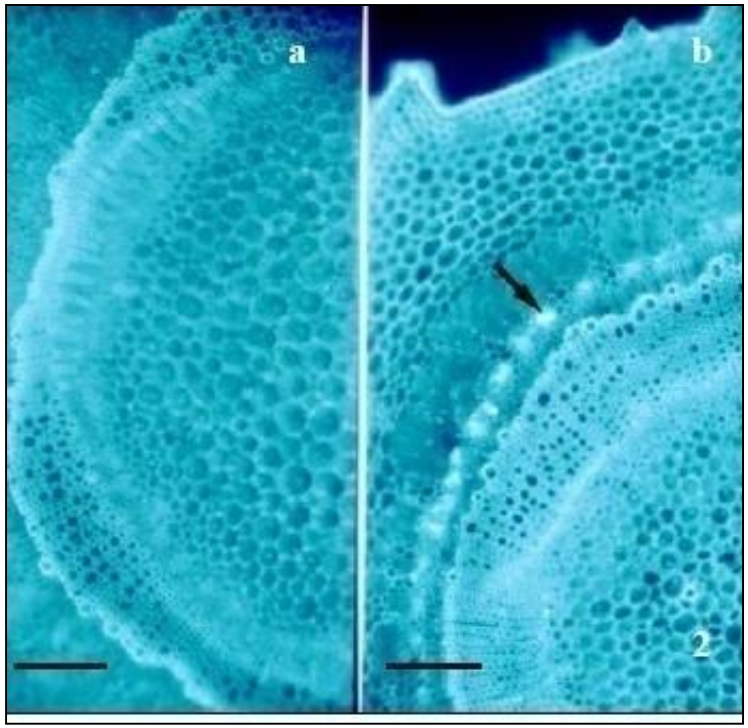
Fluorescent spots in the phloem tissue are highly correlated with the presence of phytoplasmas.

Lee,1998

DAPI: 4,6-diamidino-2-phenylidine; a fluorescent DNA binding dye; used to label chromosomes

Fluorescence Microscopy
The DAPI technique

- DAPI staining of hand cut sections of:
  a) Healthy and
  b) Phytoplasma-infected stems.
- In b, arrow indicates the fluorescent bright spots, visible at phloem level, diagnostic for the presence of phytoplasmas.
- bars=192 μ



Healthy tissue

Phytoplasma-infected tissue

Musetti *et al.*,2004

Electron Microscopy

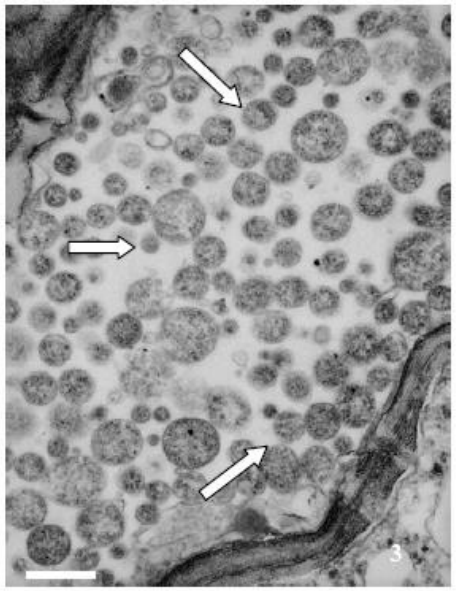
Thin section technique

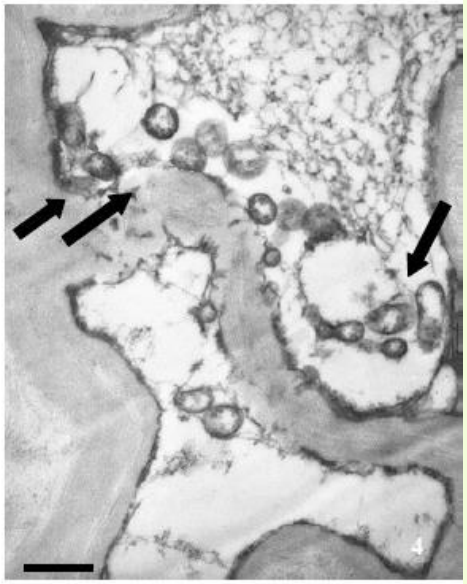
- Small pieces of leaf midribs, young stems and flowers of both healthy and phytoplasma-infected plants, were cut into 2 mm sections.
- Immediately immersed the sections in a fixative containing 5% glutaraldehyde and 4% formaldehyde.
- The samples were post-fixed in osmium tetroxide, dehydrated and embedded in a low-viscosity epoxy resin (Spurr, 1969).
- Ultrathin sections were prepared.
- Double stained with uranyl acetate and lead citrate (Reynold, 1963).
- Then examined and photographed with a Hitachi H600 electron microscope.
- The range and size means of phytoplasma bodies were determined by measuring 100 individual cells from ultra thin sections of TEM photo prints.

TEM

Thin section technique

- Phytoplasmas (arrows) in the phloem cells/tissues.
- In fruit and forest trees phytoplasmas are not uniformly distributed in the phloem of the plants.





Musetti *et al.*,2004

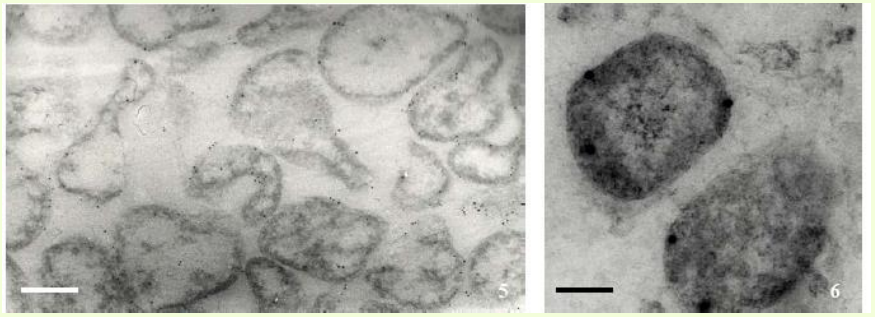
Serology
Antibodies

- A few specific polyclonal (pAbs) and monoclonal antibodies (mAbs) have been raised against a number of fruit tree phytoplasmas.
- e.g. against the WX (Western X-disease of cherry, peach, and nut trees) phytoplasma rabbit pAbs and mouse mAbs.
- While pAbs and mAbs were produced against apple proliferation (AP).

Burns,2009

Serology
Immunogold technique

- Phytoplasmas in phloem tissues of *Catharanthus roseus* L. labelled by immunogold technique.
- Primary monoclonal antibody was diluted 25 µg/ml, the secondary gold conjugated antibody 1:20.
- Using gold 15 nm in diameter, few particles are visible on phytoplasma membrane (Fig. 6), using 5 nm gold, particles are well distributed over the periphery of the phytoplasmas (Fig. 5).



Musetti *et al.*,2004

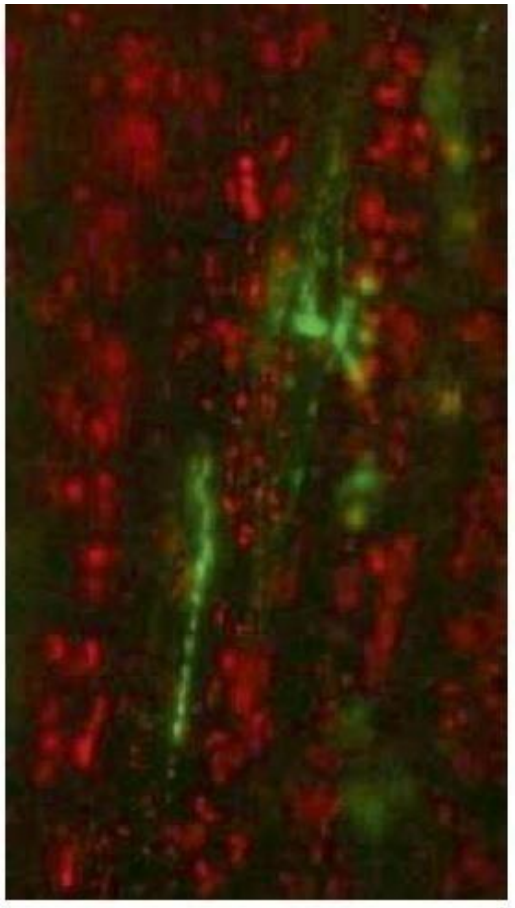
Serology
Immunofluorescence

- One-centimetre-long pieces of stems and roots are fixed in 4% paraformaldehyde in PBS, left overnight at 4°C, and then longitudinally cut by a cryomicrotome (Leitz Jung 1500) to obtain sections of 20-µm thickness.
- The sections are treated with the mab tissue culture supernatants and incubated for 1 h at 37°C.
- After washing, FITC (fluorescein-isothiocyanate)–antimouse conjugate (Sigma no. F1010) is added and incubated at 37°C for 30 min.
- Immunofluorescence is useful and more sensitive than DAPI.

Burns,2009

Serology
Immunofluorescence

- Localization of apple proliferation (AP) in phloem cells of tissue cultures of *Malus domestica* by immunofluorescence.



Burns,2009

Phytoplasma classification scheme
Based on RFLP analyses of PCR-amplified 16S rDNA sequences

- Phytoplasma taxonomic groups are based on:

1. PCR amplification of 16S rDNA followed by RFLP analysis of the amplified product (Lee *et al.*,1998a; 2000).
2. By comparison of DNA sequences from the 16s/23s spacer regions.

Wikipedia,2015;..

Phytoplasma taxonomy

1. Based on results of RFLP analyses of PCR-amplified 16S rDNA sequences

- **14 phytoplasma groups and 32 sub-groups:**
- By 1998, the international total of 34 representative phytoplasma strains were differentiated into 14 groups and 32 sub-groups based on similarity coefficients derived from RFLP analyses (Lee *et al.*, 1998; Duduk and Bertaccini, 2011).

Liu et al.,2017

Classification of phytoplasma

2. Revised schemes based on RFLP analysis

19 major phytoplasma groups and about 50 subgroups

- **19 major phytoplasma groups and about 50 subgroups:**
- The scheme based on actual(gel-based) RFLP analysis of PCR-amplified 16S rDNA sequences has been periodically updated (Lee *et al.*,1998, 2000, 2006a; Montano *et al.*,2001; Arocha *et al.*,2005; Al-Saady *et al.*,2008).
- Thus far, it comprises 19 major phytoplasma groups and about 50 subgroups.

Weintraub and Jones,2010

Classification of phytoplasma

3. Revised schemes based on RFLP analysis

33 major phytoplasma groups and about 100 subgroups

- **33 major phytoplasma groups and about 100 subgroups:**
- More recent work has extended these counts to 33 groups and at least 100 sub-groups (Dickinson and Hodgetts, 2013; Davis *et al*., 2015; Zhao and Davis, 2016).

Liu et al.,2017

Phytoplasma taxonomy

Based on results of actual and virtual RFLP analyses of PCR-amplified 16S rDNA sequences

- Each group includes at least one *Ca.* Phytoplasma species, characterized by:

1. distinctive biological,
2. phytopathological, and
3. genetic properties.

Wikipedia,2015

Phytoplasma classification scheme
Based on RFLP analysis of 16S rRNA gene
Major Phytoplasma group/subgroups

- Group and subgroup are determined on the basis of RFLP patterns of 1.2 kbp segments of 16S rDNA that are delimited by the annealing sites of PCR primers R16F2n and R16R2.
- '*Candidatus* Phytoplasma' species are distinguished on the basis of 16S rDNA nucleotide sequence homology/identity.
- If the 16S rRNA genes of two phytoplasmas share 97.5% or less nucleotide sequence identity, they are considered to represent two separate '*Candidatus* Phytoplasma' species.

Major phytoplasma group/subgroups

Based on RFLP analysis of 16S rRNA gene

Sr. no.	Common name	Phytoplasma species	Group/subgroup	Reference
1	Nigerian awka disease	[*Ca*. P. cocosnigeriae]	16SrXXII-A	Firraro, 2004
2	Tanzanian lethal disease	[*Ca*. P. cocostanzaniae]	Undetermined	Firraro, 2004
3	Loofah witches' broom	[*Ca*. P. luffae]	16SrVIII-A	Firraro, 2004
4	Palm lethal yellowing	[*Ca*. P. palmae]	16SrIV-A	Firraro, 2004
5	Western X-disease	[*Ca*. P. pruni]	16SrIII-A	Firraro, 2004
6	Stolbur phytoplasma	[*Ca*. P. solani]	16SrXII-A	Firraro, 2004
7	Flavescence doree	[*Ca*. P. vitis]	16SrV-C	Firraro, 2004
8	Papaya yellow crinkle	*Ca*. P. aurantifolia	16SrII-D	Davis et al., 1997
9	Allocasuarina yellows	*Ca*. P. allocasuarinae	Undetermined	Marcone et al., 2004
10	Potato purple top wilt	*Ca*. P. americanum	16SrXVIII	Lee et al., 2006
11	Lime witches' broom	*Ca*. P. aurantifolia	16SrII-B	Zriek et al., 1995
12	Grapevine yellows	*Ca*. P. australience	16SrXII	Davis et al., 1997
13	Balanites witches' broom	*Ca*. P. balanitae	16SrV	Win et al., 2012
14	Hibiscus witches' broom	*Ca*. P. brasiliense	16SrXV	Montano et al., 2001
15	Papaya bunchy top	*Ca*. P. caricae	16SrXVII	Arocha et al., 2005

Wikipedia,2015

Major phytoplasma group/subgroups

Based on RFLP analysis of 16S rRNA gene

Sr. no.	Common name	Phytoplasma species	Group/subgroup	Reference
16	Chestnut witches' broom	*Ca*. P. castaneae	16SrXIX	Jung et al., 2002
17	Bindweed yellows	"*Ca*. P. convolvuli"	16SrXII	Martini et al., 2012
18	Soybean stunt	*Ca*. P. costaricanum	16SXXXI	Lee et al., 2011
19	Sugarcane white leaf	*Ca*. P. cynodontis	16SrXI	Jung et al., 2003
20	Areca palm yellow leaf	*Ca*. P. cynodontis	16SrXI-A	Ramaswamy et al., 2012
21	Sugarcane white leaf	*Ca*. P. cynodontis	16SrXI-C	Lee et al., 1998
22	Leafhopper bourne BVK	*Ca*. P. cynodontis	16SrXIV	Semuller et al., 2004
23	Bermuda grass white leaf	*Ca*. P. cynodontis	16SrXIV	Marcone et al., 2004
24	Cynodon white leaf	*Ca*. P. cynodontis	Undetermined	Blanche et al., 2003
25	Sorghum grassy shoot	*Ca*. P. cynodontis	Undetermined	Blanche et al., 2003
26	Sorghum grassy shoot	*Ca*. P. cynodontis	Undetermined	Blanche et al., 2003
27	Strawberry witches' broom yellows	*Ca*. P. fragariae	16SrXII-E	Valiunas et al., 2006
28	Ash yellows	*Ca*. P. fraxini	16SrVII-A	Griffiths et al., 1999
29	Sugarcane Yellow Leaf	"*Ca*. P. graminis"	16SrXVI	Arocha et al., 2005
30	Hydrangea phyllody	*Ca*. P. japonicum	16SrXII-D	Sawayanagi et al., 1999

Wikipedia,2015

Major phytoplasma group/subgroups

Based on RFLP analysis of 16S rRNA gene

Sr. no.	Common name	Phytoplasma species	Group/subgroup	Reference
31	Parsley leaf of tomato	*Ca*. P. lycopersici"	Undetermined	Arocha et al., 2007
32	Periwinkle phyllody	*Ca*. P. malasianum	16SrXXXII	Nejat et al., 2012
33	Apple proliferation	*Ca*. P. mali	16SrX-A	Semuller et al., 2004
34	*Cassia italica* witches' broom	*Ca*. P. omanense	16SrXXIX	Saddy et al., 2008
35	Rice yellow dwarf	*Ca*. P. oryzae	16SrXI-A	Namba et al., 2009
36	Rice yellow dwarf	*Ca*. P. oryzae	16SrXI-A	Jung et al., 2003
37	Almond lethal disease	*Ca*. P. phoenicium	16SrIX-D	Verdin et al., 2003
38	Pine shoot proliferation	*Ca*. P. pini	16SrXXI	Schneider et al., 2005
39	*Prunus* X-disease	*Ca*. P. pruni	16SrIII-A	Davis et al., 2012
40	European stone fruit	*Ca*. P. prunorum	16SrX-F	Semuller et al., 2004
41	Pear decline	*Ca*. P. pyri	16SrX-C	Semuller et al., 2004
42	Buckthorn witches' broom	*Ca*. P. rhamni	16SrXX	Marcone et al., 2004
43	*Rubus* stunt	*Ca*. P. rubi	16SrV	Maher et al., 2011
44	Bois nor	*Ca*. P. solani	16SrXII	Quaglino et al., 2013
45	*Spartium* witches' broom	*Ca*. P. spartii	16SrX-D	Marcone et al., 2004

Wikipedia,2015

Major phytoplasma group/subgroups

Based on RFLP analysis of 16S rRNA gene

Sr. no.	Common name	Phytoplasma species	Group/subgroup	Reference
46	*Passiflora* witches' broom	*Ca*. P. sudamericanum	16SrIII-V	Davis et al., 2012
47	*Passiflora* witches' broom	*Ca*. P. sudamericanum	16SrVI	Davis et al., 2012
48	Salt cedar witches' broom	*Ca*. P. tamaricis	16SrXXX	Zhao et al., 2009
49	Clover proliferation	*Ca*. P. trifolii	16SrVI-A	Hiruki et al., 2004
50	Elm yellows	*Ca*. P. ulmi	16SrV-A	Lee et al., 2004
51	Jujube witches' broom	*Ca*. P. ziziphi	16SrV-B	Jung et al., 2003
52	Aster yellows	*Ca*. P. asteris	16SrI	Lee et al., 2004
53	MexiCan periwinkle virescence	Not assigned	16SrXIII-A	Wei et al., 2007
54	Bermuda grass white leaf	Not assigned	16SrXIV	Marcone et al., 2004
55	Grapevine yellow	Not assigned	16SrXXIII-A	Wei et al., 2007
56	Sorghum bunchy shoot	Not assigned	16SrXXIV-A	Wei et al., 2007
57	Weeping tea witches' broom	Not assigned	16SrXXV-A	Wei et al., 2007
58	Sugarcane yellows	Not assigned	16SrXXVI-A	Wei et al., 2007
59	Sugarcane yellows	Not assigned	16SrXXVII-A	Wei et al., 2007
60	Derbid phytoplasma	Not assigned	16SrXXVIII-A	Wei et al., 2007
61	Chinaberry yellows	Not assigned	Undetermined	Wei et al., 2007

Wikipedia,2015

The first comprehensive phytoplasma classification scheme

14 major phytoplasma groups & 32 subgroups

- Dr. Lee is best known for his pioneering studies on molecular detection, identification, and classification of phytoplasmas.
- Most diagnostic protocols for phytoplasmas include:

1. A first PCR amplification driven with universal primers;
2. Followed by a nested PCR with group specific primers;
3. RFLP analysis of the group-specific amplicon may then be required for final identification of the phytoplasma strain.

The first comprehensive phytoplasma classification scheme
Major phytoplasma group/subgroups

- The similarity coefficients of RFLP patterns between two distinct groups were 90% or below.
- An arbitrary threshold of 2.5% dissimilarity was applied as a guideline for electing a new species (IRPCM,2004).
- Based on RFLP analyses of PCR-amplified 16S rDNA sequences using 17 restriction enzymes, the system initially differentiated phytoplasmas into:

1. 14 groups, and
2. 32 subgroups.

Classification of phytoplasmas based on RFLP (or putative restriction site) analyses of 16S rRNA and ribosomal protein gene sequences comprises:

1. **14 major phytoplasma groups, and**
2. **32 subgroups.**

Lee *et al.*,1998

16Sr group	Strain	Original source	16Sr-rp sub-group	Accession no. (16Sr)	Accession no. (rp)
16SrI (Aster yellows group)					
I-A	Tomato big bud BB	Tomato: Arkansas	16SrI-A(rp-A)	L33760	L27004
I-A	New Jersey aster yellows NJAY	Lettuce: New Jersey	16SrI-A(rp-A)		
I-A	Aster yellows AY27	Aster: Canada	16SrI-A(rp-A)		
I-A	Eastern aster yellows NAY	Lettuce: Canada	16SrI-A(rp-A)		
I-A	Periwinkle little leaf CN1	Periwinkle: Connecticut	16SrI-A(rp-A)		
I-A	Oklahoma aster yellows OKAY1	Lettuce: Oklahoma	16SrI-A(rp-A)		
I-B	Michigan aster yellows MIAY	Evening primrose: Michigan	16SrI-B(rp-B)	M30970	M77470
I-B	Maryland aster yellows AY1	Periwinkle: Maryland	16SrI-B(rp-B)	L33767	
I-B	American aster yellows AAY	Periwinkle: Florida		X68373	
I-B	Dwarf aster yellows DAY	Clover: California			
I-B	Western aster yellows SAY	Celery: California	16SrI-B(rp-B)	M86340	
I-B	Aster yellows OKAY3	Carrot: Okalahoma	16SrI-B(rp-B)		
I-B	Western aster yellows TLAY	Potato: California			
I-B	Hydrangea phyllody HyPH1	Hydrangea: Italy	16SrI-B(rp-K)		
I-B	Chrysanthemum yellows CY	Chrysanthemum: Italy			
I-B	Onion yellows OAY (OA)	Onion: Japan		D12569	
I-B	European aster yellows EAY	Aster: Gemany			
I-B	Aster yellows-Koolsard KD	Cabbage: UK			
I-B	Aster yellows-Cactus CC	Cactus: UK			
I-B	Primula yellows PY	Primula: Germany			
I-B	Gladiolus yellows GLY	Gladiolus: UK			
I-B	Hydrangea virescence	Hydrangea: Belgium			
I-B	Mitsuba witches'-broom JHW	*Cryptotaenia*: Japan			
I-B	Garland chrysanthemum WB GCW	*Chrysanthemum coronarium*: Japan			
I-B	Eggplant dwarf ED	Eggplant: Japan			
I-B	Tomato yellows TY	Tomato: Japan			
I-B	Marguerite yellows MY	*Chrysanthemum frutescens*: Japan			
I-B	Ipomoea witches'-broom IOB	*Ipomoea* sp.: Taiwan	16SrI-B(rp-F)		
I-B	Maize bushy stunt MBS	Corn: Ohio, Mexico	16SrI-B(rp-L)		
I-B	Mulberry dwarf MD	Mulberry: Japan, China			
I-C	Clover phyllody CPh	Clover: Canada	16SrI-C(rp-C)	L33762	
I-C	Strawberry green petal SGP	Strawberry: Canada	16SrI-C(rp-C)		
I-C	Ranunculus phyllody RPh	Ranunculus: Italy	16SrI-C(rp-C)		
I-D	Paulownia witches'-broom PaWB	Paulownia: Taiwan	16SrI-D(rp-D)		
I-E	Blueberry stunt BBS1 BBS3	Blueberry: Michigan, Arkansas	16SrI-E(rp-E)		
I-F	Apricot chlorotic leaf roll ACLR-AY	Apricot: Spain		X68383	
I	Grey dogwood witches'-broom GD1	Grey dogwood: USA	16SrI(rp-M)		
16SrII (Peanut WB group)					
II-A	Peanut witches'-broom PnWB	Peanut: Taiwan		L33765	
II-A	Sweet potato witches'-broom SPWB	Sweet potato: Taiwan		L33770	
II-A	Sunhemp witches'-broom SUNHP	Sunhemp: Thailand		X76433	
II-B	Witches'-broom of lime WBDL '*Candidatus* Phytoplasma aurantifolia'	Lime: Arabic Peninsula		U15442	
II-C	Faba bean phyllody FBP	Faba bean: Sudan		X83432	
II-D	Sweet potato little leaf SPLL	Sweet potato: Australia			
16SrIII (X-disease group)					
III-A	X-disease CX	Peach: Canada	16SrIII-A(rp-A)	L33733	L27016
III-A	X-disease WX	Peach: California	16SrIII-A(rp-B)	L04682	L27047
III-A	Peach yellow leaf roll PYLR	Peach: California	16SrIII-A(rp-B)		
III-A	X-disease CCX	Choke cherry: New York	16SrIII-A(rp-B)		
III-B	Clover yellow edge CYE	Clover: Canada	16SrIII-B(rp-C)	L33766	L27019
III-B	Vaccinium witches'-broom VAC	Vaccinium: Germany		X76430	
III-B	Tsuwabuki witches'-broom TW	Farfugium: Japan		D12580	
III-B	Gentian witches'-broom GW	Gentian: Japan			
III-C	Pecan bunch PB	Pecan: Georgia	16SrIII-C(rp-G)		
III-D	Goldenrod yellows GRY (GR1)	Goldenrod: New York	16SrIII-D(rp-E)		
III-E	Spiraea stunt SP1	Spiraea: New York	16SrIII-E(rp-F)		
III-F	Milkweed yellows MWY (MW1)	Milkweed: New york	16SrIII-F(rp-D)		
III-G	Walnut witches'-broom WWB	Walnut: Georgia	16SrIII-G(rp-B)		
III-H	Poinsettia branch-inducing PoiBI	Poinsettia: US			
16SrIV (Coconut lethal yellows group)					
IV-A	Coconut lethal yellows LY, LY3	Palm: Florida		U18747	
IV-B	Yucatan coconut lethal decline LDY	Palm: Mexico		U18753	
IV-C	Tanzanian coconut lethal decline LDT	Palm: Africa		X80117	
16SrV (Elm yellows group)					
V-A	Elm yellows EY1	Elm: New York	16SrV-A(rp-A)	L33763	L27022
V-A	Elm yellows ItaEY	Elm: Italy			
V-A	Elm witches'-broom ULW	Elm: France		X68376	
V-B	Cherry lethal yellows CLY	Cherry: China	16SrV-B(rp-B)		
V-B	Jujube witches'-broom JWB	Jujube: China	16SrV-B(rp-C)		
V-C	Rubus stunt RS	Rubus: Italy			
V-C	Alder yellows AlY	Alder: Germany			

Continued…

Classification of phytoplasmas based on RFLP (or putative restriction site) analyses of 16S rRNA and ribosomal protein gene sequences comprises:

1. **14 major phytoplasma groups,**
2. **32 subgroups.**

Table 1. *(cont.)*

16Sr group	Strain	Original source	16Sr-rp sub-group	Accession no. (16Sr)	Accession no. (rp)
V-C	Spartium witches'-broom (EY)	Spartium: Italy			
V-C	Eucalyptus little leaf	Eucalyptus: Italy			
V-C	Flavescence dorée FD	Grapevine: France	16SrV-C(rp-D)	X76560	
16SrVI (Clover proliferation group)					
VI-A	Clover proliferation CP	Clover: Canada		L33761	L27011
VI-A	Periwinkle virescence VR, BLTVA	Periwinkle: California			
VI-A	Tomato big bud TBB	Tomato: California			
VI-A	Potato witches'-broom PWB	Potato: Canada			
VI-A	Potato yellows	Potato: North Dakota			
16SrVII (Ash yellows group)					
VII-A	Ash yellows AshY	Ash: New York		X68339	L26999
VII-A	Lilac witches'-broom LiWB	Lilac: New York			
16SrVIII (Loofah witches'-broom group)					
VIII-A	Loofah witches'-broom LfWB	Loofah: Taiwan		L33764	L27027
16SrIX (Pigeon pea witches'-broom group)					
IX-A	Pigeon pea witches'-broom PPWB	Pigeon pea: Florida		U18763	L27036
16SrX (Apple proliferation group)					
X-A	Apple proliferation AT, AP-A	Apple: Germany, Italy		X68375	L27994
X-B	Apricot chlorotic leaf roll ACLR (Ita)	Apricot: Italy			
X-B	Plum leptonecrosis PLN	Japanese plum: Italy			
X-B	European stone fruit yellows PPER	Peach: Germany		X68374	
X-C	Pear decline PD	Pear: Italy, UK			
X-D	Spartium witches'-broom SPAR	Spartium: Italy		X92869	
X-E	Black alder witches'-broom BAWB (Buckthorn witches'-broom BWB)	Black alder (Buckthorn): Germany		X76431	
16SrXI (Rice yellow dwarf group)					
XI-A	Rice yellow dwarf RYD	Rice: Japan, India		D12581	
XI-B	Sugarcane white leaf SCWL	Sugarcane: Thailand		X76432	
XI-B	Sugarcane grassy shoot SCGS	Sugarcane: India			
XI-C	Leafhopper-borne BVK	*Psammotettix cephalotes*: Germany		X76429	
16SrXII (Stolbur group)					
XII-A	Stolbur STOL	*Capsicum annum*: Serbia		X76427	
XII-A	Grapevine yellows	Grapevine: Germany		X76428	
XII-A	Celery yellows CelY	Celery: Italy			
XII-B	Australian grapevine yellows AUSGY '*Candidatus* Phytoplasma australiense'	Grapevine: Australia		L76865	
XII-B	Phormium yellow leaf PYL	New Zealand flax: New Zealand		U43570	
16SrXIII	(Mexican periwinkle virescence group)				
XIII-A	Mexican periwinkle virescence MPV	Periwinkle: Mexico			
XIII-B	Strawberry green petal (Florida)	Strawberry: Florida			
16SrXIV	(Bermudagrass white leaf group)				
XIV-A	Bermudagrass white leaf BGWL	Bermudagrass: Thailand			
XIV-A	Annual blue grass white leaf ABGWL	*Poa annua*: Italy			

Lee *et al.*,1998

The first comprehensive phytoplasma classification scheme

14 major phytoplasma groups & 32 subgroups

- **PCR-RFLP analyses can detect phytoplasmas at group/subgroups level:**

1. The subsequent nested-PCR with phytoplasma group-specific primers followed by
2. RFLP analyses and the 16S ribosomal gene sequencing, allowed classification of the detected phytoplasmas in the different group/subgroups.

- PCR amplification and RFLP analysis of the amplified product, e.g. 16S rRNA gene, allows the rapid identification of phytoplasma isolates.

Example:

16SrI-A:

- 16SrI group (Group I/aster yellows group) and I-A subgroup. Tomato big bud is included in 16SrI-A.

PCR analysis

1. Universal primers

Designed for (all) phytoplasma detection

- General and specific primers, located in the 16S rDNA, intergenic spacer (IS) and the 23S rDNA region of the phytoplasma genome, are presently used.
- e.g. The universal primer pair P1/P7 can be used to prime the amplification of a 1.8 kb product of 16S ribosomal RNA (rRNA) gene, the spacer region between the 16S and 23S rRNA gene, and the start of the 23S rRNA gene regions of the phytoplasma genome.
- Two more frequently used universal primers are:
- R16F2/R16R2(1200 bp),
- R16mF2/R16mR1(1435 bpb).

PCR analysis

Universal primers

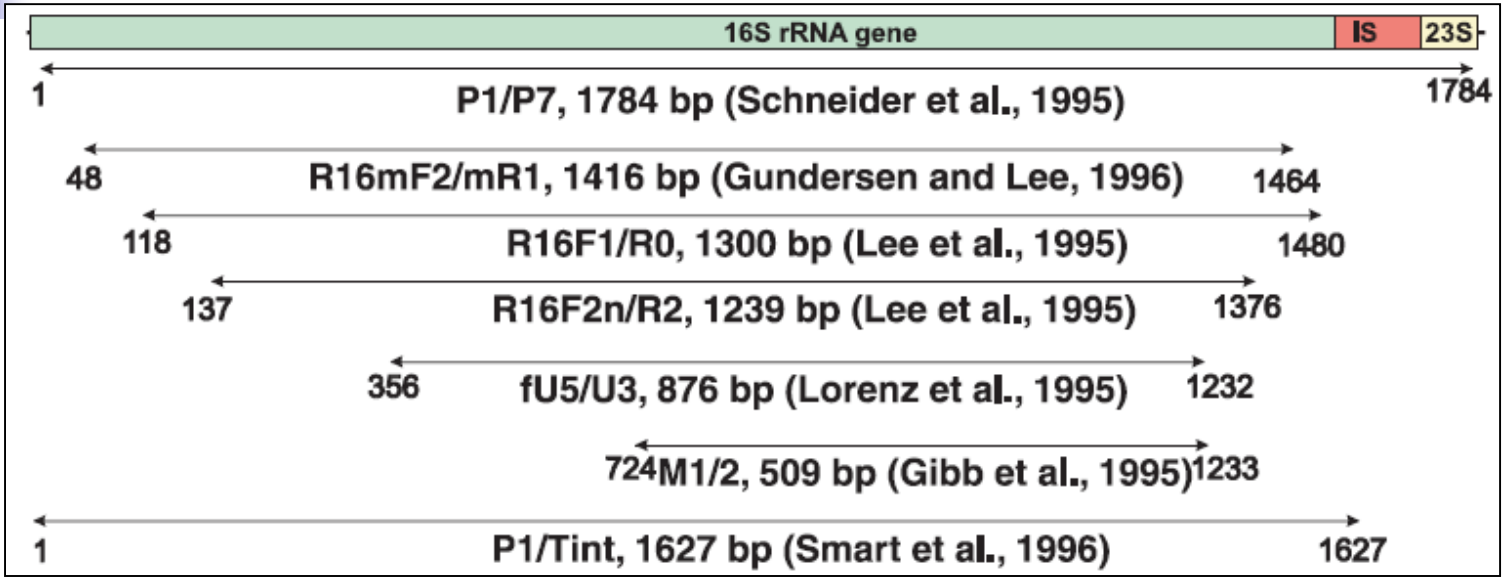
Designed for (all) phytoplasma detection

Primer pair	Target[a]	Primer sequence (5'-3')[b]	Expected size of PCR product (bp)	Specificity
Pl/P7	16SrDNA SR	aagagtttgatcctggctcaggatt (11-30)/ cgtccttcatcggctctt (23S rDNA))	1800	All phytoplasmas
RI6F1/RI6R0	16SrDNA	aagacgaggataacagttgg (129-149)/ ggataccttgttacgacttaacccc (1483-1458)	1354	All phytoplasmas
RI6mF2/ R16mR1	16SrDNA	catgcaagtcgaacgga (53-69)/ cttaaccccaatcatcgac (1487-1469)	1435	All phytoplasmas
RI6F2/ R16R2	16SrDNA	acgactgctgctaagactgg (152-168)/ tgacgggcggtgtgtacaaaccccg (1397-1373)	1248	All phytoplasmas
RI6F2n/ R16R2	16SrDNA	gaaacgactgctaagactgg (149-168)/ tgacgggcggtgtgtacaaaccccg (1397-1373)	1248	All phytoplasmas
fU5/rU3	16SrDNA	cggcaatggaggaaact (369-386)/ ttcagctactctttgtaaca (1251-1231)	882	All phytoplasmas
Pl/Tint	16SrDNA, SR	aagagtttgatcctggctcaggatt (11-30)/ tcaggcgtgtgctctaaccagc (SR-t RNA)	1600	All phytoplasmas
SN910601/ SN920204	16SrDNA	gtttgatcctggctcaggatt (1-21)/ cctcagcgtcagtaa (746-732)	746	All phytoplasmas
16R758F/ 16R1232R	16SrDNA	gtctttactgacgctgaggc (738-758)/ cttcagctacccttttgtaac (1251-1232)	513	All phytoplasmas

Schaad *et al.*,2001

Genomic location of several general and fruit tree specific primers

Primers



Heinrich *et al.*,2004

DNA extraction from infected tissues

Cetyl-trimethyl-ammonium bromide (CTAB) procedure

- Nucleic acids can be extracted from fresh or frozen (20°C or 80°C) tissues or tissues freeze-dried or dried over calcium chloride [leaf veins, vascular tissue (phloem) from bark or roots].
- For material that has been freeze-dried or dried over calcium chloride, the quantity needed for DNA extraction should be adapted depending on the weight loss during dry process.
- Grind approximately 1 g of tissue in 10 mL of 3% CTAB buffer (3% CTAB in 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 1.4 M NaCl) at room temperature. Transfer 1 mL of the suspension to an Eppendorf tube, add 2 µL of 2-mercaptoethanol (for a final concentration of 0.2%). Vortex briefly and incubate for 20 min at 65°C.
- Then, add an equal volume of chloroform:isoamyl alcohol (24:1). Vortex and centrifuge at 10 000 g for 10 min. Recover the aqueous phase and precipitate the nucleic acids with an equal volume of cold isopropanol.
- Shake by inversion and centrifuge at 10 000 g for 15 min to recover the precipitate. Wash the pellet with 70% ethanol, air dry and dissolve in 100 µL o f TE buffer (10 mM Tris, 1 mM EDTA, pH 8) or nuclease-free water.

OEPP/EPPO Bulletin,2018

DNA extraction from infected tissues

Stolbur phytoplasmas in *Prunus avium*(cherry)

Cetyl-trimethyl-ammonium bromide (CTAB) procedure

- Samples from symptomatic (Wilting, dying, and phloem necrosis) and asymptomatic cherry were collected.
- Total DNA was extracted from leaf midribs, secondary roots and scrape of phloem from small branches or trunk (cherry) and from roots and leaves (bindweed) using a CTAB method (Doyle and Doyle, 1990) and the Plant DNeasy mini kit (Qiagen GmbH, Hilden).
- Preliminary identification was carried out performing PCR assays with universal phytoplasma rDNA primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995), followed by nested PCR using the universal primers U3/U5 (Lorenz *et al.*, 1995).
- Reference controls included DNA extracts from asymptomatic cherry, naturally infected tomato samples showing typical stolbur symptoms, naturally infected apple showing apple proliferation symptoms, and PEY (Pichis echioides yellows, subgroup 16SrIX-C) and CX (X disease from peach, subgroup 16SrIII-A) infected Catharanthus *roseus*.
- Tubes without DNA were used as negative controls.

Avramov *et al.*,2011

PCR/nested PCR analyses

Universal Primers

Method

1. Phloem tissue from stems and leaf midribs of infected plants were subjected to DNA extraction.
   - 20 ng of each DNA preparation was added to the PCR reaction mix in a final reaction volume of 25 µl.
2. Amplify the DNA by 35 cycles in a Perkin Elmer 480 thermal cycler.
3. Dilute these PCR products with sterile distilled water (1:39) prior to amplification by nested PCR using general (R16F2/R2) and group-specific primer pairs(R16(I)F1/R1).
4. Observe PCR products in 1% agarose gel and photograph with the Gel Doc.

PCR-RFLP analyses

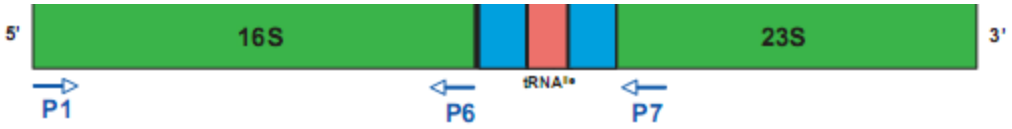
Detection at subgroups level

Method of RFLP analyses of the PCR product

5. Three to 10 µl (about 200 ng of DNA) of each positive nested PCR product observed after 1% agarose gel electrophoresis were separately digested RFLP analyses.
6. Digestion were undertaken with 2.5 U of each 17 restriction enzymes:
   - *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I),*Dra*I, *EcoR*I, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I and *Taq*I (Lee *et al.*,1998; Wei *et al.*,2008b).
7. Digestion reactions were left at the required temperature for at least 16 h.
8. The restriction patterns were compared after electrophoresis on a 5% polyacrylamide gel followed by ethidium bromide staining, and photographed under UV at 312 nm using a transilluminator.
9. The same procedure was performed with reference phytoplasma strains belonging to other subgroups such as 16SrI-B, 16SrI-C, etc.

PCR/nested PCR analyses
Phytoplasma universal primers P1/P6

- Not only phytoplasma DNA could be amplified with these primers.
- DNA from Entomoplasmatales and *Acholeplasma palmae*, from insects or plant surfaces were amplified and gave products with a size similar to the product obtained with phytoplasma DNA (~1496 pb).
- *Leifsonia xyli*, gram-positive bacteria (Actinomycetales) also gave a PCR product with a very similar size.



