Technical Manual no. 1

TECHNIQUES FOR DIAGNOSIS OF

Pseudomonas solanacearum

AND FOR RESISTANCE SCREENING AGAINST

groundnut bacterial wilt

International Crops Research Institute for the Semi-Arid Tropics

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Abstract Bacterial wilt, caused by *Pseudomonas solanacearum*, is a widespread and destructive disease of such economically important crops as banana, eggplant, groundnut, pepper, potato, tobacco, and tomato, in the tropics, subtropics, and warm temperate regions. The pathogen has a wide host range and an exceptional ability to survive in the roots of nonhost plants and in the soil.

This manual presents techniques for detection, isolation, and identification of all variants of the wilt pathogen. The use of enzyme-linked immunosorbent assay (ELISA) to detect the bacterium in plant tissues, seed, and soil is emphasized. DNA-based diagnostics for *P. solanacearum* are described, as are an infectivity titration technique to determine the virulence of the wilt pathogen, and several inoculation techniques used in evaluating host-plant resistance to groundnut bacterial wilt.

摘　要　由 *Pseudomonas solanacearum* 引起的细菌性枯萎病是热带、亚热带及温带地区一些具有重要经济意义的作物如香蕉、茄子、花生、辣椒、马铃薯、烟草、番茄的一种分布广泛的毁灭性病害。该病原菌有很宽的寄主范围，并且在非寄主植物的根内和土壤中有很强的存活能力。

这一手册介绍了青枯病的检测、分离和鉴定技术。重点阐述了检测植物组织、种子和土壤中细菌的酶联免疫(ELISA)技术，描述了青枯菌的核酸诊断方法。对于能确定青枯菌毒性水平的侵染滴定以及评价花生青枯病抗性的几种接种方法也作了阐述。

Résumé *Techniques de diagnostic de* Pseudomonas solanacearum, *et de criblage pour la résistance contre le flétrissement bactérien de l'arachide.* Le flétrissement bactérien de l'arachide, causé par *Pseudomonas solanacearum*, est une maladie très répandue des cultures de valeur économique telles le banane, l'aubergine, l'arachide, le poivre, la pomme de terre, le tabac et la tomate, dans les tropiques, les sous-tropiques et dans les régions chaudes tempérées. L'agent pathogène se trouve sur plusieurs hôtes et possède une capacité exceptionnelle à survivre dans les racines des plantes non-hôtes et dans le sol.

Ce manuel présente les techniques permettant la détection, l'isolement et l'identification de toutes les variantes de l'agent pathogène du flétrissement. Une attention particulière est portée à l'emploi du test immunoenzymatique ELISA pour la détection de la bactérie dans les tissus végétaux, les graines et le sol. Le diagnostic à base de l'ADN pour *P. solanacearum* est exposée, ainsi qu'une technique de titration pour déterminer la virulence de l'agent pathogène du flétrissement et plusieurs techniques d'inoculation utilisées dans l'évaluation de la résistance au flétrissement bactérien de l'arachide.

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EDITED BY

V K Mehan and D McDonald

ICRISAT

International Crops Research Institute for the Semi-Arid Tropics
Patancheru 502 324, Andhra Pradesh, India

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Contributors

R Black, **A Sweetmore** — Natural Resources Institute (NRI), Central Avenue, Chatham Maritime, Kent ME4 4TB, UK.

V K Mehan — Crop Protection Division, ICRISAT Asia Center, Patancheru 502 324, Andhra Pradesh, India.

D McDonald — 'Dunslair', Glentress by Peebles, Borders EH 45 8 NB, Scotland, UK.

A C Hayward — Department of Microbiology, The University of Queensland, Brisbane 4072, Queensland, Australia.

A Robinson-Smith — Institute of Arable Crops Research (IACR), Rothamsted Experimental Station, Harpenden, Herts, AL5 2JQ, UK.

L Y He, **Y L Xie** — Institute of Plant Protection, Chinese Academy of Agricultural Sciences (CAAS), Beijing 100094, China.

S Seal — Natural Resources Institute (NRI), Central Avenue, Chatham Maritime, Kent ME4 4TB, UK.

K Y Lum — Malaysian Agricultural Research and Development Institute (MARDI), PO Box 12301, 50774 Kuala Lumpur, Malaysia.

Y J Tan, **B S Liao** — Oil Crops Research Institute (OCRI), Wuhan, Hubei 430 062, China.

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Dr Kang Yaowei of the Institute of Plant Protection, CAAS, China, provided the photograph of virulent and avirulent *Pseudomonas solanacearum* colonies on page 24.

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Foreword

Bacterial wilt is a serious constraint to groundnut production in East and Southeast Asian countries, and in response to the perceived need for international cooperation to address this problem, a Groundnut Bacterial Wilt Working Group was formed in 1990 to coordinate research efforts by various international and national research institutions. At the second meeting of the Working Group in Taiwan in 1992, it was recommended that in-country training courses should be organized to facilitate technology exchange. Accordingly, a training course on 'Techniques for diagnosis of *Pseudomonas solanacearum*, and for resistance screening against groundnut bacterial wilt' was held in Wuhan, China, in July 1994. The Faculty for this course included experts drawn from Australia, China, Malaysia, and UK, and from ICRISAT. Their lecture notes, and additional information provided by them, formed the basis of this manual.

I compliment the editors and authors for their efforts in bringing out this manual and hope it will be useful to researchers and students working on the bacterial wilt pathogen and will facilitate research efforts towards solving the bacterial wilt problems of groundnut and the many other crops affected by *Pseudomonas solanacearum*.

J G Ryan
Director General
ICRISAT

Introduction

Bacterial wilt, caused by *Pseudomonas solanacearum*, one of the most important plant diseases of bacterial origin, is widespread and destructive in the tropics, subtropics, and warm temperate regions of the world. The disease limits production of such economically important crops as banana, eggplant, groundnut, pepper, potato, tobacco, and tomato.

The pathogen comprises different strains/races, has a wide host range, and has exceptional ability to survive in the roots of nonhost plants and in the soil. Five races have been described, which differ in host ranges, geographic distribution, and ability to survive under different environmental conditions. The bacterium is also divided into five biovars based on their ability to oxidize/utilize carbohydrates. Pathotypes of the groundnut bacterial wilt pathogen have also been reported.

In this manual, techniques for detection, isolation, and identification of all variants of the wilt pathogen are presented, and their underlying principles explained. While several of these techniques have been in use for some time without major modifications, others are of relatively recent origin. Emphasis has been placed on the use of enzyme-linked immunosorbent assay (ELISA) to detect the bacterium in plant tissues, in seed, and in soil. The principles underlying the major forms of ELISA are outlined, as also are the protocols for the production of polyclonal antibodies, and for ELISA. DNA-based diagnostics for *P. solanacearum* are also described. An infectivity titration technique to determine the virulence of the wilt pathogen is included, and several inoculation techniques used in evaluating host-plant resistance to groundnut bacterial wilt are discussed.

It is hoped that this manual will be useful to researchers in the detection of *P. solanacearum* and its biovars and pathotypes, and in screening germplasm of groundnut and other plant species for resistance to bacterial wilt.

V K Mehan and **D McDonald**

An Integrated System for the Identification of Bacteria

R Black and A Sweetmore

The BACTID System: Introduction and Background

Bradbury (1970) commented on the general neglect of bacteriology in plant pathology laboratories. This applies especially to laboratories functioning as diagnostic and advisory services ('plant clinics') in developing countries. According to Bradbury, the major problem is lack of knowledge rather than practical difficulties encountered. He proposes a key to simplifying the isolation and preliminary study of bacteria from plants. Certainly, lack of training in plant bacteriology results in a large number of submissions to identification services of saprophytes and other non-pathogens. Another difficulty is the wide range of media and reagents required for definitive identification of species of plant pathogens, in spite of the small number of species commonly encountered. This is a consequence of the need to define bacterial taxa on the basis of biochemical properties. With limited budgets and scarce foreign exchange, the necessary chemicals could be hard to procure. Some reagents have to be procured in quantities far in excess of what is used, so that most may sit around on the shelf, often unopened, beyond their expiry date.

The publication of *Methods for the Diagnosis of Bacterial Diseases of Plants* by Lelliott and Stead (1987) was a considerable achievement, and provided a definitive treatment of bacterial plant pathogens from a practical standpoint. The book stresses the importance of good isolation techniques and of symptomology, with the need to avoid spending time on saprophytes incorrectly assumed to be significant. It presents the concepts of presumptive and confirmed diagnosis as full identification is not always necessary.

However, Lelliott and Stead (1987) emphasize that diagnosis and identification of plant diseases in temperate regions is resource-intensive. The BACTID system presented here is essentially designed for diagnosis of plant diseases of the tropics and subtropics, typically in plant clinics, bearing in mind the resources likely to be available; the level of identification required for pest management decisions; and the need to eliminate saprophytes while ending with isolates for reliable and significant identification of pathogens.

BACTID aims to provide enough initial information to permit decisions in routine pest management advisory work. Further identification work could be done, if necessary. In the methods presented here, the BACTID systems for preliminary identification are combined with metabolic profiling for more detailed identification and characterization.

BACTID takes a standardized approach with a set of media and reagents for routine use. Kits can be used rather than conventionally prepared media. For more thorough identification, other tests can be included for specific organisms which are prevalent, or are the target of specific research projects (e.g., biovar tests for *Pseudomonas solanacearum*).

Level of identification required for diagnosis

Lelliott and Stead (1987) use the concept of presumptive diagnosis to determine the identification requirements; diagnosis should be sufficiently rigorous to enable good advice after a few days. However, the requirements for presumptive diagnosis vary greatly from species to species; depending on location, crops grown, and prevalent diseases, it may never be necessary to use some of these tests.

Full identification—This is done either by completing the classical array of tests, by modern methods of profiling (fatty acids, proteins, metabolism of many substrates), serological or molecular methods [restriction fragment length polymorphism (RFLP) probes, polymerase chain reaction (PCR)].

Confirmed diagnosis—These include host tests to establish pathogenicity, and are obviously time consuming.

The BACTID Scheme

The BACTID scheme for preliminary identification of plant pathogenic bacteria provides rapid processing to a level indicating likely significance, and eliminates saprophytes/nonpathogens along the way. Originally inspired by Bradbury's (1970) scheme, it is reoriented to suit the main species encountered in the tropics, with some additional tests for further differentiation. The original scheme was developed as a flow chart (Fig. 1) rather than as a key.

In the BACTID scheme, a standardized approach to preliminary identification is used. Some of the tests (or combinations of tests) could be considered as identifying certain genera. Other tests are used to approach presumptive diagnosis.

Starting point

It is essential to start with a pure culture of an isolate showing uniform and, preferably, recognizable characteristics of an organism (e.g., yellow, domed, mucoid colonies for *Xanthomonas*).

Principles of differentiation and tests employed

Colony color—*Xanthomonas* spp are mostly yellow, but so is *Erwinia herbicola*, a common saprophyte. Pathogenic fluorescent and nonfluorescent pseudomonads are not yellow.

Gram reaction and microscopic examination—This is fundamental to the identification of bacteria. Most Gram-positive isolates are likely to be nonpathogenic unless there are very specific host indications (e.g., *Clavibacter*). Microscopic examination reveals morphological characteristics for further identification.

Oxidase, nitrate, and tetrazolium chloride (TTC) tests—Results can be used later for differentiation, but these tests should be done routinely in the beginning, to save time, and using fresh cultures.

Blue-green fluorescence for Gram-negative isolates—Fluorescent pseudomonads. LOPAT tests to differentiate pathogenic and nonpathogenic species.

Nonfluorescent Gram-negative—Differentiate by oxidation/fermentation (O/F) test, then soft rot (potato rot test) as appropriate. *Alcaligenes* and some other nonpathogens (including *Pseudomonas*) may be non-acid-producing aerobically (in O/F test).