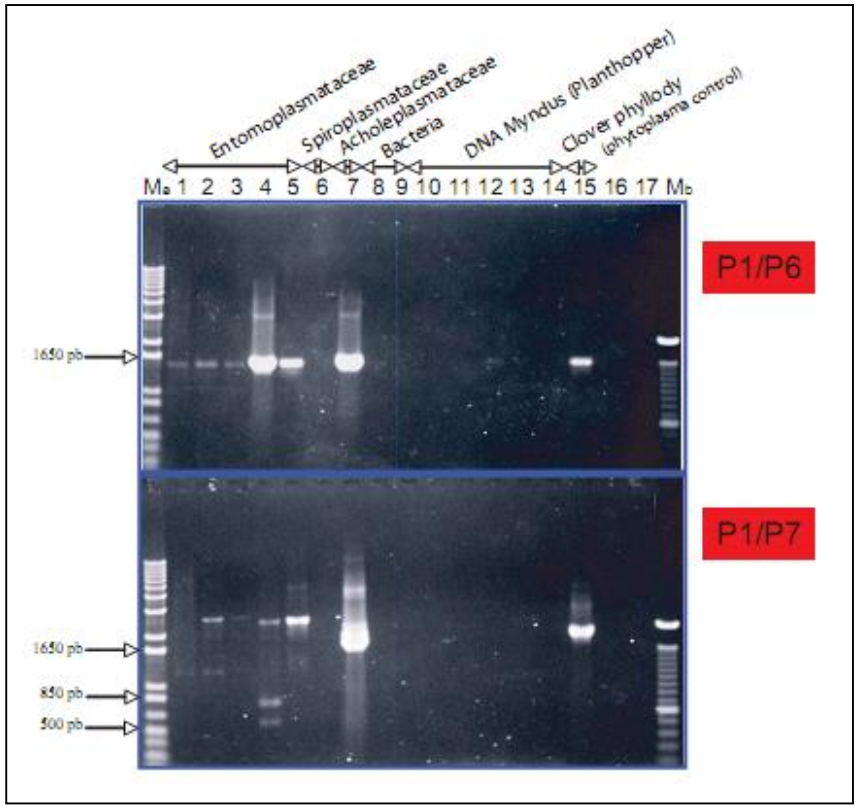
Dollet and co-workers

PCR/nested PCR analyses

Phytoplasma universal primers

P1/P7 and P1/P6

- Amplifications were also obtained with primers P1/P7, even though the amplicons were not always of the expected size.
- When using primers P1/P7 and P1/P6 not only phytoplasma DNA could be amplified but also some mollicutes or bacterium DNAs.



Dollet and co-workers

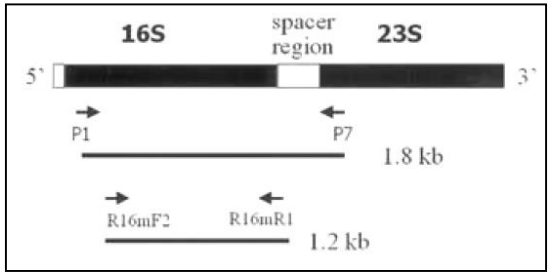
Phytoplasma detection methods

Based on direct and nested-PCR assays

Primers P1/P7 and R16mF2/R16mR1

- The direct-PCR products thus obtained were subsequently used in nested-PCR.
- The nested-PCR was sensitive enough to reamplify the direct-PCR product (1.8 kb), resulting in a DNA fragment of 1.2 kb.
- Below is an example of oligonucleotide primer sequences used for PCR(direct and nested-PCR assays) amplification as well as schematic diagram of a phytoplasma rRNA operon showing the 16S and 23S genes and intergenic spacer region.

Primer	Location	Oligonucleotide sequence
P1	16S	5′ AAGAGTTTGATCCTGGCTCAGGATT 3′
P7	23S	5′ CGTCCTTCATCGGCTCTT 3′
R16mF2	16S	5′ CATGCAAGTCGAACGA 3′
R16mR1	16S	5′ CTTAACCCCAATCATCGAC 3′



Khan *et al.*,2004

PCR-RFLP analyses

2-Group-specific primers

Designed for sub-groups detection

RI6(I)Fl/ R16(I)R1	16SrDNA	taaaagacctagcaatagg (278-297)/ caatccgaactgagactgt (1297-1279)	1020	Group 16SrI (AY)
SN920203/ SN910502	16SrDNA	tttaagcaattaaacttta (63-81)/ aaccccgagaacgtattcacc (1368-1348)	1306	16SrI
P1/AYint	16SrDNA, SR	aagagtttgatcctggctcaggatt (11-30)/ tacaatttgcaagcaagttac (SR)	1500	16SrI
R16(III)F2/ R16(III)R1	16SrDNA	aagagtggaaaaactccc (457-474)/ tccgaactgagattga (1355-1340)	899	16SrIII
Pl/WXint	16SrDNA, SR	aagagtttgatcctggctcaggatt (11-30)/ gacagtgcttataacttta (SR)	1600	16SrIII
RI6(V)Fl/ R16(V)Rl	16SrDNA	ttaaaagaccttcttcgg (204-221)/ ttcaatccgtactgagactacc (1359-1338)	1156	16SrV
fB1/rULWS1	16SrDNA, SR	gacccttcaaaaggtcttag (73-91)/ cgtcttttatataagagaaaca (SR)	1500	16SrV
Pl/BLTVAint	16SrDNA, SR	aagagtttgatcctggctcaggatt (11-30)/ gatgattttagtatatatagtcc (SR)	1450	16SrVI
fBl/rASHYS	16SrDNA, SR	gacccttcaaaaggtcttag (73-91)/ gcaggaccgtttatattaatc (SR)	1500	16SrVII
R16(X)F1/ R16(X)R1	16SrDNA	gacccgcaagtatgctgagagatg (210-233)/ caatccgaactgagactgt (1358-1339)	1149	16SrX

Schaad *et al.*,2001

PCR-RFLP analyses

Group-specific primers

Designed for sub-groups detection

Primer pair	Target[a]	Primer sequence (5'-3')[b]	Expected size of PCR product (bp)	Specificity
fAT/rAS	16SrDNA SR	ccatcatttagttgggcactt (1113-1131)/ ggccccggaccattatttatt (SR)	500	16SrX
P1/PYLRint	16SrDNA, SR	aagagtttgatcctggctcaggatt (11-30)/ cccggccattattaatttttatc (SR)	1550	16SrX-B, C
fPD/rPDS	16SrDNA, SR	gacccgtaaggtatgctga (205-324)/ cccggcaattattaatttta (SR)	1400	16SrX-C
SN910601/ SN920202	16SrDNA	gtttgatcctggctcaggatt (1-21)/ atgcaccacctgtatcc (1031-1015)	1031	16SrXI
rpF1/rpR1	rp genes	ggacataagttaggtgaattt (rps19)/ acgatatttagttctttttgg (rpl16)	1232 1350	16SrI, V, VII, VIII, X 16SrIII
rpF2/rpR2	rp gene	tctcgtacttttcgtgg (rps19)/ acctttagctcttggaa (rpl22)	1200	16SrVI,X
rp3/rp4	rp gene	aacttctagcacaaacttgc(rpl22)/ gtctgttaggagtgttagaa(rps3)	750	16SrI-D(rp-D)
IIIrpFl/ IIIrpR1	rp gene	agaaaggcattaaacatatgaat/ cgctgttcataattttagcct	1300	16SrIII
VrpF1/rpR1	rp gene	tcgcggtcatgcaaaaggcg/ acgatatttagttctttttgg	1200	16SrV
CN1-10F1/ CN1-10R1	Cloned non-rDNA	gggttaaggctagaaatggatcttg/ tatcagatatcattgggcgaaggaat	960	16SrI

Schaad *et al.*,2001

PCR-RFLP analyses

Group-specific primers

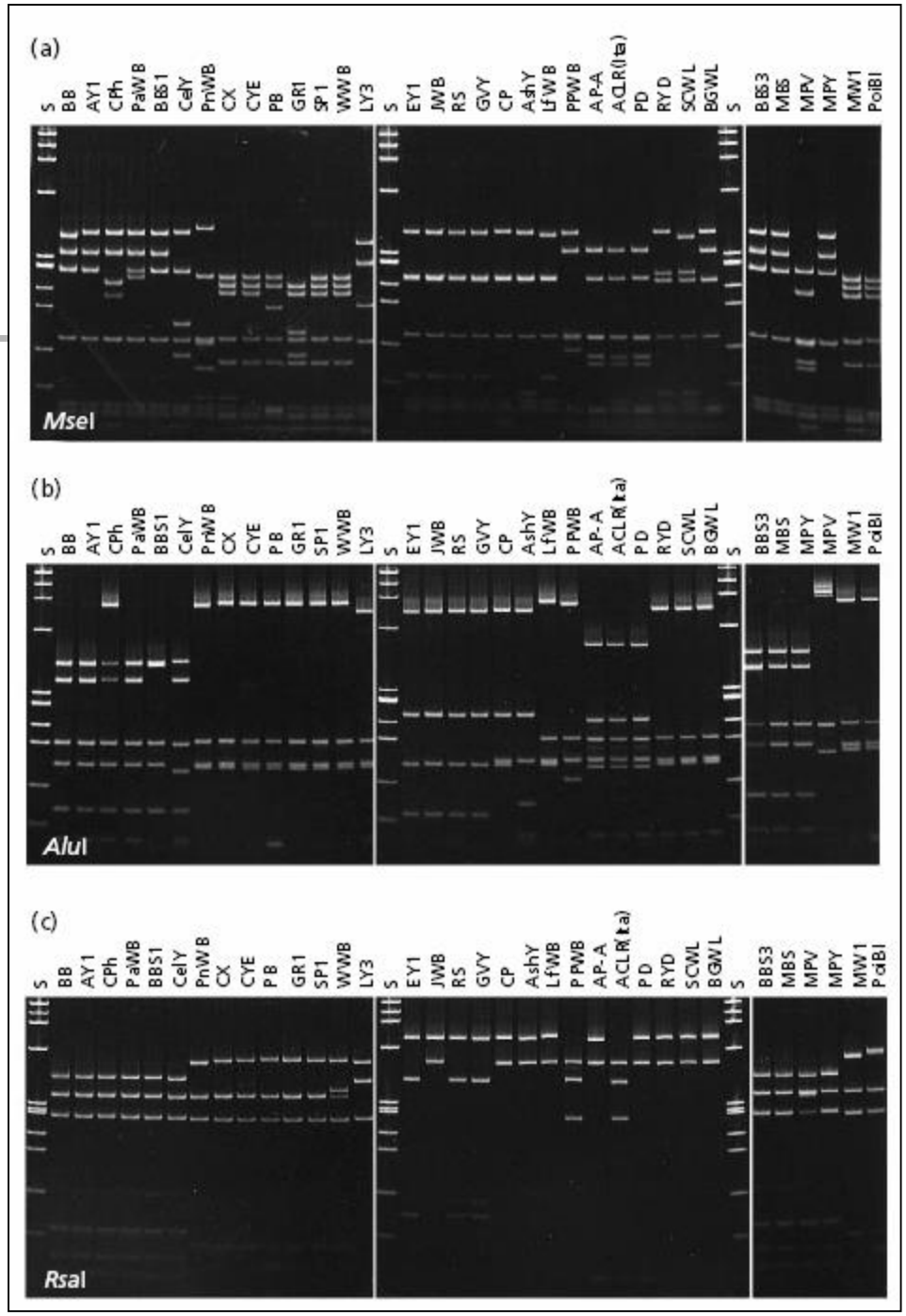
Designed for sub-groups detection

BB88F1/ BB88R1	Cloned non-rDNA	caccatcacacgcgtgatgaccgcctttcc/ gccttacaactacaccatcagtttgaggag	740	16SrI
CK6/CK8	Plasmid DNA	cctctttaggcccaatagcag/ ctcatgttttcagcttgggttc	235	16SrI
AY18p/AY18m	Cloned non-rDNA	aacccaaactatcaaaag/ tgtttctacttcttcttg	1600	16SrI-A, B, C
AY19p/AY19m	Cloned non-rDNA	taacatcagaataaatgg/ gacttacgttggtgaagg	1100	16SrI-A, B
G35p/G35m	Cloned non-rDNA	taacactgtggaagctca/ cgtcaatggctaatcgat	1200	Some 16SrI subgroups
MBS-F1/MBS-R1	Cloned non-rDNA	aatgtcgaactaacaggcgg/ ttggcgatttggttttgg	740	16SrI(rp-L) (Maize bushy stunt)
EY11-F1/ Ey11-R1	Cloned non-rDNA	gactcteggatgagagtcttgggtg/ cttgggattatggccctgttataccag	1100	16SrV-A
1A/1B	Cloned non-rDNA	tcttttacctaaattttgaggtaattc/ ttgtgttatcgcaagggctttagg	196	16SrVI-A
LY-F1/LY-R1	Cloned non-rDNA	catattttatttcctttgcaatctg/ tcgttttgataatctttcatttgac	1000	16SrVII-A (Coconut lethal yellows)
fStol/rStol	Cloned non-rDNA	gccatcattaagttgggga/ agatgtgacctattttggtgg	500	16SrXII

Schaad *et al.*,2001

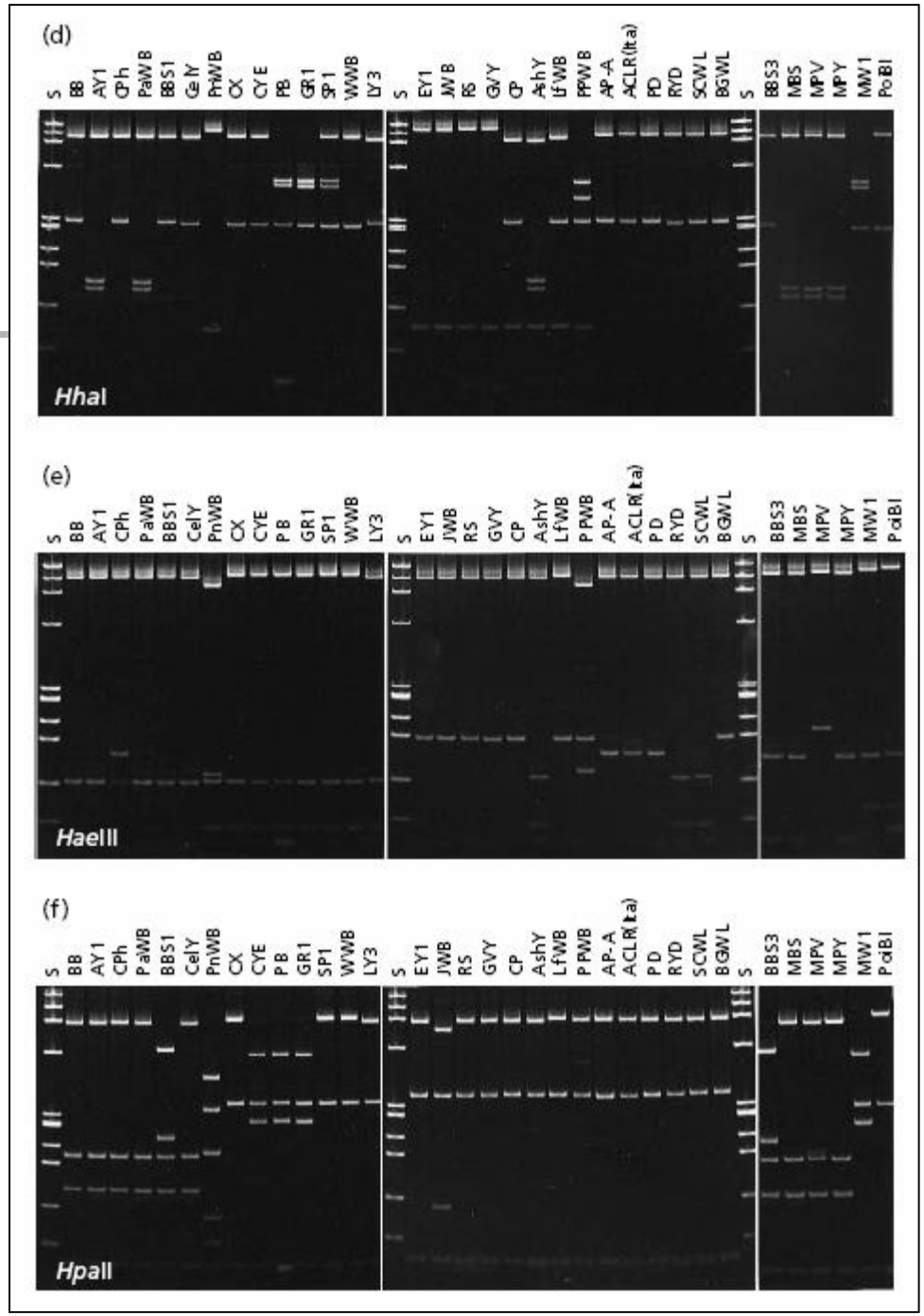
RFLP analyses of 34 phytoplasma 16S rDNAs

- The presence of upper two bands seen in lanes PB and PoiBI in (I) were due to incomplete digestion.
- Lane S, uX174 RFI DNA *Hae*III digest, fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.
- Other abbreviations are mentioned earlier.



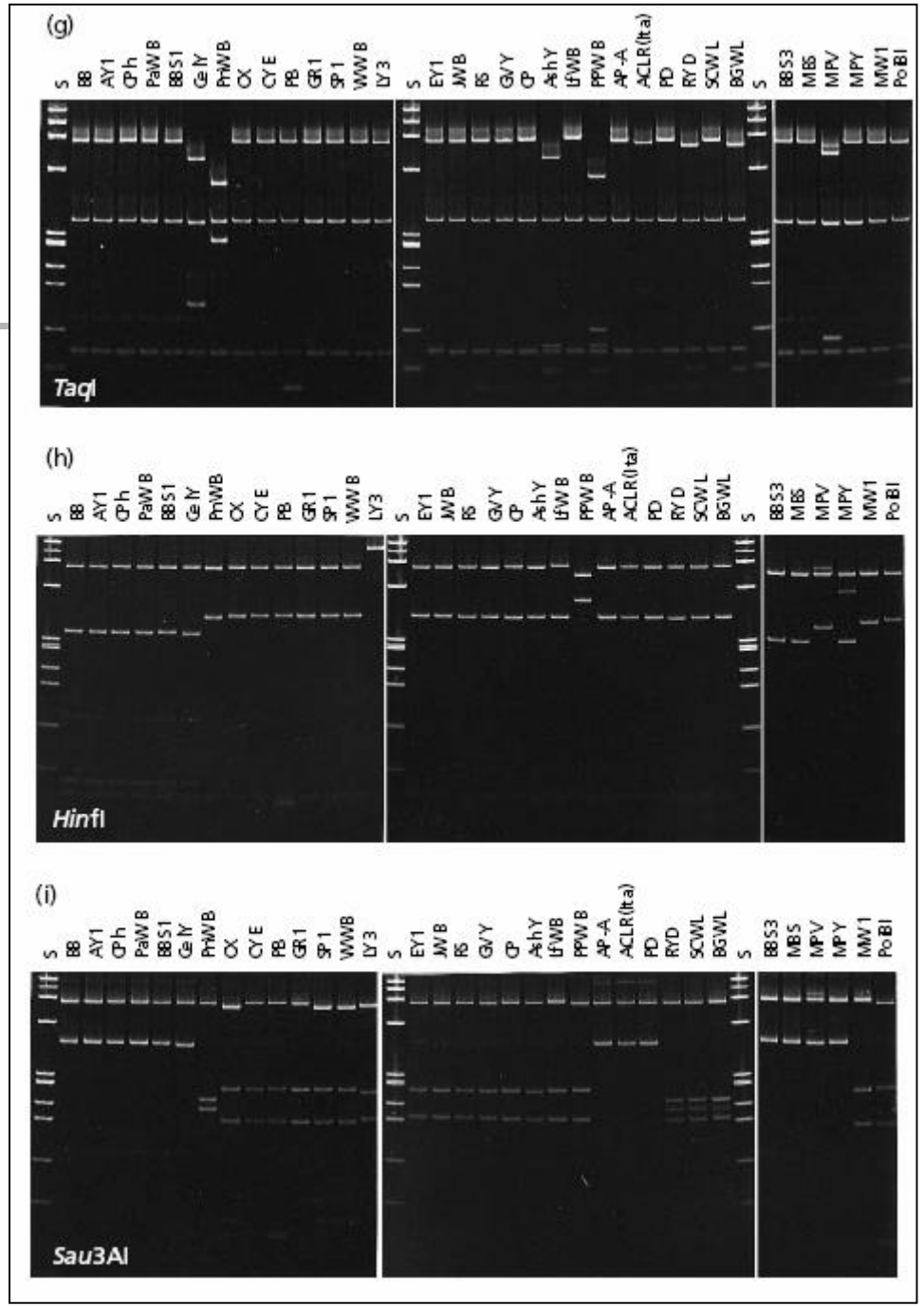
Lee *et al.*,2001

RFLP analyses of 34 phytoplasma 16S rDNAs



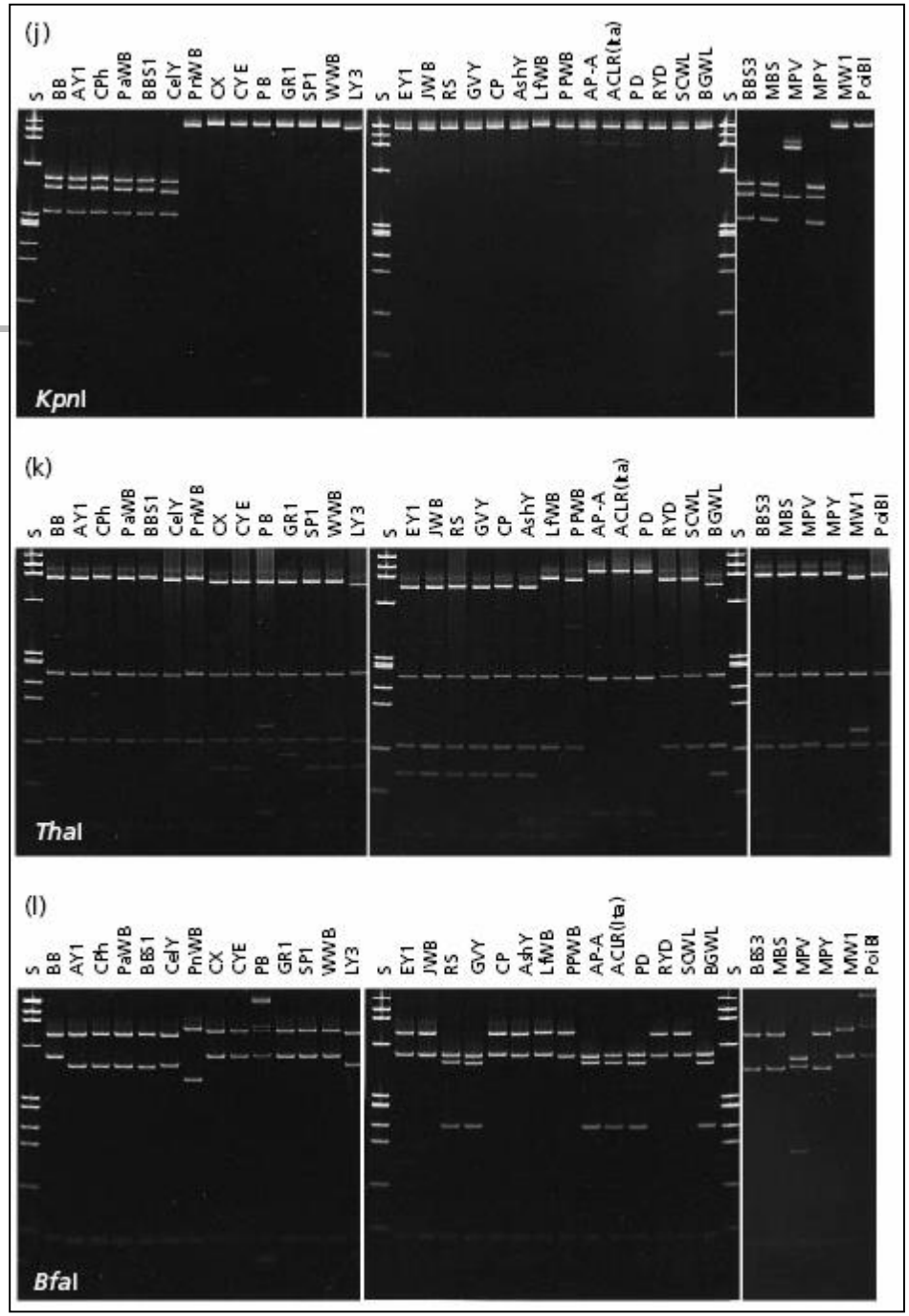
Lee *et al.*,2001

RFLP analyses of 34 phytoplasma 16S rDNAs



Lee *et al.*,2001

RFLP analyses of 34 phytoplasma 16S rDNAs



Lee *et al.*,2001

Phytoplasma taxonomy

Classification based on RFLP analyses of alternate genes

Genes other than 16S rRNA

- Because of its highly conserved nature, the 16S rRNA gene is inadequate for finer differentiation of closely related but distinct phytoplasmas strains.
- To overcome this limit, several universal and group-specific primers were developed on the basis of:
- 16S-23S rRNA intergenic spacer region (ISR);
- *Tuf* gene (encoding the elongation factor, EF-Tu, is another highly conserved gene with a central role in translation);
- Ribosomal protein (rp) genes;
- Immunodominant membrane protein (*imp*) gene;
- *SecA* gene and stress associated protein 11(SAP11)gene. These new molecular markers were used in identification and classification of phytoplasmas.

Phytoplasma taxonomy

List of molecular markers for phytoplasma classification

Marker	Primers (5′-3′)	Amplicon size (bp)	Variable sites	Informative sites	References
16S rRNA	[R16F2n] GAAACGACTGCTAAGACTGG [R16R2] TGACGGGCGGTGTGTACAAACCCCG	1,246	15	6	Gundersen and Lee, 1996
Ribosomal proteins (*rpl22* and *rps3*)	[rpF1] GGACATAAGTTAGGTGAATTT [rpR1] ACGATATTTAGTTCTTTTTGG	1,240	70	25	Lim and Sears, 1992
Ribosomal proteins (*rpl22* and *rps3*)	[rpF1C] ATGGTDGGDCAYAARTTAGG [rp(I)R1A] GTTCTTTTTGGCATTAACAT	1,236	70	25	Martini et al., 2007
Preprotein translocase SecY (*secY*)	[AYsecYF1] CAGCCATTTTAGCAGTTGGTGG [AYsecYR1] CAGAAGCTTGAGTGCCTTTACC	1,358	105	62	Lee et al., 2006
Chaperonin 60 (*groEL/cpn60*)	[H279p] GATIIIGCAGGIGATGGAACMACIAC [H280p] TGRTTITCICCAAAACCAGGIGCATT	605	21	12	Dumonceaux et al., 2014
Chaperonin 60 (*groEL/cpn60*)	[AYgroelF] GGCAAAGAAGCAAGAAAAG [AYgroelR] TTTAAGGGTTGTAAAAGTTG	1,396	59	34	Mitrović et al., 2011
Elongation factor Tu (*tuf*)	[fTufu] CCTGAAGAAAGAGAACGTGG [rTufu] CGGAAATAGAATTGAGGACG	842	39	23	Schneider and Gibb, 1997
Replication initiation protein DnaD (*dnaD*)	[F] CACAAGAAAAATTAGAAGCTC [R] ATAAGTTAAAAGCACATTGAC	767	77	60	This study
DegV family protein (*degV*)	[F] GTAGTTGATTCTACTTGCGG [R] ACTACAGGAGAAATAGCACC	785	57	36	This study
TIGR00282 family metallophospho-esterase	[F] AGATATTTACGGAAACCCAG [R] AGGTTTAAGAGTGACAAGTAAA	708	47	30	This study
Preprotein translocase SecY (*secY*)	[F] AATTGTTGTTTCGATGAGCC [R] TTGGCAGTAGCTTTGATGCG	711	64	36	This study
RluA family pseudouridine synthase (*rluA*)	[F] AAAGAGTTTCTTATTTCTGCCAG [R] GACCTAAAGGAGTAATATGGTG	778	80	54	This study

Cho *et al.*,2020

Phytoplasma taxonomy

Classification based on RFLP analyses of alternate genes

Genes other than 16S rRNA

- **Examples:**

1. **16S-23S rRNA intergenic spacer region (ISR):**

- ISR (about 232 bp) can serve as a useful tool for differentiation of phytoplasma groups and subgroups.
- Taxonomic groupings based on the analysis on the 16S/23S spacer regions which shows greater variation than the normally used 16srRNA gene results in classification similar to that derived from 16S rRNA data but with more detailed subdivisions.

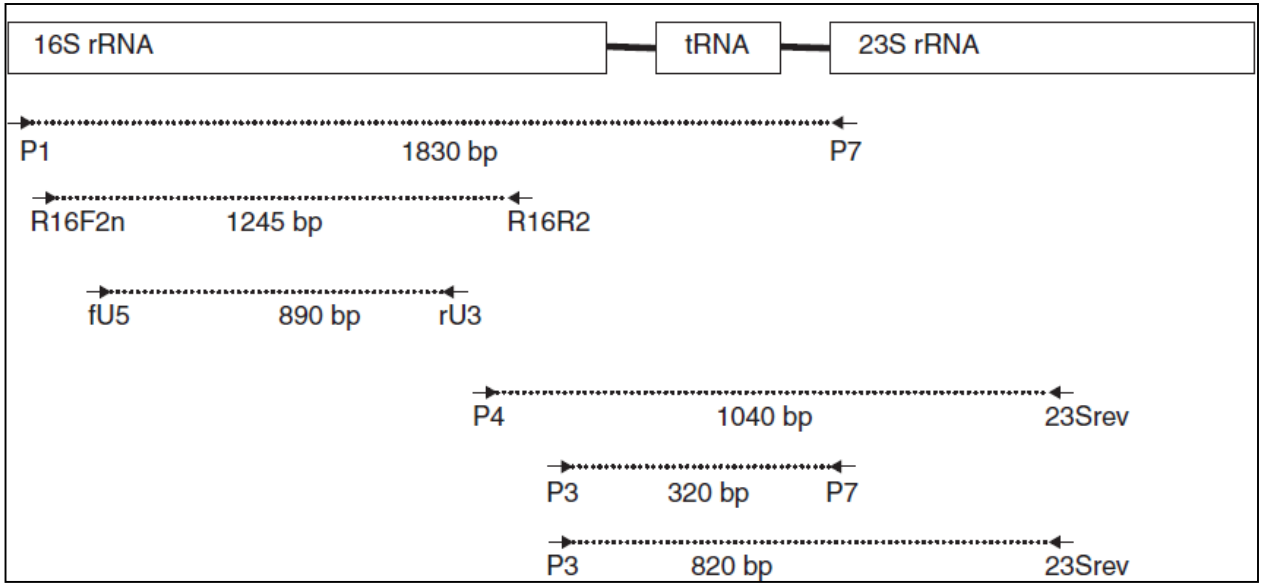
Weintraub and Jones,2010

Phytoplasma taxonomy

Classification based on RFLP analyses of alternate genes

Genes other than 16S rRNA

- Diagrammatic representation of the 16S-23S rRNA operon, showing the position of some of the various universal primers that have been developed for PCR amplification of this region from phytoplasmas.
- Primer names are given under the arrows and the sizes of the expected amplicons are shown between the dotted lines. Not drawn to scale.



Weintraub and Jones,2010

Ribosomal gene sequencing

16Sr DNA and 16S-23S rDNA spacer regions

- The 16Sr DNA and 16S-23S rDNA spacer regions of all tested phytoplsma were sequenced.
- Sequencing can be done directly (PCR products) or after cloning.
- Sequencing of samples were obtained aligning PCR products generated by amplification with primers:
- P1/U3 (position 6-1230),
- R16F2/R2 (position 152-1397), and
- 16R758/P7 (position 758-1818).
- PCR products were sequenced in both directions using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK).
- Both sequences were aligned with those of phytoplasmas available in GenBank using the World Wide Web service CLUSTAL W (http://www2.ebi.ac.uk/clustalw/) or BLAST (http://www.ncbi.nlm.nih.gov).

Ribosomal gene sequencing

Alignment of the 16S-23S rRNA intergenic spacer region sequences AY phytoplasma from different hosts

- Alignment of the 16S-23S rRNA intergenic spacer region sequences AY phytoplasma from different hosts:
- (X) Queen Anne's lace,
- (Y) poker statice, and
- (Z) AY phytoplasma from parsley.

1. Between Queen Anne's lace and parsley, nucleotides 97, 99, 101, 105 and 130 and 231 differ.
2. Between Queen Anne's lace and poker statice, nucleotides 97, 101, 105, 119, 130 and 231 differ.
3. Between poker statice and parsley, nucleotides 99 and 119 differ.

- Despite these differences, all were highly homologous with each other and therefore could be classified in the same phylogenetic group.

```
X     CAAGGTATCCCTACCGGAAGGTGGGGATGGATCACCTCCTTTC            43
Y     CAAGGTATCCCTACCGGAAGGTGGGGATGGATCACCTCCTTTC            43
Z     CAAGGTATCCCTACCGGAAGGTGGGGATGGATCACCTCCTTTC            43

X     TAAGGAAACAATTATCATCTTCAGTTTTGAGAGACTTAAGAAAGTTTTTC     93
Y     TAAGGAAACAATTATCATCTTCAGTTTTGAGAGACTTAAGAAAGTTTTTC     93
Z     TAAGGAAACAATTATCATCTTCAGTTTTGAGAGACTTAAGAAAGTTTTTC     93

X     ATT TAA TTG TTGCAAATTGTATTTGCAACATTT AATCTTTTTAAGA    143
Y     ATTGTAACTTGCTTGCAAATTGTAT TGCAACATTTTAATCTTTTTAAGA    143
Z     ATTGT ACTTGCTTGCAAATTGTATTTGCAACATTTTAATCTTTTTAAGA    143

X     TTAAGGGCCTATAGCTCAGTTGGTTAGAGCACACGCCTGATAAGCGTGAG   193
Y     TTAAGGGCCTATAGCTCAGTTGGTTAGAGCACACGCCTGATAAGCGTGAG   193
Z     TTAAGGGCCTATAGCTCAGTTGGTTAGAGCACACGCCTGATAAGCGTGAG   193

X     GTCGGTGGTTCAAGTCCATTTAGGCCCACCATAACCA AAATAGGCAAAA   243
Y     GTCGGTGGTTCAAGTCCATTTAGGCCCACCATAACCACAAATAGGCAAAA   243
Z     GTCGGTGGTTCAAGTCCATTTAGGCCCACCATAACCACAAATAGGCAAAA   243

X     TCTTAAAAAAAGCTCTTTGAAAAGTAGATAAACGAAGGTTAAAAAATCAAA 293
Y     TCTTAAAAAAAGCTCTTTGAAAAGTAGATAAACGAAGGTTAAAAAATCAAA 293
Z     TCTTAAAAAAAGCTCTTTGAAAAGTAGATAAACGAAGGTTAAAAAATCAAA 293

X     GGAACTAAGGGCGCACAGTGGATGCCTTGGCACT                   327
Y     GGAACTAAGGGCGCACAGTGGATGCCTTGGCACT                   327
Z     GGAACTAAGGGCGCACAGTGGATGCCTTGGCACT                   327
```

Phytoplasma taxonomy

Classification based on RFLP analyses of alternate genes

Genes other than 16S rRNA

- **Examples:**

2. ***secA*** (Encode SecA protein, an essential components of the Sec system for protein secretion):

- It was recently employed for classification of phytoplasmas (Hodgetts *et al.*,2008).
- A portion of the gene sequence, about 480 bp, was PCR-amplified from various phytoplasma strains representing 12 16Sr groups.

Weintraub and Jones,2010

Phytoplasma taxonomy

Classification based on RFLP analyses of alternate genes

Genes other than 16S rRNA

3. Ribosomal protein (rp) genes:

- Ribosomal protein (rp) genes are more variable than 16S rRNA genes and have more phylogenetically informative characters, which substantially enhance the resolving power in delineating distinct phytoplasma strains.
- Recently, Martini *et al.*,2007 constructed a comprehensive phylogenetic tree based on the analysis of two ribosomal protein genes:

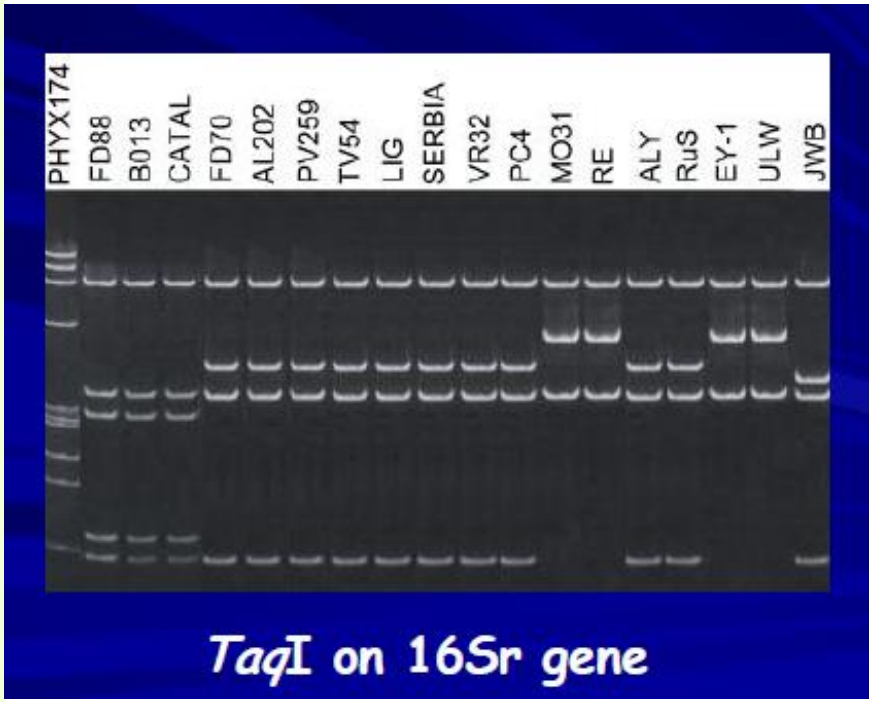
1. *rplV* (*rpl22*), and
2. *rpsC* (*rps3*), from 46 phytoplasma strains representing 12 16Sr groups.

- This rp gene-based phylogenetic tree delineated more distinct phytoplasma subclades and distinct lineages than those resolved by the 16S rRNA gene-based tree.

Weintraub and Jones,2010

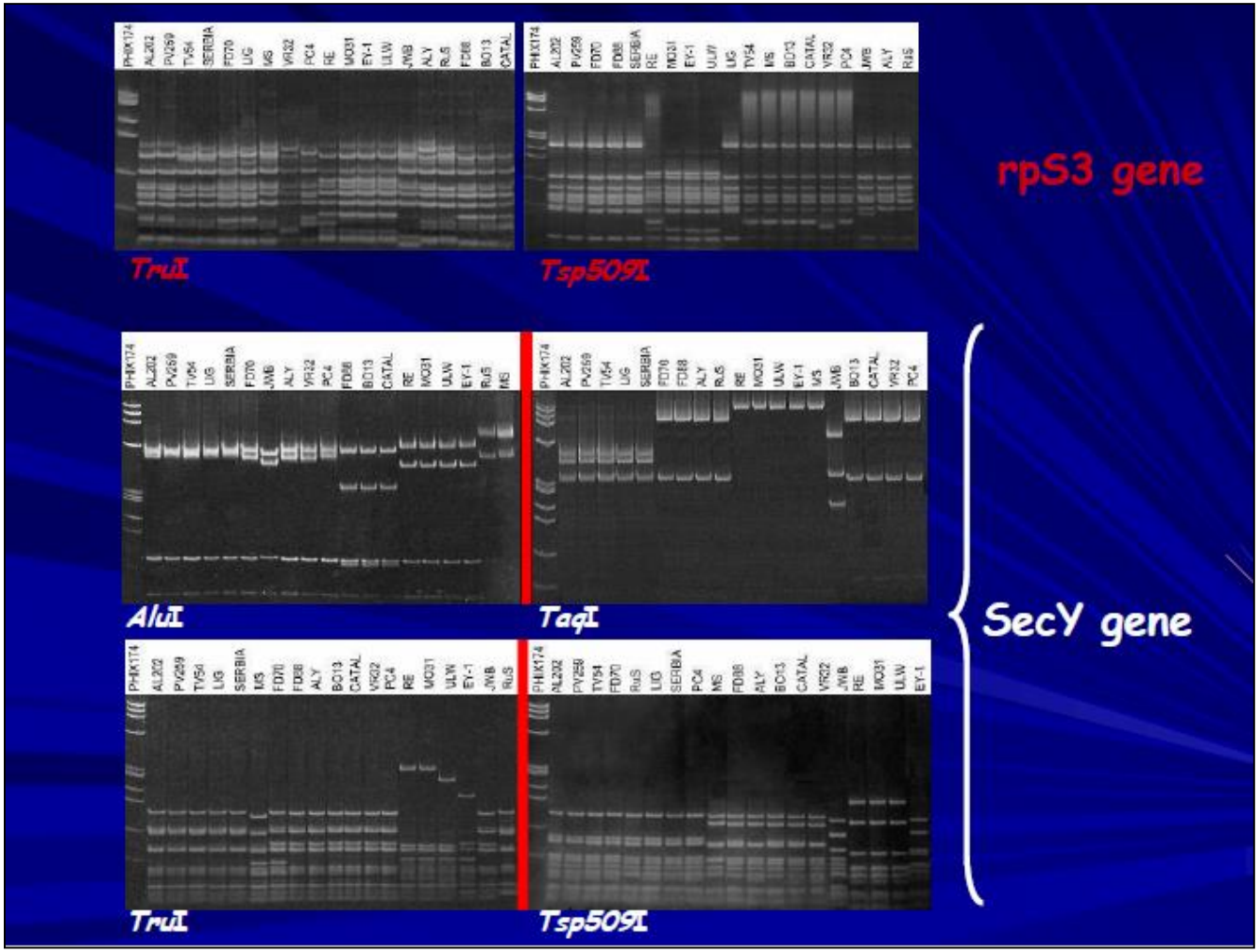
Polymorphisms as shown by RFLP analyses with selected restriction enzymes on three genes of 16Sr gene, rpS3 gene and SecY gene

- **16Sr gene:**
- Polymorphisms as shown by RFLP analyses with selected restriction enzymes on three genes of different strains of phytoplasmas related to Flavescence dorée.



Bonants,2008

Polymorphisms as shown by RFLP analyses with selected restriction enzymes on three genes of 16Sr gene, rpS3 gene and SecY gene



Phytoplasma taxonomy
Actual or Virtual RFLP analyses?

- In contrast to conventional or actual RFLP analysis, which has typically been done in the absence of prior nucleotide sequence information, virtual RFLP analysis is a nucleotide-sequence-based operation.
- Due to this and some following reasons, actual RFLP analysis will remains useful for phytoplasma differentiation and classification:
- Already established phytoplasma 16S rDNA RFLP patterns have served as standard keys for phytoplasma strain identification and classification.

Weintraub and Jones,2010

Phytoplasma taxonomy
Actual or Virtual RFLP analyses?

- Since virtual RFLP analysis is a nucleotide-sequence-based analysis, any error in an input sequence which misrepresents the phytoplasma strain under study could result in erroneous group/subgroup classification.
- New pattern types(new phytoplasmas) derived from virtual RFLP analysis should be confirmed by actual enzymatic digestions followed by gel electrophoresis.

Weintraub and Jones,2010

Phytoplasma taxonomy

Classification of phytoplasma based on Virtual RFLP analysis of 16S rDNA (Computer-simulated RFLP analysis or *in silico* RFLP analysis of 16S rRNA gene sequences)

- Sequencing of the 16S rRNA gene is required for performing virtual RFLP analysis.
- The scheme, accompanied by illustrated RFLP patterns of all representative strains in print or online, has essentially provided the most comprehensive list of reference phytoplasma strains.
- By comparison with these patterns, one can identify an unknown phytoplasma strain either:
- Through actual (for preliminary identification) or computer-simulated virtual RFLP analysis of the 16S rRNA gene sequence.

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.

Phytoplasma taxonomy

Classification based on Virtual RFLP analysis of 16S rDNA

Computer-simulated RFLP analysis or *in silico* RFLP analysis of 16S rRNA gene sequences

- Computer-simulated RFLP analysis uses a vast collections of phytoplasma 16S rRNA gene sequences that were reported and deposited in GenBank.
- In fact, by mimicking laboratory restriction enzyme digestion and subsequent gel electrophoresis, computer-simulated 16S rDNA analysis produces virtual RFLP patterns from virtual digestions by each enzyme allowing high-throughput differentiation and identification of phytoplasma strains.

Wei *et al.*,2007; Weintraub and Jones,2010

Phytoplasma taxonomy

Classification based on Virtual RFLP analysis of 16S rDNA

Computer-simulated RFLP analysis or *in silico* RFLP analysis of 16S rRNA gene sequences

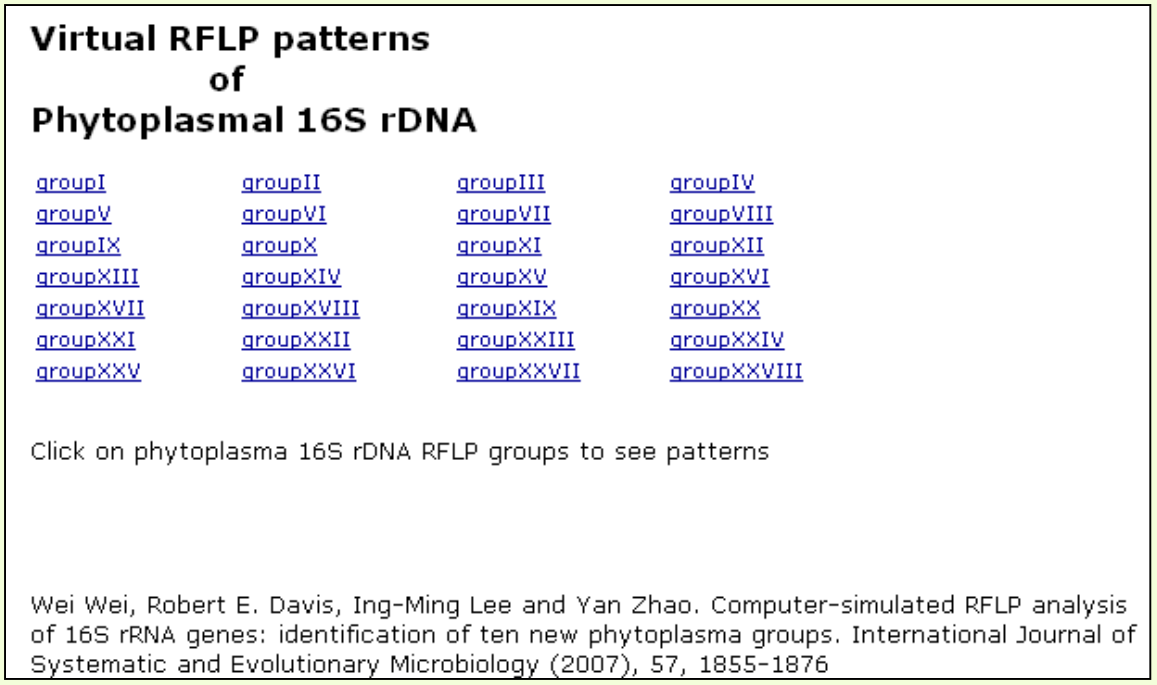
- Cladistic and virtual RFLP analysis of over 900 available phytoplasma 16S rDNA sequences revealed that phytoplasma strains can be classified into:
- 29 groups (16Sr groups), and
- 89 subgroups.
- More recent work has extended these counts to
- 36 groups (16Sr groups), and
- at least 150 sub-groups.

Wei *et al.*,2007; Weintraub and Jones,2010; Zhao and Davis,2016
Naderali *et al.*,2017; Jardim *et al.*,2021

Computer-simulated RFLP analysis of 16S rRNA genes

Virtual RFLP

- Recent work involving computer simulated restriction digests of the 16Sr gene suggest there may be up to 28 groups, whereas other papers argue for less groups, but more subgroups.



Virtual RFLP patterns of Phytoplasmal 16S rDNA

groupI	groupII	groupIII	groupIV
groupV	groupVI	groupVII	groupVIII
groupIX	groupX	groupXI	groupXII
groupXIII	groupXIV	groupXV	groupXVI
groupXVII	groupXVIII	groupXIX	groupXX
groupXXI	groupXXII	groupXXIII	groupXXIV
groupXXV	groupXXVI	groupXXVII	groupXXVIII

Click on phytoplasma 16S rDNA RFLP groups to see patterns

Wei Wei, Robert E. Davis, Ing-Ming Lee and Yan Zhao. Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. International Journal of Systematic and Evolutionary Microbiology (2007), 57, 1855-1876

Wikipedia,2015

Issues of *rrn* interoperon sequence heterogeneity

Ribosomal RNA operon (rrn)

- Many phytoplasma strains have two rRNA operons:
- *rrn*A
- *rrn*B
- While *rrn*A and *rrn*B may be identical or nearly identical in some phytoplasma strains, apparent *rrn* interoperon sequence heterogeneity exists in other strains.

Wei *et al.*,2007

Classification of phytoplasmas based on *in silico* RFLP analysis of 16S rRNA gene

Virtual RFLP

16Sr Group	Strain	16Sr Group	Strain
16SrI: Aster yellows Group		I-F	Aster yellows phytoplasma strain ACLR-AY
I-A	Aster yellows witches'-broom phytoplasma (AYWB) *rrn* A	16SrII: Peanut WB group	
I-A	Aster yellows witches'-broom phytoplasma (AYWB) *rrn* B	II-A	Peanut witches'- broom phytoplasma
I-B	Onion yellows phytoplasma mild strain (OY-M) *rrn* A	II-B	'*Ca.* Phytoplasma aurantifolia'
I-B	Onion yellows phytoplasma mild strain (OY-M) *rrn* B	II-C	Cactus witches'- broom phytoplasma
I-B	'*Ca.* Phytoplasma asteris'	II-D	'*Ca.* Phytoplasma australasiae'
I-C	Clover phyllody phytoplasma strain CPh	16SrIII: X-disease group	
I-D	Aster yellows phytoplasma strain PaWB	III-A	Western X-disease phytoplasma
I-E	Blueberry stunt phytoplasma strain BBS3	III-B	Clover yellow edge phytoplasma

The new subgroup 16SrII-W associated with *Crotalaria* witches' broom diseases in Oman(Al subhi *et al.*,2017)

Wei *et al.*,2007

Classification of phytoplasmas based on *in silico* RFLP analysis of 16S rRNA gene

Virtual RFLP

16Sr Group	Strain	16Sr Group	Strain
16SrIV: Coconut lethal yellows group		16SrVI: Clover proliferation group	
IV-A	Coconut lethal	VI-A	'*Ca.* Phytoplasma trifolii'
IV-B	Phytoplasma sp. LfY5(PE65)-Oaxaca	16SrVII: Ash yellows group	
IV-D	*Carludovica palmata* leaf yellowing phytoplasma	VII-A	'*Ca.* Phytoplasma fraxini'
16SrV: Elm yellows group		16SrVIII: Loofah witches'-broom group	
V-A	'*Ca.* Phytoplasma ulmi'	VIII-A	Loofah witches'- broom phytoplasma
V-B	'*Ca.* Phytoplasma ziziphi' strain JWBG1	16SrIX: Pigeon pea witches'-broom group	
V-C	Alder yellows phytoplasma strain ALY882	IX-A	Pigeon pea witches'- broom phytoplasma
V-G	'*Ca.* Phytoplasma ziziphi'-related strain JWB-Kor1	IX-D	'*Ca.* Phytoplasma phoenicium

Classification of phytoplasmas based on *in silico* RFLP analysis of 16S rRNA gene

Virtual RFLP

16Sr Group	Strain	16Sr Group	Strain
16SrX: Apple proliferation group		XII-C	Strawberry lethal yellows phytoplasma
X-A	'Ca. Phytoplasma mali'	XII-D	'Ca. Phytoplasma japonicum'
X-C	'Ca. Phytoplasma pyri'	XII-E	'Ca. Phytoplasma fragariae'
X-D	'Ca. Phytoplasma spartii'		
X-F	'Ca. Phytoplasma prunorum'	16SrXIII: Mexican periwinkle virescence group	
16SrXI: Rice yellow dwarf group		XIII-A	Mexican periwinkle virescence phytoplasma
XI-A	Ca. Phytoplasma oryzae'	16SrXIV: Bermudagrass white leaf group	
16SrXII: Stolbur group		XIV-A	'Ca. Phytoplasma cynodontis'
XII-A	'Ca. Phytoplasma solani'	16SrXV: Hibiscus witches'-broom group	
XII-B	'Ca. Phytoplasma australiense'	XV-A	'Ca. Phytoplasma brasiliense'

Classification of phytoplasmas based on *in silico* RFLP analysis of 16S rRNA gene

Virtual RFLP

16Sr Group	Strain	16Sr Group	Strain
16SrXVI: Sugar cane yellow leaf syndrome group		16SrXX: Buckthorn witches' broom group	
XVI-A	'*Ca.* Phytoplasma graminis	XX-A	'*Ca.* Phytoplasma rhamni'
16SrXVII: Papaya bunchy top group		16SrXXI: Pine shoot proliferation group	
XVII-A	'*Ca.* Phytoplasma caricae'	XXI-A	'*Ca.* Phytoplasma pini'
16SrXVIII: American (TX+NE) Potato purple top wilt group		16SrXXII: Nigerian coconut lethal decline (LDN) group	
XVIII-A	'*Ca.* Phytoplasma americanum'	XXII-A	Phytoplasma sp. strain LDN
16SrXIX: Japanese chestnut witches'-broom group		16SrXXIII: Buckland Valley grapevine yellows group	
XIX-A	'*Ca.* Phytoplasma castaneae'	XXIII-A	Buckland valley grapevine yellows phytoplasma

Classification of phytoplasmas based on *in silico* RFLP analysis of 16S rRNA gene

Virtual RFLP

16Sr Group	Strain	16Sr Group	Strain
16SrXXIII: Buckland Valley grapevine yellows group		16SrXXVI: Mauritius sugar cane yellows D3T1 group	
XXIII-A	Buckland valley grapevine yellows phytoplasma	XXVI-A	Sugar cane phytoplasma D3T1
16SrXXIV: Sorghum bunchy shoot group		16SrXXVII: Mauritius sugar cane yellows D3T2 group	
XXIV-A	Sorghum bunchy shoot phytoplasma	XXVII-A	Sugar cane phytoplasma D3T2
16SrXXV: Weeping tea tree witches'-broom group		16SrXXVIII: Havana derbid phytoplasma group	
XXV-A	Weeping tea witches'-broom phytoplasma	XXVIII-A	Derbid phytoplasma

Classification of phytoplasmas based on *in silico* RFLP analysis of 16S rRNA gene

Virtual RFLP

16Sr Group	Strain		
16SrXXIX: Cassia witches' broom group			
XXIX-E	'*Ca.* Phytoplasma omanense'		
16SrXXX: Salt cedar WB			
	'*Ca.* Phytoplasma tamaricis'		
16SrXXXI: Soybean stunt phytoplasma group			
	'*Ca.* Phytoplasma costaricanum'		
16SrXXXII: Malaysian periwinkle virescence phytoplasma group			
	'*Ca.* Phytoplasma malaysianum'		

Abbreviations are as follows:
AY, aster yellows; WB, witches'-broom; WL, white leaf, rrn, rRNA operons.

Classification of phytoplasmas based on *in silico* RFLP analysis of 16S rRNA gene

Virtual RFLP

16Sr Group	Strain		
16SrXXXIII: Allocasuarina phytoplasma group			
	'Ca. Phytoplasma allocasuarinae'		
16SrXXXVI: Foxtail palm phytoplasma group			
	'Ca. Phytoplasma wodyetiae'		
16SrXXXVII: Stylosanthes little leaf (StLL) phytoplasma group			
	'Candidatus Phytoplasma stylosanthis'		

Abbreviations are as follows:
AY, aster yellows; WB, witches'-broom; WL, white leaf, rrn, rRNA operons.

Computer-simulated RFLP analysis of 16S rDNA

Sequence retrieval, alignment, and cladistic analysis

Details of methods

- Wei *et al.*,2007 expanded the (old) RFLP analysis of 16S rDNA classification scheme through the use of computer-simulated RFLP analysis, achieving rapid differentiation and classification of phytoplasmas.
- Over 900 Phytoplasma 16S rRNA gene sequences were retrieved online from the National Center for Biotechnology Information (NCBI)'s nucleotide sequence database at http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi.
- For the purpose of cladistic analysis, 16S rDNA sequences from representative taxa of non-phytoplasma mollicutes as well as Gram-positive low G+C walled bacteria were also retrieved from the nucleotide sequence database.
- The 16S rDNA sequences, compiled in FASTA format, were aligned using ClustalX (V1.83) program.
- The aligned 1.25 kb fragments were exported to pDRAW32 software for *in silico* restriction digestion and virtual gel plotting.

Wei *et al.*,2007

Computer-simulated RFLP analysis of 16S rDNA

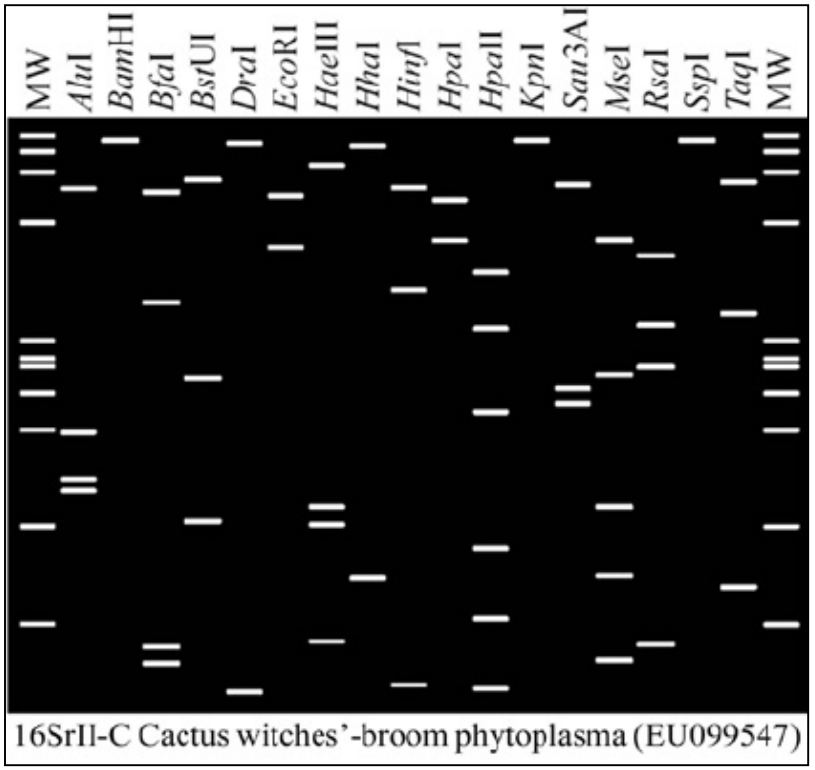
Details of method to classify phytoplasma 16Sr groups based on RFLP pattern similarity coefficient

- NCBI nucleotide sequence database, in which the restriction digestion profiles are simulated for a broad range of enzymes using *in silico* methods such as the AcaClone pDRAW32 system (http://acaclone.com).
- Based on numbers of similar and dissimilar fragments, the program calculates a similarity coefficient (F) for each pair of phytoplasma strains according to the formula of Nei and Li,1979 (Dice coefficients).
- Similarity coefficients for the restriction fragments reflect the number of shared and distinct fragments between any given strains, and, based on a similarity coefficient of 0.85, new 16Sr groups have been assigned.

Weintraub and Jones,2010

Virtual RFLP analysis of DNA sequences and automated calculation of similarity coefficients

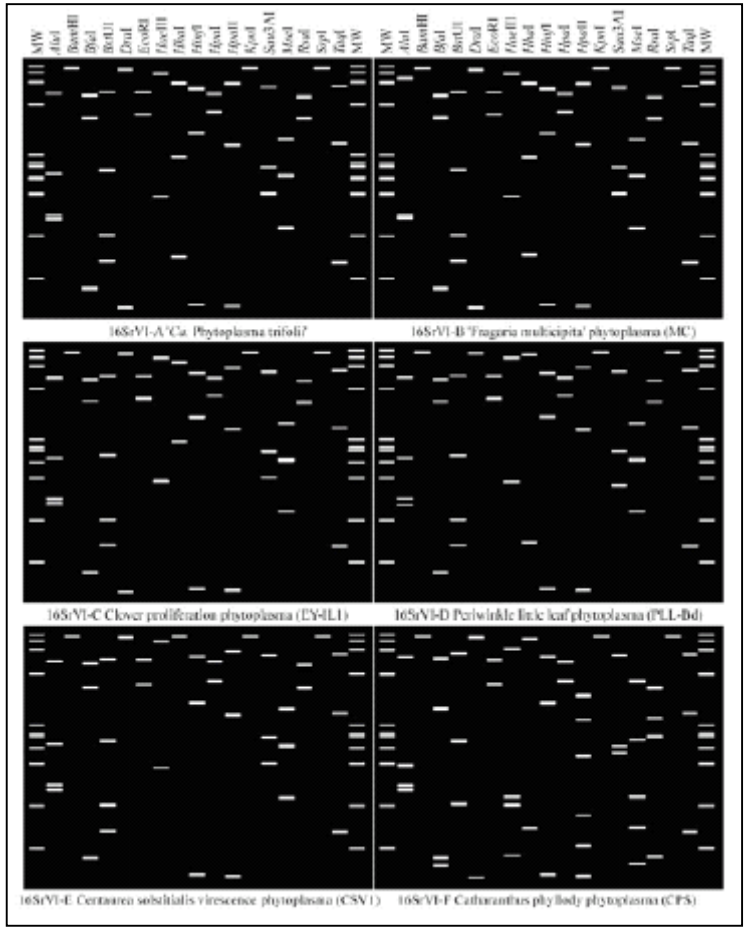
- This is an example of virtual RFLP patterns of a 16S rDNA nucleotide sequence that was "digested" *in silico* with 17 restriction endonuclease enzymes.
- The nucleotide sequence of this 16S rDNA (from cactus witches'-broom phytoplasma) was obtained from the GenBank database (GenBank accession number EU099547).
- This phytoplasma was originally classified, in 16S rDNA RFLP group 16SrII, on the basis of RFLP patterns that were obtained after actual, wet lab (actual wet laboratory) enzymatic digestion of 16S rDNA amplified in the polymerase chain reaction (PCR).



16SrII-C Cactus witches'-broom phytoplasma (EU099547)

Virtual RFLP analysis of 16S rDNA sequences Identifies new subgroups in the clover proliferation phytoplasma group

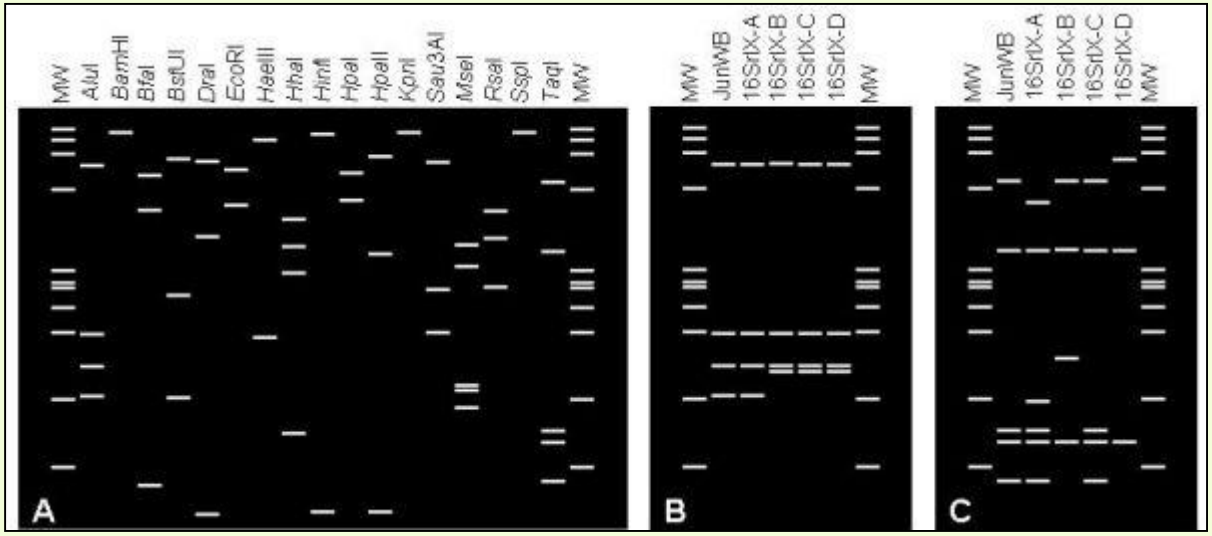
- Virtual RFLP patterns from *in silico* digestions of 16S rDNA F2nR2 fragments.
- Each aligned 16S rDNA sequence was trimmed to an approximately 1.25 kb fragment termed the F2nR2 region.
- Patterns of six phytoplasma strains representing three previously and three newly delineated 16SrVI subgroups are shown.
- Recognition sites for the following 17 restriction enzymes were used in the simulated digestions:
- *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I.
- MW: φX174 DNA-*Hae*III digestion.



Wei *et al.*,2007

Virtual RFLP analysis of DNA sequences and automated calculation of similarity coefficients

- Virtual RFLP patterns derived from in silico digestions of 1.2 kb 16S rDNA fragments.
- **(A)** Patterns from juniper witches'-broom (JunWB) phytoplasma; digestion using 17 restriction endonuclease enzymes;
- **(B) and (C)** differentiation of JunWB from phytoplasmas belonging to previously designated subgroups in group 16SrIX by digestion of 16S rDNA with (B) *Alu*I and (C) *Taq*I.
- Restriction fragments were resolved by in silico electrophoresis through 3% agarose gel.
- MW, ΦX174 DNA-HaeIII digestion.



Davis *et al.*,2010

Phytoplasma detection methods
Real time (or TaqMan) PCR

- The previous diagnostic procedure (PCR followed by RFLP) is laborious and requires several post amplification steps.
- To overcome these problems, several approaches have been developed, amongst which universal and group-specific real-time PCR protocols have been proposed since 2004.
- Real-time PCR has recently replaced the traditional (conventional) PCR in efforts to increase specific, sensitive and quantitative detection.

Weintraub and Jones,2010

Phytoplasma detection methods
Real-time PCR

- During a real-time PCR run, accumulation of newly generated amplicons is monitored at each cycle by fluorescent detection methods, and so there is no need for post-PCR manipulation such as electrophoresis, which is required at the end of regular PCR.
- The amount of fluorescence, monitored at each amplification cycle, is proportional to the log of concentration of the PCR target.
- Real-time PCR amplicons are visualized through several labelling techniques, most of which specifically bind to a target sequence on the amplicon, while others aspecifically stain double-stranded (ds) DNA amplicons.
- TaqManR probes are the most commonly used ones for the diagnosis of phytoplasmas.

Weintraub and Jones,2010

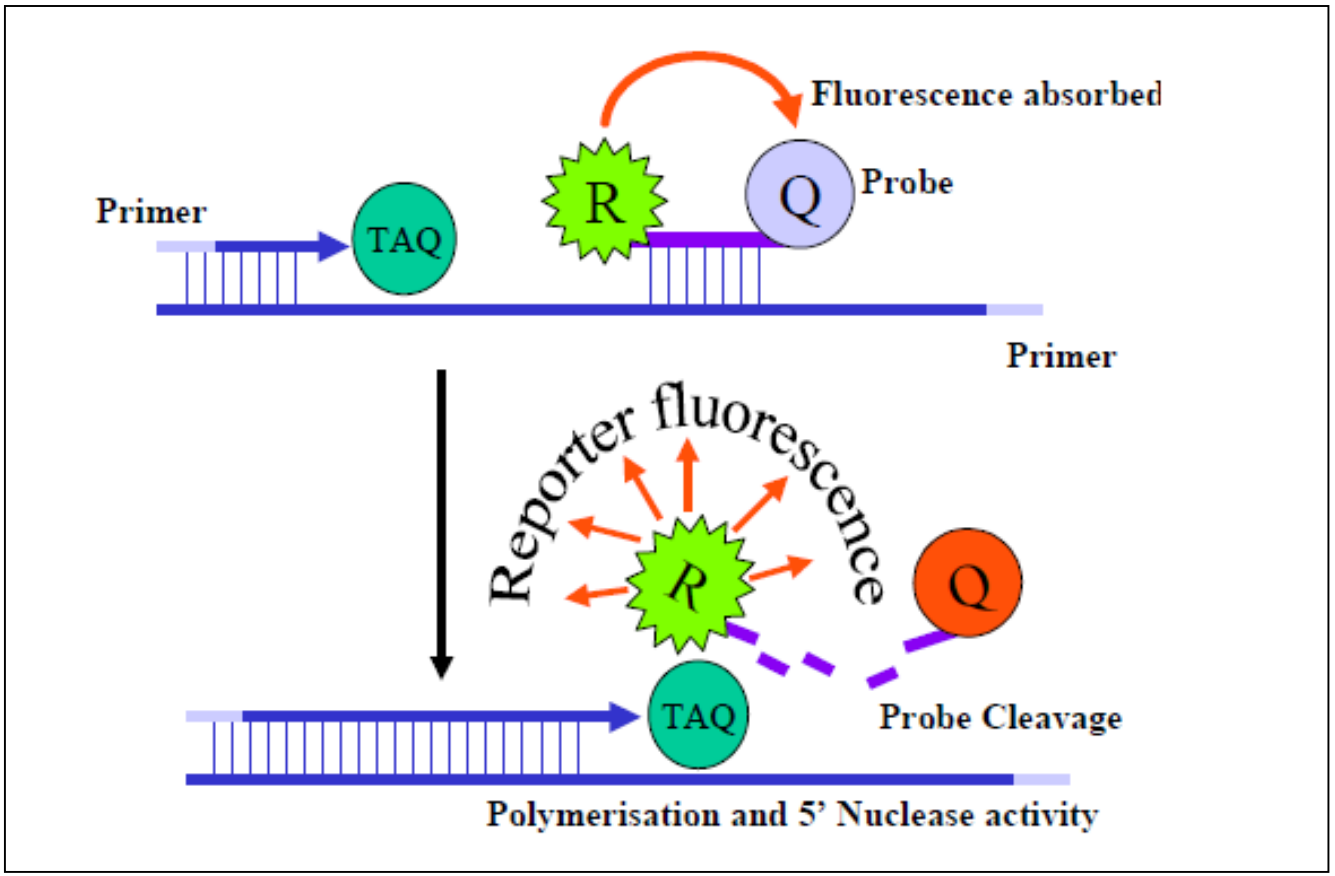
Phytoplasma detection methods
Real-time PCR(Taqman) chemistry

- Although several real-time PCR chemistries exist, we generally use the TaqMan™ real-time PCR chemistry which exploits the 5' nuclease activity of *Taq* DNA polymerase in conjunction with fluorogenic DNA probes.
- Each probe:

1. Designed to hybridise specifically to the target PCR product,
2. Is labelled with a fluorescent reporter dye, and
3. A quencher dye.

Weller *et al.*,2006

Phytoplasma detection methods
Real-time PCR(Taqman) chemistry



Real-time PCR
Universal phytoplasma detection

- Recently three protocols for the universal diagnosis of phytoplasmas using direct real-time PCR amplification of the 16S rDNA gene have been developed.
- All of them exploited a TaqMan probe for detection i.e. real-time PCR protocol with a TaqMan probe.
- 16S rDNA-based primer/probe systems can be used to detect phytoplasmas belonging to several ribosomal subgroups with sensitivity similar to that of conventional nested PCR.

Weintraub and Jones,2010

Name and sequence of primers and probes designed for the universal or group-specific detection of phytoplasma DNA by real-time PCR

Specificity	Target gene	Forward primer 5′–3′	Reverse primer 5′–3′	Probe 5′–3′	Reference
Universal	16S rDNA	CGTACGCAAGTATGAA ACTTAAAGGA	TCTTCGAATTAAACAACAT GATCCA	TGACGGGACTC CGCACAAGCG	Christensen *et al.*, 2004
Universal	16S rDNA	CYS2Fw AGGTTGAACGGCCACATTG	CYS2Rv TTGCTCGGTCAGAGTT TCCTC	CYS2 Probe ACACGGCCCAAAC TCCTACGGGA	Galetto *et al.*, 2005
Universal	16S rDNA	UniRNA Forward AAATATAGTGGAGGTTATC AGGGATACAG	UniRNA Reverse AACCTAACATCTCACGAC ACGAACT	UniRNA Probe ACGACAACCATGC ACCA	Hren *et al.*, 2007
FD Flavescence doree	16S rDNA	fAY GCACGTAATGGTGGGGACTT	rEY GCTTCAATTCGGTGAC GAAAG	/	Galetto *et al.*, 2005
FD	16S rDNA	Flavescence dorée Forward AAGTCGAACGGAGACCCTTC	Flavescence dorée Reverse TAGCAACCGTTTCCGATTGT	Flavescence dorée Probe AAAAGGTCTTAGT GGCGAACGGGT	Angelini *et al.*, 2007
FD	sec Y	FDgen Forward TTATGCCTTATGTTACTGCTT CTATTGTTA	FDgen Reverse TCTCCTTGTTCTTGCCAT TCTTT	FDgen Probe ACCTTTTGACTCA ATTGA	Hren *et al.*, 2007
FD	16S rDNA	F1024 GTGAGATGTTAGGTT AAGTCCTAAAACGA	R1112 TTGGCAGTCTCGCTAA AGTCC	iProbe AACCCCTGTCGC TAGTTGCCAGC	Bianco *et al.*, 2004
BN	Genomic fragment	StolFw AACCGCTCGCAAACAGC	StolRev ATTAGCGCCTTAGCTGTG	/	Galetto *et al.*, 2005
BN	16S rDNA	Bois noir Forward GGTTAAGTCCCGCAACGAG	Bois noir Reverse CCCACCTTCCTCCAATT TATCA	Bois noir Probe AACCCTTGTTGTT AATTGCCATCATTAAG	Angelini *et al.*, 2007
BN bois noir	Genomic fragment	BNgen Forward AAGCAGGTTTAGCGAT GGTTGT	BNgen Reverse TGGTACCGTTGCTTCAT CATTT	BNgen Probe TTAATACCACCTTC AGGAAA	Hren *et al.*, 2007

(*continued*)

Weintraub and Jones,2010

Name and sequence of primers and probes designed for the universal or group-specific detection of phytoplasma DNA by real-time PCR Continued

Specificity	Target gene	Forward primer 5′–3′	Reverse primer 5′–3′	Probe 5′–3′	Reference
AP Apple proliferation	Nitro-reductase	fAP_2 AAGAGCAATTCGTACTTTCG	rAP_2 GCCGAACTAGTTTCTAAT TGAC	/	Galetto *et al.*, 2005
AP	Genomic fragment	AP3 GAAACATGTCCTATTGGTGG	AP4 CCAATGTGTGAAATCTGTAG	/	Jarausch *et al.*, 2004
AP	16S rDNA	qAP-16S-F CGAACGGGTGAGTAAC ACGTAA	qAP-16S-R CCAGTCTTAGCAGTCGTT TCCA	qAP-16S TAACCTGCCTCTTA GACG	Baric and Dalla-Via, 2004
AP	16S rDNA	qAP-16S-F CGAACGGGTGAGTAAC ACGTAA	qAP-16S-R CCAGTCTTAGCAGTCGTT TCCA	AP-MGB CTGCCTCTTAGA CGAGG	Aldaghi *et al.*, 2007
AP	16S rDNA	fAT CATCATTTAGTTGGGCACTT	rATRT CGCTTCAGCTACTCTTTGTG	TaqMan Probe CCCTTATGACCTGG GCTACA	Bisognin *et al.*, 2008
ESFY	Ribosomal protein	rpLNS2f GTGCTGAAGCTAATTTATTG	rpLNS2r2 CAATATGGCTAGTTCTTTTT	/	Martini *et al.*, 2007
16SrX	16S rDNA	P1 AAGAGTTTGATCCTGG CTCAGATT	R16(X)F1r CATCTCTCAGCATACTT GCGGGTC	/	Torres *et al.*, 2005
'*Ca.* P. asteris' (onion yellows)	tuf	Tuf1 GCTAAAACTTGTCCACG TTGTACG	Tuf2 CGGAAATAGAATTGAGG ACGGT	TGTTTTAACTAAAA GAAGAAGGAGGAC GTCACACTGCCTT TTTCTCTC	Wei *et al.*, 2004
'*Ca.* P. asteris' (aster yellows)	16S rDNA	Aster yellows Forward TTGGGTTAAGTCCCGCAAC	Aster yellows Reverse CCCACCTTCCTCCAAT TTATCA	Aster yellows Probe CCAGCACGTAATGGTG GGGACTT	Angelini *et al.*, 2007
'*Ca.* P. asteris' (aster yellows)	16S rDNA	AACCCTCACCAGGT CTTGACA	CACGAGCTGACGACA ACCAT	/	Hollingsworth *et al.*, 2008
Beet leafhopper-transmitted virescence agent	16S rDNA	16Sp303F AGGGCCTATAGCTCAGTT GGTTAGA	16Sp378R GTGGGCCTAAATGGA CTTGAAC	16TM329 CACACGCCTGATAAGC GTGAGGTCG	Crosslin *et al.*, 2006

Weintraub and Jones,2010

Phytoplasma taxonomy

iPhyClassifier: An online tool for real-time identification and classification of phytoplasmas

- Recently, an interactive phytoplasma research tool, iPhyClassifier, has been launched on the internet, transforming phytoplasma classification from individual laboratory procedures to a real-time World Wide Web operation.
- The iPhyClassifier server is equipped with a suite of bioinformatic programs and 16S rDNA databases.
- The framework of iPhyClassifier can be easily expanded to accommodate other genes or multiple DNA loci.
- The online tool can be accessed at http://www.ba.ars.usda.gov/data/mppl/iPhyClassifier.html.

Weintraub and Jones,2010

Phytoplasma taxonomy

iPhyClassifier: An online tool for real-time identification and classification of phytoplasmas

- Besides implementing the concepts and programs that we described previously (Wei *et al.*,2007,2008b), iPhyClassifier integrates additional functions that we developed in the present study.
- Such new functions include:
- Overall sequence comparison and similarity score calculation,
- Intelligent trimming of input sequences, and Publication-ready virtual gel plotting.

Weintraub and Jones,2010

Phytoplasma taxonomy

16Sr group/subgroup classification and *Candidatus* Phytoplasma' species assignment based on RFLP pattern similarity coefficient and overall sequence similarity scores

- iPhyClassifier performs:
- Sequence similarity analysis;
- Simulates laboratory restriction enzyme digestions and subsequent gel electrophoresis, and
- Generates virtual restriction fragment length polymorphism (RFLP) profiles.
- Based on calculated RFLP pattern similarity coefficients and overall sequence similarity scores, iPhyClassifier makes instant suggestions on:

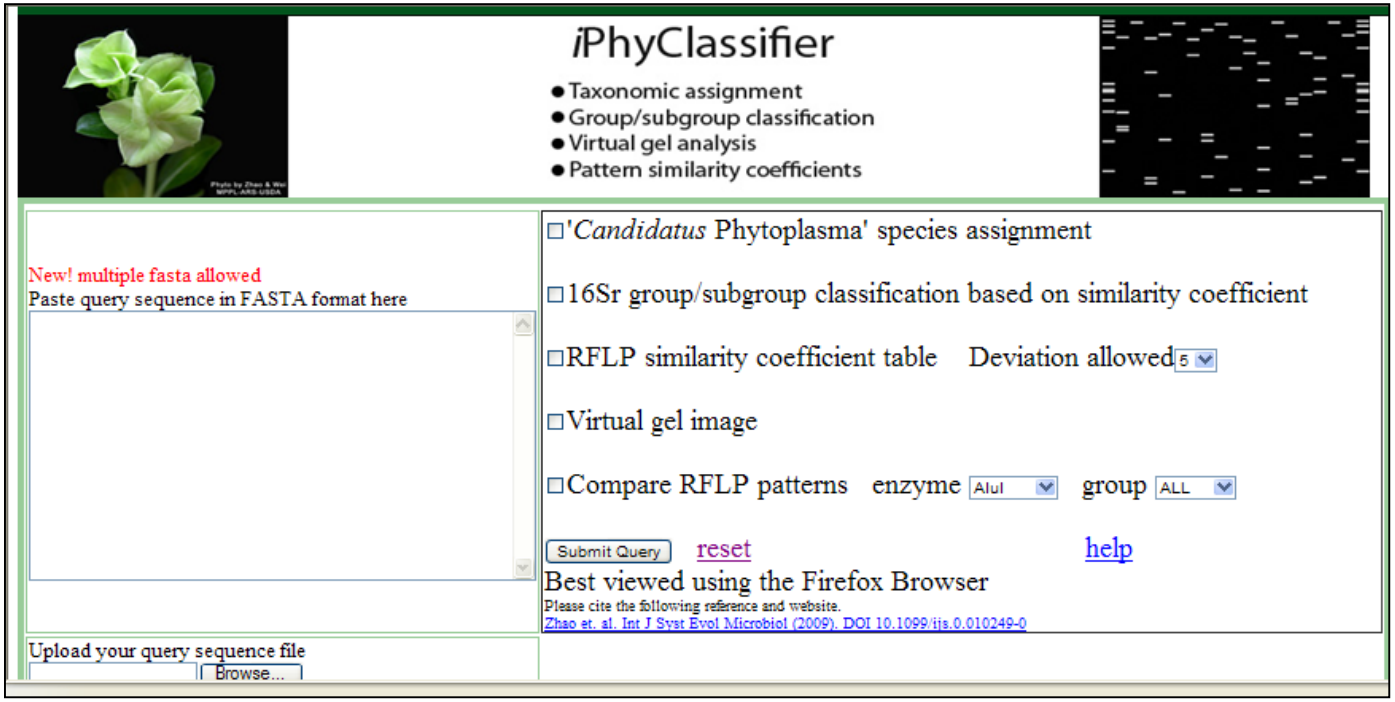
1. Tentative phytoplasma 16Sr group/subgroup classification status, and
2. '*Candidatus* Phytoplasma' species assignment.