Additional tests—Additional tests could be done to confirm *Xanthomonas* and nonfluorescent white pseudomonads. *Agrobacterium* can

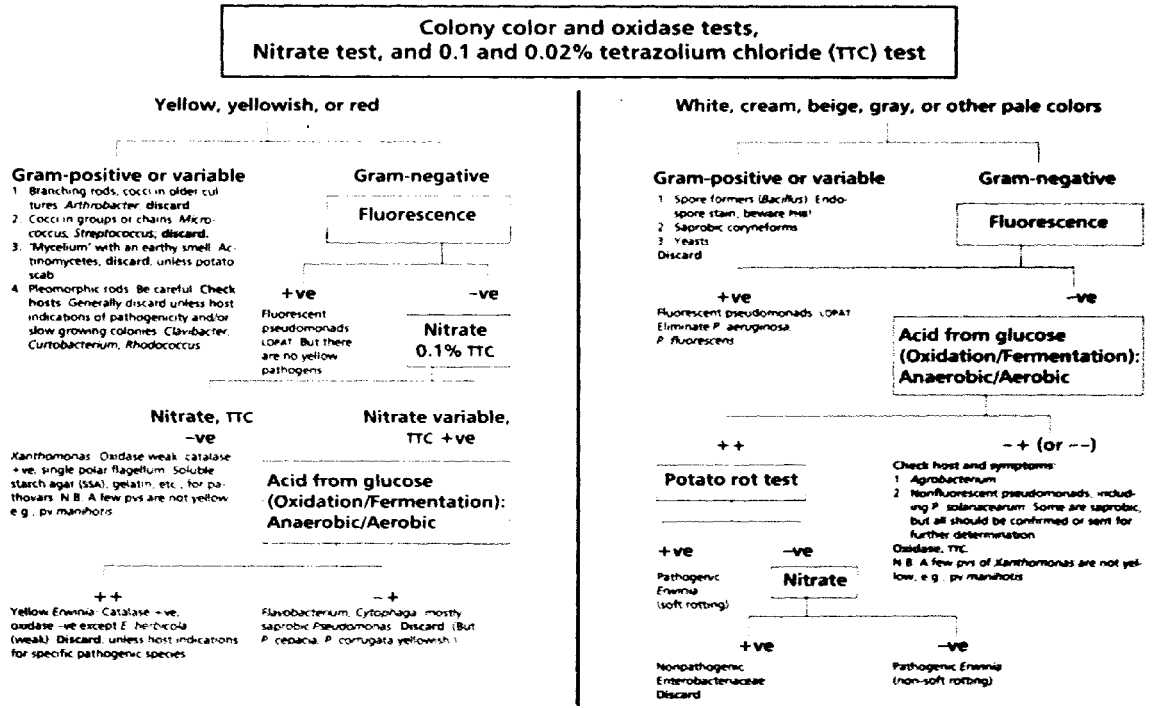

Figure 1—Flow chart of scheme for preliminary identification of plant pathogenic bacteria in the tropics, emphasizing early elimination of nonpathogens. (Originally devised by senior author and J Kolkowsky of International Mycological Institute.)

be distinguished in the first instance from potentially pathogenic non-fluorescent *Pseudomonas* by symptoms, but further biochemical tests can be done for confirmation.

Caution—The results must be related back to the host/symptom data and isolation plates. If a bacterium has been consistently isolated and comes out as 'saprophyte', but there are no other likely candidates, it should be investigated further: there is always the possibility of rare, obscure or new diseases caused by Gram-positive bacteria or by species of Enterobacteriaceae.

Using the BACTID Scheme

Using conventional media and commercial kits—These tests can be set up using conventional media and the scheme illustrated in the flow chart. Commercially available kits such as the API 20NE® strip (Bio Mérieux) or the Roche Enterotube® (Becton Dickinson), which provide some of the important tests in a convenient ready-made format, can also be used. These are expensive, hence the BACTID kits described below.

BACTID kits—These have been designed as do-it-yourself kits of all necessary tests for preliminary identification. Besides the tests for using the BACTID scheme itself (colony color, Gram reaction, tetrazolium chloride, oxidase, fluorescence, nitrate, oxidation/fermentation, and potato rot tests) there are some additional tests to confirm the identification (catalase, gelatin hydrolysis, starch hydrolysis). The BACTID Training Kit, based on a 96-well microtiter plate, allows up to twelve isolates to be tested at one time. In the BACTID Tube Kit, each test is provided conveniently in an Eppendorf tube (or equivalent); there are also more tests for *Pseudomonas* spp, especially *P. solanacearum*. The above tests are described in Tables 1 (preliminary identification) and 2 (additional tests for *Pseudomonas*).

BACTID software—The BACTID Scheme has been developed into a computer program (of the type called an expert system). After entering the test results, preliminary identification of the isolate is made according to the scheme. The BACTID program also incorporates information on the bacteria (such as confirmatory tests), full details of the media and reagents used, and instructions to prepare the BACTID Training Kit. Further details are given under the heading 'BACTID Software' in this chapter.

BACTID Training Kit

The BACTID Training Kit consists of a 96-well microtiter plate containing the media for eight tests arranged in rows. The wells then act as minute petri dishes which can mostly be inoculated and read in the normal way. By preparing in advance, the kit will be ready for use as soon as bacteria need to be identified. The plate can be stored in a refrigerator for at least 6 months if properly sealed, and each plate allows 12 cultures to be inoculated (as columns). This overcomes one of the main problems of bacteriology in small plant clinics which do not deal with bacteriology regularly.

Gram reaction, catalase, and oxidase tests are done with loopfuls of cultures grown on a nutrient agar (NA) slope in Eppendorf tubes. The oxidation/fermentation (O/F) test is included as paired tubes of the usual medium, one for aerobic and the other for anaerobic conditions. At least one column should be reserved as noninoculated control. The layout of the kit is shown in Figures 2 and 3.

Table 1—BACTID kits (Training Kit and Tube Kit): media and tests for preliminary identification.

Medium/Test	Observation	Training Kit[1]	Tube Kit
Nutrient agar (NA) (catalase test)	Add a drop of 3% hydrogen peroxide. Bubbling indicates positive reaction.	Well[2]	Slope
Tetrazolium chloride (TTC) (tolerance test; 0.1, 0.02%)	Bright red smear indicates tolerance to TTC	Well	Slope
King's medium B (KMB) (fluorescence test)	Blue-green fluorescence under ultra violet (UV) light, or in bright sunlight is positive.	Well	Slope
Sucrose nutrient agar (SNA) (levan test)	Thick, creamy, mucilaginous growth indicates levan-positive strain.	Well	Slope
Soluble starch agar (SSA) (starch hydrolysis test)	Add iodine. Clear zones in the blue iodine reaction indicate starch hydrolysis.	Well	Slope
Nitrate reduction test	One drop of each of the nitrate test reagents should be added in the usual way. Alternatively, nitrate/nitrite test strips (Merckoquant 10 020 or 10 050) could be used.	Well (stab)	Slope
Potato rot test	Positive strains will break down the plug of freeze-dried potato.	Well (stab)	Stab
Nutrient agar (NA) (color, Gram reaction)	Observe color on the NA slope. Use a loop to remove culture from the slope for the Gram reaction. (The 3% potassium hydroxide solubility test is recommended).	Slope[2]	Slope
NA for oxidase test	Use oxidase touch sticks on NA slope. A deep purple coloration within 30 seconds indicates a positive result.	Slope	Slope
Gelatin hydrolysis test	Gelatin-positive strains will liquefy the medium. Compare with noninoculated control and/or a known positive.	Tube/stab	Stab
Oxidation/fermentation test	After stab inoculation, the anaerobic tube is closed tight. Change from blue to yellow indicates acid production. Some bacteria may not metabolize glucose (aerobic tube positive).	Tube/stab	Stab

1 At least one column should be a noninoculated control. If all 12 columns of the plate are seldom used, use of the Tube Kit is preferable.

2 Smear the surface of the medium in well or tube, unless otherwise indicated.

Table 2—Additional media and tests for identification of Pseudomonas spp (especially P. solanacearum): BACTID Tube Kit only.

Medium/Test	Observation
Sucrose nutrient agar/Nile blue	Production of polyhydroxybutyric acid (and therefore RNA Group II pseudomonads) is indicated by strong fluorescence under UV light.
2% sodium chloride/salt tolerance	Look for growth. *Pseudomonas solanacearum* does not tolerate 2% sodium chloride (NaCl).
Biovar substrates (plus glucose as control)	Observe acid production on maltose, lactose, cellobiose, mannitol, sorbitol, and dulcitol. The differential patterns of substrate use indicate the biovar.
Nitrate medium in sealed tube/Gas from nitrate	Biovars 3, 4, 5 usually produce (nitrogen) gas profusely from nitrate. Biovar 1 reduces nitrate to nitrite but not to gas. Most biovar 2 isolates do not reduce nitrate.

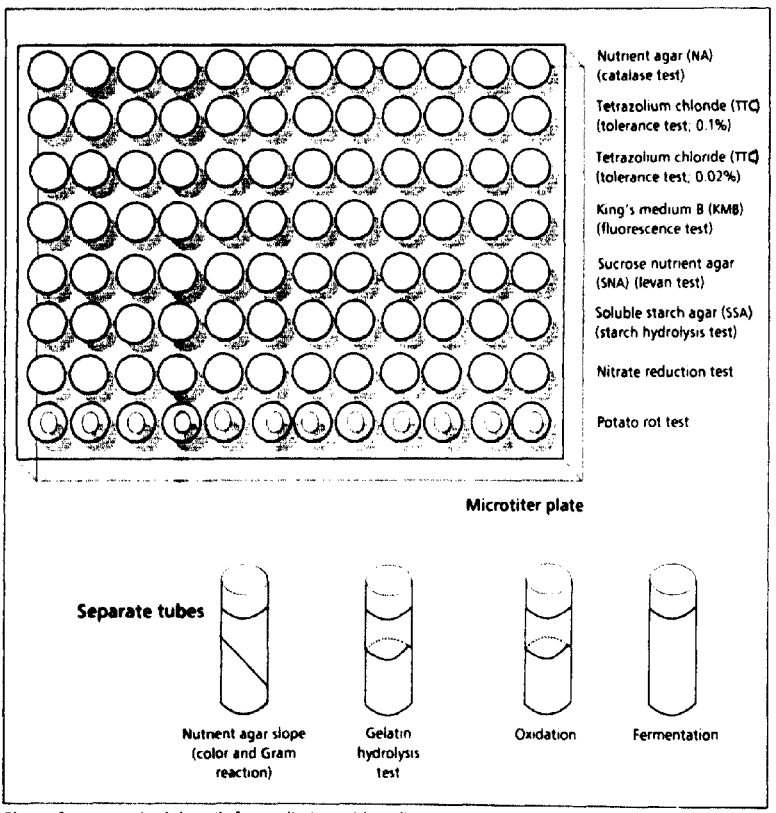

Figure 2—BACTID Training Kit for preliminary identification.

BACTID Training Kit

Template for Bacteriological Identification

Date inoculated _____ Dates read _____

Main plate			1	2	3	4	5	6	7	8	9	10	11	12
Isolate details														
A	Nutrient agar (NA) (catalase)	Smear												
B	Tetrazolium chloride (TTC) (tolerance; 0.1%)	Smear												
C	Tetrazolium chloride (TTC) (tolerance; 0.02%)	Smear												
D	King's medium B (KMB) (fluorescence)	Smear												
E	Sucrose nutrient agar (SNA) (levan)	Smear												
F	Soluble starch agar (SSA) (starch hydrolysis)	Smear												
G	Nitrate reduction	Stab												
H	Potato rot	Stab												
Separate tubes	Nutrient agar (NA) slope for: Color and Gram reaction	Streak												
	Oxidase (touch sticks)													
	Gelatin hydrolysis	Stab												
	Oxidation/fermentation	Streak												

Figure 3—Proforma for the BACTID Training Kit.

Preparing and inoculating the BACTID plate

Use sterile microtiter plates. Most of the media are prepared in the usual way. Molten media are poured into the well so that a 2-mm space is left at the top. This works out to approximately 200 µL well^{-1}. After preparation, the plates are sealed (using a bag sealer if available) and stored in a refrigerator. Slopes of nutrient agar (NA) prepared in plastic tubes are inoculated for observation of color and Gram reaction, catalase, and oxidase tests.

Inoculation—The wells for nitrate and potato rot tests, and the gelatin and oxidation/fermentation tubes are stab inoculated. The NA slope is streaked. The remaining wells are inoculated by smearing the surface (see Fig. 3).

Tetrazolium chloride (TTC) salt tolerance tests—Use a clean loop to avoid carrying the TTC reagent on to other media.

Oxidation/fermentation (O/F) test—This test is used to indicate whether a particular carbohydrate (usually glucose) is utilized oxidatively or fermentatively by the test bacterium. Carbohydrate utilization resulting in acid formation is determined under aerobic and anaerobic conditions.

Pairs of plastic tubes of nutrient agar are used since the wells are too small to avoid diffusion of oxygen. For the anaerobic test, the tube is filled right to the top, and the cap sealed. For the aerobic test, the tube should be three-quarters full. After stab inoculation, the tube for the anaerobic test should again be sealed.