Zaho *et al.*,2009

Phytoplasma taxonomy

16Sr group/subgroup classification and *Candidatus* Phytoplasma' species assignment based on RFLP pattern similarity coefficient and overall sequence similarity scores

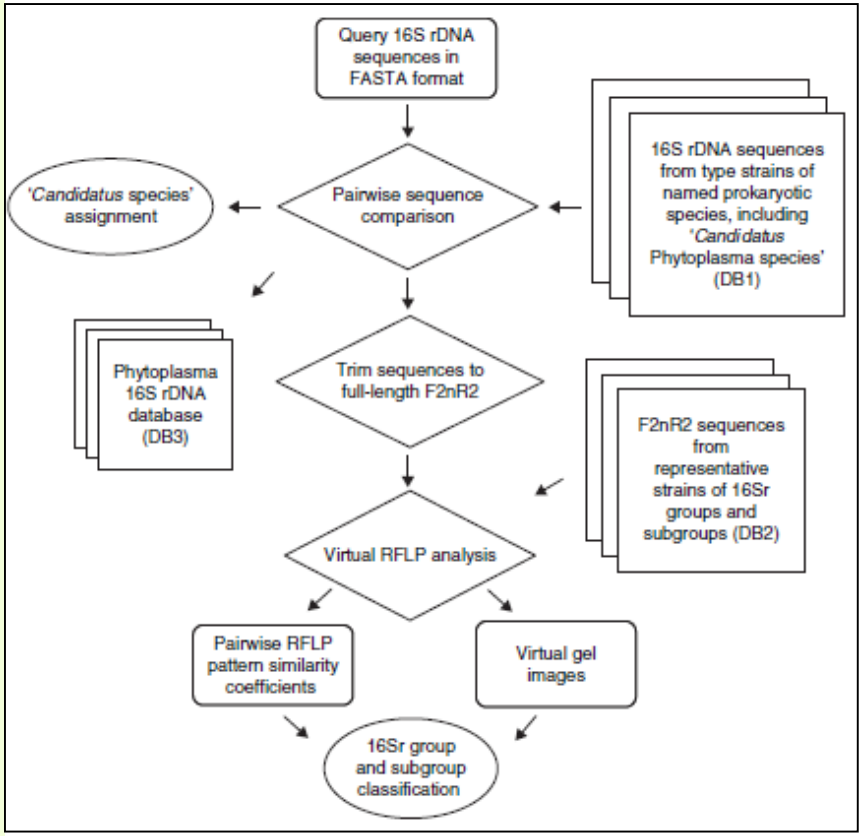
- The virtual RFLP patterns of reference strains of all phytoplasma 16Sr groups and subgroups are available online at the *i*PhyClassifier web site.



Zaho *et al.*,2009

Diagrammatic representation of the operational process of iPhyClassifier

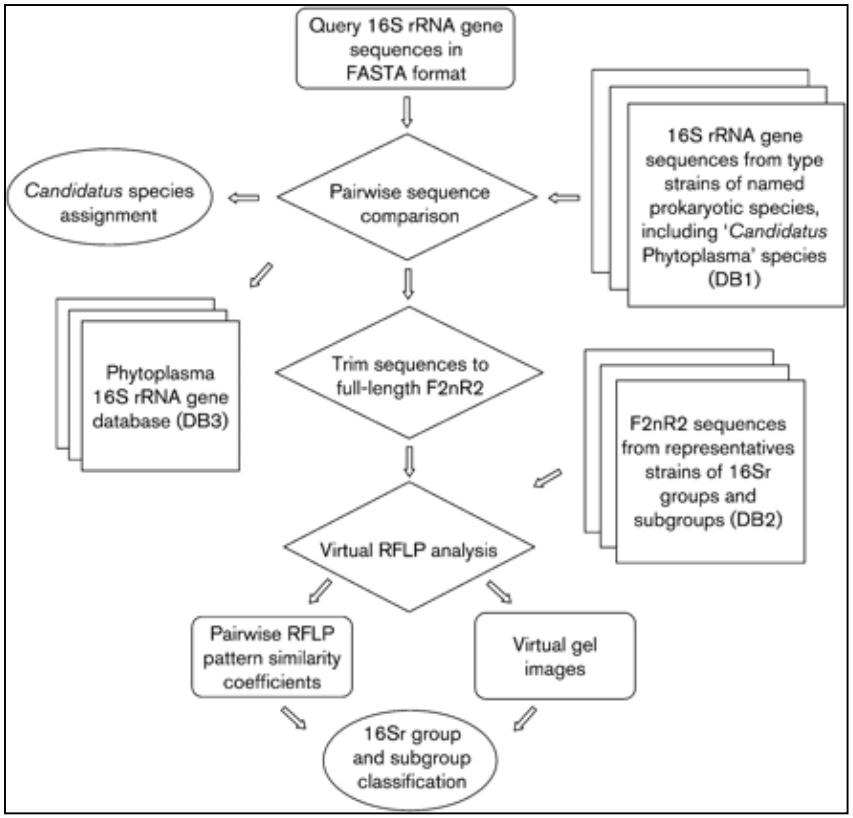
- Rectangles represent input and output files;
- Squares represent databases;
- Diamonds represent computational operations, and
- Ovals represent recommendations on tentative 16Sr group/subgroup classification status and '*Ca.* Phytoplasma' species assignment.



Zaho *et al.*,2009

Diagrammatic representation of the operational process of *i*PhyClassifier

- DB1, a set of full- or near-full-length 16S rRNA gene sequences from reference strains of all formally described '*Candidatus* Phytoplasma' species.
- DB2, a set of F2nR2 sequences (*rrn* operons) from representative strains of established phytoplasma 16Sr groups and subgroups; and
- DB3, a set of F2nR2 sequences compiled from all phytoplasma 16S rRNA sequences currently deposited in the GenBank.



Weintraub and Jones,2010

Phytoplasma Nomenclature
Candidatus status

- In 1992, *Subcommittee on the Taxonomy of Mollicutes* proposed the use of the name Phytoplasma in place of the use of the term MLO (Mycoplasma-like organism) "for reference to the phytopathogenic mollicutes".
- In order to provide a formal description of the genus 'Ca. Phytoplasma', the IRPCM Phytoplasma/Spiroplasma Working Team Phytoplasma Taxonomy Group has published a list of 16S rRNA gene sequences of strains related to 'Ca. Phytoplasma' species (Firrao,2004).

The International Committee on Systematics of Prokaryotes-Subcommittee on the Taxonomy of Mollicutes.

List of 16S rRNA gene sequences of strains related to '*Ca*. Phytoplasma' species

The trivial name '*Candidatus* Phytoplasma'

Phylogenetic group	'*Candidatus* Phytoplasma' species	Reference of the species description paper	GenBank accession no.	Database entry description
Aster yellows group (16SrI)	'*Ca.* Phytoplasma asteris'	Lee *et al.* (2004a)	M30790	Mycoplasma-like organism (strain OAY)
			M86340	Mycoplasma-like sp.
			AF177384	Alfalfa stunt phytoplasma
			U96616	*Phytoplasma* sp. STRAWB2
			L33760	Tomato big bud mycoplasma-like organism
			AF268403	Aster yellows phytoplasma A isolate 98UW159
			AF268404	Aster yellows phytoplasma A isolate 98UW166A
			AF268405	Aster yellows phytoplasma O isolate 98UW166B
			AF503568	Aster yellows phytoplasma B
			AY075038	Mulberry dwarf phytoplasma
			AF217247	Potato purple top phytoplasma
			AF200431	*Cirsium* yellows phytoplasma
			AF245439	Aster yellows phytoplasma
			AF222064	Tomato big bud phytoplasma
			AF222063	Aster yellows phytoplasma
			AF222065	Clover phyllody phytoplasma strain CPh rrnA
			AF222066	Clover phyllody phytoplasma strain CPh rrnB
			X68373	Mycoplasma-like organism (substrain AAY)
			U89378	*Phytoplasma* sp.
			L33762	Clover phyllody mycoplasma-like organism
			D12569	Group I phytoplasma
			X68338	Mycoplasma-like organism (substrain ACLR)
			AF268408	Aster yellows phytoplasma A isolate 99UW111
			AF268409	Aster yellows phytoplasma B isolate 99UW108
			AF322644	Aster yellows phytoplasma strain AY1 clone 14 A
			AF291682	Carrot proliferation phytoplasma
			AF335107	*Rehmannia glutinosa* var. *purpurea* phytoplasma
			AF453328	*Phytoplasma* sp. PY1
			AF279271	Paulownia witches'-broom phytoplasma
			AF356846	Cactus phytoplasma 'Martinez-Soriano 2001'
			AF322645	Aster yellows phytoplasma strain AY1 clone 10
			L33767	Aster yellows mycoplasma-like organism
			AF268407	Aster yellows phytoplasma B isolate 99UW89
			AF268406	Aster yellows phytoplasma A isolate 99UW93
	'*Ca.* Phytoplasma japonicum'	Sawayanagi *et al.* (1999)	AB010425	*Phytoplasma* sp.

Table 1. cont.

Phylogenetic group	'*Candidatus* Phytoplasma' species	Reference of the species description paper	GenBank accession no.	Database entry description
			AF147707	Chayote witches'-broom phytoplasma ChWBIII (Mor5)
			AF173558	Clover yellow edge phytoplasma
			AF236121	Peach rosette phytoplasma
			AF236122	Little peach phytoplasma
			AF189288	Clover yellow edge phytoplasma
			AF236123	Red suture phytoplasma
			AF190223	Poinsettia branch-inducing phytoplasma
			AF190226	Walnut witches'-broom phytoplasma rrnA
			AF190227	Walnut witches'-broom phytoplasma rrnB
			AF190228	*Spiraea* stunt phytoplasma
			AF244363	Black locust witches'-broom phytoplasma
			L33733	Canadian peach X mycoplasma-like organism
			D12580	Group II phytoplasma
			X77482	Mycoplasma-like organism (Italian clover phyllody)
			L33766	Clover yellow edge mycoplasma-like organism
			AF302841	Black raspberry witches'-broom phytoplasma clone BRWB7
			AF274876	Strawberry leafy fruit phytoplasma
Coconut lethal yellowing group (16SrIV)	To be described as '*Ca.* Phytoplasma palmae'*	Suggested name†	U18747	Coconut lethal yellowing phytoplasma
			AF237615	*Carludovica palmata* leaf yellowing phytoplasma
			AF434989	Texas Phoenix palm phytoplasma
			U18753	Yucatan coconut lethal decline phytoplasma
			AF361020	Florida Panus decline phytoplasma
	To be described as '*Ca.* Phytoplasma cocostanzaniae'*	Suggested name‡	X80117	*Phytoplasma* sp. strain LD
	To be described as '*Ca.* Phytoplasma cocosnigeriae'*	Suggested name‡	Y14175	*Phytoplasma* sp. strain LDN
			Y13912	*Phytoplasma* sp. strain LDG
	'*Ca.* Phytoplasma castaneae'	Jung *et al.* (2002)	AB054986	'*Candidatus* Phytoplasma castaneae'
Elm yellows group (16SrV)	'*Ca.* Phytoplasma ziziphi'	Jung *et al.* (2003a)	AY072722	*Ziziphus* jujube witches'-broom phytoplasma
			AF279272	*Ziziphus* jujube witches'-broom phytoplasma
			AF305240	*Ziziphus* jujube witches'-broom phytoplasma
	To be described as '*Ca.* Phytoplasma vitis'*	Suggested name†	AF176319	Flavescence dorée phytoplasma
			X76560	Mycoplasma (MLO; FD) transm. from *V. vinifera* to *V. faba*
	'*Ca.* Phytoplasma ulmi'	Lee *et al.* (2004b)	AF122910	Elm yellows phytoplasma strain EY1

Table 1. cont.

Phylogenetic group	'*Candidatus* Phytoplasma' species	Reference of the species description paper	GenBank accession no.	Database entry description
			AF122912	Phytoplasma HD1
			AF122911	Elm yellows phytoplasma strain WVEY
			AF189214	Elm yellows phytoplasma
			L33763	Elm yellows mycoplasma-like organism (rDNA)
			Y16395	*Rubus* stunt phytoplasma
			X68376	Mycoplasma-like organism (substrain ULW)
			AF305198	Virginia creeper phytoplasma
			AY028789	Alder yellows phytoplasma
			Y16387	Alder yellows phytoplasma
Clover proliferation group (16SrVI)	'*Ca.* Phytoplasma trifolii'	Hiruki & Wang (2004)	AY390261	'*Ca.* Phytoplasma trifolii'
			L33761	Clover proliferation mycoplasma-like organism (rDNA)
			AF036354	*Fragaria multicipita* phytoplasma
			AF190224	*Fragaria multicipita* phytoplasma rrnA
			AF190225	*Fragaria multicipita* phytoplasma rrnB
			X83431	*Mollicutes* sp. from *S. melongena*
			AF228052	Brinjal little leaf phytoplasma
			AF228053	Periwinkle little leaf phytoplasma
			AF409069	Clover proliferation phytoplasma strain EY-IL 2
			AF409070	Clover proliferation phytoplasma strain EY-IL 1
Ash yellows group (16SrVII)	'*Ca.* Phytoplasma fraxini'	Griffiths *et al.* (1999)	AF092209	Ash yellows phytoplasma
			AF105316	Ash yellows phytoplasma strain AshY5
			AF105315	Ash yellows phytoplasma strain AshY3
			AF105317	Ash yellows phytoplasma strain LWB3
			AF189215	Ash yellows phytoplasma
			X68339	Mycoplasma-like organism (substrain ASHY)
			L33759	Ash yellows mycoplasma-like organism (rDNA)
Loofah witches'-broom group (16SrVIII)	To be described as '*Ca.* Phytoplasma luffae'*	Suggested name†	AF086621	Loofah witches'-broom phytoplasma
			AF353090	Loofah witches'-broom phytoplasma str. LfWB clone1 rrnB
			AF248956	Loofah witches'-broom phytoplasma
			L33764	Loofah witches'-broom mycoplasma-like organism
Pigeon pea witches'-broom group (16SrIX)	'*Ca.* Phytoplasma phoenicium'	Verdin *et al.* (2003)	AF515636	'*Candidatus* Phytoplasma phoenicium'
			AF515637	'*Candidatus* Phytoplasma phoenicium'
			AF248957	Pigeon pea witches'-broom phytoplasma
			AF455038	Almond witches'-broom phytoplasma strain AlmWB3
			AF455041	Almond witches'-broom phytoplasma strain AlmWB-N1

Table 1. cont.

Phylogenetic group	'*Candidatus* Phytoplasma' species	Reference of the species description paper	GenBank accession no.	Database entry description
			AF455040	Almond witches'-broom phytoplasma strain AlmWB-P1
			AF390136	Almond witches'- broom phytoplasma strain AlmWB1
			AF390137	Almond witches'- broom phytoplasma strain AlmWB2
			AF455039	Almond witches'-broom phytoplasma strain AlmWB4
			L33735	Pigeon pea witches'-broom mycoplasma-like organism
			Y18052	*Knautia arvensis* phyllody phytoplasma 23S
			Y16389	*Picris echioides* yellows phytoplasma
			U18763	Caribbean PPWB mycoplasma-like organism
			AF361017	Honduran *Gliricidia* little leaf phytoplasma
			AF361019	Florida *Rhynchosia* little leaf phytoplasma
Apple proliferation group (16SrX)	'*Ca.* Phytoplasma mali'	Seemüller & Schneider (2004)	AJ542541	Apple proliferation phytoplasma AP15
			AJ542542	Apple proliferation phytoplasma AP1/93
			AF248958	Apple proliferation phytoplasma
			X68375	Mycoplasma-like organism (substrain AT)
			X72206	Apple proliferation MLO
	'*Ca.* Phytoplasma pyri'	Seemüller & Schneider (2004)	AJ542543	Pear decline phytoplasma PD1
			X76425	*Mollicutes*
			Y16392	Pear decline phytoplasma
			Y16394	Peach yellow leafroll phytoplasma
	'*Ca.* Phytoplasma prunorum'	Seemüller & Schneider (2004)	AJ542544	European stone fruit yellows phytoplasma ESFY-G1
			AJ542545	European stone fruit yellows phytoplasma ESFY-G2
			AY029540	European stone fruit yellows phytoplasma
			X77372	Mycoplasma-like organism (plum leptonecrosis)
			X68374	Mycoplasma-like organism (substrain PPER)
			Y11933	*Phytoplasma* sp.
	'*Ca.* Phytoplasma spartii'	Marcone *et al.* (2004a)	X92869	*Phytoplasma* sp.
	'*Ca.* Phytoplasma rhamni'	Marcone *et al.* (2004a)	X76431	*Mollicutes* (from *R. frangula*)
	'*Ca.* Phytoplasma allocasuarinae'	Marcone *et al.* (2004a)	AY135523	'*Allocasuarina muelleriana*' phytoplasma
Rice yellow dwarf group (16SrXI)	'*Ca.* Phytoplasma oryzae'	Jung *et al.* (2003b)	D12581	Group III phytoplasma
Stolbur group (16SrXII)	'*Ca.* Phytoplasma australiense'	Davis *et al.* (1997)	L76865	Australian grapevine yellows phytoplasma
			U43570	*Phormium* yellow leaf phytoplasma rrnB
			U43569	*Phormium* yellow leaf phytoplasma rrnA
			AJ243045	Strawberry lethal yellows phytoplasma
			AJ243044	Strawberry green petal phytoplasma
	To be described as '*Ca.* Phytoplasma solani'*	Suggested name†	AF248959	Stolbur phytoplasma
			X76427	*Mollicutes* (from *C. annuum* to *C. roseus*)
			X76428	*Mollicutes* (from *V. vinifera*)

Table 1. cont.

Phylogenetic group	'*Candidatus* Phytoplasma' species	Reference of the species description paper	GenBank accession no.	Database entry description
BGWL group (16SrXIV)	'*Ca.* Phytoplasma cynodontis'	Marcone *et al.* (2004b)	AJ550984	Bermuda grass white leaf phytoplasma
			AJ550985	Bermuda grass white leaf phytoplasma
			AJ550986	Bermuda grass white leaf phytoplasma
			AF248961	Bermuda grass white leaf phytoplasma
			AF509321	*Cynodon* white leaf phytoplasma
'*Ca.* Phytoplasma brasiliense' group (16SrXV)	'*Ca.* Phytoplasma brasiliense'	Montano *et al.* (2001)	AF147708	*Hibiscus* witches'-broom phytoplasma strain HibWB26
Other phytoplasmas not related to the above *Candidatus* species according to 16S rRNA gene sequences:				
Mexican periwinkle virescence group (16SrXIII)	No name suggested		AF248960	Mexican periwinkle virescence phytoplasma
			U96614	*Phytoplasma* sp. STRAWB1
Not assigned	No name suggested		AF495882	Chinaberry yellows phytoplasma strain CbY1
			AJ289195	Vigna little leaf phytoplasma
			Y16391	Bindweed yellows phytoplasma
			AJ310849	*Phytoplasma* sp. PinP
			Y17055	*Phytoplasma* sp. (strain StLL)
			AJ289192	*Stylosanthes* little leaf phytoplasma
			X76429	*Mollicutes* (from *C. roseus*)
			X76432	*Mollicutes* (from *S. officinarum*)
			X83438	*Mollicutes* sp. 16S rRNA gene and tRNA-Ile
			AF509324	Sorghum grassy shoot phytoplasma variant I
			AF509325	Sorghum grassy shoot phytoplasma variant II
			Y15865	*Phytoplasma* sp. (strain GaLL)

*According to Rule 28b of the Bacteriological Code, this is an incidental citation and does not constitute prior citation.
†Name proposed by the IRPCM Phytoplasma Working Team at the X International Congress of the International Organization of Mycoplasmology, Bordeaux, 1994.
‡Name proposed by the IRPCM Phytoplasma/Spiroplasma Working Team at the XIV International Congress of the International Organization of Mycoplasmology, Vienna, 2002.

IRPCM Phytoplasma taxonomy group,2004

Fruit tree phytoplasmas

Group-, species-, and even strain-specific primers

- Increasing knowledge of molecular components and information about more isolates and sequences allowed the development of group-, species-, and even strain-specific primers.
- Ribosomal and non-ribosomal primers are too specific to detect all strains of apple proliferation(AP) group.
- Or
- Ribosomal protein genes (*rpl22* and *rps3*) are more variable markers useful for differentiating phytoplasma strains below the genus level.
- The primer pair rpF1/rpR1 was designed to amplify a segment of the ribosomal protein gene operon.

Fruit tree phytoplasmas

Most frequently used species-specific primer combinations for the detection of temperate fruit tree phytoplasmas

- Fruit tree phytoplasma-specific primers were developed to detect phytoplasmas in the apple proliferation(AP) group such as apple proliferation, Apricot chlorotic leaf roll, pear decline, etc. (Next Table).
- The primer pair P1/PYLRint amplifies rDNA from all phytoplasmas in the apple proliferation group.
- The most specific of these primer pairs is fPD/rPDS, detecting only German pear decline, California pear decline, and peach yellow leaf roll.

Burns,2009

Fruit tree phytoplasmas

Most frequently used species-specific primer combinations for the detection of temperate fruit tree phytoplasmas

Phytoplasma	Primer	Location	Oligonucleotide sequence	product (bp)
AP	fAT/rAS	16S/IS	5′-CATCATTTAGTTGGGCACTT-3′	500
			5′-GGCCCCGGACCATTATTTATT-3′	
	P1/PYRLint	16S/IS	5′-AAGAGTTTGATCCTGGCTCAGGATT-3′	1,550
			5′-CCCGGCCATTATTAATTTTTATC-3′	
	fO1/rO1	16S	5′-CGGAAACTTTAGTTTCAGT-3′	1,071
			5′-AAGTGCCCAACTAAATGAT-3′	
	qAP16SF/qAP16SR	16S	5′-CGAGGTGAGTAACACGTAA-3′	75
			5′-CCATTAGCAGTCGTTTCC-3′	
	AP5/AP4	NRL-protein gene	5′-TCTTTTAATCTTCAACCATGGC-3′	483
			5′-CCAATGTGTGAAATCTGTAG-3′	
	AP3/AP4	NRL-protein gene	5′-GAAACATGTCCTATTGGTGG-3′	162
			5′-CCAATGTGTGAAATCTGTAG-3′	
	RpAP15f/rpAP15r	S10-operon	5′-AGTGCTGAAGCTAATTTGG-3′	920
			5′-TGCTTTTTATAGCAAAAGGTT-3′	
	RpAP15f2/rp(I)R1A	S10-operon	5′-CTCCTAAATCAGCTTCAAGT-3′	1,036
			5′-TTCTTTTTGGCATTAACAT-3′	
	rp(I)F1A/rp(I)R1A	S10-operon	5′-TTTTCCCCTACACGTACTTA-3′	1,200

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Fruit tree phytoplasmas

Most frequently used species-specific primer combinations for the detection of temperate fruit tree phytoplasmas (Contiuned)

			5′-GTTCTTTTTGGCATTAACAT-3′	
ESFY	fAT/rPRUS	16S/IS	5′-CATCATTTAGTTGGGCACTT-3′	500
			5′-GGCCCAAGCCATTATTGATT-3′	
	P1/PYRLint	16S/IS	5′-AAGAGTTTGATCCTGGCTCAGGATT-3′	1,550
			5′-CCCGGCCATTATTAATTTTTATC-3′	
	PA2F/PA2R	16S/IS	5′-GCC CCG GCT AAC TAT GTG C-3′	1,187
			5′-TTG GTGGGC CTA AAT GGA CTC-3′	
	NPA2F/NPA2R	16S/IS	5′-ATGACC TGG GCT ACA AAC GTG A-3′	485
			5′-GGT GGG CCT AAA TGGACT CG-3′	
	fO1/rO1	16S	5′-CGG AAACTTTTAGTTTCAGT-3′	1,071
			5′-AAG TGCCCAACTAAATGA T-3′	
PD	fPD/rPDS	16S/IS	5′-GACCCGTAAGGTATGCTGA-3′	1,400
			5′-CCCGGCCATTATTAATTTTA-3′	
	fAT/rPRUS	16S/IS	5′-CATCATTTAGTTGGGCACTT-3′	500
			5′-GGCCCAAGCCATTATTGATT-3′	
	fAT/rAS	16S/IS	5′-CATCATTTAGTTGGGCACTT-3′	500
			5′-GGCCCCGGACCATTATTTATT-3′	
	P1/PYRLint	16S/IS	5′-AAGAGTTTGATCCTGGCTCAGGATT-3′	1,550
			5′-CCCGGCCATTATTAATTTTTATC-3′	
	fO1/rO1	16S	5′-CGG AAACTTTTAGTTTCAGT-3′	1,071
			5′-AAG TGCCCAACTAAATGA T-3′	

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Fruit tree phytoplasmas

Most frequently used species-specific primer combinations for the detection of temperate fruit tree phytoplasmas (Contiuned)

Phytoplasma	Primer	Location	Oligonucleotide sequence	Expected product (bp)
PYLR	P1/PYRLint	16S/IS	5′-AAGAGTTTGATCCTGGCTCAGGATT-3′	1,550
			5′-CCCGGCCATTATTAATTTTTATC-3′	
WX	P1/WXint	16S/IS	5′-AAGAGTTTGATCCTGGCTCAGGATT-3′	1,600
			5′-GACAGTGCTTATAACTTTTA-3′	
	fU2W/rWX	16S	5′-ATAATGGAGGTCATCAG-3′	430
			5′-CGAAGTTAGGTGACCGCTTTG-3′	
	fU3/Tmod	16S/IS	5′-CTGTTACAAAGRGTAGCT-3′	420–422
			5′-ATCAGGCGTGTGCTCTA-3′	

- Apple proliferation (AP),
- Pear decline (PD),
- Peach yellow leaf roll (PYLR)
- Western X-disease (WX) of cherry

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Appendix 1

Basic keys for identification of phytopathogenic bacteria

Goszczynaska *et al.*,2000

Media and diagnostic tests

Agar media for plating plant extracts

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

Diagnostic tests

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

Key tests used to characterize some genera of bacteria

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

Key No. 1

Beans, Peas

Pod spot, leaf spot and blight

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

Key No. 1
(Contd.)

Beans, Peas

Pod spot, leaf spot and blight

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

Key No. 1
(Contd.)

Beans, Peas

Pod spot, leaf spot and blight

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

V = variable reaction.

Key No. 2

Cowpea

Leaf spot or leaf blight

Key No. 2 — Cowpea

LEAF SPOT OR LEAF BLIGHT

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

Key No. 3

Tomato

No.	Description	Go to
1	Wilt	Key No. 5
	Galls	Key No. 7
	Canker, browning of vascular tissue	Key No. 4
	Soft rot of fruit and stems	Key No. 6
	Leaf, fruit and stem spots	2
	Pith necrosis, plate on MT, KBC, TGA	12
2	KBC	Key No. 1, point 2
	SCM	Key No. 4, point 4
	MT	3
3	Off-white, semi-transparent colonies, Gr– rods	4
	Yellow colonies, Gr– rods	9
4	Colonies fluorescent under UV light	5
	Colonies non-fluorescent	Discard
5	Clear zone around colonies	6
	No zone	6
6	LOPAT (– – + – +)	*Pseudomonas viridiflava*
	LOPAT (– + – – +)	*Pseudomonas chicorii*
	LOPAT (+ – – – +)	7
7	Carbohydrate utilisation Sucrose Inositol Mannitol Erythitol Sorbitol Homoserine	
	+ + + – + –	8
	+ + + + + –	*Pseudomonas syringae pv. syringae*

Key No. 3
(Contd.)

Tomato

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

Key No. 4

Tomato

Canker and wilt

Key No. 4 — Tomato

CANKER AND WILT

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

Key No. 5

Potato

Wilt

No.	Description	Go to
1	Milky exudate from stems after 30 minutes	2
	No milky exudate from stems	3
3	Isolate on CVP, TGA and TZC as in 2	5
4	**TZC:** mucoid, drop-shaped white colonies with pink, half-moon-shaped centres	6
	TGA: mucoid, white colonies	
5	**CVP:** pith formation, Gr– rods	6
	TGA: circular, white colonies, Gr– rods	
	TZC: mucoid, drop-shaped white colonies with pink, half-moon-shaped centres, Gr– rods	6
	TGA: mucoid, white colonies, Gr– rods	
6	Hugh-Leifson test – oxidative	7
	Hugh-Leifson test – fermentative	11
7	OX +	8
	OX –	Discard
8	Colonies non-fluorescent on KB	9
	Colonies fluorescent on KB	Discard
9	PHB inclusions present	10
	No PHB inclusions	Discard
10	Pathogenicity test at 28 °C – wilting	*Ralstonia solanacearum* see Table 4 (p. 30) for biovar classification
	Pathogenicity test – negative	Discard
11	Potato soft rot positive	*Erwinia*, see Table 7 (p. 35) for further classification
	Potato soft rot negative	Discard

Key No. 6

Fruits, Tubers, Bulbs and Leaves

Soft rots

No.	Description	Go to
1	Plate on CVP, TGA and in the case of potatoes include TZC	2
2	**CVP**: pith formation	3
	TGA: circular, white or cream-coloured colonies	
	TZC: mucoid, drop-shaped, white colonies with pink half-moon-shaped centres	Key No. 5, point 6
	TGA: mucoid, white, fluidal colonies	6
3	Gr– rods	4
	Gr+, Gr– cocci	Discard
4	Potato soft-rot positive	5
	Potato soft-rot negative	Discard
5	Hugh-Leifson test – fermentative	*Erwinia*, see Table 7 (p. 35) for further classification
	Hugh-Leifson test – oxidative	6
6	Colonies fluorescent on KB	7
	Colonies non-fluorescent on KB	Discard
7	Utilisation of sucrose and trehalose	8
	No utilisation of sucrose and trehalose	9
8	LOPAT (+ + + + –)	*Pseudomonas marginalis*
9	LOPAT (– – + – +)	*Pseudomonas viridiflava*

Key No. 7

Galls

Key No. 7 — Galls

☛ include at least 6 isolates from each gall.

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

Key No. 8

Crucifers

Leaf spots, black rot and soft rot

Key No. 8 — Crucifers

LEAF SPOT, BLACK ROT AND SOFT ROT

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

Appendix 2

Primary key for screening and identification of bacteria from plants

Bradbury, J.F.,1970; revision, 1988

1. **Gram-positive**	2
Gram-negative	5
2. Cocci	*Micrococcus* or *Sarcina*, not a plant pathogen.
Rods or cocco-bacilli	3
3. Spores present (visual or heat test)	*Bacillus*
Spores absent	4
4. Cells more than 0.8µ wide	Not a plant pathogen, discard
Cells less than 0.8µ wide	Coryneforms, *Brevibacterium*
5. Cocci (all cells round)	*Micrococcus* or *Sarcina*, not a plant pathogen.
Rods or cocco-bacilli	6
6. Spores present (visual or heat test)	*Bacillus*
Spores absent	7
7. Colonies whitish, or grayish to buff	8
Colonies yellow	16
Colonies other colors	17
8. Produces fluorescent on KB medium	*Pseudomonas*
No fluorescent pigment	9
9. Oxidase negative	10
Oxidase positive	14
10. Acid from glucose aerobically & anaerobically	11

Acid from glucose aerobically	*Achromobacter* , *Acinetobacter*, and other non-plant pathogens, some less common plant pathogen, may key out here.
No acid from glucose	13
11. Soft rots potato tissue	*Pectobacterium* (*carotovora* group)
No soft rots of potato	12
12. Nitrate reduced	*Enterobacter*, *Escherichia*, possibly *Pectobacterium*
Nitrate not reduced	*Erwinia* (*amylovora* group)
13. Curved rods becoming cocci with age	*Arthrobacter*, not a plant pathogens
Straight rods	*Achromobacter*, *Cellulomonas*, possibly *Pseudomonas* (few if any plant pathogens).
14. Acid from glucose both aerobically & anaerobically	*Azotomonas*, *Aeromonas*, not a plant pathogen.
Acid from glucose aerobically only	*Pseudomonas* (incl. some plant pathogens), *Agrobacterium*.
No acid from glucose	*Alcaligenes*, *Pseudomonas* (few plant pathogens).

Primary key…
Continued

15. Acid from glucose both aerobically & anaerobically	*Erwinia* (incl. *Erwinia stewartii, E. uredovora*, and *E. herbicola*)
Acid from glucose both aerobically only	16
No acid from glucose	Probably *Flavobacterium*, not a plant pathogen
16. Nitrate not reduced to nitrite, Kovacs oxidase negative(positive reaction delayed 15-60 sec.)	*Xanthomonas*
Nitrate reduced, Kovacs oxidase positive	*Flavobacterium* ,*Cytophaga*
17. Colonies reddish, Kovacs oxidase negative, Acid produced both aerobically & anaerobically from glucose, nitrate reduced, soft rots potato, causes soft rot of rhubarb,	*E. rhapontici*
Others	Discard

Appendix 3

Key to bacteria likely to be associated with plants
In details

Bradbury, J.F. 1988

Key to bacteria likely to be associated with plants

Key to bacteria likely to be associated with plants

The following key is constructed using the characteristics that should have been discovered by use of the limited number of tests described in this paper. Obviously a larger number of tests, or special tests for particular groups of bacteria will give a more certain result. Uncertainty will arise both because of variability of bacteria and when difficult judgements have to be made by workers with little experience. The results obtained with this key will usually require some confirmation, depending on the level of certainty required. It might be considered sufficient to compare a description of the bacterium with the information gathered so far. In other cases some confirmatory tests will be needed. For suspected plant pathogens a successful pathogenicity test is usually the best confirmation. If facilities and/or materials are available, more sophisticated techniques may be used, such as serology, bacteriophage or bacteriocin typing, fatty acid profiles or protein electrophoresis patterns.

1.	Gram positive		2
	Gram negative		7
2.	Cocci at all stages of growth	Micrococcus, Sarcina, possibly Staphylococcus	
	Cocci in older cultures, but definite cycle of rods to cocci	Arthrobacter, Cellulomonas, Kurthia	
	Rods		3
3.	Spores present (heat test or observed)	Bacillus, possibly Clostridium	
	Spores absent		4

4.	Regular shape, facultative anaerobe	*Lactobacillus* (common in silage)
	Irregular shape	5
5.	Obligate aerobe	6
	Facultative anaerobe	*Cellulomonas*, *Oerskovia*, *Agromyces* etc.
6.	Motile, nitrate not reduced	*Curtobacterium*
	Non-motile, nitrate not reduced	*Clavibacter*
	Non-motile, nitrate reduced	*Rhodococcus*
7.	Cocci (all cells round)	*Micrococcus* etc.(usually Gram positive or variable)
	Distinctly curved rods	*Vibrio*, spirochaetes etc. (unusual on plants)
	Rods straight or nearly so	8
8.	Spores present	*Bacillus*, possibly *Clostridium*
	Spores absent	9
9.	Colonies whitish or greyish to buff	10
	Colonies yellow	24
	Colonies other colours	27
10.	Diffusible fluorescent pigment produced on King's medium B	11
	No fluorescence on KB	14
11.	Oxidase negative	*Pseudomonas syringae* group, *P. viridiflava* (Fluorescent groups I or II of Lelliott *et al*.1966)
	Oxidase positive	12
12.	Arginine negative, potato not rotted	*Pseudomonas cichorii*, *P. agarici* (Fluorescent group III)
	Arginine positive	13
13.	Potato rotted, levan positive, nitrate reduced	*Pseudomonas marginalis*, *P.* spp. (soft rotting)
	Potato not rotted, levan and nitrate negative	*Pseudomonas tolaasii*, *fluorescens* (some biovars)
	Not as above	*Pseudomonas fluorescens* (some biovars), *P. aeruginosa*
14.	Oxidase negative	15
	Oxidase positive	20
15.	Acid from glucose aerobically and anaerobically	16
	Acid from glucose aerobically only	18
	No acid from glucose	19
16.	Potato rotted	*Erwinia* (soft rot group)
	Potato not rotted	17
17.	Nitrate reduced	*Enterobacter*, *Citrobacter*, *Klebsiella*, *Erwinia cypripedii*
	Nitrate not reduced	*Erwinia amylovora*

18.	Peritrichous flagella	*Agrobacterium*, *Rhizobium*, *Acetobacter*, *Acinetobacter*, "*Achromobacter*"
	Polar flagella	*Pseudomonas* spp. (including *P. andropogonis*, *P. avenae* and *P. ficuserectae*)
19.	Rods, becoming cocci with age	Probably *Arthrobacter* (usually Gram-positive)
	Straight rods	"*Achromobacter*", *Cellulomonas*, possibly *Pseudomonas* (not a plant pathogen)
20.	Acid from glucose aerobically and anaerobically	*Aeromonas*, *Azotomonas* (unlikely on plants)
	Acid from glucose aerobically only	21
	No acid from glucose	23
21.	Nitrate reduced	22
	Nitrate not reduced, arginine negative	*Pseudomonas* spp. (including *P. amygdali*, *cissicola*, *gladioli*, and *meliae*), *Agrobacterium*
22.	Arginine negative	*Pseudomonas* spp. (including *P. cepacia*, *corrugata*, *glumae*, *rubrilineans*, *rubrisubalbicans*, *solanacearum*, *Agrobacterium*
	Arginine positive	*Pseudomonas* spp. (including *P. caryophylli*)
23.	Peritrichous flagella	*Alcaligenes*
	Polar flagella	*Pseudomonas acidovorans*, *P. pseudoalcaligenes*
24.	Acid from glucose aerobically and anaerobically	25
	Acid from glucose aerobically only	26
25.	Motile	*Erwinia herbicola*, *E. ananas*, *E. uredovora*
	Non-motile	*Erwinia stewartii*
26.	Nitrate reduced, oxidase strongly positive, peritrichous flagella or non-motile	*Flavobacterium*
	Nitrate not reduced, oxidase weak or negative, single polar	*Xanthomonas*
27.	Colonies red or reddish	28
	Colonies violet	*Chromobacterium*
	Colonies other colours	Discard or refer to textbooks (not likely to be a plant pathogen)
28.	Oxidase positive, aerobic	*Pseudomonas*, "*Protaminobacter*"
	Oxidase negative, facultative anaerobe	29
29.	Soft rots potato and rhubarb, or causes pink wheat or browning of hyacinth bulbscales	*Erwinia rhapontici*

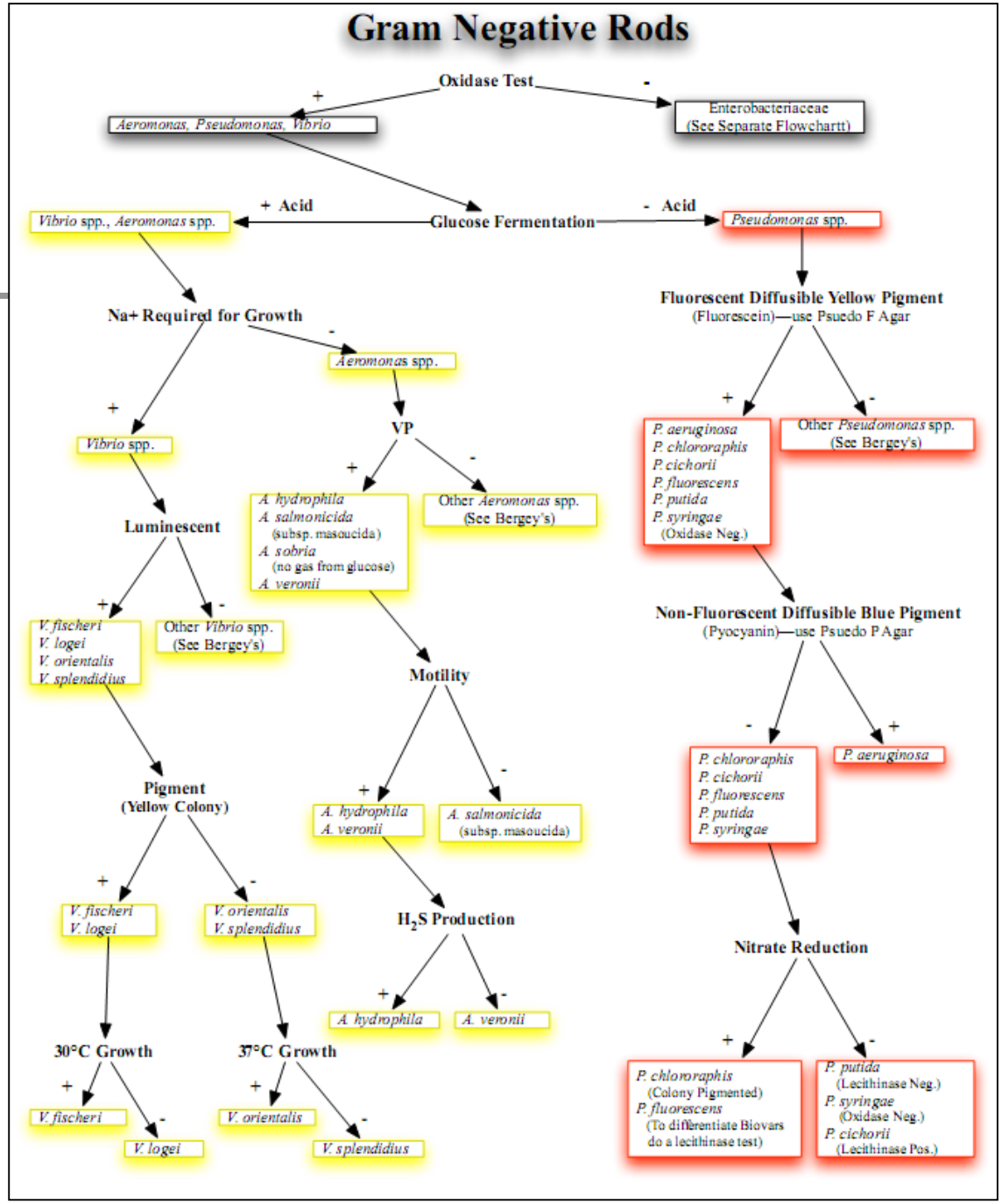
Appendix 4

Bacterial flow charts

Characteristics of miscellaneous bacteria

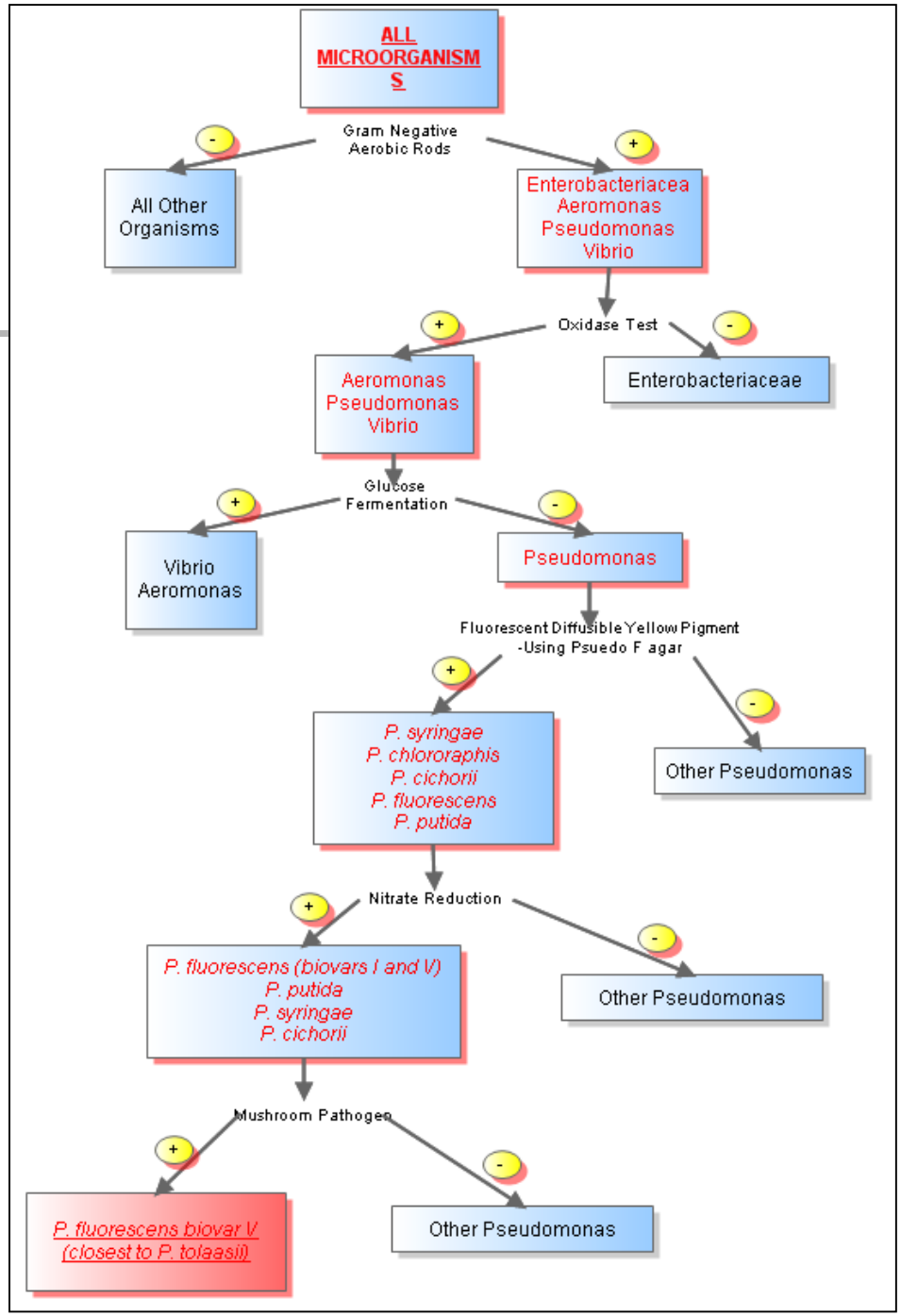
Identification flowcharts

Gram Negative Rods ID Flowchart: *Pseudomonas* *Aeromonas* *Vibrio*



Steffens,2011

Gram Negative Rods ID Flowchart: *Pseudomonas Aeromonas Vibrio*



The genus *Aeromonas*

Characteristics of genus *Aeromonas*

- The normal habitat of *Aeromonas* species is the aquatic environment.
- Gram-negative rods or cocco-bacilli (0.3-1.0 mm by 1.0-3.5 mm);
- Singly, in pairs, or short chains;
- Facultative anaerobes;
- Oxidase positive;
- Non-spore forming;
- Motile with a single polar flagellum (*A. media* and *A. salmonicida* are psychrophilic and nonmotile).