Potato rot test—Cores of potato tissue (5 mm diameter) are cut aseptically with a cork borer, and 8-mm-thick disks are cut out. The disks can be kept at -10°C in bulk (in a deep freeze, preferably after freeze drying) and added to the plates when they are prepared. Immediately before inoculation, 150 µL of sterile water is added to each well. The potato disks are then stab inoculated. (The disks may discolor during storage or after adding water; this does not matter).

Freeze-dried potato disks will keep indefinitely when the prepared plates are stored in a refrigerator. If a freeze drier is not available, store potato cores in a freezer until the plates are used. Otherwise, prepare potato cores aseptically immediately before use.

Options for portability and convenience

Reagents—Oxidase touch strips (Unipath); nitrate/nitrite test strips (Merck/BDH).

Equipment—Disposable sterile plastic loops (suitable for the catalase test and to use Kovacs' reagent in conventional form).

BACTID Tube Kit

In the BACTID Tube Kit, each test is provided conveniently in an Eppendorf tube (or equivalent). This has advantages when it is not necessary to use a whole plate:

- when not all the tests need to be done to confirm a particular group of bacteria. For example, with *Xanthomonas* it would not be necessary to do the O/F and KMB tests for confirmation;
- when less than 12 isolates, replicates, and controls require testing;
- Eppendorf tubes could be re-autoclaved and used again.

In addition, some tests have been added for further identification of nonfluorescent *Pseudomonas* spp classified in RNA homology group II (Palleroni et al. 1973), called the genus *Burkholderia* by some authors. Production of polyhydroxybutyric acid (PHB), which distinguishes this group from other nonfluorescent *Pseudomonas* spp is tested for in BACTID by an SNA Nile blue medium; PHB is visualized by UV light. One member of this group which is of particular importance, *P. solanacearum*, can be distinguished from the other by the absence of growth in 2% sodium chloride (NaCl). The format of the kit is shown in Figures 4 and 5.

Preparing the Tube Kit for preliminary identification

All the media are prepared in 1.5-mL microcentrifuge tubes (Eppendorf type). The media for preliminary identification (following the BACTID Scheme, Table 1) are mostly prepared, with about 0.75 mL medium in each tube. The exception is the anaerobic tube of the O/F pair, which is filled up to the top. The tubes are inoculated, and then used in the same way as wells or tubes in the BACTID Training Kit.

Additional tests for *Pseudomonas*

SNA-Nile blue medium—Nile blue staining is the most reliable test for the presence of PHB granules. This can be used as a test for *P. solanacearum* and similar species. Normally, bacterial cultures are grown on a medium promoting PHB production (e.g., SNA or medium containing β-hydroxybutyric acid), from which a smear is prepared, stained with Nile blue and then examined under the microscope with green fluorescence for orange PHB granules. However, PHB can also be tested for in BACTID using a one-step test without microscopy: bacteria are inoculated onto slopes of SNA medium supplemented with 0.001% Nile blue in microcentrifuge tubes. After incubation, the tubes are examined under UV light (e.g., bacteriological UV light) with a gel-scanner, or in a thin-layer chromatography plate visualizer. PHB-positive isolates fluoresce brightly; there may be very weak fluorescence in non-PHB isolates and controls. This medium was developed by Quiroz Salazar (1994); it is similar to that of Pierce and Schroth (1994).

Growth in 2% sodium chloride—Sodium chloride (NaCl) is added to nutrient broth at the required concentration before autoclaving in microcentrifuge tubes for the BACTID Tube Kit. Cloudiness after several days incubation indicates tolerance of NaCl. Ordinary nutrient broth should be used as a control for each culture.

Distinguishing biovars of P. solanacearum—The media and tests described by Hayward (1964) and by Hayward et al. (1990) can be used in this format. They include tests for utilization of disaccharides and sugar alcohols to distinguish the five biovars, and the gas from the nitrate test to distinguish biovars 3, 4, and 5 from biovars 1 and 2. The media are also described by Hayward in this publication.

BACTID software

The BACTID program will be published in 1995/96 by the Natural Resources Institute (NRI), UK, as part of a package of integrated systems for bacterial identification. An interim version is available from the senior author for evaluation. BACTID is an expert system: information on the bacterial groups and test methods is incorporated in it as hypertext.

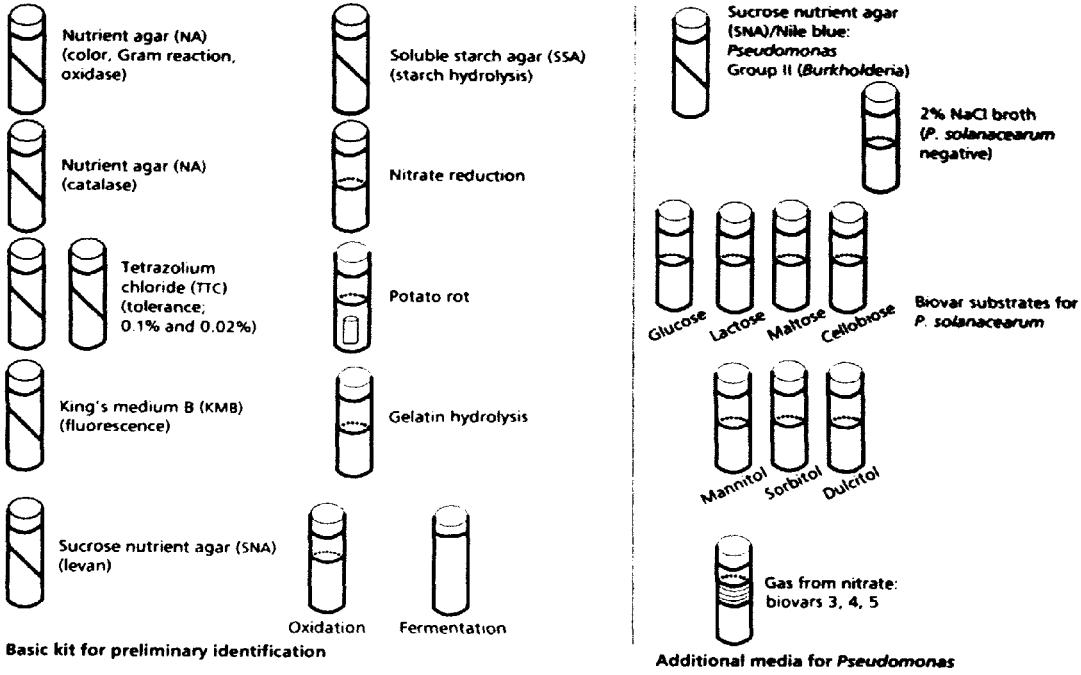

Figure 4—BACTID Tube Kit: media for the basic scheme and further identification.

BACTID Tube Kit

Template for Bacteriological Identification

Isolate details .....

Main system (Main bacterial groups)						**Nonfluorescent Pseudomonads/ *Burkholderia/Pseudomonas solanacearum***					
Date inoculated		Date(s) read				Date inoculated		Date(s) read			
Nutrient agar (color and Gram reaction)	Streak										
						Sucrose nutrient agar/ Nile blue	Streak				
Oxidase (touch sticks)	Streak					2% sodium chloride	Stab				
Nutrient agar (catalase)	Streak					Biovar Maltose	Stab				
Tetrazolium chloride (TTC) (tolerance, 0.1%)	Streak					Lactose	Stab				
Tetrazolium chloride (TTC) (tolerance, 0.02%)	Streak					Cellobiose	Stab				
King's medium B (KMB) (fluorescence)	Streak					Mannitol	Stab				
Sucrose nutrient agar (SNA) (levan)	Streak					Sorbitol	Stab				
Soluble starch agar (SSA) (starch hydrolysis)	Streak					Dulcitol	Stab				
Nitrate reduction	Stab					Gas from nitrate	Stab				
Potato rot	Stab										
Gelatin hydrolysis	Stab										
Oxidation/fermentation	Stab										

Figure 5—Proforma for the BACTID Tube Kit.

BACTID can be used in several ways for bacterial identification:

- following the flow chart for the sequence of tests. This is particularly useful when no particular bacterial group is suspected (using tests in a conventional format).
- performing selected tests in any desired sequence. This is useful when a particular group is suspected (e.g., *Xanthomonas*).
- performing all necessary tests at one time. The BACTID program should be used this way with the BACTID Training Kit, which provides tests in a convenient, portable format.

Where data are missing, more than one bacterial group may be possible. Throughout the program, hypertext screens can be viewed to obtain more information about the diagnostic tests and the characteristics of the different bacterial groups (e.g., pressing the F4 key displays the flow chart).

Recipes for media and reagents, and practical details of the text are read from an external ASCII file (METHODS.TXT). This file can be edited by a DOS text editor if variations need to be inserted. However, the 'Warning' should be read carefully before attempting to edit this file.

BACTID was written with the Knowledge Pro* expert system shell (Knowledge Garden Inc., 473a Malden Bridge Road, Nassau, NY 12123, USA). The interim release in use is a runtime version in the public domain which cannot be edited.

Identification of bacteria by metabolic profiles

Biolog* identification system

Patterns of substrate use (metabolic profiles) are characteristic of species and some subspecific taxa. The Biolog* system (Biolog Inc., 3938 Trust Way, Hayward, CA 94545, USA) presents 95 substrates (and a blank control) separately in the wells of a microtiter plate. If a given substrate is used, this is indicated by a color reaction since each well also includes a tetrazolium redox dye. The pattern of substrate use (the metabolic profile or fingerprint) is matched with profiles of known taxa in a database using identification software.

Plates can be read by eye or by a microtiter plate reader. There can be manual data entry, entry of data files from the plate reader, or the identification software can control a plate reader for direct entry of data. The Biolog system now has different databases (and different plates) for Gram-negative bacteria, Gram-positive bacteria, yeasts, and other groups of organisms.

Biolog was evaluated for plant pathogens and other bacteria associated with plants, particularly for the tropics, concentrating on its suitability for small plant pathology laboratories (Black and Sweetmore 1994). For further discussion of adapting Biolog for use in these situations and suitability of databases, see Black and Sweetmore (1993, 1994).

Recommended methods for using Biolog

Inoculation of plates—

- *Active cultures.* Incubate pure cultures overnight in nutrient agar on a shaking incubator; 50–100 mL depending on size of flask and holder available; 10–12 isolates in one batch is convenient.
- *Preparation of inoculum.* Washing off exogenous nutrients: Use 25-mL disposable sterile Universal bottles (graduated). Alternatively, autoclavable or disposable centrifuge tubes of the same capacity are suitable. Centrifuge at 4000 rpm (2800 g) for 30 min at 15°C. Pour off supernatant (to be autoclaved before disposal), resuspend with 20 mL sterile distilled water, homogenize in a vortex mixer, and centrifuge again. Repeat this procedure so that the cultures are washed twice in water, and finally resuspend in 20 mL water. If the suspension looks too thin, resuspend in about 16 mL water.
- *Initial optical density (OD) (turbidity).* Suspensions should have OD_{590} 0.4–0.6. The requirement is 15 mL of final suspension (96 × 150 µL). Determine the OD, and dilute accordingly using a turbidimeter, spectrophotometer, or determine the OD by eye with turbidity standards provided by Biolog.
- *Inoculation. Bring plates to room temperature. Inoculate each well* with 150 µl of suspension; use either an 8-channel pipette or a repeating pipette, and sterile pipette tips.

- ***Incubation times.*** Incubate at 28°C. Biolog Inc. advises reading after 4 h and 24 h. The Biolog GN database includes profiles for these times. However, 4 h is not commonly used with plant pathogens, but cultures could grow very rapidly and produce too many positives by 24 h. A flexible approach is suggested: check after 4 h and read. Check again from 18 h and use all 18 h+ readings as 24 h. Some bacteria could be slow growing and require longer incubation times. Use the 24 h database.

Reading plates—There are three general difficulties:

- Borderline results;
- High background (including colored control well) makes positives difficult to read;
- Spurious readings of pale, milky suspension rather than intense opaque violet.

Visual reading—

- Fully positive: medium in the well is deep violet.
- Borderline: wells incompletely filled, but medium is deep violet.
- Fully negative (and control): medium clear, colorless.
- Other readings: if necessary, record with a special code.