Spencer and Spencer,2004

Characteristics of Fermentative, Oxidase-Positive Gram-Negative Rods

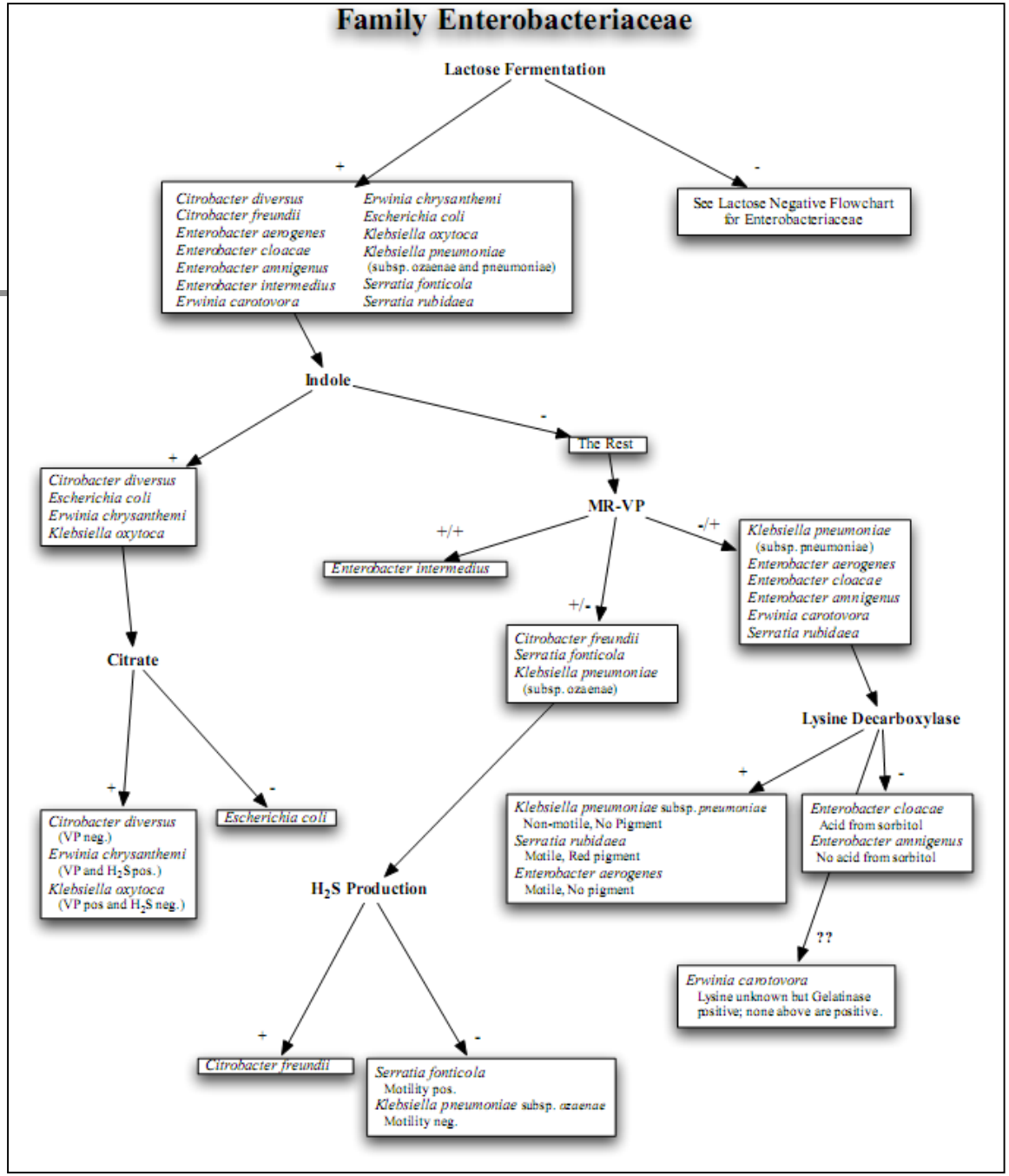
Aeromonas

	Aeromonas hydrofila	*Aeromonas sobria*	*Aeromonas caviae*	*Aeromonas salmonicida*			
				Subsp. *salmonicida*	Subsp. *achromogenes*	Subsp. *masoucida*	Atypical
Motility	+	+	+	–	–	–	–
Growth at 37°C	+	+	+	–	–	–	–
Diffusible brown pigment	–	–	–	+	–	–	D
β-galactosidase	+	+	+	+	+	+	D
Arginine dihydrolase	+	+	+	+	+	+	D
Lysine decarboxylase	D	D	D	D	–	+	–
Ornithine decarboxylase	–	–	–	–	–	–	–
Simmon's citrate	D	D	D	–	–	–	–
H_2S production	+	+	–	–	–	+	D
Urease	–	–	–	–	–	–	–
Indole	+	+	+	–	D	+	D
Voges-Proskauer reaction	+	D	–	–	–	+	D
Gelatin hydrolysis	+	+	+	+	–	+	D
Aesculin hydrolysis	+	–	+	+	–	+	D
Gowth in KCN	+	–	+	–	–	–	•
Acid from:							
Glucose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	–	+	D
Inositol	–	–	–	–	–	–	–
Sorbitol	D	D	D	–	–	–	–
Sucrose	+	+	+	–	+	+	D
Arabinose	+	+	+	+	–	+	D

D, variable reaction; •, not known.

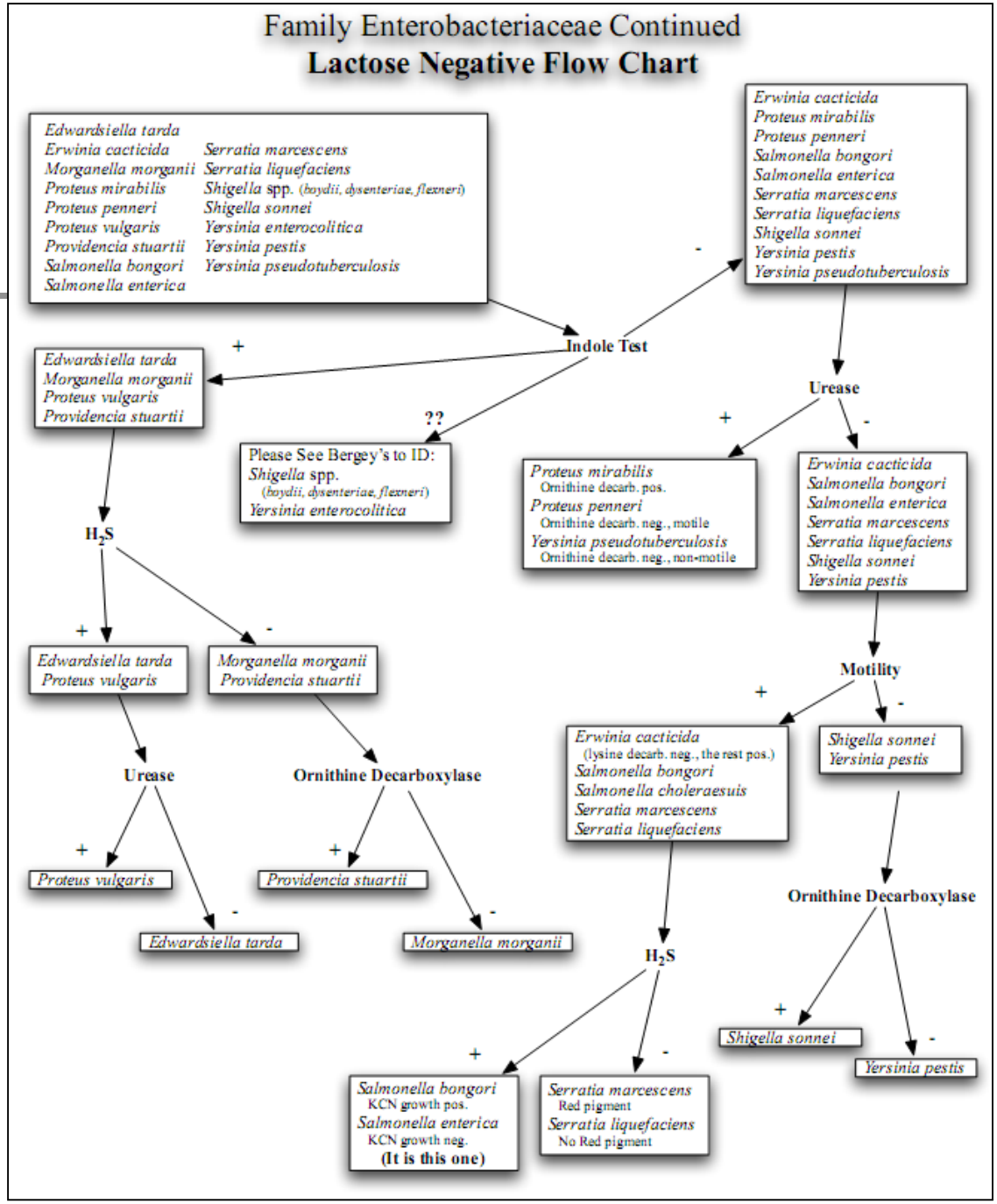
Spencer and Spencer,2004

Family Enterobacteriaceae Lactose Positive ID Flowchart



Steffens,2011

Family Enterobacteriaceae Lactose Positive ID Flowchart (continued)



Steffens,2011

The genus *Bacillus*

***Bacillus* spp. ID Flowchart**

Simplified key for the tentative identification of typical strains of *Bacillus* species.

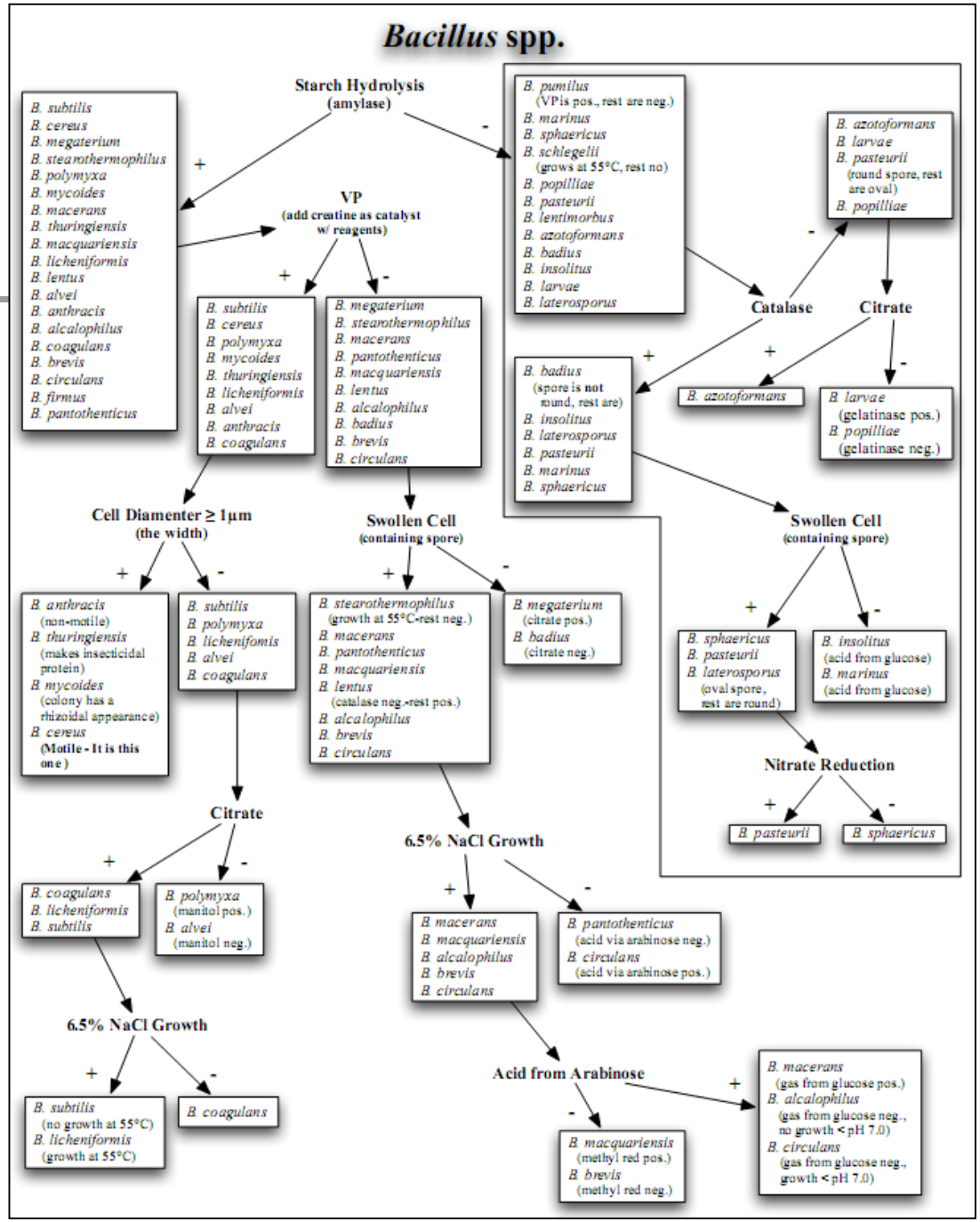
1.	Catalase: positive	2
	negative	17
2.	Voges-Proskauer: positive	3
	negative	10
3.	Growth in anaerobic agar: positive	4
	negative	9
4.	Growth at 50°C: positive	5
	negative	6
5.	Growth in 7% NaCl: positive	*B. licheniformis*
	negative	*B. coagulans*
6.	Acid and gas from glucose (inorganic N): positive	*B. polymyxa*
	negative	7
7.	Reduction of NO_3 to NO_2: positive	8
	negative	*B. alvei*
8.	Parasporal body in sporangium: positive	*B. thuringiensis*
	negative	*B. cereus*
9.	Hydrolysis of starch: positive	*B. subtilis*
	negative	*B. pumilus*
10.	Growth at 65°C: positive	*B. stearothermophilus*
	negative	11
11.	Hydrolysis of starch: positive	12
	negative	15
12.	Acid and gas from glucose (inorganic N): positive	*B. macerans*
	negative	13
13.	Width of rod 1.0µm or greater: positive	*B. megaterium*
	negative	14
14.	pH in V-P broth <6.0: positive	*B. circulans*
	negative	*B. firmus*
15.	Growth in anaerobic agar: positive	*B. laterosporus*
	negative	16
16.	Acid from glucose (inorganic N): positive	*B. brevis*
	negative	*B. sphaericus*
17.	Growth at 65°C: positive	*B. stearothermophilus*
	negative	18
18.	Decomposition of casein: positive	*B. larvae*
	negative	19
19.	Parasporal body in sporangium: positive	*B. popilliae*
	negative	*B. lentimorbus*

[a]Numbers on the right indicate the number (on the left) of the next test to be applied until the right-hand number is replaced by a species name.
From Norris et al. (1981).

The Prokaryotes (chapter 1.2.16),2006

The genus *Bacillus*

Bacillus spp. ID Flowchart



Steffens,2011

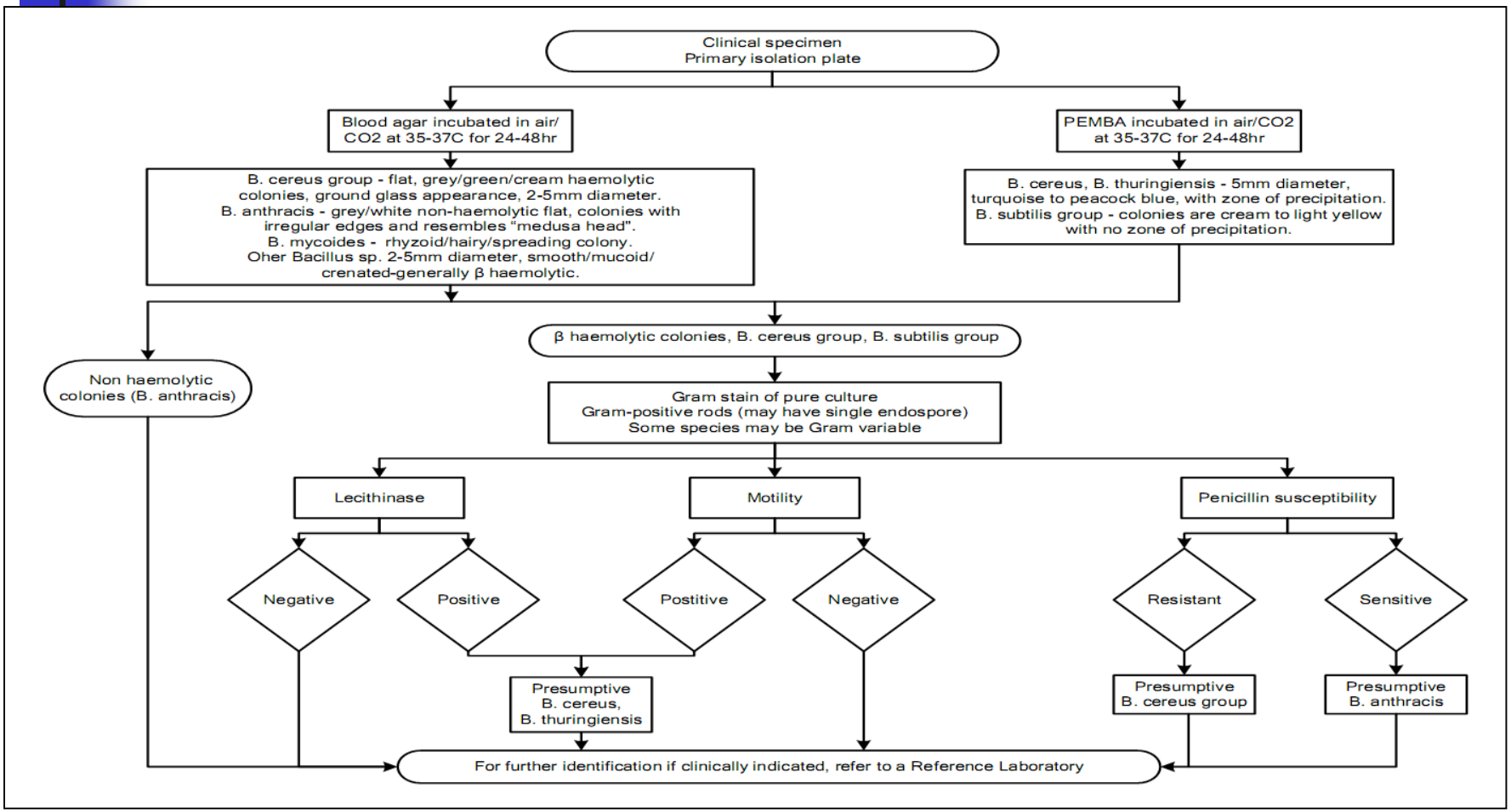
Identification of bacteria species

Isolate code	Gram Reaction	Cellular Morphology	Catalase	Oxidase Test	Indole Prod.	Motility Test	Methyl Red Test	Voge's Proskaver Test	Citrate Utilization	Urease Activity	Starch Hydrolysis	Gelatin Hydrolysis	NO_3^- Reduction	Spore test	Growth on MacConkey	Glucose	Arabinaose	Xylose	lactose	Sucrose	Raffinose	Galactose	Salicin	Maltose	Mannitol	Probable Identity
1	+	Cocci	+	+	-	-	-	-	-	+	-	+	-	-	-	+	-	+	-	+	+	-	-	-	-	*Micrococcus leteus*
2	-	Rods	+	+	-	+	-	-	-	+	-	+	-	-	-	+	-	-	-	+	-	-	-	-	+	*Flavobacterium rigense*
3	+	Rods	+	+	-	+	+	-	-	-	-	+	-	+	-	+	-	+	-	-	-	+	-	-	-	*Bacillus brevis*
4	-	Rods	+	-	-	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-	+	-	+	*Enterobacter cloacae*
5	+	Rods	+	-	-	+	-	+	+	-	+	+	+	+	+	+	+	-	-	+	-	+	-	+	+	*Bacillus licheniformis*
6	-	Rods	+	+	+	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	*Alcaligenes eutrophs*
7	-	Rods	+	-	+	-	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	+	+	*Klebsiella aerogenes*
8	-	Rods	+	+	+	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	*Alcaligenes eutrophs*
9	-	Rods	+	-	-	+	+	+	-	-	+	+	-	-	-	+	-	+	+	+	-	-	+	+	+	*Serratia liquefasciens*
10	+	Rods	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	+	*Bacillus subtilis*
11	+	Rods	+	-	-	+	-	-	-	-	+	+	+	+	-	+	+	-	+	+	-	-	+	-	+	*Bacillus macerans*
12	-	Rods	+	-	-	-	-	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	*Acinetobacter moffi*
13	+	Rods	+	+	-	-	-	+	-	-	+	-	+	+	-	+	-	-	+	+	-	-	-	-	+	*Bacillus mycoides*
14	-	Rods	+	-	-	-	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+	*Acinetobacter mallei*
15	+	Rods	+	+	-	+	-	+	-	-	+	-	+	+	+	+	-	+	+	+	+	+	-	+	-	*Bacillus coagulans*
16	+	Rods	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Bacillus polymyxa*
17	+	Rods	+	-	-	+	-	+	+	-	+	+	+	+	+	+	+	-	-	+	-	+	-	+	+	*Bacillus licheniformis*
18	+	Rods	+	+	-	+	-	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	-	+	-	*Bacillus cereus*
19	-	Rods	+	+	-	+	-	-	+	-	-	+	+	-	+	+	-	-	-	-	+	-	-	+	-	*Chromobacterium violaceum*
20	+	Cocci	+	+	-	+	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	+	+	*Micrococcus roseus*
21	-	Rods	+	+	-	+	-	-	+	-	+	+	+	-	+	+	+	-	+	+	+	-	-	+	-	*Pseudomonas aeruginosa*
22	+	Rods	+	-	-	-	-	-	-	+	+	-	+	-	-	+	-	-	-	-	-	-	-	+	-	*Corynebacterium pilosum*
23	+	Cocci	+	+	-	-	-	-	+	-	-	-	-	-	+	+	+	+	-	+	-	-	-	+	-	*Micrococcus varians*
24	+	Rods	+	+	-	+	-	-	+	-	+	+	-	+	+	+	+	+	-	+	-	-	-	-	+	*Bacillus megaterium*
25	-	Rods	+	+	-	+	-	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	*Alcaligenes faecalis*

Olajuyigbe and Ajele,2005

The genus *Bacillus*

ID Flowchart



Standard Unit,2015

Appendix 5

Symptoms and their Expression

Key to pathogens

Lelliott and Stead,1987

Plant Host/Symptoms Index

Key to pathogens

Ronald Alexander Lelliott and David E. Stead,1987

Host genus	Symptom	Possible pathogen(s)
Aechinea	Rotting of leaves and stems	*Erwinia chrysanthemi*
Agloanema	Rotting of leaves and stems	*E. chrysanthemi*
Agropyron	Leaf striping	*Pseudomonas avenae*
	Leaf spotting	*P. avenae*
Agaricus	Blotching of caps	*P. gingeri*
		P. tolaasii
	Oozing from gills	*P. agarici*
	Pitting of caps	*Bacillus polymyxa*
Allium	Soft skin, rotting	*Pseudomonas cepacia*
	Soft rotting	*P. gladioli* pv. *alliicola*
		Erwinia carotovora subsp. *carotovora*
		E. herbicola
	Leaf necrosis	*Pseudomonas syringae* pv. *porri*
		P. syringae pv. *syringae*
Ananas	Fruit rotting	*Erwinia ananas*
		E. chrysanthemi
	Pink discoloration	*Gluconobacter oxydans*
Anthurium	Watersoaking, leaf and stem necrosis	*Xanthomonas campestris* pv. *dieffenbachiae*
Antirrhinum	Leaf spotting and necrosis	*Pseudomonas syringae* pv. *antirrhini*
Apium	Leaf spotting and necrosis	*P. syringae* pv. *apii*
		P. cichorii
		P. marginalis
Arachis	Wilting	*P. solanacearum*

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Key to pathogens

Host genus	Symptom	Possible pathogen(s)
Areca	Leaf spotting and/or striping	*Xanthomonas campestris* pv. *vasculorum*
Avena	Leaf and sheath spotting	*Pseudomonas syringae* pv. *coronafaciens* *P. s.* pv. *striafaciens*
	Glume spotting	*P. s.* pv. *coronafaciens*
	Leaf striping	*P. s.* pv. *striafaciens*
	Leaf death	*P. s.* pv. *striafaciens*
Axonopus	Leaf striping	*Xanthomonas axonopodis*
	Leaf drop	*X. axonopodis*
	Stunting and withering	*X. axonopodis*
Begonia	Stunting and wilting	*Erwinia chrysanthemi* *Xanthomonas campestris* pv. *begoniae*
	Leaf spotting and necrosis	*X. c.* pv. *begoniae* *Pseudomonas cichorii*
	Soft rotting of leaves and stems	*Xanthomonas campestris* pv. *begoniae*
Berberis	Leaf spotting	*Pseudomonas syringae* pv. *berberidis*
	Shoot dieback	*P. s.* pv. *berberidis*
Beta	Rounded galling usually at soil level on root	*Agrobacterium tumefaciens*
	Silvering and wilting of leaves of red beet, usually of seed plants	*Curtobacterium flaccumfaciens* pv. *betae*
	Rotting of roots	*Erwinia carotovora* *E. chrysanthemi* *Pseudomonas marginalis*
Boerhavia	Leaf necrosis	*Xanthomonas campestris* pv. *campestris*
	Veinal necrosis	*X. c.* pv. *campestris*
	Leaf yellowing	*X. c.* pv. *campestris*
Bougainvillea	Leaf spotting	*Pseudomonas andropogonis*
	Leaf striping	*P. andropogonis*
Brachutica	Stunting, yellowing, wilting	*Erwinia chrysanthemi*
Brassica	Leaf spotting	*Pseudomonas syringae* pv. *maculicola* *P. viridiflava*
	Leaf marginal necrosis	*Xanthomonas campestris* pv. *campestris* *X. c.* pv. *armoraciae*

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Key to pathogens

Host genus	Symptom	Possible pathogen(s)
	Leaf yellowing	*X. c.* pv. *campestris*
	Veinal necrosis	*X. c.* pv. *campestris*
		X. c. pv. *armoraciae*
	Soft rotting of stems and roots	*Erwinia carotovora*
		Pseudomonas marginalis
		P. viridiflava
Bromus	Leaf striping	*P. avenae*
	Leaf spotting	*P. avenae*
		Xanthomonas campestris pv. *cerealis*
Camellia (C. japonica)	Leaf spotting	*Pseudomonas syringae* pv. *theae*
	Shoot dieback	*P. s.* pv. *theae*
Capsicum	Leaf spotting	*Xanthomonas campestris* pv. *vesicatoria*
	Fruit spotting	*Clavibacter michiganense* subsp. *michiganense*
		Xanthomonas campestris pv. *vesicatoria*
	Fruit rot	*Erwinia carotovora*
	Wilting	*Pseudomonas solanacearum*
		Clavibacter michiganense subsp. *michiganense*
Chrysanthemum	Stunting and wilting	*Erwinia chrysanthemi*
	Leaf and stem necrosis	*E. chrysanthemi*
		Pseudomonas syringae pv. *syringae*
		P. cichorii
		P. marginalis
		P. viridiflava
	Galling of leaves and stems	*Agrobacterium tumefaciens*
	Fasciation	*Rhodococcus fascians*
Cichorium	Leaf necrosis	*Pseudomonas cichorii*
Citrus	Leaf spotting	*P. syringae* pv. *syringae*
		Xanthomonas campestris pv. *citri*
	Branch cankering	*Pseudomonas syringae* pv. *syringae*
		Xanthomonas campestris pv. *citri*
Coffea	Leaf spotting	*Pseudomonas syringae* pv. *garcae*

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Key to pathogens

Host genus	Symptom	Possible pathogen(s)
	Shoot dieback	*P. s.* pv. *garcae*
Colocasia	Wilting and rotting	*Erwinia chrysanthemi*
Corylus	Leaf spotting	*Xanthomonas campestris* pv. *corylina*
	Fruit spotting	*X. c.* pv. *corylina*
	Shoot dieback	*X. c.* pv. *corylina*
	Bud necrosis	*X. c.* pv. *corylina*
Cotoneaster	Blossom blight, leaf and shoot necrosis	*Erwinia amylovora*
Crataegus	Blossom blight, leaf and shoot necrosis, cankering	*E. amylovora*
Cucumis	Wilting	*E. tracheiphila*
	Leaf and stem spotting	*Pseudomonas syringae* pv. *lachrymans*
		Xanthomonas campestris pv. *cucurbitae*
	Fruit spotting	*Pseudomonas syringae* pv. *lachrymans*
Cucurbita	Leaf spotting	*Xanthomonas campestris* pv. *cucurbitae*
		Pseudomonas syringae pv. *lachrymans*
Cyamopsis	Leaf and stem necrosis	*P. s.* pv. *syringae*
Cypripedium	Leaf spotting	*Erwinia cypripedii*
	Leaf and stem necrosis	*E. cypripedii*
Dactylis	Yellow slime on upper parts	*Clavibacter rathayi*
Dahlia	Stunting and wilting	*Erwinia chrysanthemi*
	Leaf necrosis	*E. chrysanthemi*
		Pseudomonas cichorii
		P. viridiflava
	Fasciation	*Rhodococcus fascians*
Delphinium	Black leaf spotting	*Pseudomonas syringae* pv. *delphinii*
		P. viridiflava
Dianthus	Stunting and wilting	*Erwinia chrysanthemi*
		Pseudomonas caryophylli
	Stem cracking	*Erwinia chrysanthemi*
		Pseudomonas caryophylli
	Leaf spotting	*P. andropogonis*
Dictyosperma	Leaf spotting and striping	*Xanthomonas campestris* pv. *vasculorum*

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Key to pathogens

Host genus	Symptom	Possible pathogen(s)
Dieffenbachia	Soft rotting	*Erwinia chrysanthemi*
	Leaf and stem necrosis	*Xanthomonas campestris* pv. *dieffenbachiae*
Echinocloa	Leaf streaking	*X. c.* pv. *translucens*
Eriobotrya	Stem canker and bud necrosis	*Pseudomonas syringae* pv. *eriobotryae*
Euchlaena	Leaf and sheath spotting	*P. avenae* *P. andropogonis*
	Leaf striping	*P. avenae* *P. andropogonis*
	Stalk rotting	*P. avenae*
Euphorbia	Stunting and wilting	*Erwinia chrysanthemi*
	Watersoaking, defoliation and leaf spotting	*Curtobacterium flaccumfaciens* pv. *poinsettiae*
	Leaf spotting	*Xanthomonas campestris* pv. *poinsettiicola*
Fragaria	Leaf spotting and necrosis	*Xanthomonas fragariae*
	Wilting	*X. fragariae*
	Leaf galling	*Rhodococcus fascians*
Fraxinus	Stem cankering	*Pseudomonas syringae* subsp. *savastanoi*
Freesia	Corm scabbing, leaf necrosis	*P. gladioli* pv. *gladioli*
Geranium	Leaf spotting	*Xanthomonas campestris* pv. *pelargonii*
Gladiolus	Corm scabbing, leaf spotting	*Pseudomonas gladioli* pv. *gladioli*
Glycine	Leaf spotting	*P. syringae* pv. *glycinea* *P. s.* pv. *phaseolicola* *P. s.* pv. *tabaci* *Xanthomonas campestris* pv. *glycines*
Gossypium	Leaf spotting	*X. c.* pv. *malvacearum*
	Stem spotting/rotting	*X. c.* pv. *malvacearum*
	Ball spotting/rotting	*X. c.* pv. *malvacearum*
Hedera	Leaf spotting	*X. c.* pv. *hederae*
Helianthus	Leaf spotting	*Pseudomonas syringae* pv. *helianthi*
Hibiscus	Leaf and stem necrosis	*P. s.* pv. *syringae*
Hordeum	Leaf spotting	*P. avenae*

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Key to pathogens

Host genus	Symptom	Possible pathogen(s)
		P. syringae pv. *atrofaciens*
		Xanthomonas campestris pv. *hordei*
	Leaf striping	*Pseudomonas avenae*
	Glume blotch and blackening	*P. syringae* pv. *atrofaciens*
		Xanthomonas campestris pv. *hordei*
	Seed blackening	*Pseudomonas syringae* pv. *atrofaciens*
Humulus	Rounded galling on stem, usually at soil level or on root	*Agrobacterium tumefaciens*
Hyacinthus	Bulb rotting	*Xanthomonas campestris* pv. *hyacinthi*
		Erwinia rhapontici
	Leaf necrosis	*Xanthomonas campestris* pv. *hyacinthi*
Juglans	Stem cankering	*Erwinia nigrifluens*
		E. rubrifaciens
	Exudation	*E. nigrifluens*
		E. rubrifaciens
	Leaf and shoot spotting and necrosis	*Pseudomonas syringae* pv. *syringae*
		Xanthomonas campestris pv. *juglandis*
	Fruit spotting	*Pseudomonas syringae* pv. *syringae*
		Xanthomonas campestris pv. *juglandis*
Lablab	Leaf, stem and/or pod spotting	*Pseudomonas syringae* pv. *pisi*
Lactuca	Leaf spotting and necrosis	*P. viridiflava*
		P. cichorii
		P. marginalis
		Xanthomonas campestris pv. *vitians*
	Vascular necrosis	*Pseudomonas viridiflava*
		P. marginalis
	Butt rotting	*P. viridiflava*
		P. marginalis
	Soft rotting	*Erwinia carotovora*
		Pseudomonas marginalis
		P. viridiflava

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Key to pathogens

Host genus	Symptom	Possible pathogen(s)
Lathyrus	Leaf, stem and/or pod spotting	*P. syringae* pv. *pisi*
Leersia	Leaf striping	*Xanthomonas campestris* pv. *oryzae*
	Leaf yellowing and/or withering	*X. c.* pv. *oryzae*
Lolium	Leaf streaking and wilting	*X. c.* pv. *graminis*
Lycopersicon	Wilting of leaves	*Clavibacter michiganense* subsp. *michiganense*
		Pseudomonas corrugata
		P. solanacearum
	Wilting of whole plant	*P. solanacearum*
		P. corrugata
	Irregular yellow-brown necrotic areas on leaves	*Clavibacter michiganense* subsp. *michiganense*
		Xanthomonas campestris pv. *vesicatoria*
	Mealiness of leaves/stems	*Clavibacter michiganense* subsp. *michiganense*
	Scorching of leaves	*C. michiganense* subsp. *michiganense*
	Cankering	*C. michiganense* subsp. *michiganense*
		Xanthomonas campestris pv. *vesicatoria*
	Yellow discoloration of vascular system	*Clavibacter michiganense* subsp. *michiganense*
	Brown discoloration of vascular system	*Pseudomonas solanacearum*
		P. corrugata
	Pith necrosis	*P. corrugata*
	Marbling of young green fruit	*Clavibacter michiganense* subsp. *michiganense*
	Spotting of fruit	*Xanthomonas campestris* pv. *vesicatoria*
		Clavibacter michiganense subsp. *michiganense*
		Pseudomonas syringae pv. *tomato*
	Production of adventitious roots	*P. solanacearum*
		P. corrugata
	Leaf and stem spotting and necrosis	*Xanthomonas campestris* pv. *vesicatoria*
		Pseudomonas syringae pv. *tomato*
		P. cichorii