Using a plate reader—In the majority of cases, results as good as or better than visual reading can be obtained with a plate reader. A 590-nm filter is recommended, but one close to this wavelength will give satisfactory results. Only with very poor plates (high background), or overgrowth of a rapidly growing species, is it possible to extract some results by eye where a plate reader fails. Integral or PC-mounted software will have data reduction facilities to convert absorbance readings to well reactions. The Biolog system uses standard thresholds to convert OD_{590} readings to positive, negative, and borderline:

Threshold borderline A1 × 1.4

Threshold positive A1 × 2.1

These are set as defaults in MicroLog software versions supplied by Biolog which accept data files or direct plate readings. High background readings can cause problems if absorbance readings in the plate reader are converted to substrate reactions by internal or external software with the standard Biolog thresholds. The solution recommended originally was to use fixed thresholds of 1.3 for borderline, and 2.1 for positive (Black and Sweetmore 1993). However, recent versions of MicroLog which accept absorbance data files, or can read the plate reader directly, will interpret poor plates satisfactorily in most cases.

Bacterial identification software

MicroLog programs and databases—MicroLog 1 is the basic version of Biolog software, enabling data to be entered manually and profiles matched against the MicroLog GN Database. This database cannot be edited. Using the more advanced versions of MicroLog (2/2N, 3/3N), databases can be edited and compiled from the user's own profiles. A further advantage of these versions is the facility to accept data in the form of absorbance data files from the plate reader and/or direct plate reading.

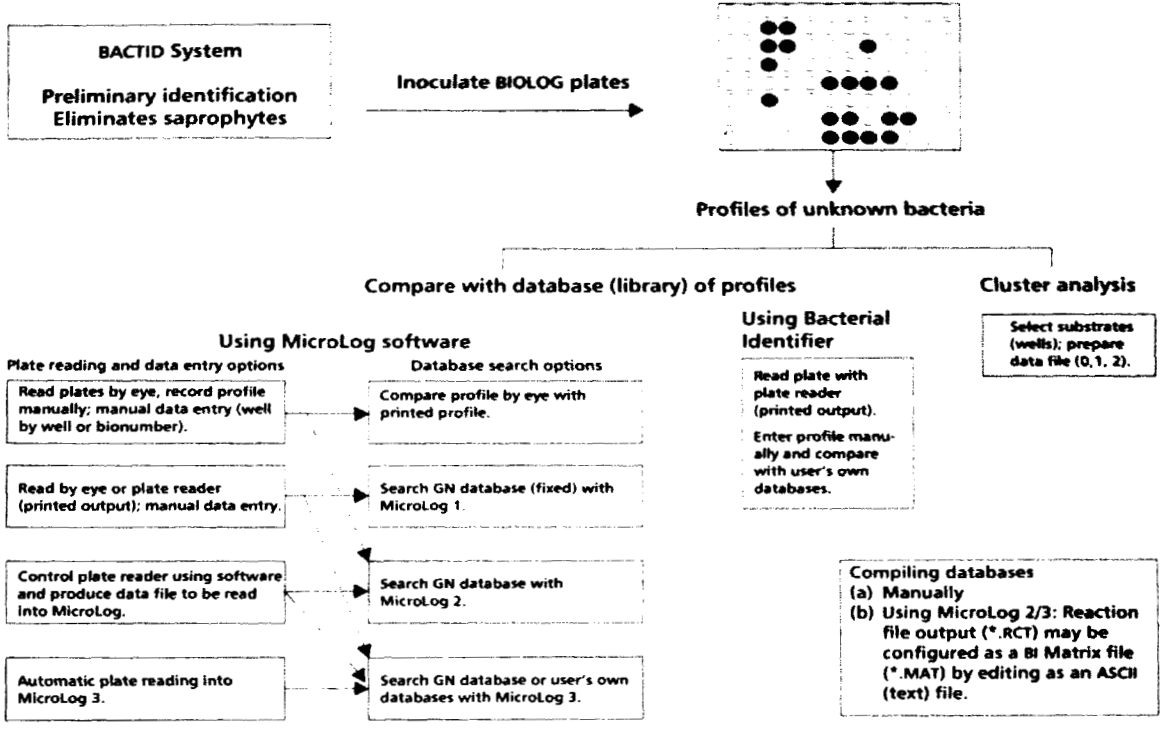

Figure 6—Integration of BACTID system and metabolic profiling for identification of bacteria: software options.

Bacterial identifier program

Blackwell's Bacterial Identifier program (Blackwell Scientific Publications Ltd) has been adapted for use with Biolog metabolic profiling (Black and Sweetmore 1993, 1994). User-defined databases can be used for a much lower purchase price than the equivalent MicroLog programs, and the program is statistically superior. However, only manual data entry is possible. It is convenient to use MicroLog to compile databases; the MicroLog database files cannot be edited or used in other programs but the reaction file (*.RCT) can be treated as an ASCII file and edited into the format for a matrix file (*.MAT) in Bacterial Identifier. The options for bacterial identification software and databases in an integrated system are given in Figure 6.

Cluster analysis of metabolic data

Metabolic profiling lends itself to analysis of relationships among isolates for taxonomy, identification or ecological studies. Substrate utilization data can be used on their own or with other characteristics. Advanced versions of MicroLog software have a cluster analysis facility, but this only compares a test isolate with taxa in a database and the number of strains or species compared is limited. It is preferable to use an independent multivariate statistical package (e.g., NTSYS-pc, MVSP-Plus) to analyze substrate-use data. Most programs have data entry facilities but experience has shown that an independent spreadsheet (e.g., Excel® or Lotus®) is the best means of preparing primary data. This could then be input directly into the program or first converted into an ASCII file. Cluster analysis is done in two stages: generation of a matrix of coefficients, and actual cluster analysis. There are many different coefficients and methods of clustering available. One factor in the choice is whether borderline values will be entered as missing values or treated as half-way values between negative and positive. This depends on the statistical program available. The authors use simple matching coefficients in the former case, and Euclidean distance coefficients in the latter with the Ungrouped Pair Method of Analysis (UGPMA).

Most programs will produce a dendrogram on screen which can be dumped to a printer. However, it is preferable to produce a special output for a graphics program so that the chart can be edited for labeling, title, etc., for a better presentation.

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Isolation and Identification of *Pseudomonas solanacearum* ■

V K Mehan

Collection of Samples of Wilt-Affected Plants

Diseased plants with early but well-established symptoms should be selected. Infection should be at an early stage, before secondary invaders colonize the decaying plant tissue. It is advisable to collect diseased plants from several sites within the field. Samples are packed in paper bags and labeled with the place and date of collection, and other useful information such as host variety, growth stage, etc. The time between sampling and processing should be as short as possible. Therefore, it is important that preparations in the laboratory are done before sampling.

Isolation of *Pseudomonas solanacearum*

Pseudomonas solanacearum can be isolated from the infected stem/root of a wilt-affected plant by the following procedure:

1 Cut the infected stem/root into 3–4 cm long pieces.

2 Wash these thoroughly in sterile water, and then dry them on sterile blotting paper.

3 Place the individual stem/root pieces in test tubes containing 5 mL sterile distilled water. Within a short time, bacteria can usually be seen to ooze from the cut ends.

4 After 3–5 min, take a loopful of the bacterial suspension and streak it onto an appropriate agar medium [e.g., sucrose peptone agar (SPA), tetrazolium chloride agar (TZCA)] in petri dishes, and incubate the dishes at 28–30°C.

5 Observe the colonies of the bacterium after 48–72 h of incubation.

Note—Single colonies are most easily obtained by the 3-streak method: a small drop of bacterial suspension is placed on the petri dish, the loop is resterilized, cooled down by touching the medium, and three lines drawn across the surface of the medium away from the original inoculum. This sequence—sterilization, cooling, streaking of previous inoculum—is repeated to achieve serial dilution of the original inoculum.

On the TZCA medium, the virulent isolates of the bacterium form irregularly round, fluidal, creamy white colonies with light pink centers. Avirulent colonies are round, butyrous, and uniformly deep red on this medium (Fig. 1).

Detection of *Pseudomonas solanacearum*

Detection methods have concentrated on detection in soil, using semiselective media. Several conventional methods are suitable for the detection of *P. solanacearum* from soil samples; they help enumerate either

Figure 1*—Pseudomonas solanacearum *colonies on tetrazolium chloride agar medium: virulent (fluidal, white colonies with pink centers; petri dish at right) and avirulent (deep red colonies; petri dish at left).

the culturable population or the total population. A count of the culturable population is obtained after growth on a suitable medium. These procedures use serial dilutions of the sample in appropriate media. Sampling plans are very important for the efficiency of these procedures.

Several nonselective media are suitable for isolation and growth of *P. solanacearum*—sucrose peptone agar (Hayward 1960), King's medium B (King et al. 1954), tetrazolium chloride agar (Kelman 1954). The composition of these media, and colony characteristics (Table 1) are given below.

Table 1*—Appearance of colonies of** Pseudomonas solanacearum ***on three nonselective agar media (incubated for 48–72 h at 30°C).

Medium	Cultural characteristics
Sucrose peptone agar (SPA)	Cream-colored, round colonies
King's medium B (KMB)	White, fluidal, nonfluorescent colonies
Tetrazolium chloride agar (TZCA)	Fluidal, white colonies with pink centers

Sucrose peptone agar (SPA)

Composition (g L^{-1})—

Sucrose	20
Peptone	5
Potassium hydrogen phosphate (K_2HPO_4)	0.5
Magnesium sulfate heptahydrate ($MgSO_4.7\ H_2O$)	0.25
Agar	15

pH is adjusted to 7.2–7.4 using 40% sodium hydroxide (NaOH).

King's medium B (KMB)

Composition (g L^{-1})—

Proteose peptone (Difco no. 3)	20
Potassium hydrogen phosphate (K_2HPO_4)	1.5
Magnesium sulfate heptahydrate ($MgSO_4.7\ H_2O$)	1.5
Glycerol	10
Agar	15

pH is adjusted to 7.2 using 40% NaOH.

Tetrazolium chloride agar (TZCA)

Composition (g L^{-1})—

Peptone	10
Casein hydrolysate	1
Glucose	5
Agar	15
2,3,5-triphenyl tetrazolium chloride	0.05

Note—Tetrazolium chloride (TZC) is added as 1 mL of a filter-sterilized 0.5% solution per 100 mL of molten sterilized medium (60°C) before pouring into petri dishes.

TZCA is used as a general growth and isolation medium for *P. solanacearum*. It is suitable to differentiate wild colony types (white with pink centers) from low-virulence mutants or avirulent mutants that could occur on subculturing. Mutant colonies usually take up the formazen produced on reduction of tetrazolium chloride to form deep red colonies.

Selective Media

Several selective media for the isolation of *P. solanacearum* have been developed (Nesmith and Jenkins 1979, Karganilla and Buddenhagen 1972, Granada and Sequeira 1983). These media can detect 10^4 cells g^{-1} soil; some are suitable only for certain strains. In general, these media are useful for ecological and epidemiological studies of the wilt pathogen. They increase plating efficiency and reduce interference from saprophytic soil microorganisms. For diagnosis, these media should be used in conjunction with nonselective isolation media.

Other Detection Tests

Serological tests based on specific antigen-antibody reactions can also be used to detect the bacterium in infected plant tissues and seeds. Among the various serological tests, dot immunobinding assay (DIBA), enzyme-linked immunosorbent assay (ELISA), and immunosorbent electron microscopy (ISEM) are most suited for this purpose, ELISA being the most preferred. ISEM requires access to an electron microscope, and large numbers of samples cannot be easily handled. In developing countries, DIBA is more difficult to adopt than ELISA, because the materials and reagents required for the test are not easily available. DNA-based diagnostics can also be used to detect the wilt pathogen in plant tissues, but their use in developing countries is very limited at present.

In recent years, several simple, sensitive, and highly specific ELISAs of *P. solanacearum* have been developed. These detection methods do not require purification or culture of the bacterium. They are more rapid and

sensitive, and simpler to use than conventional detection methods. Several polyclonal and monoclonal antibodies against *P. solanacearum* have been produced in laboratories at the University of Hawaii, USA, the Rothamsted Experimental Station, UK, the Institute of Plant Protection, Beijing, China, and the International Potato Center, Peru. Most of these antibodies show cross-reactions with several closely related bacterial species (e.g., *Pseudomonas syzygii, P. pickettii, P. cepacia*). Some highly specific monoclonal antibodies produced at the University of Hawaii, and at the Rothamsted Experimental Station do not show any cross-reactivity with these bacterial species (Robinson 1993, Alvarez et al. 1993).

ELISA is now being adopted for the detection of *P. solanacearum* because of its simplicity, sensitivity, speed, and the ability to quantify pathogen biomass in plant tissues and other matrices. It is different from the classical serological tests (e.g., Ouchterlony double diffusion test) in which immunoprecipitin reactions occur. Immunospecificity is recognized through the action of the associated enzyme label with a suitable substrate rather than by observing the precipitate formed by the insoluble antigen-antibody complex.

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Phenotypic Methods for the Differentiation of *Pseudomonas solanacearum*: Biovars and Supplementary Observations

A C Hayward

Introduction

The bacterial wilt pathogen, *Pseudomonas solanacearum* (Smith) Smith, is variable in some phenotypic properties such as utilization of carbon sources, nitrate metabolism, and pectolytic activity. Some of the key properties for the identification of *P. solanacearum* are described below, with emphasis on the utilization and/or oxidation of hexose alcohols and disaccharides which enables separation of isolates into five biovars (Hayward 1964, Hayward et al. 1990, He et al. 1983).

Biovar Differentiation

For determination of biovars in *P. solanacearum*, the medium of Ayers et al. (1919) is modified as follows:

Basal medium for oxidation/ fermentation tests

Peptone (Difco Bacto)	1 g
Ammonium dihydrogen phosphate ($NH_4H_2PO_4$)	1 g
Potassium chloride (KCl)	0.2 g
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	0.2 g
Agar (Difco Bacto)	3 g
Bromothymol blue	0.08 g
Distilled water	1 L

The pH is adjusted to 7.0–7.1 (an olivaceous green color) by dropwise addition of 40% sodium hydroxide (NaOH) solution. The medium is steamed to melt the agar and dispensed as 90 mL or 18 mL per bottle. It is then sterilized by autoclaving at 121°C for 30 min.

This medium is used for distinguishing the biovars of *P. solanacearum*, based on utilization of various carbohydrates (Table 1).

Preparation of carbohydrate solutions

- Prepare 10% aqueous solutions of the test carbohydrates. (Dulcitol is poorly soluble at 20–30°C; dissolve it by heating in a water bath followed by cooling).
- For D-glucose, mannitol, sorbitol, and dulcitol, sterilize in an autoclave at 110°C for 20 min as 10-mL quantities.
- For lactose, maltose, and D (+) cellobiose, sterilize by membrane filtration and dispense in 10-mL quantities into presterilized containers.

Addition of carbohydrates to basal medium

Sufficient carbohydrate solution is added to the basal medium (at 60°C) to give a final concentration of 1% in the medium (i.e., 10 mL of 10% solution to 90 mL basal medium or 2 mL to 18-mL quantities of the basal medium). After mixing, the still molten medium is dispensed as 3-mL quantities to sterilized test tubes (150 mm × 10 mm internal diameter).

Table 1—Differentiation of **Pseudomonas solanacearum** *biovars.*

Test	Biovars				
	1	2	3	4	5
Oxidation/utilization of					
Mannitol	–	–	+	+	+
Sorbitol	–	–	+	+	–
Dulcitol	–	–	+	+	–
Oxidation of					
Lactose[1]	–	+	+	–	+
Maltose[1]	–	+	+	–	+
Cellobiose[1]	–	+	+	–	+
Nitrite from nitrate	+[2]	+[2]	+	+	+
Gas from nitrate	–[3]	–[3]	+	+	+

1 Disacchrides are oxidized to bonic acids, but are not utilized as a source of carbon and energy
2 There are rare exceptions which fail to reduce nitrate to nitrite.
3 There are rare exceptions which produce gas from nitrate

Inoculum preparation

- Add several loopfuls of 24–48 h culture from agar medium to 3–5 mL sterile distilled water to make a suspension of light milky opacity.
- Add 0.02 mL of the bacterial suspension to each tube using a micropipette.
- Incubate the inoculated tubes at 28–32°C.
- Examine the tubes at 3, 7, 14, and 28 days after inoculation for change in pH (examine from the top of the medium downward).

Note—A tube containing only the basal medium should also be inoculated to serve as control.

With the glucose and hexose alcohols, a change to yellow (acid pH, <6) indicating oxidation of the carbohydrate occurs within 3–5 days; those biovars capable of oxidizing the disaccharides could take a few days longer to give a clear positive result. The tubes should be compared with the control tube to observe pH changes (in some cases there could be a slight change to alkaline pH in some of the tubes containing carbohydrates that are not oxidized).

Oxidative versus fermentative metabolism

To confirm oxidative or fermentative metabolism of carbohydrates:

- Stab inoculate duplicate tubes of glucose medium (to the base of the tube) with a straight wire loaded with inoculum.
- Seal one tube with molten vaspar (a 50:50 mixture of vaseline and paraffin wax) or 3% agar (to a depth of 2 cm).
- Examine the tubes after 3–4 days for yellow (pH ≤6) or green (pH 7.1).

A fermentative reaction is shown by members of the Enterobacteriaceae such as *Escherichia coli* and *Erwinia carotovora*. This reaction could occur with or without gas production. Any gas produced will be trapped as bubbles in the semisolid agar or displace the seal on the top of the tube.

Organisms with a fermentative metabolism of glucose are invariably strict or facultative anaerobes; acid is produced throughout the depth of the medium in both open and sealed tubes. Organisms with an oxidative (respiratory) metabolism of glucose produce acid only at the surface of the open tube where conditions are aerobic. An oxidative reaction is given by *Pseudomonas*, *Xanthomonas*, *Agrobacterium*, and *Rhizobium*.

Supplementary tests with additional sugars

Further differentiation of isolates of biovar 2 could be achieved by adding the sugars D-ribose, trehalose, and *meso*-inositol. Bacterial wilt of potato in temperate and subtropical regions and at high altitudes in the tropics worldwide is caused by biovar 2 (race 3) with the phenotype D-ribose-, trehalose-, and *meso*-inositol+. A distinct phenotype of biovar 2 occurring in parts of Chile and Colombia, South America, has the phenotype D-ribose-, trehalose+, and *meso*-inositol- (Hayward et al. 1990). Most of these isolates do not produce nitrite from nitrate, which is a property universal among isolates of biovar 2 from potato of the alternate and more widely distributed phenotype. There is a third distinct phenotype of biovar 2 occurring in Peru and Brazil which has the phenotype D-ribose+, trehalose+, and *meso*-inositol+.

Nitrate metabolism

Production of gas from nitrate is found in more than 90% of the isolates of biovars 3, 4, and 5, whereas this property is rarely found in biovars 1 and 2. Inability to grow anaerobically in the presence of nitrate, or produce nitrite from nitrate, is rare and almost entirely limited to biovar 2 (Hayward et al. 1990). The choice of medium is critical. In certain media, the production of gas from nitrate is erratic or absent. Reliable results can be obtained using the medium of Van den Mooter et al. (1987):

Medium of Van den Mooter et al.

Composition (g L^{-1})—

Potassium dihydrogen phosphate (KH_2PO_4, anhydrous)	0.5
Dipotassium hydrogen phosphate (K_2HPO_4, anhydrous)	0.5
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	0.2
Sodium succinate (or glycerol)	2
Potassium nitrate (KNO_3)	3
Yeast extract (Difco)	5
Agar (Difco Bacto)	3

pH is 6.8–7.0 without addition of acid or alkali.

Procedure—

- Heat at 100°C to melt the agar and dispense quantities of 3–4 mL into test tubes (150 mm × 10 mm internal diameter either capped or plugged).
- Sterilize the prepared tubes in an autoclave at 121°C for 30 min.
- Store the medium at room temperature (if stored at 4°C for long periods the medium should be remelted and reset immediately before use).
- Stab inoculate duplicate tubes of the semisolid medium (to the base of the tube) two or three times using a straight wire loaded at the point with inoculum from an agar plate.
- Seal one of the tubes with 2–3 mL of 3% water agar or vaspar.

- Incubate the tubes at 28–32°C.
- After 3–7 days, test the unsealed tube for the presence of nitrite by adding starch iodide reagents prepared according to Skerman (1967) as described below.

Starch iodide spot test for nitrite

Reagents—

- **Starch iodide solution**

Starch	0.4 g
Zinc chloride ($ZnCl_2$)	2 g
Distilled water	100 mL

Dissolve $ZnCl_2$ in 10 mL water. Boil and add starch. Dilute to 100 mL, allow to stand for 1 week, and filter. Add an equal volume of a 0.2% solution of potassium iodide (KI).

- **Hydrochloric acid**

Concentrated hydrochloric acid (HCl)	16 mL
Distilled water	84 mL

Procedure—Using clean, glass dropping pipettes, place 1 drop of each reagent in the depression of a white spot test plate. Add 1 drop of the culture. A blue color indicates the presence of nitrite.

The test depends on the formation of nitrous acid and its subsequent reaction with potassium iodide with the liberation of iodine, which turns the starch blue. The test is not entirely specific. Control tests should be made on a noninoculated medium. Several other reagents are available to test for production of nitrite from nitrate (Lelliott and Stead 1987).

A negative reaction for nitrite could indicate either that the nitrate has not been reduced, or that the nitrate has been reduced beyond nitrite. To differentiate between these possibilities, a speck of zinc dust is added to the tubes in which no reaction for nitrite, or a weak reaction, has occurred. If the nitrate has not been reduced by the bacterium, there is a reaction after addition of the zinc dust; if there has been a weak reaction which does not intensify even after addition of zinc dust, then most of the nitrate has been reduced beyond nitrite. A weak reaction that intensifies after the addition of zinc dust indicates that a little of the nitrate has been reduced to nitrite.

The tube sealed with agar or vaspar should be examined each day for 7 days, for the presence of gas bubbles trapped in the medium or beneath the seal. The reaction is sometimes weak and slow to appear. A stronger reaction could be obtained if the isolates are subcultured several times through a medium containing nitrate in order to enhance the activity of the enzyme nitrate reductase (Stanier et al. 1966). False positives can sometimes be obtained if the medium is stab inoculated when solid. Bubbles of 'gas' can appear on the stab marks in the agar, which in fact are caused by the method of inoculation. A more subtle cause of error could arise when the medium has been prepared and dispensed long before use and stored at 4°C. Gas is released into the medium when it is subsequently incubated at 28–32°C. This effect can be avoided if the medium is remelted before use. The effect could be due to the greater solubility of certain gases at 4°C than at higher temperatures, the gases being released into the medium on subsequent incubation.

Poly-β-hydroxybutyrate (sudanophilic) inclusions

Pseudomonas solanacearum in culture, or in ooze from affected plants, exhibits bipolar staining when the bacterial cells are treated with a dilute solution of safranine. The lack of affinity for basic dyes which results in bipolar staining is caused by the presence in the cells of massive inclusions of poly-β-hydroxybutyrate (PHB). There are three complementary observations that indicate the presence of this substance: a) bipolar staining of bacterial cells when heat-fixed smears of bacteria are stained with a dilute solution of safranine; b) the appearance of refractile, phase-bright inclusions, under a phase-contrast microscope; and c) staining of the inclusions by a solution of Sudan black B according to the method of Burdon (1946).

Safranine stock solution—

Safranine	0.25 g
Ethanol (95%)	100 mL

For use, dilute 10 mL of stock solution with 90 mL of distilled water. The stock solution is stable and can be stored for months.

The following procedure for Sudan staining could be applied to heat-fixed smears of bacterial ooze expressed from the host plant or smears prepared from cultures on an agar medium.

Sudan black B stain—

Sudan black B powder	0.3 g
Ethanol (70%)	100 mL

Shake thoroughly at intervals and stand overnight before use. Keep in a well-stoppered bottle.

Procedure—

1 Make a film of bacteria on a microscope slide, dry in air, and fix by flaming.

2 Cover the entire slide with Sudan black B stain and leave at room temperature for 15 min.

3 Drain off excess stain, blot, and dry in air.

4 Rinse thoroughly with water under the tap and again blot dry.

5 Counterstain lightly by covering with 0.5% aqueous safranine or dilute carbol fuchsine for 5–10 sec; rinse with tap water, blot and dry. Lipid inclusion granules are stained blue-black or blue-grey, while the bacterial cytoplasm is stained light pink.

The method given above is that of Burdon (1946), except that in Step 4, rinsing is done with tap water instead of xylol. Maximum inclusions of PHB are formed on media of high carbon-nitrogen ratio. The following notes on methods are taken from Lelliott and Stead (1987).

Grow cultures on 5% sucrose nutrient agar or sucrose peptone agar, or preferably in shake-culture in the basal medium of Ayers et al. (1919) containing 0.5% β-hydroxybutyrate (sodium salt). Production of PHB is more certain in the latter medium.

When good growth has been obtained (after about 2 days), examine smears or wet preparations under a phase-contrast microscope, and look for granules which are hyaline, circular, one or more to a cell and occupying much of the cell volume. PHB granules also show up well in the electron microscope as electron-dense bodies. Cells which have accumu-

lated PHB granules do not usually Gram stain well. Since spores cannot be easily differentiated from polymer granules by this method, it should only be used when it is known from other evidence that the culture being observed is of a non-spore-forming bacterial species.