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Key to pathogens

Host genus	Symptom	Possible pathogen(s)
		P. marginalis *P. viridiflava*
	Stem necrosis	*P. marginalis* *P. viridiflava*
Mallotus	Leaf spotting Shoot blight	*Erwinia mallotivora* *E. mallotivora*
Malus	Root proliferation on stems or roots	*Agrobacterium rhizogenes*
	Rounded galls on stem, usually at soil level or on root	*A. tumefaciens*
	Blossom blighting	*Erwinia amylovora* *Pseudomonas syringae* pv. *syringae*
	Branch cankering	*Erwinia amylovora* *Pseudomonas syringae* pv. *syringae*
	Dieback of shoots	*Erwinia amylovora* *Pseudomonas syringae* pv. *syringae*
	Shoot wilting Fruit rotting Fruit blistering	*Erwinia amylovora* *E. amylovora* *Pseudomonas syringae* pv. *papulans*
Mangifera	Leaf spotting	*Xanthomonas campestris* pv. *mangiferaeindicae*
	Fruit spotting	*X. c.* pv. *mangiferaeindicae*
	Stem cankering	*X. c.* pv. *mangiferaeindicae*
Manihot	Leaf spotting	*X. c.* pv. *manihotis* *X. c.* pv. *cassavae*
	Wilting and shoot dieback	*X. c.* pv. *manihotis*
Matthiola	Leaf necrosis Veinal necrosis	*X. c.* pv. *campestris* *X. c.* pv. *campestris* *X. c.* pv. *incanae*
	Leaf yellowing	*X. c.* pv. *campestris* *X. c.* pv. *incanae*
Medicago	Leaf and stem spotting	*X. c.* pv. *alfalfae* *Pseudomonas marginalis*
	Damping off	*Xanthomonas campestris* pv. *alfalfae*
	Wilting and stunting	*Clavibacter michiganense* subsp. *insidiosum*

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Key to pathogens

Host genus	Symptom	Possible pathogen(s)
Morus	Rounded galls on stem, usually at soil level or on root	*Agrobacterium tumefaciens*
	Leaf spotting	*Pseudomonas syringae* pv. *mori*
	Cankering	*P. s.* pv. *mori*
Mucuna	Leaf spotting	*P. andropogonis*
Musa	Wilting, necrosis	*P. solanacearum*
Nerium	Stem cankering and leaf galling	*P. syringae* subsp. *savastanoi*
Nicotiana	Leaf spotting	*P. s.* pv. *tabaci* *P. cichorii* *P. marginalis*
	Wilting	*P. solanacearum*
Olea	Stem cankering, and knotting	*P. s.* subsp. *savastanoi*
Oryza	Leaf and stem spotting	*P. avenae* *P. s.* pv. *oryzicola*
	Leaf striping	*P. avenae* *Xanthomonas campestris* pv. *oryzicola*
		X. c. pv. *oryzae*
	Leaf yellowing and/or withering	*X. c.* pv. *oryzae* *X. c.* pv. *oryzicola*
Panicum	Stunting, yellowing, wilting	*Erwinia chrysanthemi*
	Leaf striping	*Pseudomonas avenae*
Papaver	Leaf necrosis	*P. cichorii*
Paspalum	Leaf striping	*P. rubrilineans*
	Top rotting	*P. rubrilineans*
Pastinaca	Necrosis, rotting	*P. marginalis*
Pelargonium	Leaf spotting	*Xanthomonas campestris* pv. *pelargonii*
	Leaf zonal necrosis	*X. c.* pv. *pelargonii*
	Stem rotting	*X. c.* pv. *pelargonii*
	Leafy galling	*Rhodococcus fascians*
Pennisetum	Stunting, yellowing, wilting	*Erwinia chrysanthemi*
	Leaf blighting	*Pseudomonas syringae* pv. *syringae*
Persea	Leaf and shoot necrosis	*P. s.* pv. *syringae*
Phaseolus	Leaf, stem and pod spotting	*P. s.* pv. *phaseolicola* *P. s.* pv. *syringae*

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Key to pathogens

Host genus	Symptom	Possible pathogen(s)
		P. viridiflava *Xanthomonas campestris* pv. *phaseoli* *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*
	Leaf mosaic and malformation	*P. s.* pv. *phaseolicola*
	Leaf necrosis	*P. s.* pv. *phaseolicola* *P. s.* pv. *syringae* *P. viridiflava* *Xanthomonas campestris* pv. *phaseoli*
	Stem galling	*Pseudomonas viridiflava*
	Wilting	*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*
Philadelphus	Leaf spotting	*P. syringae* pv. *philadelphii*
Philodendron	Rotting of leaves and stems	*Erwinia chrysanthemi*
Phleum	Leaf streaking	*Xanthomonas campestris* pv. *phlei*
Physalis	Leaf spotting	*X. c.* pv. *vesicatoria*
Piper	Leaf and shoot necrosis	*Pseudomonas syringae* pv. *syringae*
Pisum	Leaf stem and pod spotting	*P. s.* pv. *pisi* *P. s.* pv. *syringae* *P. viridiflava*
Poa	Leaf spotting	*Xanthomonas campestris* pv. *poae*
Populus	Swelling or cankering on shoots and branches	*X. populi* pv. *populi*
	Bark necrosis	*X. campestris* pv. *populi*
	Dieback	*Erwinia salicis*
Primula	Leaf spotting	*Pseudomonas syringae* pv. *syringae* *P. s.* pv. *primulae* *P. viridiflava*
Prunus	Rounded galls on stem, usually at soil level or on root	*Agrobacterium tumefaciens*
	Cankering on branches and shoots	*Pseudomonas amygdali* *P. syringae* pv. *syringae*

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Key to pathogens

Host genus	Symptom	Possible pathogen(s)
		P. syringae pv. *morsprunorum*
		Xanthomonas campestris pv. *pruni*
	Swellings on twigs and branches	*Pseudomonas amygdali*
		Xanthomonas campestris pv. *pruni*
	Leaf spotting	*Pseudomonas syringae* pv. *morsprunorum*
		P. s. pv. *syringae*
		P. s. pv. *persicae*
		Xanthomonas campestris pv. *pruni*
	Fruit spotting	*X. c.* pv. *pruni*
	Dieback of shoots	*Pseudomonas syringae* pv. *syringae*
		P. s. pv. *morsprunorum*
		P. s. pv. *persicae*
		Xanthomonas campestris pv. *pruni*
	Bud death	*Pseudomonas syringae* pv. *syringae*
		P. s. pv. *morsprunorum*
	Blossom blight	*P. s.* pv. *syringae*
Pyracantha	Blossom blight, leaf and shoot necrosis	*Erwinia amylovora*
Pyrus	Root proliferation on root or stem	*Agrobacterium rhizogenes*
	Rounded galls on stem usually at soil level or on root	*A. tumefaciens*
	Blossom blight	*Erwinia amylovora*
		Pseudomonas syringae pv. *syringae*
	Branch cankering	*Erwinia amylovora*
		P. s. pv. *syringae*
	Dieback of shoots	*E. amylovora*
		P. s. pv. *syringae*
	Shoot wilting	*E. amylovora*
	Fruit rotting	*E. amylovora*
		Gluconobacter oxydans
Quercus	Exudation from acorns and cups	*Erwinia quercina*
Raphanus	Leaf necrosis	*Xanthomonas campestris* pv. *campestris*

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Key to pathogens

Host genus	Symptom	Possible pathogen(s)
	Veinal necrosis	*X. c.* pv. *campestris*
	Leaf yellowing	*X. c.* pv. *campestris*
Rheum	Crown rotting	*Erwinia rhapontici*
Ricinus	Leaf spotting and necrosis	*Xanthomonas campestris* pv. *ricini*
	Stem necrosis	*X. c.* pv. *ricini*
Rosa	Root proliferation on root or stem	*Agrobacterium rhizogenes*
	Rounded galls on stem mainly at soil level or on roots	*A. tumefaciens*
	Leaf and shoot necrosis	*Pseudomonas syringae* pv. *syringae*
Roystonea	Leaf spotting and/or striping	*Xanthomonas campestris* pv. *vasculorum*
Rubus	Root proliferation on root or stem	*Agrobacterium rhizogenes*
	Rounded galls on stem mainly at soil level or on roots	*A. tumefaciens* *A. rubi*
	Erumpent longitudinal galls on canes	*A. tumefaciens*
Saccharum	Leaf mottling	*Erwinia chrysanthemi*
	Leaf mosaic	*E. chrysanthemi*
	Leaf striping	*Pseudomonas rubrilineans* *P. rubrisubalbicans*
	Top rotting	*P. rubrilineans* *Xanthomonas campestris* pv. *vasculorum*
	Leaf scalding	*X. albilineans* *X. campestris* pv. *vasculorum*
	Yellow slime	*X. c.* pv. *vasculorum*
Saintpaulia	Rotting of leaves and petioles	*Erwinia chrysanthemi* *E. carotovora*
Salix	Rounded galls on stem, usually at soil level, or on root	*Agrobacterium tumefaciens*
	Wilting	*Erwinia salicis*
	Reddening of leaves	*E. salicis*
Secale	Leaf and stem spotting	*Xanthomonas campestris* pv. *secalis*

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Key to pathogens

Host genus	Symptom	Possible pathogen(s)
Sesamum	Leaf spotting	*Pseudomonas syringae* pv. *sesami* *Xanthomonas campestris* pv. *sesami*
	Stem lesions	*Pseudomonas syringae* pv. *sesami* *Xanthomonas campestris* pv. *sesami*
	Capsule blackening	*Pseudomonas syringae* pv. *sesami* *Xanthomonas campestris* pv. *sesami*
Setaria	Leaf striping	*Pseudomonas avenae*
Solanum	Stunting	*Erwinia carotovora* *E. chrysanthemi*
	Wilting	*E. carotovora* *E. chrysanthemi* *Pseudomonas solanacearum* *Clavibacter michiganense* subsp. *sepedonicum*
	Chlorosis of leaves, leaf rolling	*E. carotovora* *E. chrysanthemi* *Clavibacter michiganense* subsp. *sepedonicum*
	Stem lesions	*Erwinia carotovora* *E. chrysanthemi* *Pseudomonas solanacearum*
	Tuber rotting	*Erwinia carotovora* *Clavibacter michiganense* subsp. *sepedonicum* *Pseudomonas solanacearum* *P. marginalis* *P. viridiflava*
Sorbus	Blossom blight, leaf and shoot necrosis	*Erwinia amylovora*
Sorghum	Leaf spotting	*Pseudomonas andropogonis* *P. syringae* pv. *syringae*
	Leaf striping	*P. andropogonis* *P. syringae* pv. *syringae* *Xanthomonas campestris* pv. *holcicola*

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Key to pathogens

Host genus	Symptom	Possible pathogen(s)
Stranvaesia	Blossom blight, leaf and shoot necrosis	*Erwinia amylovora*
Syringa	Leaf necrosis	*Pseudomonas syringae* pv. *syringae*
	Dieback of shoots	*P. s.* pv. *syringae*
Tagetes	Leaf spotting	*P. syringae* pv. *tagetis*
Thysanolaena	Leaf spotting and/or striping	*Xanthomonas campestris* pv. *vasculorum*
Trifolium	Leaf and stem spotting	*Pseudomonas andropogonis* *P. syringae* pv. *syringae* *Xanthomonas campestris* pv. *alfalfae*
Triticum	Yellow slime on upper parts	*Clavibacter tritici*
	Yellow or white spots on leaves and sheaths	*C. tritici*
	Pink grain	*Erwinia rhapontici*
	Leaf spotting and streaking	*Pseudomonas syringae* pv. *atrofaciens* *P. s.* pv. *syringae* *Xanthomonas campestris* pv. *undulosa*
	Glume blotching	*Pseudomonas syringae* pv. *atrofaciens* *Xanthomonas campestris* pv. *undulosa*
	Seed blackening	*Pseudomonas syringae* pv. *atrofaciens*
Tulipa	Spotting and cracking of leaves	*Curtobacterium flaccumfaciens* pv. *oortii*
	Stunting and veinal silvering	*C. flaccumfaciens* pv. *oortii*
	Spotting and necrosis of scales	*C. flaccumfaciens* pv. *oortii*
Vicia	Leaf spotting	*Pseudomonas andropogonis* *P. syringae* pv. *pisi* *P. s.* pv. *syringae*
	Stem lesions	*P. andropogonis* *P. s.* pv. *pisi* *P. s.* pv. *syringae*
Vigna	Leaf spotting	*Pseudomonas syringae* pv. *pisi* *P. s.* pv. *syringae* *P. viridiflava*

Lelliott and Stead,1987

Key to pathogens

Host genus	Symptom	Possible pathogen(s)
	Stem and pod lesions	*P. s.* pv. *pisi* *P. s.* pv. *syringae*
Vitis	Leaf spotting	*P. s.* pv. *syringae* *P. viridiflava* *Xanthomonas ampelina*
	Dieback	*Pseudomonas syringae* pv. *syringae* *Xanthomonas ampelina*
	Blighting	*X. ampelina*
	Stem galling	*Agrobacterium tumefaciens*
Zea	Stunting and wilting	*Erwinia chrysanthemi* *E. stewartii* *Clavibacter michiganense* subsp. *nebraskense*
	Necrosis or soft rotting of leaves	*Erwinia chrysanthemi*
	Leaf striping	*E. stewartii* *Xanthomonas campestris* pv. *vasculorum* *Pseudomonas avenae* *P. andropogonis* *P. rubrilineans* *Clavibacter michiganense* subsp. *nebraskense* *Xanthomonas campestris* pv. *holcicola*
	Top death	*Erwinia stewartii* *Pseudomonas avenae* *P. rubrilineans* *Xanthomonas campestris* pv. *vasculorum*
	Stalk rotting	*Erwinia chrysanthemi* *Pseudomonas avenae*
	Root rotting	*Clavibacter michiganense* pv. *nebraskense*
	Seedling blight	*Erwinia stewartii*
Zingiber	Wilting	*Pseudomonas solanacearum*
Zinnia	Leaf spotting	*Xanthomonas campestris* pv. *zinniae*
Zizania	Leaf striping	*X. c.* pv. *oryzae*
	Leaf yellowing and/or withering	*X. c.* pv. *oryzae*

Lelliott and Stead,1987

Appendix 6

The software CABIQ

Computer-assisted identification system of phytopathogenic bacteria

Poliakoff *et al.*,2004 & 2005

CABIQ

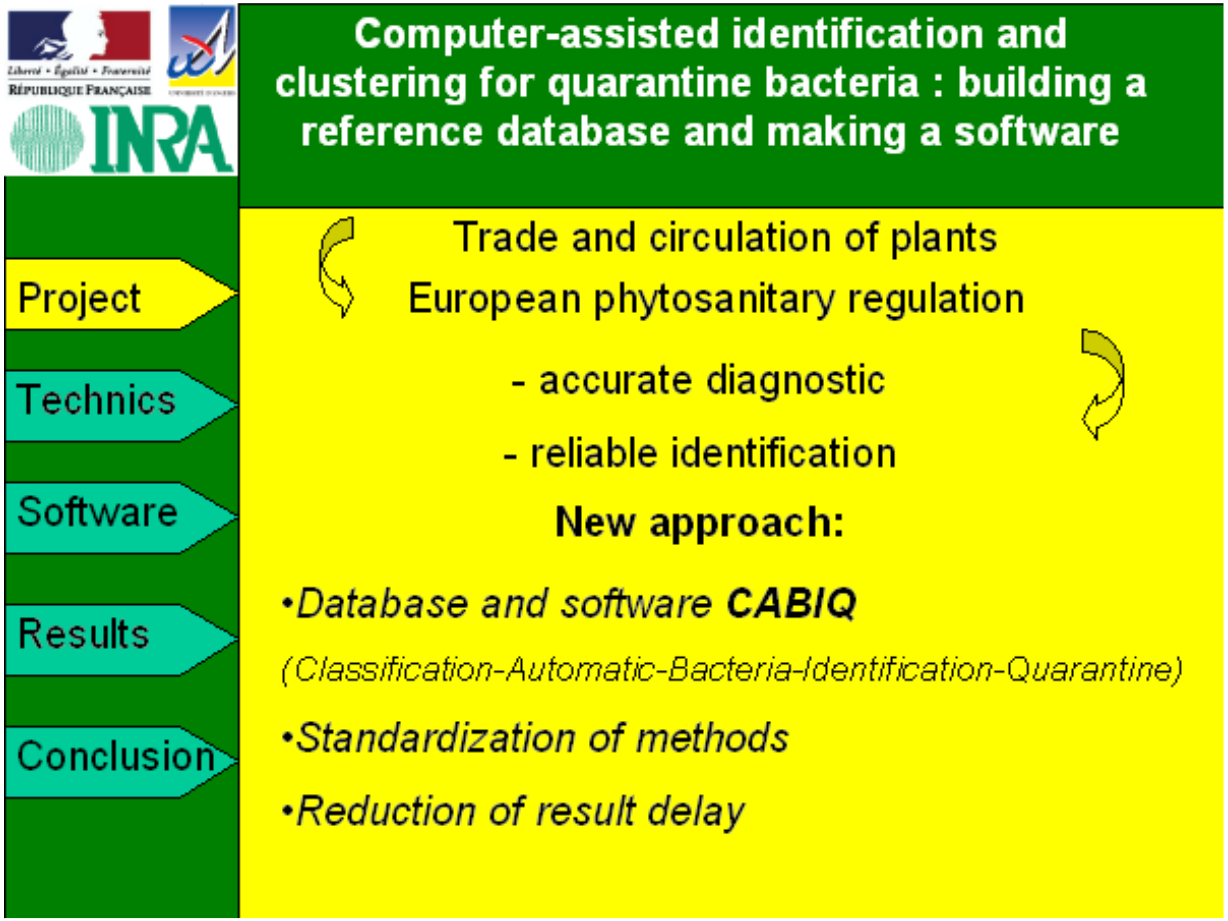
Windows-based system for computerized identification of quarantine bacteria

- This tool will be released as a Windows-based system called CABIQ ('Classification Automatique Bactéries Identification Quarantaine') incorporating the database.
- This tool is innovative because it combines:
- Traditional identification tests, and
- Molecular methods in a single system to provide a more rapid and reliable identification.
- It proposes an user-friendly interface to:
  - Obtain detailed statistics for each genus, species and strip compared to a reference;
  - Identify an isolate and validate its identification,
  - Classify automatically an isolate within a dendrogram (UPGMA).

M., Sutra .Poliakoff F., Caffier D., François C., Rivoal C., Fischer-Le Saux L., Perret S., Bonneau S.& G. Hunault 2004 & 05.

CABIQ

Computer-assisted identification system of phytopathogenic bacteria



Poliakoff *et al.,* 2004. Presented in EPPO Conference on Quality of Diagnosis and New Diagnostic Methods for Plant Pests.

CABIQ

Specific computer-assisted identification system of phytopathogenic bacteria

- About 500 reference strains have been used to initiate the database, including:

1. Conventional phenotypic tests, and
2. The Biotype 100 (BioMérieux) galleries.

- The CABIQ system, with its database and reference matrices, is a guide on the tests to be done when identifying new isolates.

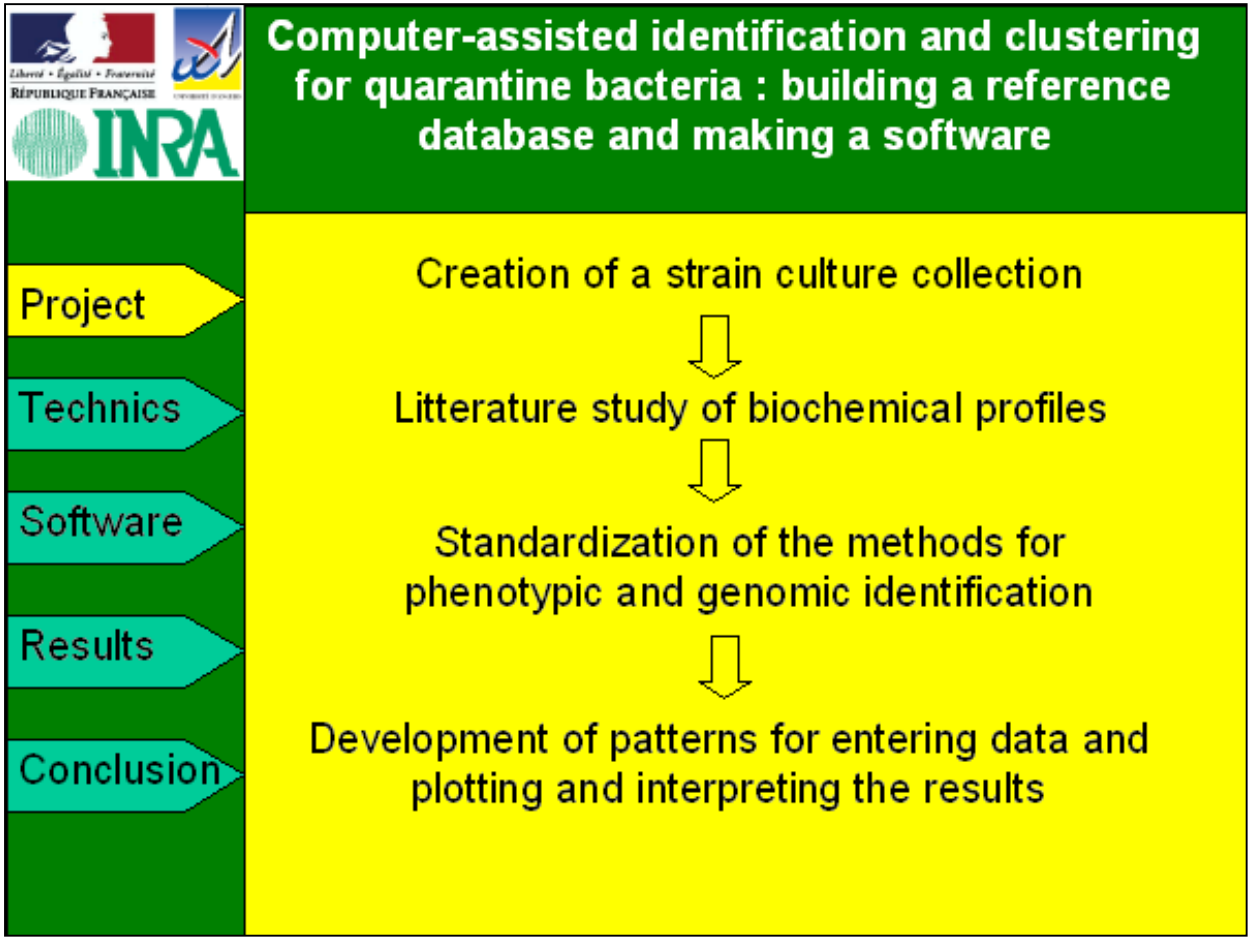
3. Results on repetitive PCR will soon be added to the system.

- This system was designed for phytosanitary regulations of the European Union to survey for certain quarantine pests and to prohibit their introduction and spread into the EU.

Poliakoff *et al.*,2005

CABIQ

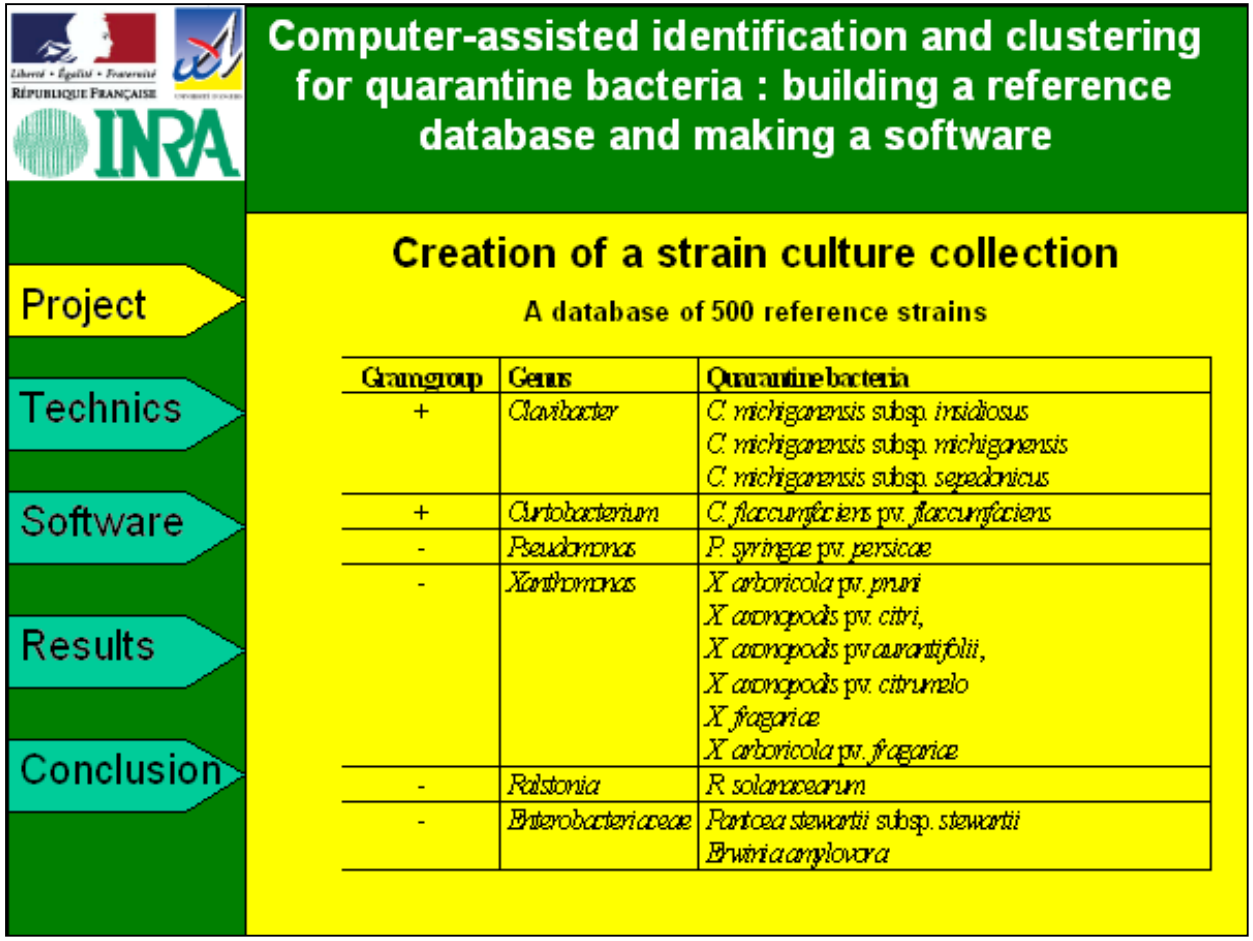
Computer-assisted identification system of phytopathogenic bacteria



Poliakoff *et al.*,2005

CABIQ

Computer-assisted identification system of phytopathogenic bacteria

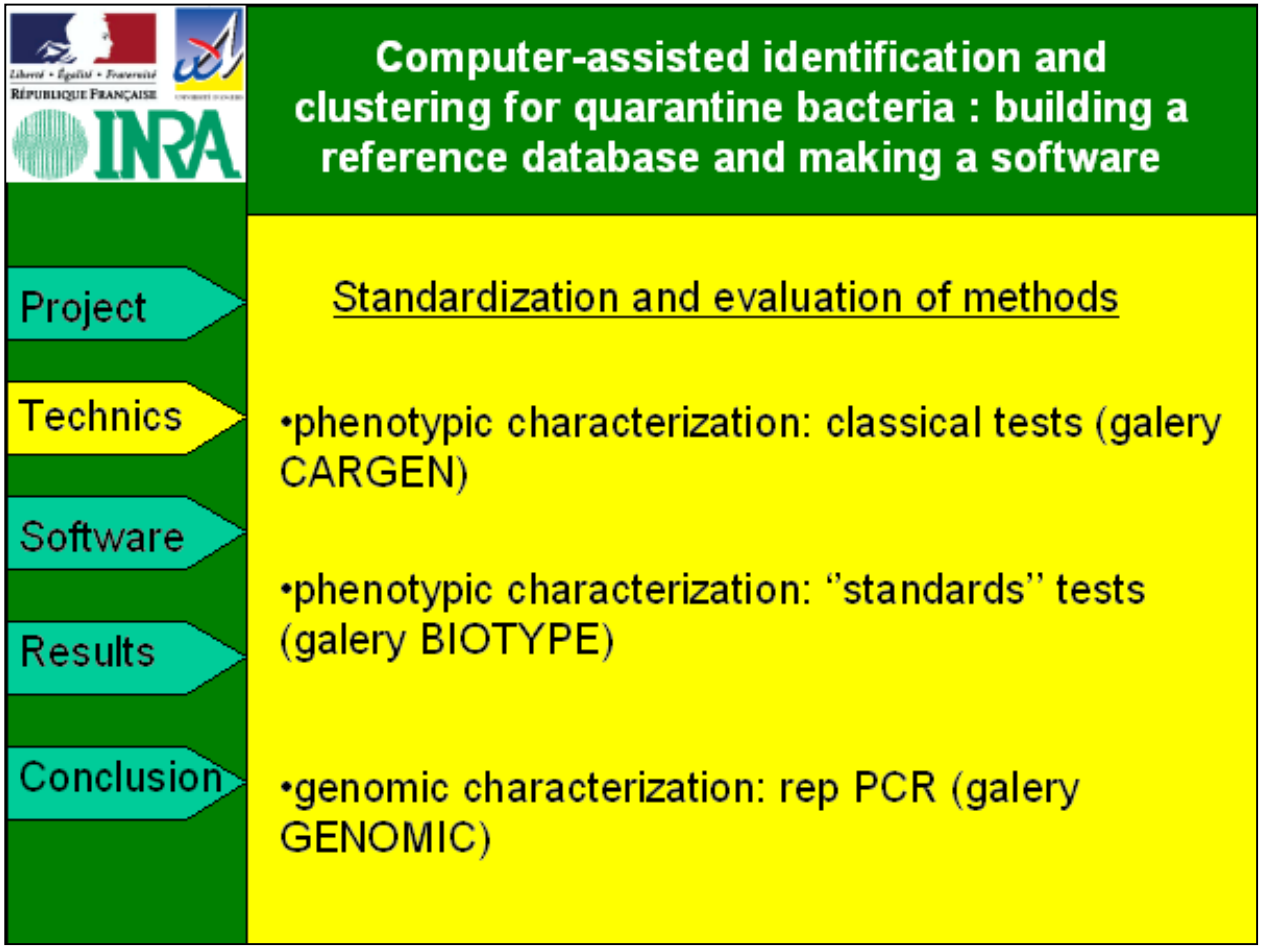


Gramgroup	Genus	Quarantine bacteria
+	*Clavibacter*	*C. michiganensis* subsp. *insidiosus* *C. michiganensis* subsp. *michiganensis* *C. michiganensis* subsp. *sepedonicus*
+	*Curtobacterium*	*C. flaccumfaciens* pv. *flaccumfaciens*
-	*Pseudomonas*	*P. syringae* pv. *persicae*
-	*Xanthomonas*	*X arboricola* pv. *pruni* *X axonopodis* pv. *citri*, *X axonopodis* pv *aurantifolii*, *X axonopodis* pv. *citrumelo* *X fragariae* *X arboricola* pv. *fragariae*
-	*Ralstonia*	*R solanacearum*
-	*Enterobacteriaceae*	*Pantoea stewartii* subsp. *stewartii* *Erwinia amylovora*

Poliakoff *et al.*,2004

CABIQ

Computer-assisted identification system of phytopathogenic bacteria



Poliakoff *et al.*,2004

CABIQ

Computer-assisted identification system of phytopathogenic bacteria

Computer-assisted identification and clustering for quarantine bacteria : building a reference database and making a software

Project

Technics

Software

Results

Conclusion

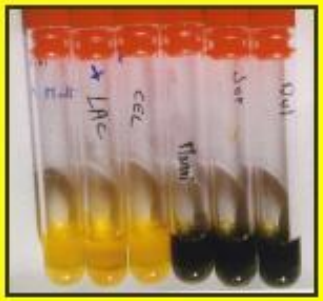
Classical identification tests for bacteria:

- General characteristics: Gram stain, morphological, and growth characteristics.
- Orientation tests for each bacterial group: physiological, enzymatics
- Diagnostic tests linked to genus:

metabolical and biochemical

characteristics:

carbon source utilization



Poliakoff *et al.*,2004

CABIQ

Computer-assisted identification system of phytopathogenic bacteria

Computer-assisted identification and clustering for quarantine bacteria : building a reference database and making a software

Project

Technics

Software

Results

Conclusion

"Standards" tests : Galerie biotype 100

Standardized method (Biomerieux) to establish metabolical profiles of bacteria

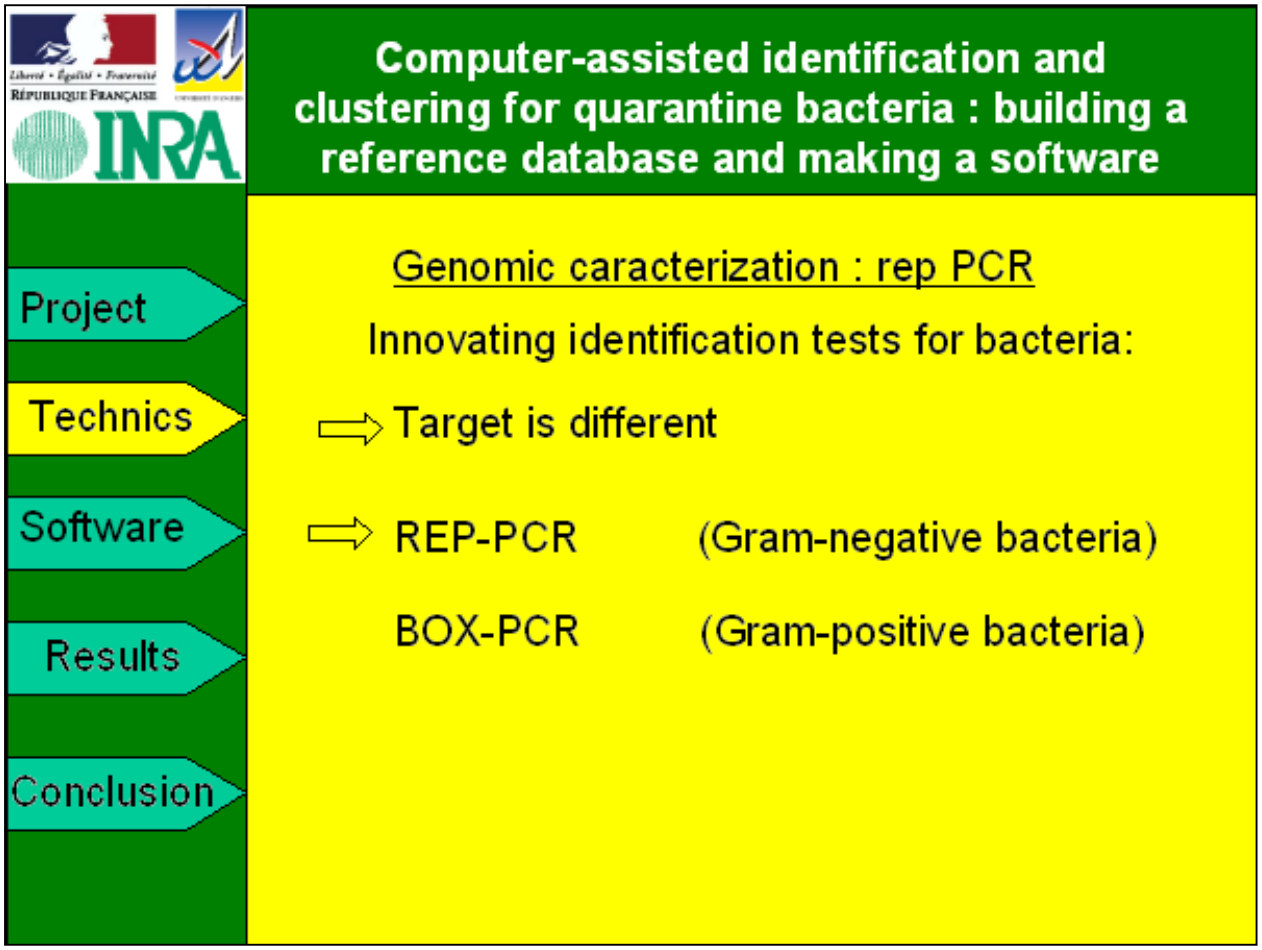
⇨ Description:

- strips with 100 tubes
- assimilation medium (biotype medium 1 and 2)
- incubation (28°C and 24°C)
- strips are read at 2, 4, 6 days

Poliakoff *et al.*,2004

CABIQ

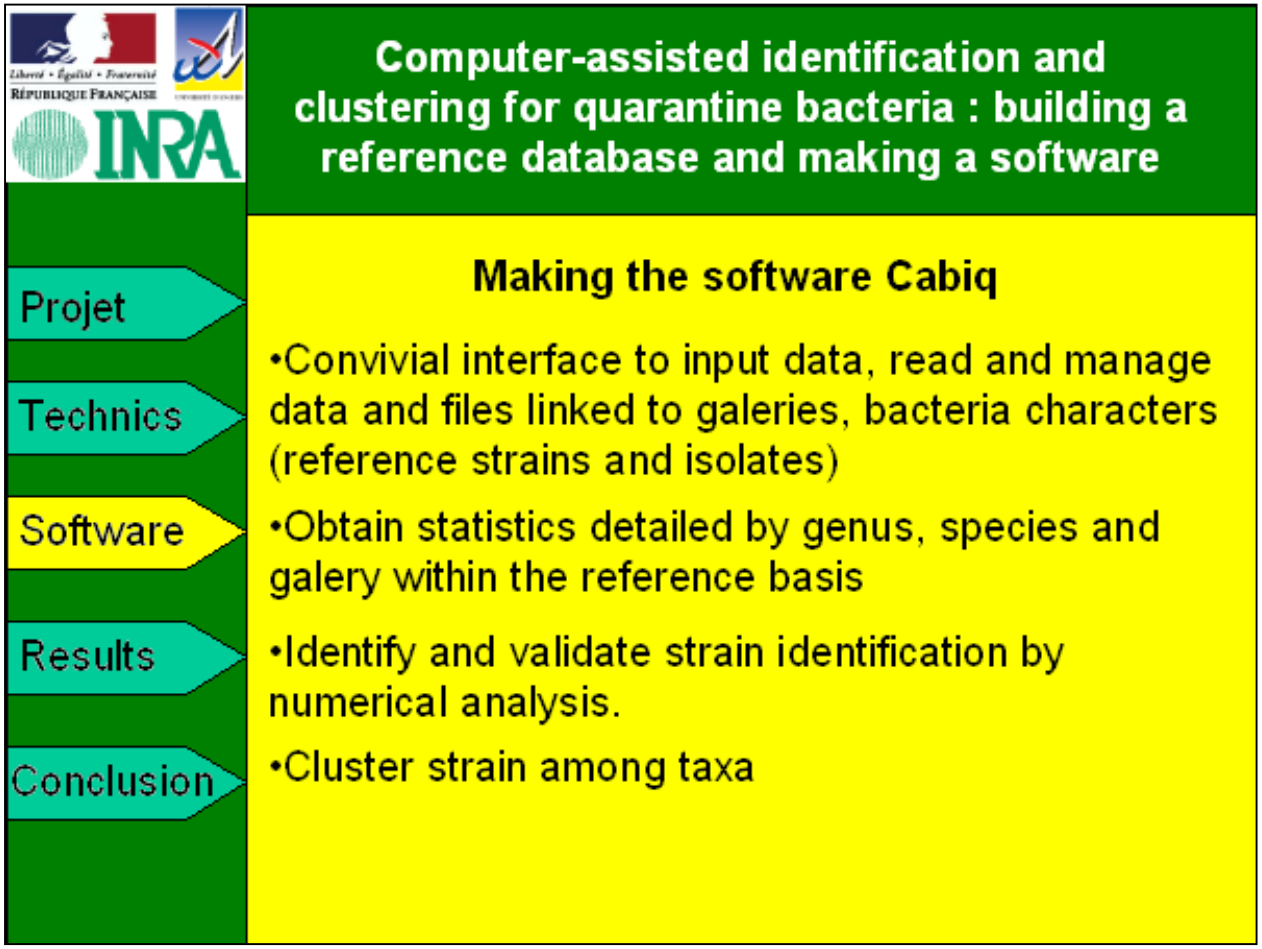
Computer-assisted identification system of phytopathogenic bacteria