Alternatively, flood a heat-fixed smear with Nile blue A (1% aqueous solution) for 10 min at 55°C. Wash briefly with tap water. Flood with 8% aqueous acetic acid for 1 min to remove excess stain. Wash again in tap water and blot dry. Moisten again with a drop of water and cover with a cover slip. Examine under oil immersion with epifluorescence at 450 nm. PHB granules fluoresce bright orange (Ostle and Holt 1982).

Experience has shown that the Nile blue A staining method combined with epifluorescence microscopy is superior to staining with Sudan black B, which does not always differentiate PHB granules satisfactorily (Lelliott and Stead 1987). The latter method should be used when only light microscopy is available. PHB granules are found widely among aerobic, Gram-negative bacteria; however, there are a few other bacteria causing wilt in plants in which this property is found e.g., *P. caryophylli* which causes a vascular wilt of carnation.

Kovacs' oxidase test

Various methods can be used to carry out this test. Oxidase strips available commercially should be used according to the manufacturer's instructions. The method detailed below closely follows the one originally described by Kovacs (1956).

Procedure—

- Place a Whatman no. 1 filter paper in a petri dish and add 3–4 drops of freshly prepared 1% aqueous solution of tetramethyl paraphenylenediamine dihydrochloride on the center of the paper.
- Using a platinum loop heavily charged with growth from a 24–48 h culture of *P. solanacearum* on Kelman's medium (Kelman 1954) or on nutrient agar, rub a band about 1 cm long across the reagent-impregnated paper.

With *P. solanacearum*, the change to a purple color within 10 sec of application of the culture could be regarded as a positive result. The reagent solution should be made up weekly or every 2 weeks and stored in a stoppered dark glass bottle at 4°C.

Caution—The reagent is an aromatic amine and a highly toxic substance; skin contact with the powder or solution should be avoided.

The choice of medium can be important for proper execution of this test. False positive reactions could occur where nitrite present in the medium reacts at once with the reagent to give a purple color. If, therefore, it contains nitrite, or, more probably, if the medium contains nitrate and the species in question (e.g., *P. solanacearum*) is able to reduce it to nitrite, an apparently 'positive' reaction is likely to occur.

The N,N-dimethyl-p-phenylenediamine reagent is to be preferred to the tetramethyl form as it is less sensitive to nitrites that could form in the culture medium (Hildebrand and Schroth 1972).

There is evidence that the presence of cytochrome C is correlated with a positive reaction. Examination of the absorption spectrum of several oxidase-negative bacteria has shown that they possess cytochromes of the type B, and not cytochrome C which is invariably present in oxidase-positive bacteria (Sands et al. 1967).

The oxidase reaction is negative in the Enterobacteriaceae, and positive in *Aeromonas* and most pseudomonads and related bacteria. Within the genus *Pseudomonas* the test is of differential value. It is useful for distinguishing the pathovars of *P. syringae* from *P. cichorii*, *P. marginalis*, and the saprophytic fluorescent pseudomonads. The oxidase reaction is also of differential value among the many plant pathogenic species that produce inclusions of poly-β-hydroxybutyrate (for example, *P. andropogonis* gives a negative reaction; *P. solanacearum* gives a positive reaction).

The presence of a high concentration of a utilizable carbohydrate in the medium on which *P. solanacearum* is grown for the oxidase test does not appear to affect the result. However, with other oxidase-positive species, false negative results could occur in agar media containing a concentration of glucose > 0.5%. Inhibition of the reaction has been observed on media containing 2% glucose; this could be a pH effect resulting when the pH of the medium is between 5.0 and 5.5.

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Serological Techniques for the Detection of *Pseudomonas solanacearum*

A Robinson-Smith

Background

Antibodies are protein molecules produced naturally by biological organisms against external 'nonself' molecules. These 'nonself' molecules are called antigens, and can take different forms—components of bacteria, viruses, plant pollens or foreign red blood cells. Antibodies to specific antigens may be produced by immunizing with an antigen, a warm-blooded animal, usually a mouse, rat or rabbit, and occasionally, a larger animal, e.g., horses. Antibodies are then produced by the animal and released into its bloodstream, from which they could be harvested.

Polyclonal and Monoclonal Antibodies

Antibodies are produced in B- and T-lymphocytes which comprise approximately 30% of the white blood cell count in the peripheral circulation of mammals. These B-lymphocytes are derived directly from the bone marrow precursors and have antibody receptors on their surface, usually of one specificity. When the B-lymphocyte encounters the antigen against which its antibody is directed, it differentiates into a plasma cell, which then produces large amounts of the antibody. Antigens are complex molecules or organisms (bacteria and viruses) and could have several distinct sites or epitopes against which antibodies could be specifically directed. As a result, a number of antibodies of different specificities will be produced, even though overall, they are directed against the same antigen. The antibodies produced will also have different affinities for a given antigen. So, when a vertebrate organism produces antibodies against a given antigen, the antibodies have different specificities and affinities, and the resulting blood would contain a mix of antibodies to the antigen. These antibodies are referred to as polyclonal antibodies.

Monoclonal antibody technology was developed by Köhler and Milstein in 1975, and enables the polyclonal antibody to be dissected into the sum of its monoclonal parts. The principle of monoclonal antibody production is remarkably simple, and is based on the production and selection of immortal antibody-producing hybrid cell lines, derived from the fusion of antibody-producing lymphocytes with an immortal myeloma cell line.

In all mammals, the main source of antibody-producing cells is the spleen, which can be removed and its cells fused with a myeloma line. This fusion is mediated by polyethylene glycol and dimethyl sulfoxide, and is followed by incubation in a selective medium, to destroy the unfused cells. The resulting hybridomas can then be 'cloned-out' into individual antibody-producing lines of one specificity and one affinity. These cell lines are immortal in cell culture and can therefore be grown indefinitely, producing large amounts of a given antibody. Some important features of polyclonal and monoclonal antibodies are given in Table 1.

Table 1—Some important features of polyclonal and monoclonal antibodies.

Feature	Polyclonal antibodies	Monoclonal antibodies
Cost of production	Low	Initially high
Specificity	Variable with animal and breed. Partial cross reactions with common determinants. Seldom too specific.	Standard. Unexpected cross-reactions can occur. May be too specific.
Determinants recognized	Many	Single
Yield of antibody	Up to 1 mg mL^{-1}.	Up to 100 µg mL^{-1} in culture and 20 mg mL^{-1} in ascites.
Contaminating immunoglobulins	High	None in culture, 10% in ascitic fluid.
Antigen purity required	Either pure antigen or serum absorption.	Purity desirable but not essential.

Preparation of Bacterial Antigens for Antibody Production

Bacterial antigens can be extracellular, intracellular or structural; their biochemical nature can be protein, glycoprotein, polysaccharide, lipopolysaccharide, or lipid. Immunogen preparations can therefore consist of whole cells, crude cell extracts or purified bacterial components.

Whole cells

Untreated—Bacterial cells grown on solid or in broth media can be used directly as antigens. The bacteria are grown for 24–48 h, then washed two to three times in 0.01M phosphate-buffered saline (PBS) by centrifugation and resuspension, before inoculating.

Heat-treated—Heat treatment destroys most proteins, thus permitting some degree of selection for carbohydrate antigens. This can be done either by placing the washed cells in a boiling water bath for 2 h, or by autoclaving for 2 h.

Formalin-fixed—Bacterial cells are suspended in 0.85% saline and mixed with an equal volume of 0.6% formalin. The cells are then left at room temperature for 1–2 days before being collected by centrifugation. The cells are finally resuspended in 0.3% formalized saline.

Glutaraldehyde-fixed—This is perhaps the most useful and most frequently used antigen, for not only does it stabilize the cell wall, but also decreases toxicity of some bacterial species to the host animal. Bacterial cells are washed three times in PBS and dialyzed against PBS containing 2% glutaraldehyde for 3 h at room temperature. The cells are then dialyzed against PBS for 24 h, with several changes of buffer to remove residual glutaraldehyde.

Crude antigen extracts

Glycoprotein—Bacterial cultures (48-h-old) are shaken in sterile distilled water for 30 min and the cells removed by centrifuging. The supernatant, which contains a mixture of proteins and polysaccharides from the cell surface, is then adjusted to pH 7, mixed with an equal volume of saturated ammonium sulphate, and kept overnight at 4°C. A precipitate is formed, that can be collected by centrifuging at 20 000 g for 15 min. The pellet is then dissolved in sterile distilled water and dialyzed to remove ammonium sulphate.

Purified bacterial components

Purification of many antigens such as lipopolysaccharides, ribosomes, secreted enzymes, membrane proteins, flagella, etc, is possible, but only limited use has been made of these immunogens for serology in plant pathology.

Production of Polyclonal Antibodies

Most polyclonal antibodies are produced in rabbits. Their production is relatively simple as long as the antigen is immunogenic. Bacteria are highly immunogenic, and a simple immunization program such as that detailed below can help produce polyclonal serums of a very high titer.

1 Immunize female inbred rabbits by injecting intramuscularly 5×10^8 cells in 0.5 mL antigen emulsified in 0.5 mL Freund's complete adjuvant (Difco Labs) at two sites in the inner thigh muscle.

2 Wait for 4 weeks and immunize as above, but with the antigen emulsified in Freund's incomplete adjuvant.

3 After a 2-week rest, bleed the rabbit (via the lateral ear vein), and collect the blood (up to 30 mL) in a centrifuge tube.

4 Allow the blood to clot at room temperature (could take about 10 h) and then spin at 2000 g for 15 min.

5 Carefully decant the supernatant (the serum fraction) from the tube.

6 Test the antibody titer of the serum by enzyme-linked immunosorbent assay (ELISA). If the titer is sufficiently high (1:2000), continue bleeding at 3-week intervals; otherwise repeat Step 2.

7 Continue bleeding up to 10 bleeds, or until the titer drops below 1:2000.

Storage of antiserums

Antibodies are resistant to a broad range of mildly denaturing conditions, making long-term storage relatively easy. The only problem commonly encountered is contamination with bacteria or fungi, which can be overcome by:

- freezing in aliquots at −20°C; or
- adding sodium azide to 0.02% and storing at 4°C; or
- adding glycerol to 50% and storing at 4°C.

Note—Antibody solutions should not be frozen and thawed repeatedly, as this can lead to loss of activity, because the antibody aggregates and therefore blocks the antigen-binding sites.

Purification of antiserums

Several techniques require purification of the antibody. Many methods could be used, the most conventional being precipitation followed by column chromatography. Neither of these, when used alone, will completely purify the antibody, but if used together, a relatively pure antibody will result.

Ammonium sulphate precipitation—

1 To 1 mL of antiserum, add 9 mL of distilled water in a 50-mL centrifuge tube and mix.

2 Add 10 mL of saturated ammonium sulphate solution, and leave at room temperature for 30–60 min.

3 Spin at 10 000 g for 10 min.

4 Discard the supernatant, and drain the tube by holding it upside down on a tissue.

5 Dissolve the pellet in approximately 2 mL of PBS.

6 Dialyze overnight against several changes of PBS to completely remove the ammonium sulphate.

Immunoglobulins prepared by this procedure often require further purification by ion-exchange chromatography.

DEAE-matrix chromatography—

1 Wash the DEAE-matrix with 0.5M hydrochloric acid (HCl) and then 0.5M sodium hydroxide (NaOH), followed by 20 volumes of 10 mM Tris (pH 8.5). Check the pH and repeat the washes until the pH is 8.5. Transfer the matrix to a column, using approximately 2 mL of wet matrix for each mL of serum.

2 Dialyze the serum (or ammonium sulphate purified antibodies) against three changes of 10 mM Tris (pH 8.5).

3 Pass the antibody solution down the column. Wash the column with 10 bed volumes of 10 mM Tris (pH 8.5).

4 Sequentially elute the column with increasing sodium chloride (NaCl) concentrations in the original 10 mM Tris (pH 8.5) buffer. This can be done with a gradient maker or by step buffers. Most antibodies will elute with salt concentrations below 500 mM.

5 Determine the fractions containing the antibody and pool together.

6 Regenerate the matrix by washing with 0.5 M HCl and 0.5 M NaOH.

Serological Assays

There are several different serological assays, all with their own advantages and disadvantages. The choice depends on what one requires from the assay: speed, sensitivity, specificity, etc.

Precipitin methods—In general, soluble antibodies and/or antigens diffuse independently through a gel, usually of agar (Ouchterlony double diffusion). Where the homologous antibodies and antigens meet in equivalent proportions, a precipitin reaction occurs, which can be seen as the formation of a white precipitate. Such tests can also be done in a liquid medium (ring precipitin test), but they tend to use large volumes of antibody and are often difficult to observe at high dilutions.

Agglutination methods—These can be the simplest of diagnostic methods. Basically, a drop of antigen is placed on a slide and a drop of specific antibody is added. The clumping together of antigen and antibody indicates a positive reaction. Unfortunately, a high concentration of antigen is required for a reaction to take place. Sensitivity can be improved, however, if the antigen or antibody is first absorbed onto a larger particle (e.g., blood cells, chloroplasts, latex particles, and bacteria). The large cells of the bacterium *Staphylococcus aureus* are especially suitable, as they are coated with a protein (protein A) which binds specifically the IgG molecules of the serum. Such particles act by amplifying the antigen-antibody reaction, as they also clump together.

Enzyme-linked immunosorbent assays (ELISA)

ELISA assays take a variety of forms. All of them involve a plastic multiwell plate (microplate) specially treated so that the surface of the wells will bind protein at high pH. Infected plant samples should be ground directly in a high pH buffer, pipetted into the wells and incubated. The antigen will then bind to the plastic. More commonly, wells are first treated with specific antiserum (mixed in high pH coating buffer), which is then used to trap the antigen when the samples are placed in the wells. Samples are mixed in a buffer at pH 6–7 to inhibit binding to the plastic. The antigen is then detected by a second antibody linked to an enzyme (alkaline phosphatase, horseradish peroxidase, penicillinase) which changes color when a suitable chemical substrate is added. So, a color change occurs in those wells where the antigen is present.

Dot immunobinding assay (DIBA)

The dot immunobinding assay is similar to ELISA, but sap samples are spotted directly onto a membrane, usually of nitrocellulose or nylon, which binds all proteins including the antigen as the sap samples dry. The presence of the antigen is again detected using an antiserum linked to an enzyme.

Immunofluorescence

Immunofluorescence is used mainly to locate antigens in tissues and cells. Instead of using an enzyme linked to an antibody to detect the antigen, it uses a fluorochrome such as fluorescein isothiocyanate linked to an antibody.

Immunosorbent electron microscopy (ISEM)

An antigen-containing drop of water is added to an appropriately diluted antibody on an electron microscope grid. The antigen clumps together with the antibody molecules and can be examined by transmission electron microscopy.

Polyacrylamide gel electrophoresis/ Western blotting

Plant and antigen proteins are separated on a slab of polyacrylamide and then transferred to a sheet of nitrocellulose. The presence of the antigen is again detected using an antiserum linked to an enzyme as in the case of DIBA. The prime advantage of Western blotting over Dot blotting is that antibody reactivity can be correlated with proteins/peptides of particular molecular weights.

Enzyme-Linked Immunosorbent Assays (ELISA)

In most cases, ELISA is the assay of choice for screening of infected plant samples for *Pseudomonas solanacearum*, as it is relatively rapid and sensitive, and requires only a small amount of unpurified antibody. Also, as it is carried out in a microtiter plate, a large number of samples can be screened at one time.

Types of ELISA

There are a variety of ELISAs available which differ in their layout; some rely on the binding of antigen to the plate, others on the binding of antibody.

Direct ELISA—The antigen is coated onto the plate and the enzyme used is directly conjugated to the specific antibody. This could be less sensitive than the indirect ELISA.

Indirect ELISA—The antigen is coated onto the plate and the enzyme used is conjugated to an antispecies antibody. This increases the sensitivity of the test.

Double antibody sandwich (DAS) ELISA—The plate is first coated with a specific antibody to capture the antigen. This increases the sensitivity and specificity of the test. It can be direct or indirect.

F(ab')$_2$ ELISA—As in DAS ELISA, the plate is first coated with the antibody, but instead of using the whole antibody, only the F(ab')$_2$ portion of IgG is used. The second antibody used is, however, whole. Unlike the other ELISAs, the enzyme used in this case is coupled to the protein A or the Fc portion of IgG. This binds to the Fc portion of the immunoglobulin molecule and thus overcomes the problem of cross-reaction with the first antibody.

Competitive ELISA—Similar in method to the above ELISAs, but an inhibitor antigen is included so that the amount of antigen present can be determined.

Protocol for indirect ELISA

1. Macerate a known amount of infected plant tissue 1:1 in PBS.
2. Allow to settle, and remove supernatant. Dilute 1:10–1:20 in a coating buffer.
3. Coat the wells of a suitable microtiter plate with this supernatant, using 100 µL well^{-1}. Include positive and negative controls (healthy plant extracts).
4. Incubate in a moist chamber or in a plastic box lined with moistened filter paper at 37°C for 1 h, or overnight at 4°C.
5. Wash the microtiter plate three times in a washing buffer, leaving it in the last change for 5 min.
6. Add 100 µL of a polyclonal antibody, diluted to working concentration (1:5000) in a blocking buffer, to each well. Alternatively, use a monoclonal antibody and continue as above, but with an antimouse conjugate.
7. Incubate and wash as in Steps 4 and 5.
8. Add 100 µL of the second antibody [peroxidase conjugated antirabbit, antimouse (Sigma Chemical Co.)], diluted to 1:5000 in a blocking buffer (optimum dilution to be determined), to each well.
9. Incubate and wash as in Steps 4 and 5.
10. Add 100 µL of peroxidase substrate (3,3', 5,5'-tetramethyl-benzidine) per well. Leave at room temperature until a color change (from colorless to blue) is observed (5–10 min).
11. Stop the reaction by adding 25 µL of 3 M sulfuric acid (H_2SO_4) to each well. A blue color develops indicating the termination of the reaction.
12. Assess results visually, or measure the absorbance on a spectrophotometer at 450 nm.
13. A positive result is taken as three times the mean of the negative control.

This ELISA can detect 1×10^4 cfu mL^{-1} bacterial cells in either plant or soil samples.

Materials for indirect ELISA

Carbonate coating buffer (pH 9.6)—

Sodium carbonate (Na_2CO_3)	6.36 g
Sodium bicarbonate ($NaHCO_3$)	11.72 g

Make up to 1 L with distilled water.

Phosphate-buffered saline (PBS) (concentrated stock solution)—

Sodium chloride (NaCl)	80 g
Potassium dihydrogen phosphate (KH_2PO_4)	2 g
Sodium phosphate dibasic ($Na_2HPO_4.12H_2O$)	14.2 g
Potassium chloride (KCl)	2 g

Make up to 1 L with distilled water.

Washing buffer: PBS plus Tween (PBS-T)—

PBS stock solution	100 mL
10% Tween 20	5 mL

Make up to 1 L with distilled water (buffer must be made up when required).

Blocking buffer—

10 × PBS	10 mL
Polyvinyl pyrrolidine (mw 44000)	2 g
10% Tween 20	0.5 mL
Full cream milk powder (e.g., NIDO®, Nestlé®)	0.5 g

Make up to 100 mL with distilled water (buffer must be made up when required).

Peroxidase substrate—

3,3', 5,5'-tetramethyl-benzidine (Sigma)	
@ 10 mg mL $^{-1}$ in dimethyl sulfoxide (DMSO)	100 µL
30% hydrogen peroxide (H_2O_2)	2 µL
1 M sodium acetate (pH 5.8)	1 mL

Make up to 10 mL with distilled water (buffer must be made up when required).

Reference

Köhler, G., and Milstein, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497.

Monoclonal Antibody-Based Sandwich ELISA for the Detection of Latent Bacterial Wilt Infection in Groundnut

L Y He and Y L Xie

Antibody Preparation

An IgG preparation from the polyclonal antiserum against *Pseudomonas solanacearum* is lyophilized in vials, and preserved at 4°C. The monoclonal antibodies (McAb) specific to *P. solanacearum* were produced from ascitic fluids of mice using standard procedures. The McAb is preserved in vials, each containing 0.1 mL of conjugated antibody.

Preparation of samples

Samples can be collected from diseased or artificially inoculated seedlings or seeds. Healthy seedlings and seeds should be used as controls. Seeds or stems of seedlings are cut into small pieces, placed in small plastic bags, placed in a PBS buffer (1:1 w/v), pressed, and allowed to soak. The sample is centrifuged at 1000 rpm for 5 min to remove the pellet, and then at 10 000 rpm for 10 min to remove the supernatant. The pellet is dissolved in an extraction buffer (1:10 w/v). The resulting solution can be used for the detection of bacterial infection, or stored at -20°C for later use.

Preparation of buffers

Phosphate-buffered saline (PBS) (pH 7.4)—

Sodium chloride (NaCl)	8 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 g
Sodium phosphate dibasic ($Na_2HPO_4 \cdot 12H_2O$)	2.9 g
Potassium chloride (KCl)	0.2 g
Sodium nitrate ($NaNO_3$)	0.2 g

Dissolve these chemicals in distilled water to make up 1 L.

Coating buffer (pH 9.6)—

Sodium carbonate (Na_2CO_3)	1.5 g
Sodium bicarbonate ($NaHCO_3$)	2.93 g
Sodium nitrate ($NaNO_3$)	0.2 g

Dissolve these chemicals in distilled water to make up 1 L.

Extraction buffer (pH 7.4)—

Polyvinyl pyrrolidine K-25	20 g
Tween 20	0.5 mL
1% egg albumin	10 g

Dissolve these chemicals in 1 L PBS buffer.

Washing buffer (pH 7.4)—
PBS containing 0.05% Tween 20
(1 L PBS + 0.5 mL Tween 20)

Enzyme conjugation buffer—

Ovalbumin	2 g
Tween 20	0.5 mL
Polyvinyl pyrrolidine K-25	20 g

Dissolve these chemicals in 1 L PBS buffer.

Substrate buffer (pH 9.8)—Dissolve diethanolamine (97 mL) in about 0.8 L distilled water, add 0.2 g $NaNO_3$, and adjust the pH to 9.8 with concentrated hydrochloric acid (HCl). Adjust the volume to 1 L with distilled water.

ELISA Protocol

1 Coating of the plate well:

Dilute each vial of IgG from polyclonal antiserum with 10 mL of coating buffer.

Add 100 µL of the mixture to each well and cover the plates tightly.

2 Incubate the plates at 37°C for 2 h.

3 Washing after incubation:

Remove the solution from wells and wash the plate 3 times with washing buffer. Careful washing is extremely important.

4 Addition of the sap diluted in extraction buffer:

Add 100 µL of diluted sap to each well, and add the positive and negative control samples. Cover the plates tightly.

5 Incubate the plates at 4°C for 18 h (overnight).

6 Wash the plates as described above in Step 3.

7 Addition of conjugated monoclonal antibody:

Dilute one vial of 0.1 mL of conjugated monoclonal antibody with 10 mL of conjugation buffer.

Add 100 µL of mixture solution to each well, and cover plates tightly.

8 Incubate the plates at 37°C for 1 h.

9 Wash the plates as described above in Step 3.

10 Addition of enzyme substrate:

Dissolve 7.5 mg of p-nitrophenyl-phosphate in 10 mL of substrate buffer.

Add 100 µL of the enzyme substrate to each well.

11 Incubate for 60 min at room temperature in the dark.

12 Stop the reaction with 50 µL of 3 M NaOH.

13 Measure the absorbance by an ELISA reader at 405 nm, or evaluate visually.

The reaction is positive if the absorbance value of the tested sample is twice as high as the negative control, or if color appears in the well.

Detection and Identification of *Pseudomonas solanacearum* using a Polymerase Chain Reaction Technique

S Seal

Background

The test described in this chapter is based on the detection of *Pseudomonas solanacearum* by amplification and visualization of part of the bacterium's genetic information. The latter is encoded for by a double-stranded DNA circular bacterial chromosome, and often, one or more bacterial plasmids.

The nucleic acid strand has a direction defined by the orientation of its deoxyribose-phosphate backbone. One end is termed the 5' end and the other the 3' end, and copying of DNA is carried out by enzymes (e.g., polymerases) which copy in the 5' to 3' direction. Because of specific base pairing, if the sequence of one strand is known, then the sequence of the opposite strand can be deduced, and will be generated by copying enzymes known as DNA polymerases.

When double-stranded DNA is exposed to near-boiling temperatures, the hydrogen bonds between the two strands are broken, resulting in the DNA separating into its two complementary single strands. This is called denaturation. At temperatures $<70^{\circ}C$, the strands start recombining, positioning themselves such that they form a perfect match with their complementary strand. This is called renaturation or re-annealing.