Poliakoff *et al.*,2004

CABIQ

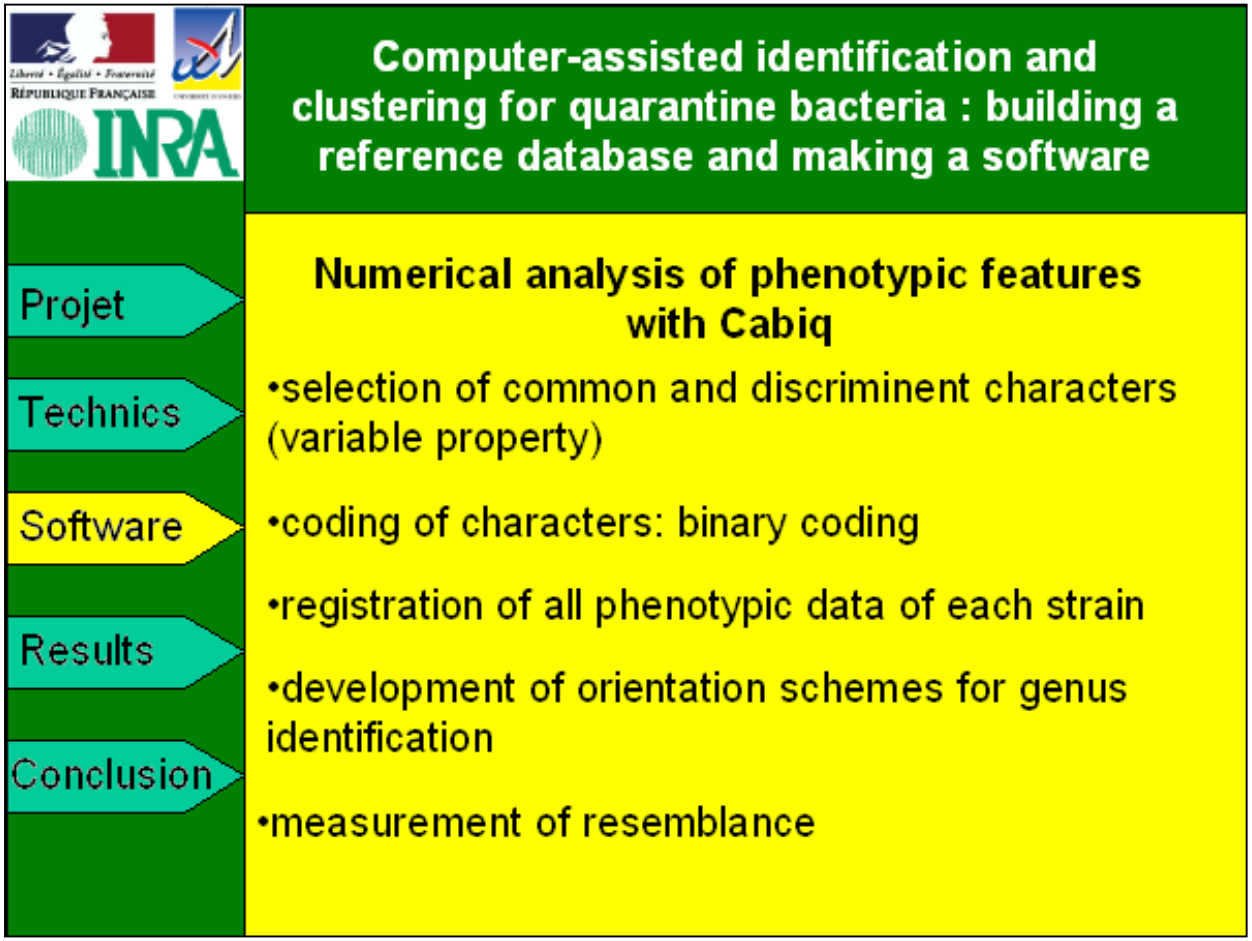
Computer-assisted identification system of phytopathogenic bacteria



Poliakoff *et al.*,2004

CABIQ

Computer-assisted identification system of phytopathogenic bacteria



Poliakoff *et al.*,2004

CABIQ

Rules to determine the probable genus of a Gram-positive bacterium

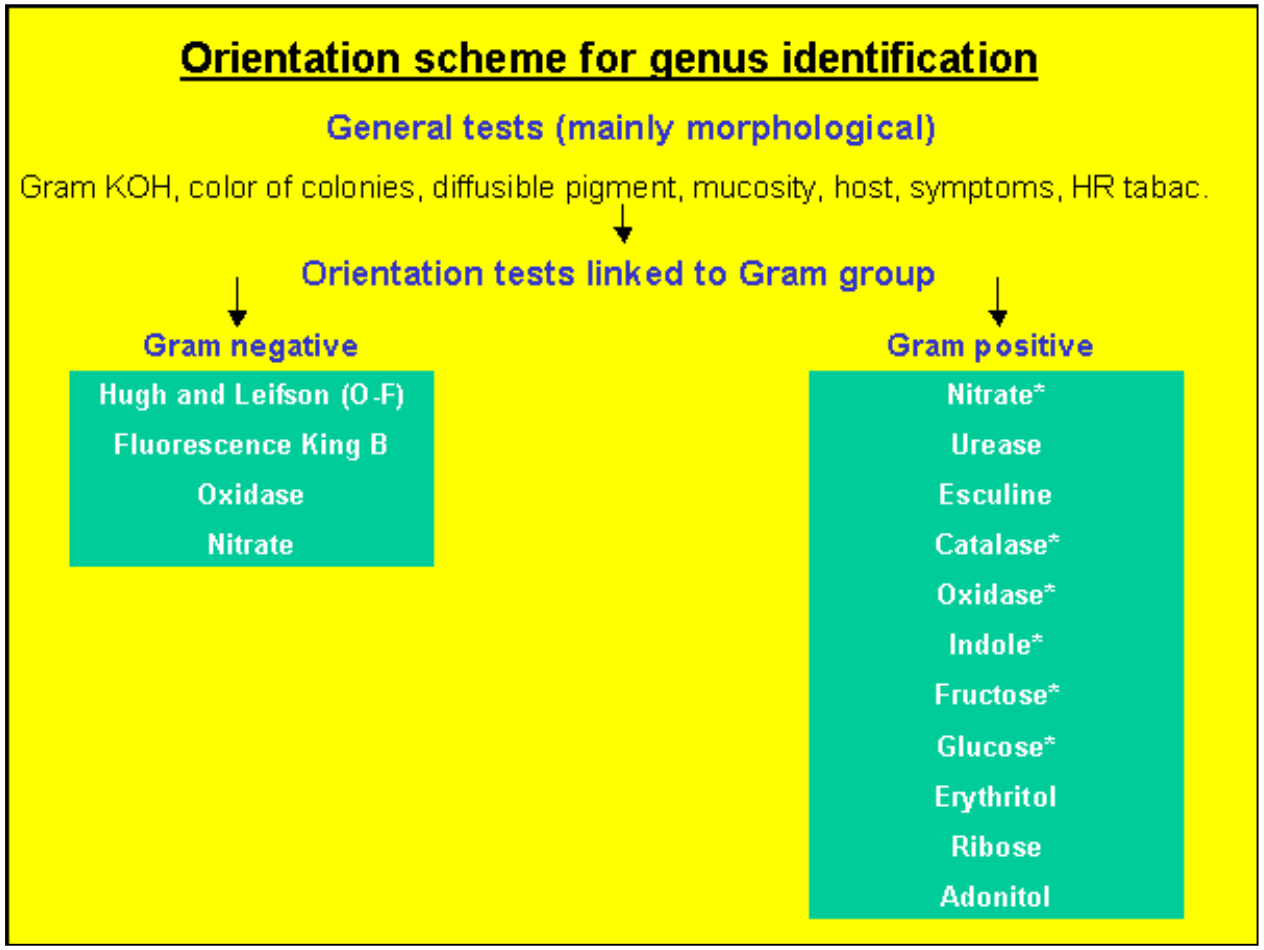
- Rules to determine the probable genus of a Gram-positive bacterium according to the results of orientation tests (O/F, enzymatic activities, production of acid from different carbon sources):

Test results	Probable genus
Urease (+), no esculin (–), orange colonies on YPGA	*Rhodococcus*
Urease (–), esculin (+), acid from erythritol (+), acid from ribose (+), acid from adonitol (+)	*Curtobacterium*
Urease (–), esculin (+), acid from erythritol (–), acid from ribose (–), acid from adonitol (–)	*Clavibacter*/*Rathayibacter*

Poliakoff *et al.*,2005

CABIQ

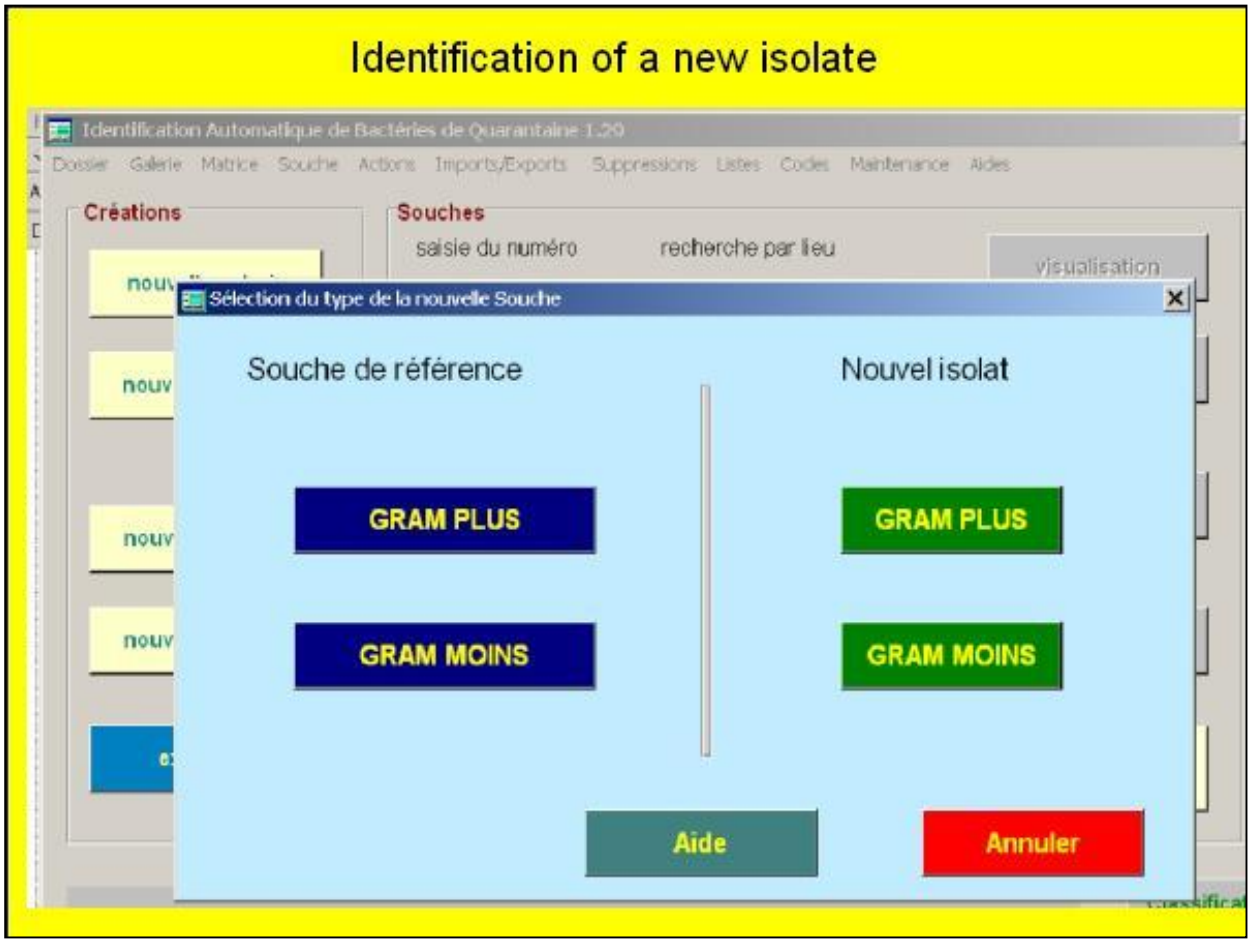
Computer-assisted identification system of phytopathogenic bacteria



Poliakoff *et al.*,2004

CABIQ

Computer-assisted identification system of phytopathogenic bacteria



Poliakoff *et al.*,2005

CABIQ

Computer-assisted identification system of phytopathogenic bacteria

Specific tests of diagnostic depending on probable genus

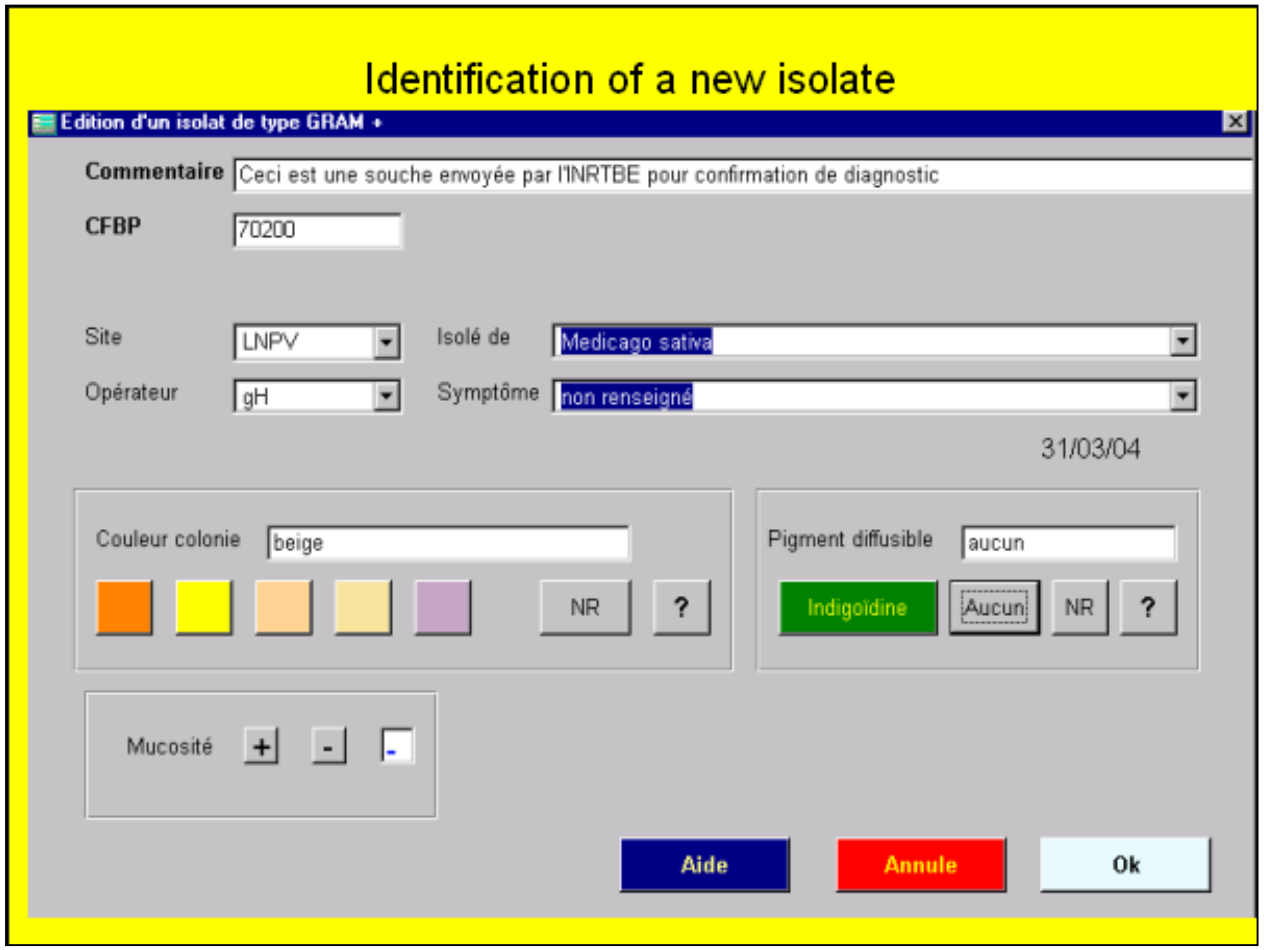
e.g. Diagnostic tests for genus fom Gram positive

Rhodococcus	Curtobacterium	Clavibacter/ Rathayibacter
None	Lactose	Lactose
	Sorbitol	Sorbitol
	Host	Mannitol
		Gelatine
		NaCl 5%
		Levane
		Host

Poliakoff *et al.*,2004

CABIQ

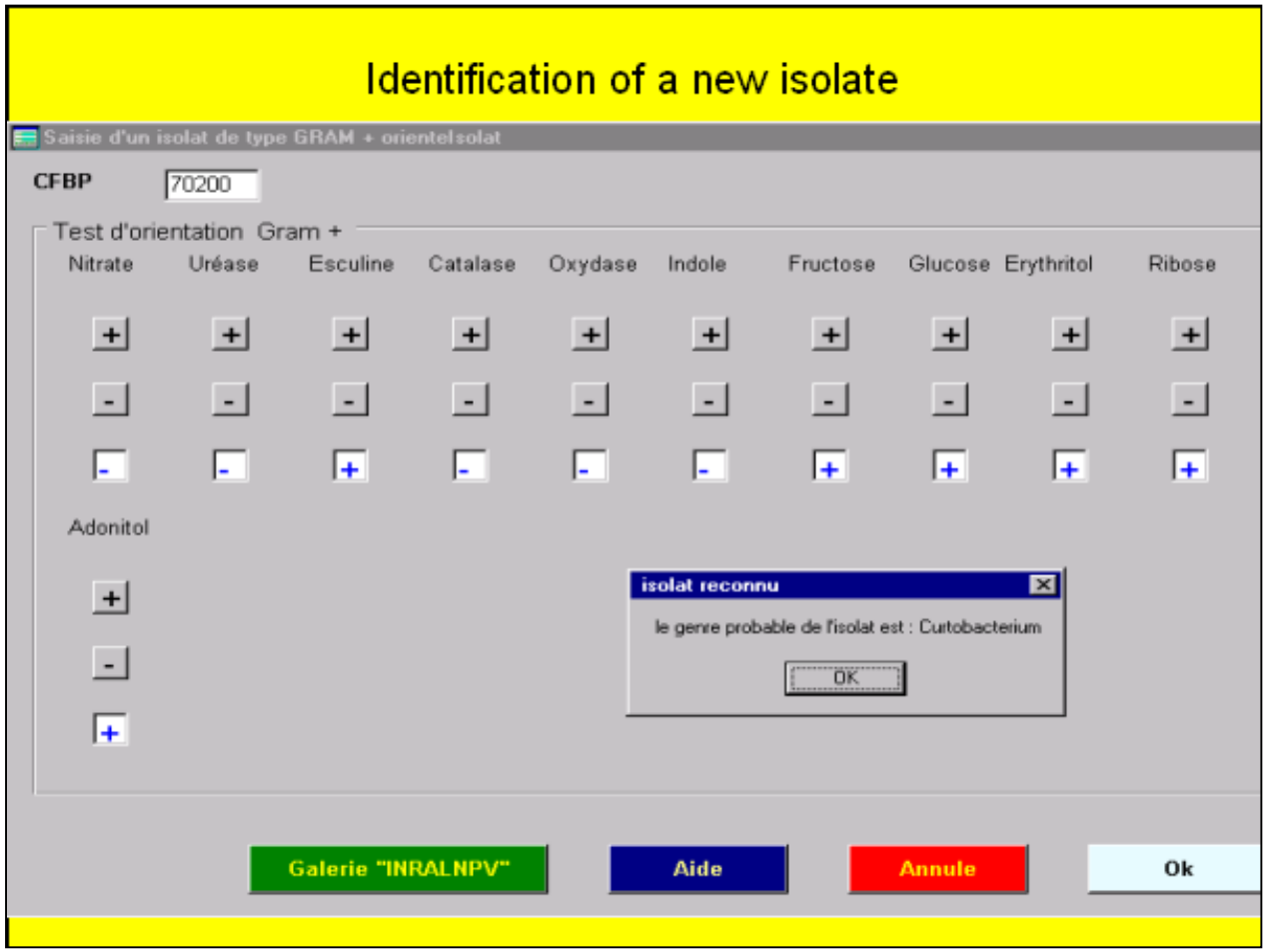
Computer-assisted identification system of phytopathogenic bacteria



Poliakoff *et al.*,2005

CABIQ

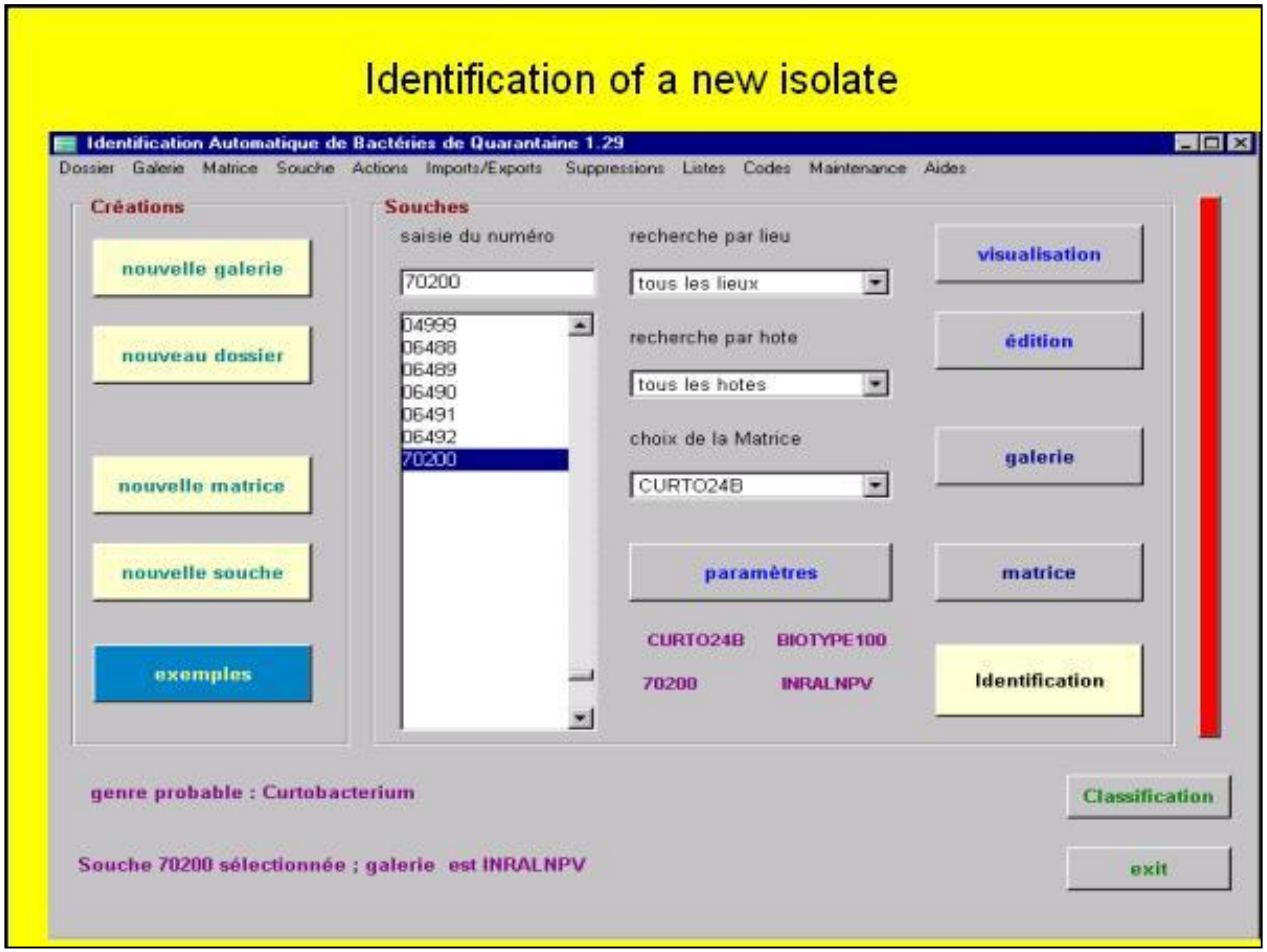
Computer-assisted identification system of phytopathogenic bacteria



Poliakoff *et al.*,2004

CABIQ

Computer-assisted identification system of phytopathogenic bacteria



Poliakoff *et al.*,2005

CABIQ
Measurement of resemblance

- A paired comparison method may be used to check which reference strains are most similar to the unknown isolate.
- After these comparisons, CABIQ calculates the interrelation between the unknown isolate and each taxon through likelihood coefficient, normalized likelihood coefficient (also called identification score) or Willcox's probability, and relative likelihood.
- For the example given in Table 7, the unknown isolate is identified as *Clavibacter michiganensis* subsp. *nebraskensis*, since this taxon has the highest identification score.
- The modal likelihood fraction proves the absolute degree of affinity to the species in the matrix.
- In the case of Table 8, the unknown isolate looks like *Clavibacter sepedonicus* but this identification is not validated.

Poliakoff *et al.*,2004

Subspecies	Oxidase	Levan	Glucose	Erythritol	Esculin
Clavibacter michiganensis subsp. *insidiosus*	0.14	0.10	0.43	0.10	0.90
Clavibacter michiganensis subsp. *michiganensis*	0.10	0.10	0.81	0.10	0.90
Clavibacter michiganensis subsp. *nebraskensis*	0.10	0.90	0.90	0.10	0.90
Clavibacter michiganensis subsp. *sepedonicus*	0.10	0.20	0.67	0.10	0.90
Clavibacter michiganensis subsp. *tessellarius*	0.10	0.80	0.90	0.10	0.90
Unknown isolate	0	1	1	0	1

Table 6 Comparison of an unknown isolate with the positivity frequency matrix of the different subspecies of *Clavibacter michiganensis* given by CABIQ

5 taxa and 19 conventional tests	Score	Modal likelihood fraction
Clavibacter michiganensis subsp. *insidiosus*	5.9%	7.3%
Clavibacter michiganensis subsp. *michiganensis*	9.0%	11.1%
Clavibacter michiganensis subsp. *nebraskensis*	98.7%	100.0%
Clavibacter michiganensis subsp. *sepedonicus*	9.0%	11.1%
Clavibacter michiganensis subsp. *tessellarius*	1.0%	1.2%
Unknown isolate	Successful identification	

Table 7 Identification score of Lapage and Wilcox and modal likelihood fraction given by CABIQ (example of validated identification)

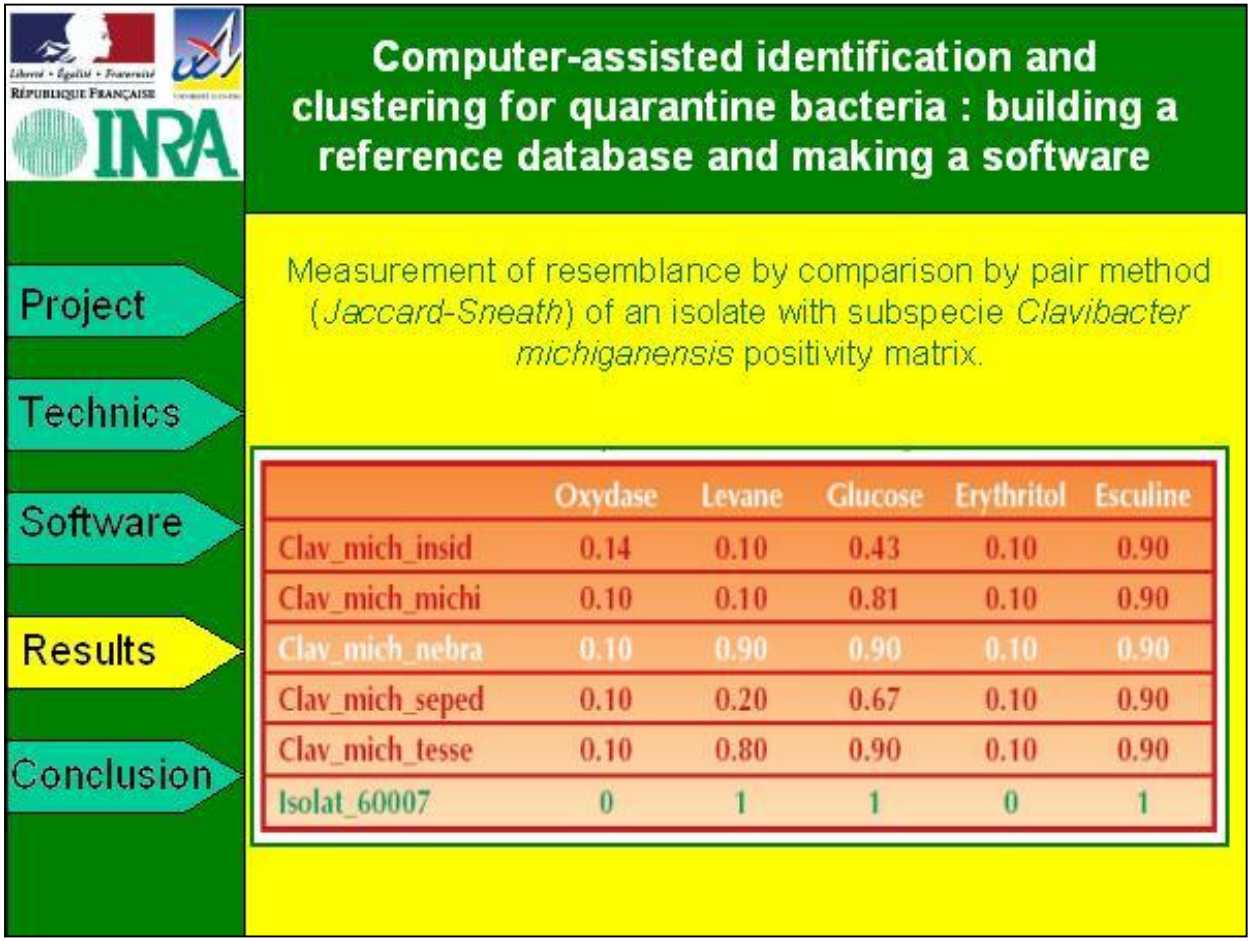
5 taxa and 19 conventional tests	Score	Modal likelihood fraction
Clavibacter michiganensis subsp. *insidiosus*	0.0%	0.0%
Clavibacter michiganensis subsp. *michiganensis*	0.0%	0.0%
Clavibacter michiganensis subsp. *nebraskensis*	14.7%	4.2%
Clavibacter michiganensis subsp. *sepedonicus*	82.1%	0.3%
Clavibacter michiganensis subsp. *tessellarius*	3.1%	1.5%
Unknown isolate	Non validated identification	

Table 8 Identification score of Lapage and Wilcox and likelihood fraction given by CABIQ (example of non-validated identification)

Poliakoff *et al.*,2004

CABIQ

Computer-assisted identification system of phytopathogenic bacteria

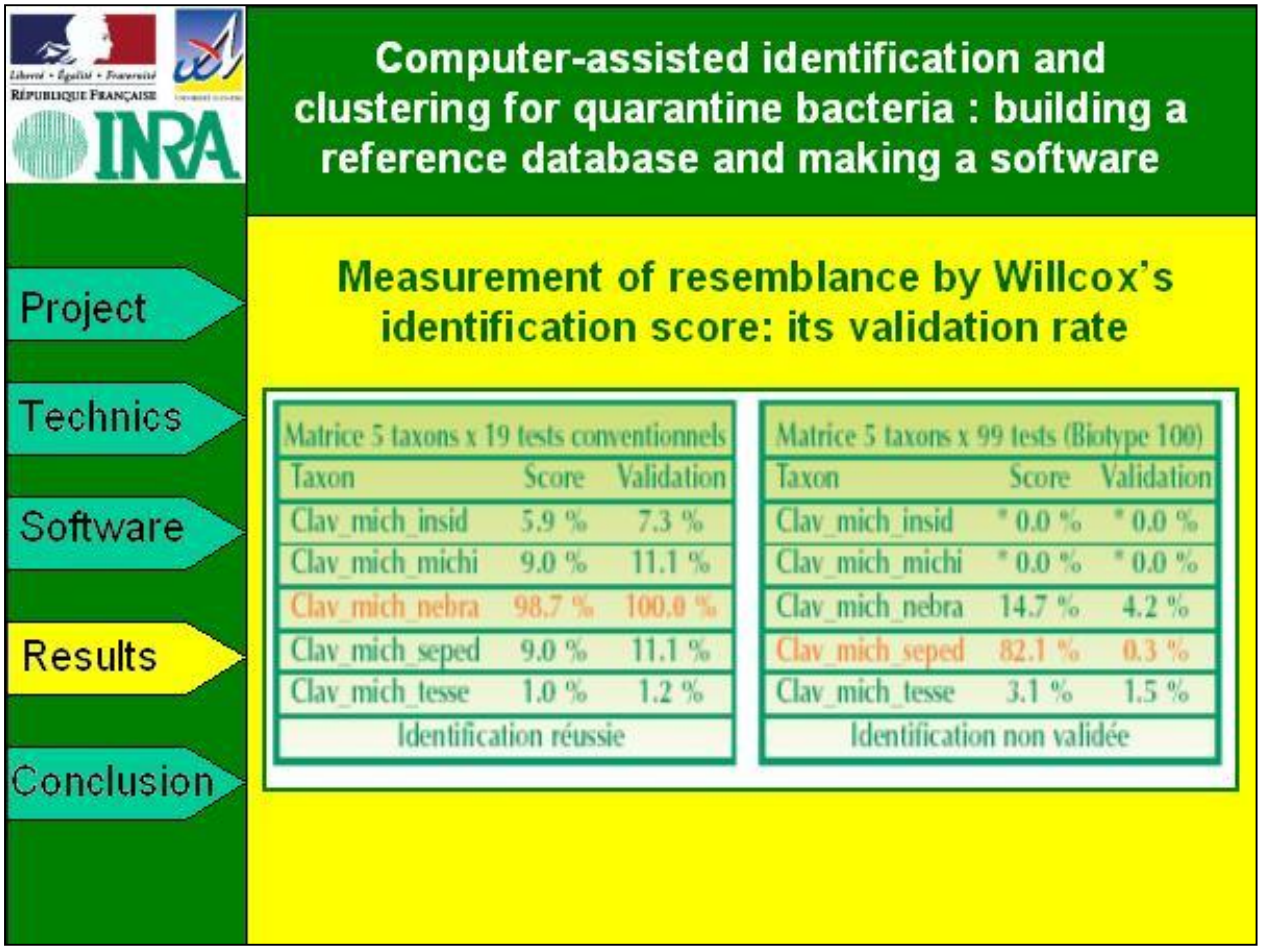


	Oxydase	Levane	Glucose	Erythritol	Esculine
Clav_mich_insid	0.14	0.10	0.43	0.10	0.90
Clav_mich_michi	0.10	0.10	0.81	0.10	0.90
Clav_mich_nebra	0.10	0.90	0.90	0.10	0.90
Clav_mich_seped	0.10	0.20	0.67	0.10	0.90
Clav_mich_tesse	0.10	0.80	0.90	0.10	0.90
Isolat_60007	0	1	1	0	1

Poliakoff *et al.*,2005

CABIQ

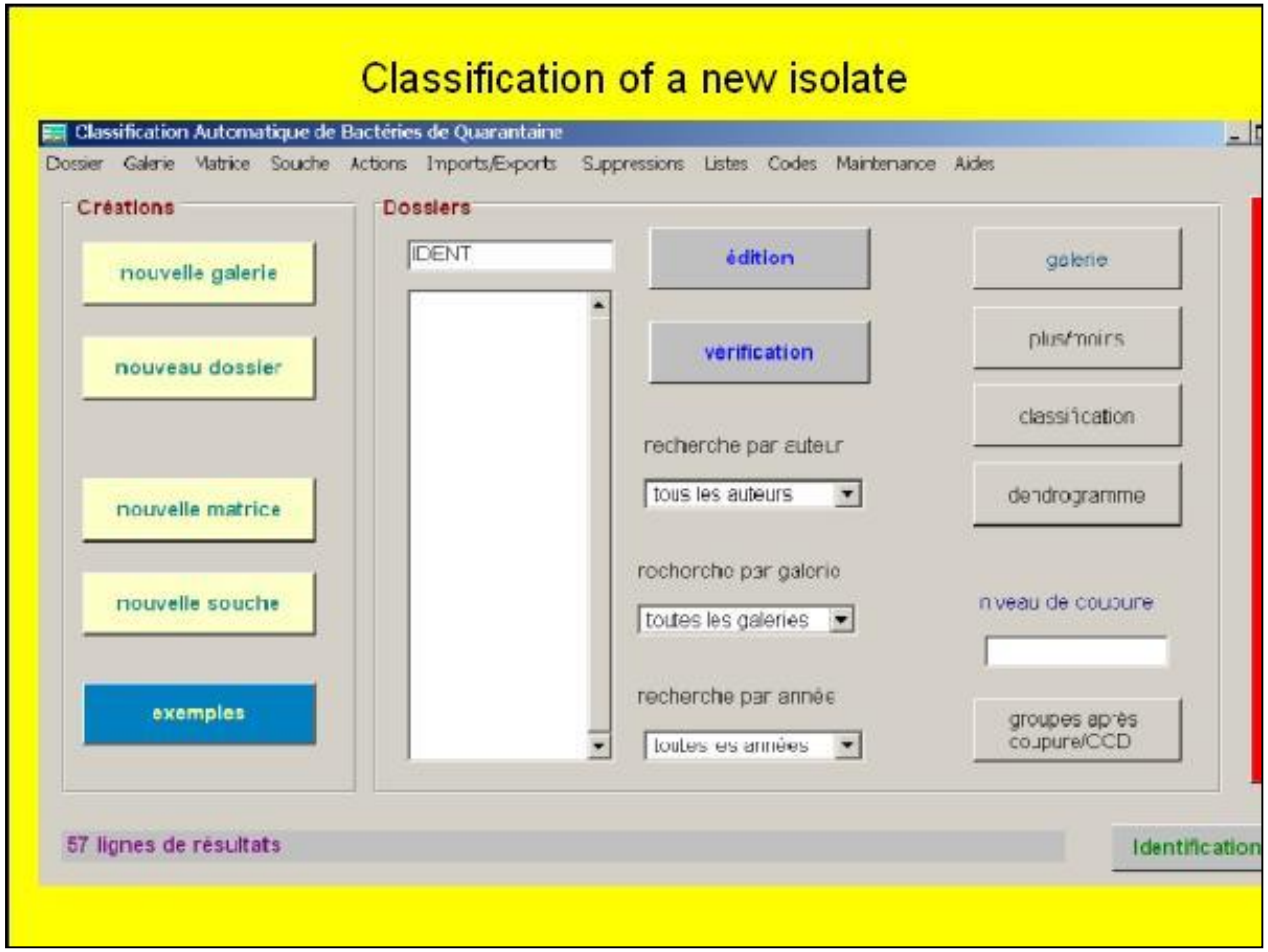
Computer-assisted identification system of phytopathogenic bacteria



Poliakoff *et al.*,2005

CABIQ

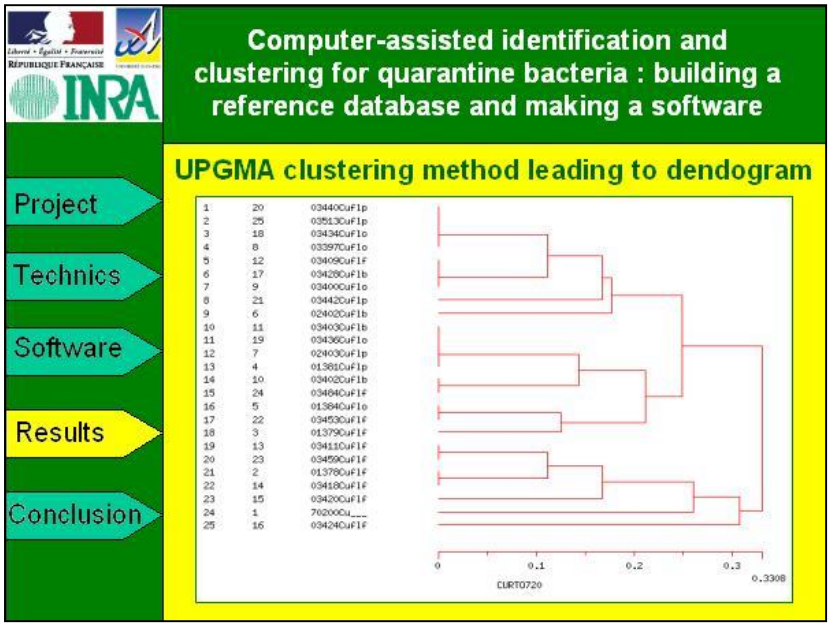
Computer-assisted identification system of phytopathogenic bacteria



Poliakoff *et al.*,2005

Numerical analysis of phenotypic features with the CABIQ software

- Import and export strains for automatic clustering using the UPGMA (Unweighted Pair Group Method with Average) leading to dendrograms and CDC (coefficients of diagnosis capacity) computations, considering bacterial strains as Operational Taxonomic Units.
- CABIQ software is moreover able to import and export data using classical formats (Dbase, Excel, text, Phylip).
- Clustering of an unknown isolate 70200 in the dendrogram of subspecies of *Curtobacterium flaccumfaciens* given by CABIQ.



Clustering of an unknown isolate

Poliakoff *et al.*,2004

Appendix 7

QBOL

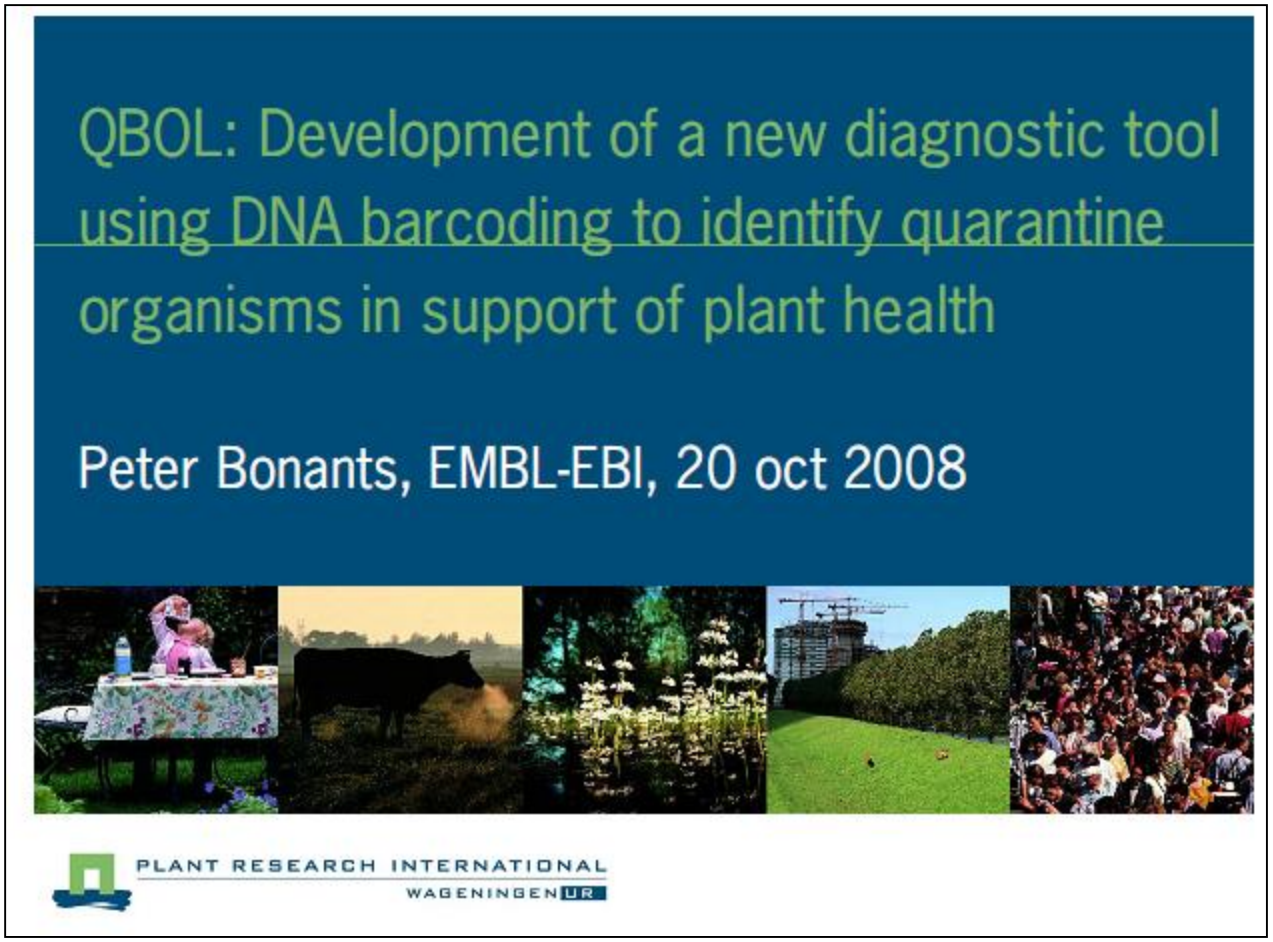
DNA-barcode identification of quarantine organisms

www.qbol.org

QBOL

Development of a new diagnostic tool using DNA Barcoding to identify quarantine organisms in support of plant health

DNA-barcode identification of quarantine organisms



Bonants,2008

QBOL

A new diagnostic tool using DNA Barcoding to identify quarantine organisms in support of plant health

DNA-barcode identification of quarantine plant pests or pathogens

- DNA barcoding is a new technique that uses a short DNA sequence from a standardized and agreed-upon position in the genome as a molecular diagnostic for species-level identification.

Bonants,2008; www.qbol.org

QBOL
Q-bank database

- QBOL is financed by the 7th Framework Program of the European Union.
- QBOL makes collections harboring plant pathogenic quarantine organisms available.
- Informative genes from selected species on the EU Directive and EPPO lists are DNA barcoded.
- Sequences, together with taxonomic features, will be included in an internet-based Q-bank database.

QBOL

Three principle QBOL objectives for DNA-barcode identification for all quarantine plant pests or pathogens

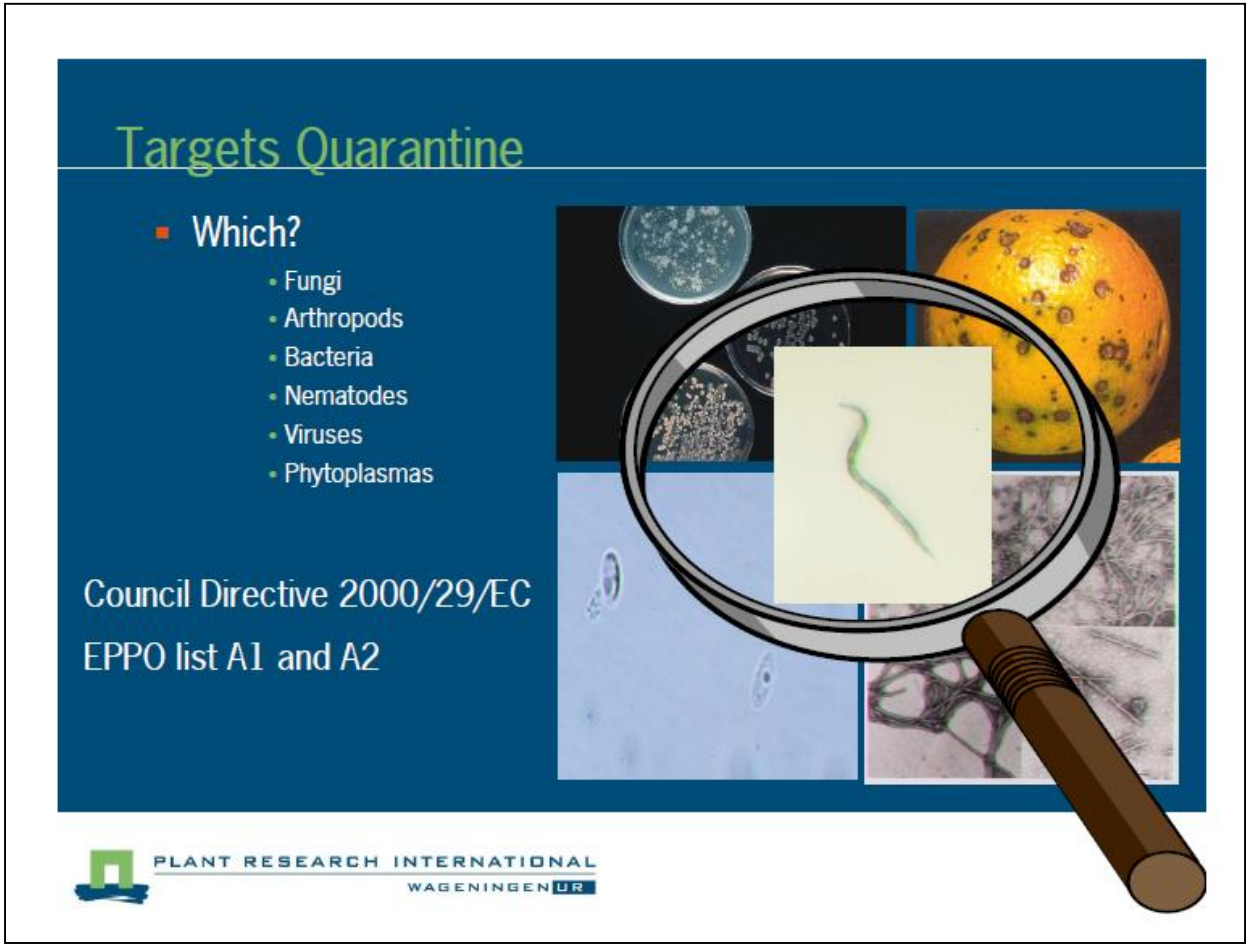
1. To DNA barcode relevant Q-organisms plus morphologically and/or taxonomically related organisms.
2. To develop a database of DNA barcode sequences plus relevant taxonomic/geographic/host data.
3. To develop a DNA bank for the selected set of Q-organisms plus morphologically and/or taxonomically related organisms.

Bonants,2008

QBOL

Barcoding of life

DNA-barcode identification of quarantine organisms

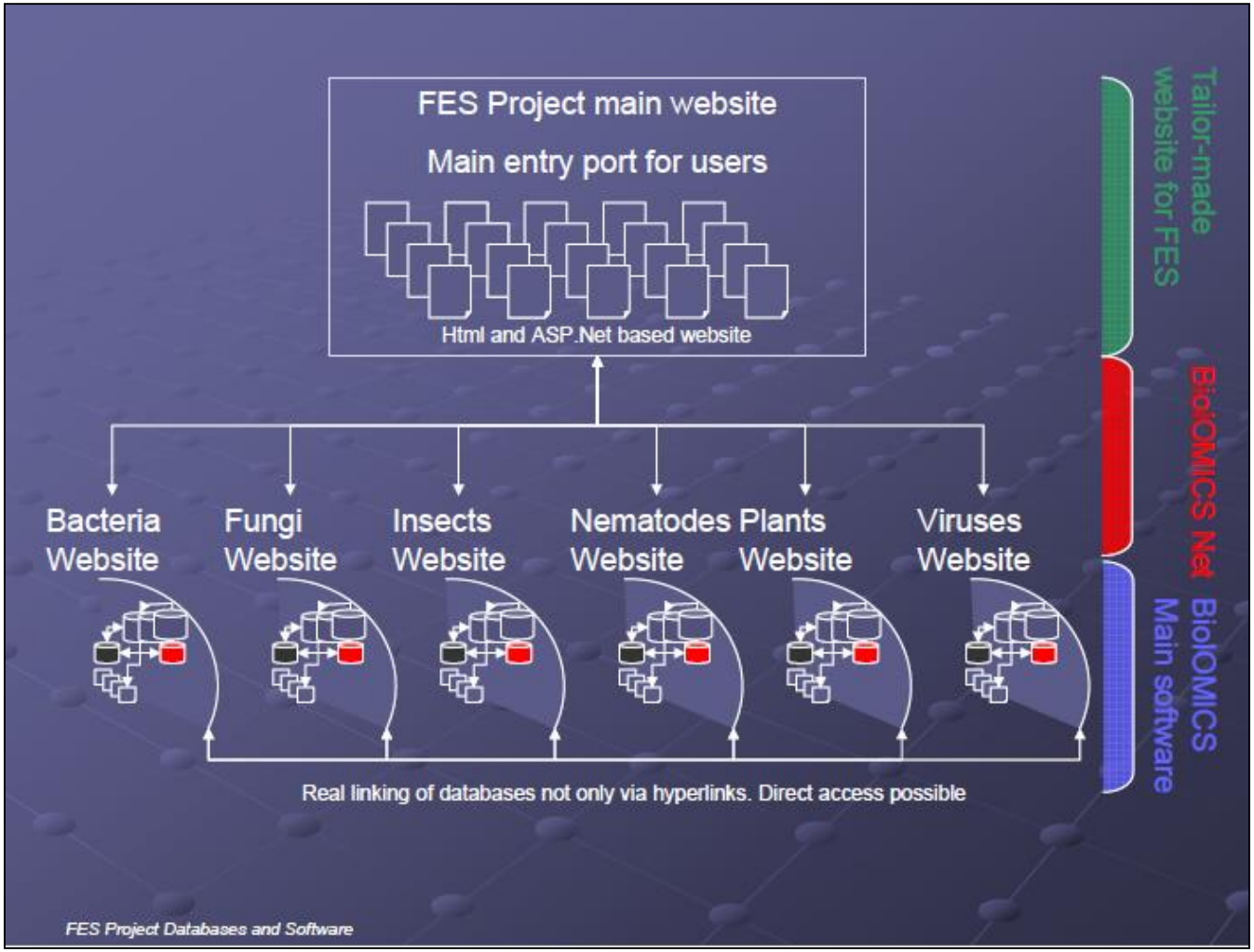


Bonants,2008

QBOL

Barcoding of life

DNA-barcode identification of quarantine organisms



QBOL
Barcoding of Bacteria

- The Q-bank Bacteria database contains DNA sequence (Barcodes), morphological, phenotypical and ecological data of more than xxx species that are of relevance to bacterial phytopathology.
- Currently, the database focuses on the bacterial genera *Clavibacter*, *Xanthomonas*, *Ralstonia* and *Xylella*.

QBOL
Barcoding of Bacteria

- To maintain and expand existing collection with relevant bacterial isolates (collection strains and recent field isolates).
- To unravel the taxonomic and phylogenetic identity of quarantine bacteria in support of EU regulation.
- To collect barcode data including genetic variation, inclusiveness and exclusiveness for the selected scope of Q-bacteria.
- To generate barcode sequences from a selected set of regions and for a selected set of relevant quarantine bacteria.
- To develop reliable routine DNA extraction protocols.
- To develop robust primers for PCR amplification of the barcode gene regions in the different taxa.

QBOL

Quarantine bacteria on EU Directive and EPPO list

Combined EU / EPPO list — Q and Q-alert bacteria

Annex I	**Part A**	**Section I**
	1 *Xyllela fastidiosa*	
Annex I	**Part A**	**Section II**
	1 *Clavibacter michiganensis* ssp. *sepedonicus*	
	2 *Pseudomonas solanacearum* = *Ralstonia solanacearum (race 1, race 3)*	
Annex I	**Part B**	
Annex II	**Part A**	**Section I**
	1 *Erwinia stewartii*	
	2 *Xanthomonas campestris* strains pathogenic to Citrus (as *Xanthomonas axonopodis* pv. *citri)*	
	3 *Xanthomonas campestris* pv. *oryzae* & *oryzicola* = *Xanthomonas oryzae* pv. *oryzae* & *oryzicola*	
Annex II	**Part A**	**Section II**
	1 *Clavibacter michiganensis* ssp. *insidiosus*	
	2 *Clavibacter michiganensis* ssp. *michiganensis*	
	3 *Erwinia amylovora*	
	4 *Erwinia chrysanthemi* pv. *dianthicola* = *Dickeya dianthicola*	
	5 *Pseudomonas caryophylli* = *Burkholderia caryophylli*	
	6 *Pseudomonas syringae* pv. *persicae*	
	7 *Xanthomonas campestris* pv. *phaseoli*	
	Xanthomonas arboricola pv. *corylina* (EPPO list)	
	8 *Xanthomonas campestris* pv. *pruni* = *Xanthomonas arboricola* pv. *pruni*	
	9 *Xanthomonas campestris* pv. *vesicatoria* = *Xanthomonas vesicatoria* & *Xanthomonas axonopodis* pv. *vesicatoria*	
	10 *Xanthomonas fragariae*	
	11 *Xylophilus ampelinus*	
Annex II	**Part B**	
	1 *Curtobacterium flacumfaciens* pv. *flaccumfaciens*	
Q-alert EPPO		
	Xanthomonas axonopodis pv. *dieffenbachiae*	
	Xanthomoans arboricola pv. *fragariae*	
	Xanthomonas axonopodis pv. *allii*	

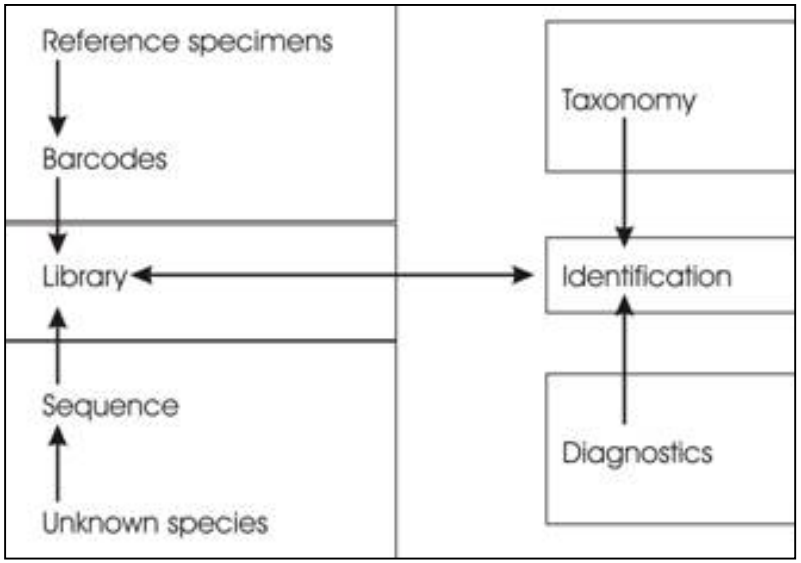
QBOL

DNA sequence (Barcodes)

Procedure of DNA-barcode identification

- DNA Barcoding projects have four components:

1. The Specimens;
2. The Laboratory Analysis;
3. The Database (e.g. BOLD, GenBank, FES Programme (NL);
4. The Data Analysis.



Bonants,2008

QBOL
Q-bank
DNA sequence (Barcodes)

www.gbol.org

QBOL
Barcoding of life
The future

Bonants,2008

Appendix 8

ABIS online

Automated Biometric Identification System

www.microrao.com/identify.htm

ABIS online

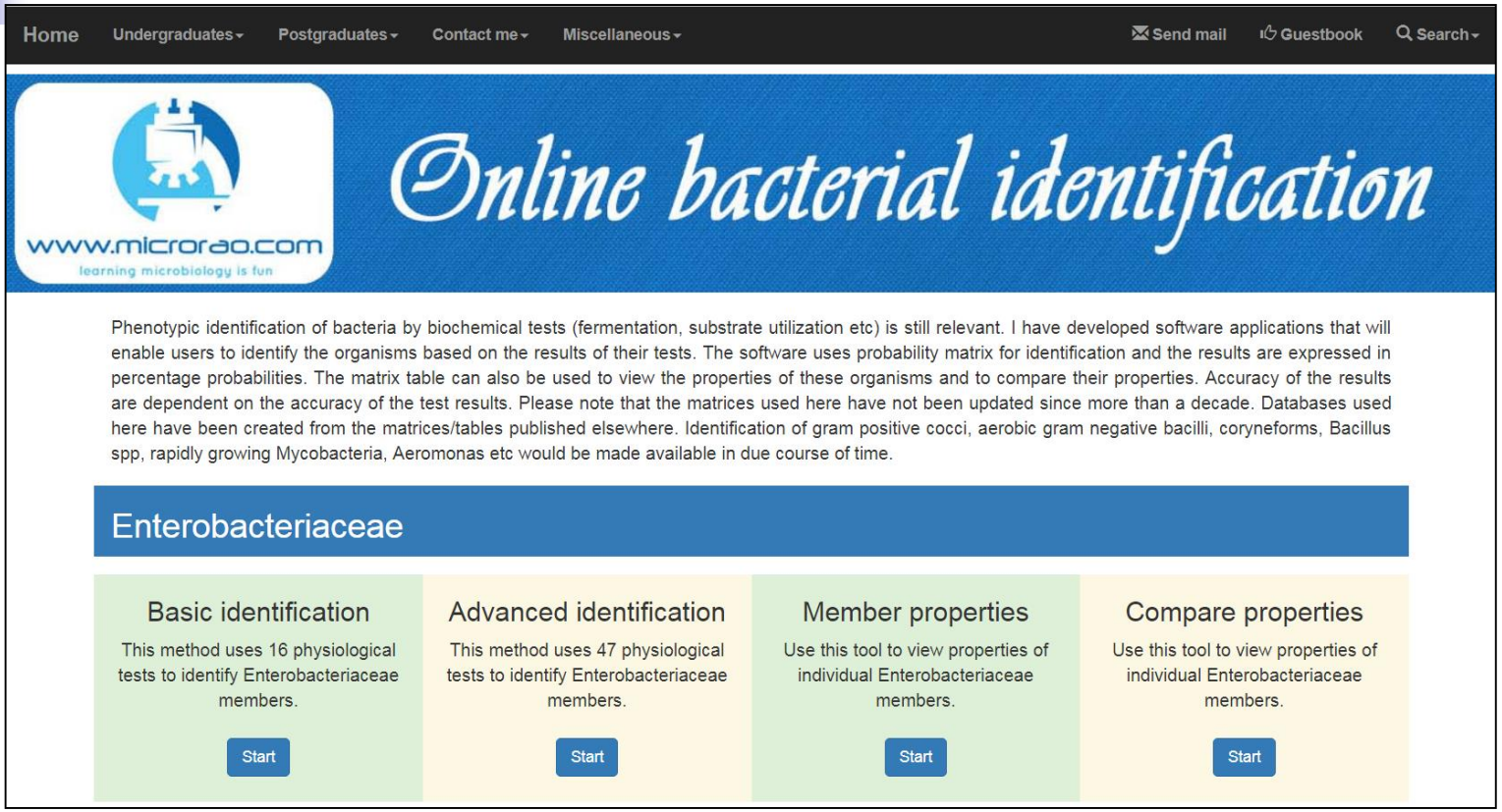
Automated Biometric Identification System

An Advanced Bacterial Identification Software

ABIS online

Automated Biometric Identification System

An Advanced Bacterial Identification Software



September 6, 2007
"ABIS 6 online" official launch with Enterobacteriaceae database.

ABIS online

Automated Biometric Identification System

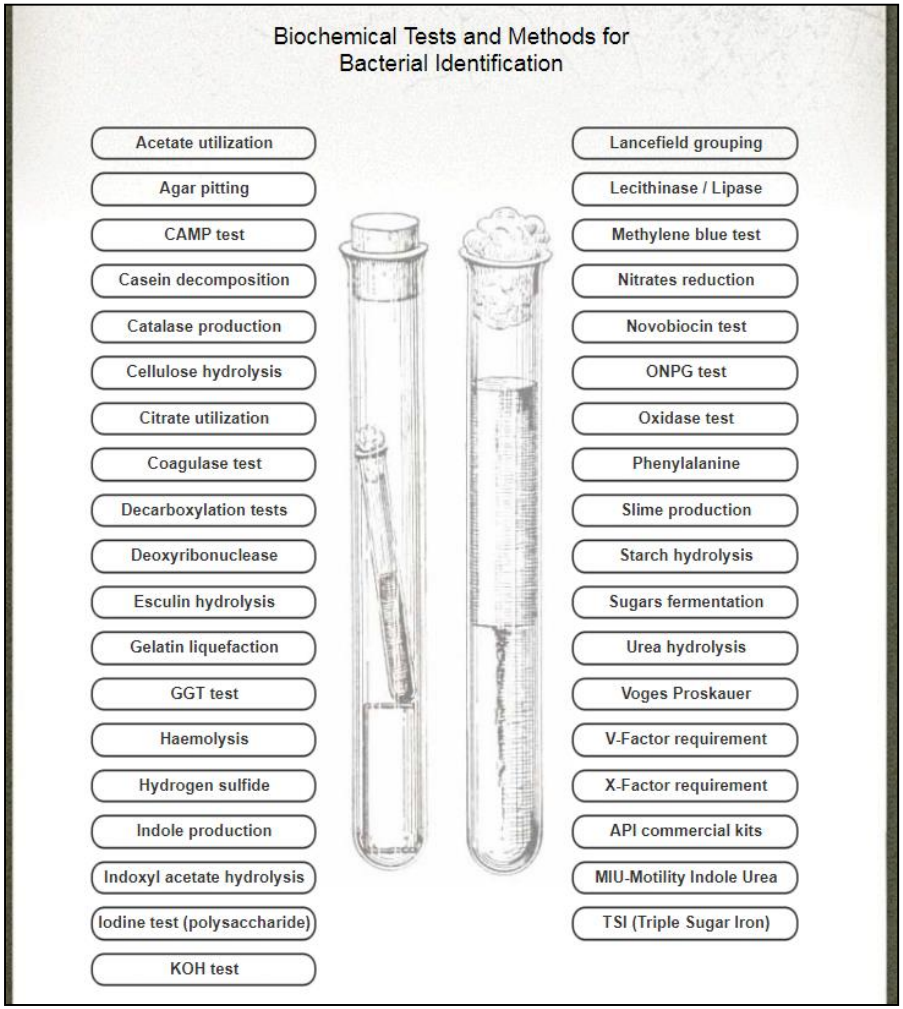
An Advanced Bacterial Identification Software

- ABIS online is a laboratory tool for bacterial identification, based on morpho-biochemical characters, cultural characteristics, growth conditions, ecology & pathogenicity data.
- The program allows a great flexibility in choosing biochemical tests and is an alternative to commercial systems or code-books.
- The software is linked with a germs Encyclopedia and an antibiogram interpreter.
- Main identifiable categories: Enterobacteriaceae, Pasteurellaceae, Campylobacteraceae, Bacillaceae, Staphylococus, Streptococcus, Aeromonas, Pseudomonas and related species.

ABIS online

Automated Biometric Identification System

Biochemical tests and methods for bacterial identification



ABIS online

Automated Biometric Identification System

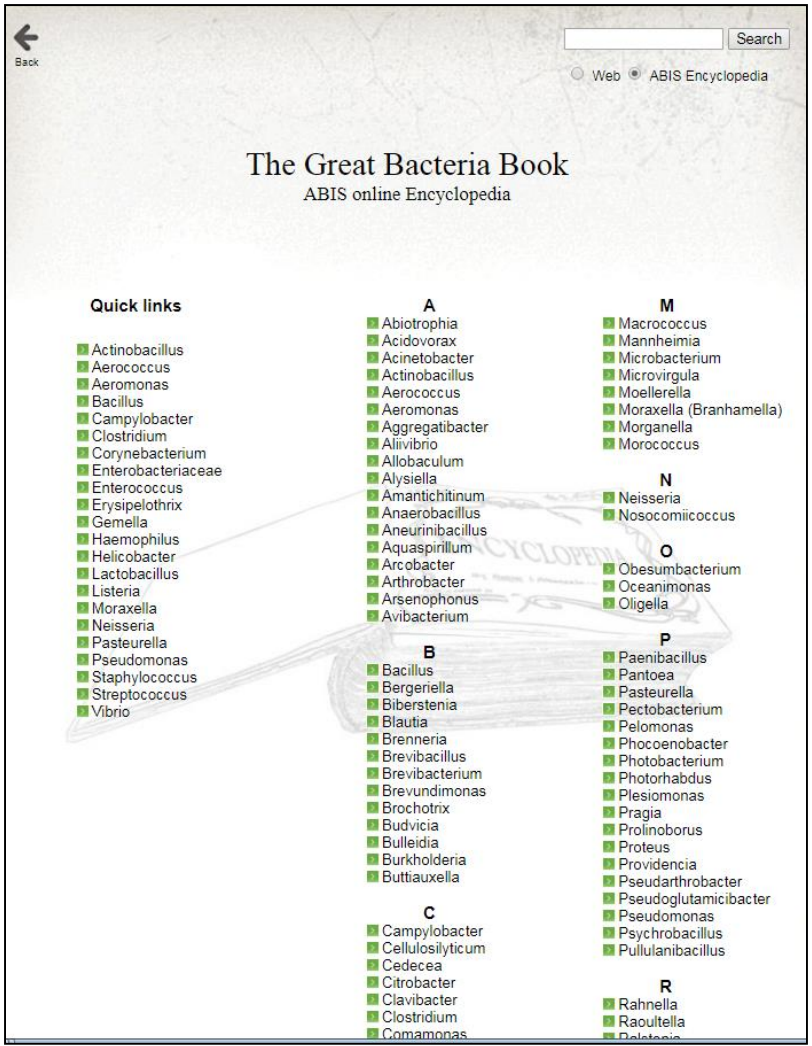
Library

- More than 700 species are included in databases and more than 800 species in encyclopedia.
- A beta version of 16S RNA gene sequence identification is also available from the main menu.
- Although we have optimized the program for Android operating system, some minor graphical issues may appear on low screen resolution devices.
- Bacteria identification (version 1118532) has a file size of 112.20 MB and is available for download from our website.

ABIS online

Automated Biometric Identification System

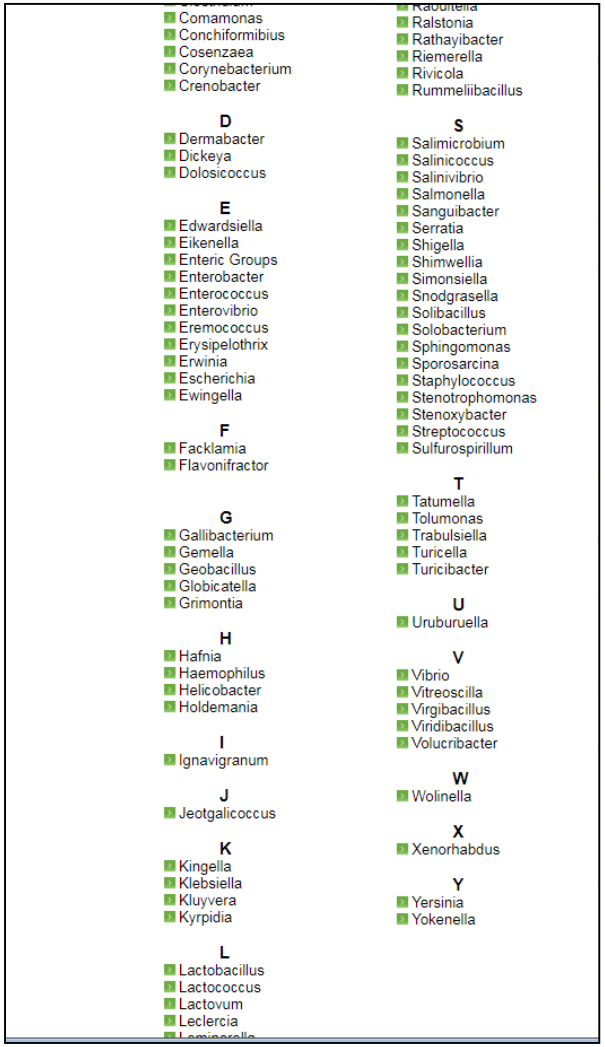
Library



ABIS online

Automated Biometric Identification System

Library



ABIS online

Automated Biometric Identification System

First enter the bacterial name e.g. *Bacillus subtilis* in the check box

ABIS online

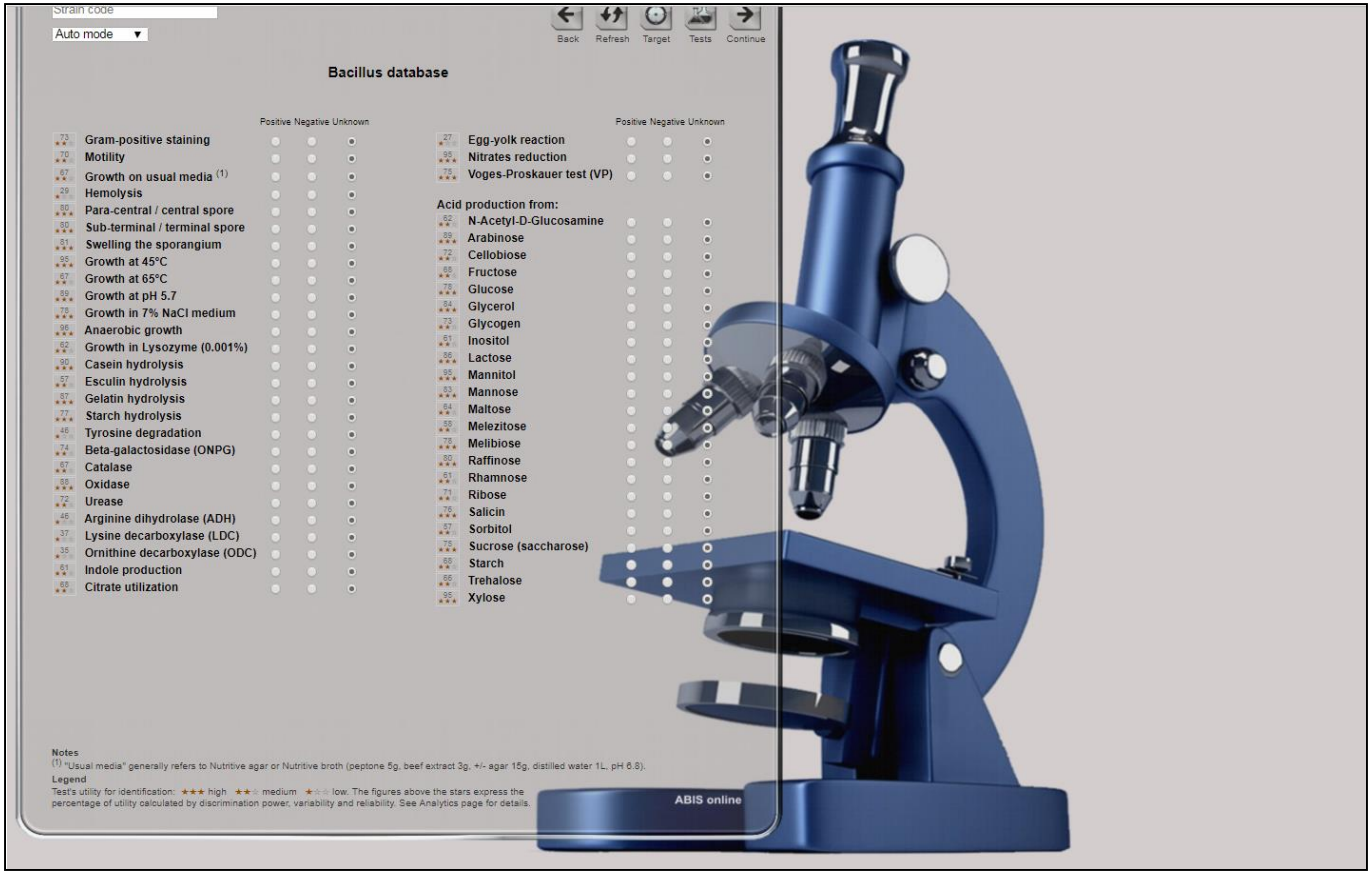
Automated Biometric Identification System

Follow the family/species names

ABIS online

Automated Biometric Identification System

Find the characteristics of Bacillus spp.



ABIS online

Automated Biometric Identification System

Validation

- Many identification systems (API, Biolog, etc.) become available in recent years, from commercial identification kits, to online software. Each of these systems has its limitations.
- To tackle these problems we have created ABIS online, a laboratory tool for bacterial identification, based on morphology, biochemical characters, cultural characteristics, ecology and pathogenicity data.
- The aim of the study was to present and validate ABIS software by comparison to a commercially available identification system (API strips and apiweb™ bioMerieux software), testing 16 reference strains and 123 wild isolates.
- APIWEB™(API databases) confirmed ABIS's results with a high percentage, showing a very good taxa identification by ABIS software.

Costin and Ionut,2017

ABIS online

Automated Biometric Identification System

Comparative reference strains identification between ABIS online and apiweb™ software

#	Strain name and code	ABIS results id %[a]	apiweb™ results id %[b]
1	*Staphylococcus aureus* ATCC 25293	*Staphylococcus aureus* subsp. *aureus* 99%	*Staphylococcus aureus* 97.8%
2	*Helicobacter pylori* ATCC 43504	*Helicobacter pylori* 94%	*Helicobacter pylori* 99.9%
3	*Bacillus thuringiensis* NCIMB 9134	*Bacillus thuringiensis* 93%	*Bacillus cereus*, possibility of *B. thuringiensis* 98.9%
10	*Escherichia coli* ATCC 8739	*Escherichia coli* 98%	*Escherichia coli* 99.9%
14	*Pseudomonas aeruginosa* ATCC	*Pseudomonas aeruginosa* 92%	*Pseudomonas aeruginosa* 98.1%
16	*Lactobacillus paracasei* CCM 1837	*Lactobacillus paracasei* subsp. *paracasei* 94%	*Lactobacillus paracasei* subsp. *paracasei* 1 / 3 48.0 / 51.0%

Legend: [a]- ABIS %id represents the percentage of similarity with taxa from the database, all databases containing a matrix where probabilistic incidence values are allocated for every taxon and their corresponding morpho-biochemical characters; [b]- apiweb™ %id is a probabilistic calculation using bioMerieux own system procedure.

Costin and Ionut,2017

ABIS online

Automated Biometric Identification System

Comparative reference strains identification between ABIS online and apiweb™ software

- Apiweb™ confirmed ABIS's results with a high id%, proving very good taxa identification by ABIS program.
- ABIS online (ABIS online - bacterial identification software version 12, http://www.tgw1916.net) is capable of correct phenotypical species identification, generates reliable results, is a powerful tool for microbiology lab and the Encyclopedia connection provides essential information about the ecological significance, pathology and other features of the identified strains.

Costin and Ionut,2017

ABIS online

Request for adequate identification

From a researcher

- I am having a bit of trouble identifying an unknown organism. any help would be appreciated!
- The following describes my organisms and the tests I have run:
  - Gram Negative
  - Coccobaccilli
  - Motility: nonmotile (subjective but almost certain)
  - FTM: facultative anarobe
  - Citrate+
  - Lysine+
  - Glucose+
  - Mannitol -
  - Lactose -
  - Sucrose -
  - Maltose -
  - Methyl red+
  - VP-
  - H2S (KIA)-
  - Indole -
  - Urea -
  - Litmus milk: purple (confirms nonlactose fermenter)
  - Gel-
  - Nitrate+:
  - Catalase+
  - Oxidase+/- (uncertain)
- I thought it might have been shigella, but shigella is both citrate AND lysine negative, which clearly conflict. Any help from experienced microbiologists (or anyone with knowledge of these) would be appreciated!

ABIS online

Requests for adequate identification

A recommendation from a reader on Research Gate

- Putting your biochemical results into PIBWIN software your organism is identified as *Escherichia blatae*. But some more biochemical tests will be required for accurate identification.
- You can try on your own with this software (freely available in net) also you can put all your results into another online identification software named ABIS online.
- I think this will solve your purpose.

Translations for "bacterium"

Alternative meanings/domain in parentheses

Afrikaan	**bakterie**. (various references)
Albanian	**bakter** (bacteria). (various references)
Arabic	جرثوم (bug). (various references)
Bulgarian	**бактерия**. (various references)
Chinese	**细菌** (Bacteria, Bacterial, bacterially). (various references)
Czech	**baktérie** (bacteria). (various references)
Danish	**bakterie**. (various references)
Dutch	**bacterie**. (various references)
Esperanto	**bakterio**. (various references)
Finnish	**bakteeri** (bacteria). (various references)
French	**bactérie** (bacteria). (various references)
Frisian	**baktearje**. (various references)
German	**bakterie** (germ), **Spaltpilz**. (various references)
Greek	**βακτηρίδιο, βακτήριο** (bacillus). (various references)
Hebrew	בקטריע (bacteria, microorganism). (various references)

Translations for "bacterium"

Alternative meanings/domain in parentheses

Hungarian	**baktérium** (bacteria, germ, microbe, wog). (various references)
Italian	**batterio** (prokaryotic). (various references)
Japanese Kanji	**細菌** (bacillus, germ). (various references)
Japanese Katakana	**きア** (a little, bacillus, ban, be equal to, be fit for, bedding, cloth, germ, gold, gold general, napkin, prohibition, quilt, serve, small quantity, unit of weight ~600g), **さいきア** (bacillus, germ, latest, most recent, nowadays, reappointment, slight flaw). (various references)
Korean	**박테리아** (Bacteria). (various references)
Manx	**bacteyr**. (various references)
Papiamen	**bakteria**. (various references)
Pig Latin	**acteriumbay**.(various references)
Portuguese	**bactéria**. (various references)
Russian	**бактерия** (bacteria). (various references)
Spanish	**bacteria** (bacteria, germ). (various references)
Swedish	**bakterie** (bacteria, bug, germ, microbe). (various references)
Turkish	**bakteri** (bacterial, germ), **bakterí** (germ, microbe). (various references)
Ukranian	**бактерія**. (various references)
Persian	**باکتری (Bacterium)**

General terms and abbreviations

- **DEPC:** Diethylpyrocarbonate (DEPC) treatment is the most commonly used method for eliminating RNase contamination from water, buffers, and other solutions.
- **Electropherogram:** is a graphical representation of data received from a sequencing machine and is also known as a trace.
- **Generic primers:** This option will design standard PCR primers according to the region input options you select. These options allow you to specify what part of a sequence you wish to amplify.
- **Prions:** Proteins that multiply themselves. An infectious agent smaller than a virus, composed primarily of protein. e.g. Mad Cow disease,…

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