The Polymerase Chain Reaction (PCR)

The PCR reaction was devised to take advantage of certain features that occur naturally during DNA replication. DNA polymerases copy DNA from a point where double-stranded DNA becomes single-stranded DNA. The enzymes need a small section of double-stranded DNA to initiate synthesis of a complementary new strand from the 3' end of this double-stranded section.

Thus, when the DNA of interest is heated to separate the strands, and a short piece of another DNA (termed an oligonucleotide or PCR primer) that is known to be a perfect match to the former, is added, the polymerase starts copying from the 3' end of the PCR primer. Both strands can be copied if a primer to each strand is supplied. However, to achieve the desired exponential PCR amplification, the primers on the opposite strands should be close enough to each other [ideally within 2 000 base pairs (bp) of each other], so that the synthesized DNA from one strand extends at least as far as the corresponding position of the primer for the opposite strand.

The starting materials for a PCR reaction are the sample or extract to be tested, two (or more) PCR primers, DNA polymerase (e.g., Taq poly-

merase), and a mixture of all four deoxynucleotides (dNTPs, i.e., dATP, dCTP, dGTP, and dTTP) in the correct concentration in a reaction buffer optimized for the DNA polymerase activity.

PCR cycling procedure

Heat the reaction mixture to 96°C for 2–3 min to separate the double-stranded DNA molecules.

Lower the temperature to one suitable to the PCR primers to anneal to the single-stranded DNA, thus giving rise to suitable templates for the DNA polymerase.

Increase the temperature to the optimum level for the activity of the DNA polymerase (for Taq polymerase, this is 72°C).

Incubate the reaction for sufficient time to allow synthesis of the DNA that lies between the two primers on opposite strands. For short products, i.e., < 500 bp, 20–30 sec at 72°C are sufficient.

The above three steps together form what is termed the first PCR cycle. Subsequent denaturation, annealing, and extension cycles are performed with the same parameters, except that the denaturation temperature and duration can be reduced, as the time required to separate the short synthesized DNA strands from their template is less than that needed to separate the two strands of the entire bacterial chromosome. Copied DNA will act as a template in subsequent cycles, resulting in exponential amplification of the sequence that lies between the primers. Under optimum conditions, this will lead to the target sequence being amplified a million-fold, after 22 cycles of amplification (e.g., with 24 cycles, the number of double-stranded DNA that will be amplified is 4 194 304).

A typical PCR is carried out for 30–50 cycles, depending on the sensitivity required.

The development of this PCR detection test for *P. solanacearum* has been published (Seal et al. 1993). The test can detect the DNA from one bacterial cell. For the protocol described below, the maximum volume that can be tested is 31.3 µL, and therefore, the sensitivity of the test is approximately 30 cfu mL^{-1} for bacterial suspensions. Sensitivities for plant extracts will not be as high, due to the presence of compounds which inhibit the polymerase enzyme used for PCR. Sensitivities of 100 cfu mL^{-1} potato tuber extract can generally be achieved. The corresponding sensitivity for groundnut extracts has not yet been determined.

Preparation of samples to be tested by PCR

Materials required—

1 small (50 mL) beaker containing domestic bleach (5–10% sodium hypochlorite)

3 large (250 mL) beakers, each containing at least 100 mL distilled water

1 beaker containing 95–100% ethanol

Two or more scalpels

Bunsen burner

Microcentrifuge

Micropipetters and sterile tips

Sterile Eppendorf tubes (preferably screw-capped type)

Sterile distilled water

Selective media plates (optional).

Sterilization and removal of DNA from scalpels between samples

1 Leave scalpel for at least 5 min in bleach solution to degrade DNAs.

2 Rinse two to three times in successive distilled water in large beakers to remove traces of bleach, then dip in alcohol in a beaker, and flame.

If two scalpels are available, one can be left in the bleach solution while the other is being used to cut a sample.

Note—Wear sterile gloves while processing samples and carrying out the assays.

Sampling plant tissue (e.g., potato tubers)

1 Wash soil off the surface of the tuber.

2 Cut a cone-shaped plug from the stolon end (i.e., the end originally closest to the plant) using a sterile, bleach-treated blade.

3 Flood the resulting hole with 200–500 µL sterile distilled water, depending on the size of the hole cut.

4 Remove water from the hole after 1 min, and concentrate bacteria by centrifugation for 5 min at 12 000 g.

5 Carefully remove water from the sample, leaving 50–100 µL in the tube.

6 If a culture of the bacterium is required, resuspend the pellet, and streak out one loopful onto a selective agar medium.

7 Boil the sample as soon as possible to reduce production of phenolics in the sample (method for boiling is given below).

Sampling stems

1 Wash the surface of the stem to remove soil.

2 Cut stem close to the bottom of the plant with a bleach-treated scalpel, and place the stem in sterile distilled water. Use the minimum volume of water necessary to cover the cut end of the stem.

3 Leave for 10 min for bacteria to ooze out, remove stem, and concentrate the suspension by centrifuging as described earlier.

If the total volume of water is more than 1 mL, repeat centrifugation, carefully removing and discarding the supernatant after each spin, and leaving 50–100 µL to avoid disruption of the pellet.

4 Finally, resuspend the pellet by vortexing in the 50–100 µL of water left after removing the supernatant with a micropipette. Boil the sample as described below.

Testing cultures

1 Pour about 1 mL of 12 to 36-h-old tryptone yeast glycerol broth (TYGB) culture into a screw-capped Eppendorf tube, and spin at full speed (12 000 g) in a microcentrifuge for 5 min. A bacterial pellet should form after centrifugation. If it does not form, add another 1 mL of culture to the tube (after decanting the supernatant), and repeat centrifugation.

2 Wash bacterial cells three to four times with 1 mL sterile distilled water, spinning and decanting the supernatant each time.

3 Resuspend cells in 50–100 µL water, and boil.

Note—Minimal media broth cultures can be tested directly without washing cells.

TYGB broth

This broth, or a *P. solanacearum* minimal media broth, should be used to grow bacterial cells to be tested by polymerase chain reaction (PCR). Cells grown in some other media, may not be lysed adequately by boiling.

Tryptone	5 g
Yeast extract	3 g
Glycerol	20 mL

Make up to 1 L with distilled water.

Divide this broth into 5–50 mL volumes, and autoclave at 121°C for 15 min.

Soil samples The PCR test is not suitable for soil samples, due to the presence of PCR-inhibitory substances. Soil extracts can be plated on selective media, and then the PCR test done on the colonies of *P. solanacearum* isolated.

Boiling the samples

1. Use boiling water, rather than a dry heating block, to provide good heat transfer.
2. Before boiling, concentrate samples, if necessary, by centrifugation (12 000 g, 5 min), and removing some of the upper liquid using a pipette. Do this carefully, and resuspend the pellet by vortexing.
3. Boil samples for 10 min as soon as possible after preparation.
4. Allow samples to return to room temperature before testing. Do not freeze or put on ice. Once the samples have cooled to room temperature, spin the tubes for a few seconds to remove condensation on the tube lid.
5. Proceed with PCR testing, or freeze samples till required.

Pseudomonas solanacearum PCR Protocol

Solutions required

dNTP mix (1.25 mM each dNTP)—

dATP (100 mM)	12.5 µL
dCTP (100 mM)	12.5 µL
dGTP (100 mM)	12.5 µL
dTTP (100 mM)	12.5 µL
Sterile distilled water	950 µL
Total volume	1 mL

10x Tris-borate-EDTA (TBE) buffer—

Dissolve in 500 mL distilled water

Tris-base	108 g
Boric acid	55 g
Add 0.5 M sodium EDTA	40 mL

Adjust to pH 8.3 with NaOH, and make up to 1 L with water.

1.5% Agarose gel in 1x TBE buffer—

1. For each 100 mL 1x TBE buffer, dissolve 1.5 g of 1.5% agarose gel by boiling over a flame or for 2 min in a microwave oven. Take extra care if a microwave oven is used, as the gel solution can get very hot, and can boil over causing severe burns. Make sure the agarose has dissolved totally, then cool to 50–60°C. Pour into a level tray, and place the comb in position ensuring that there is about 1 mm gap between the gel tray and the bottom of the comb.
2. Allow gel to set for 30–60 min. Remove the comb from the gel when it is properly set, and submerge the gel in a tank containing 1x TBE buffer.

5x Orange G loading dye (for 5 mL)—

FICOLL® 400	0.75 g
Orange G	0.0125 g
0.5M EDTA (pH 8.0)	400 µL
Sterile distilled water	4.5 mL

100 bp ladder (1 µg 10 µL^{-1})—

Sterile distilled water	150 µL
5x Orange G loading dye	40 µL
100 bp ladder stock (1 µg µL^{-1}; Pharmacia)	10 µL

Ethidium bromide stock (10 mg mL^{-1})—Dissolve one 100-mg ethidium bromide tablet (Sigma) in 10 mL sterile water. This product is carcinogenic; handle it with maximum care.

Setting up the PCR reactions

Always wear clean gloves that fit well when handling PCR reagents and tubes. This avoids contaminating the tubes with microorganisms and DNA-degrading enzymes present on the skin. The PCR reactions are carried out in 50-µL volumes, each reaction consisting of:

dNTP mix (1.25 mM each dNTP)	8 µL
10x PCR buffer	5 µL
25 mM magnesium chloride ($MgCl_2$)	3 µL
Primer OLI1 (20 µM)	1.25 µL
Primer Y2 (20 µM)	1.25 µL
Taq polymerase (5U µL^{-1})	0.2 µL
DNA sample (equivalent 25 ng DNA)	1-31.3 µL

Sterile distilled water (0-30.3 µL) to make a total volume of 50 µL.

The above reagents are generally supplied commercially at the correct concentrations, with the exception of the primer stocks, which should be diluted to 20 µM, and the dNTP mix.

Making a mix for more than one sample

It is advisable to include two positive and two negative controls in each PCR run. Hence, a minimum of five PCR tubes should be set up. It is more convenient and accurate to make a mix of all ingredients (except for the sample), and subsequently place the desired aliquot of the mix in each of the PCR tubes.

For example, if you have 2 plant samples, make a mix for 2 + 4 controls + 1 extra = 7 samples. Test 5 µL of the sample, and add 26.3 µL sterile distilled water for each sample. A mix for 7 would therefore be:

Sterile distilled water	184.1 µL
dNTP mix	56 µL
10x PCR buffer	35 µL
25 mM $MgCl_2$	21 µL
Primer OLI1 (20 µM) (5'GGGGGTAGCTTGCTACCTGCC3')	8.75 µL
Primer Y2 (20 µM) (5'CCCACTGCTGCCTCCCGTAGGAGT3')	8.75 µL
Taq polymerase (5U µL^{-1})	1.4 µL
Total volume	315 µL (= 7 × 45 µL)

Procedure Mix all the ingredients well together using 'PCR-only' micropipetters, and place 45 µL of the mix at the bottom of each of the six tubes. Then add 5 µL of the sample to each tube. For pipetting the samples, use another (non-PCR) P20 Gilson® micropipetter. This is necessary to eliminate the possibility of cross-contaminating the barrel of the micropipetter used for the PCR set up.

Add 5 µL of each sample (clear liquid, not plant material) to the mix at the bottom of each tube. Add one drop of mineral oil to each tube, and close the tubes before finally setting up the positive controls (5 µL diluted *P. solanacearum* DNA or boiled cells).

Always add 5 µL water to the negative controls first. Cover both with one drop of mineral oil, and then close the tubes before moving onto the samples. Once the oil has been added to the positive controls and the tubes have been closed, they are transferred to a Programmable Thermal Controller, with the following heating profile setting:

Initial denaturation	96°C, 2 min 30 sec × 1 cycle
Denaturation	94°C, 15–30 sec
Annealing	67–68°C, 15–30 sec (depending on machine) × 50 cycles
Extension	72°C, 20 sec
Final extension	72°C, 5 min × 1 cycle

After the cycle is completed, switch off the machine when convenient.

Add 10 µl of 5x Orange G loading dye to each sample, and mix well with the bottom phase.

Take 10–15 µL of the mixture, wipe mineral oil off the pipette tip, and load into a well of the 1.5 % agarose gel in 1x TBE.

Include 10 µl of the 100 bp ladder (Pharmacia). Run the gel at 100–120 V for about 1 h (till the Orange dye has run about 5 cm or more down the gel), and then stain in 0.5 µg mL^{-1} ethidium bromide solution (150–200 mL).

Any sample with a band at 300 base pairs (3rd band from bottom of 100 bp ladder), contains DNA of *P. solanacearum*.

Precautions During PCR Assay

A corollary of the tremendous amplification power of PCR is that minute amounts of contamination of the samples can lead to false positive results. The most common vehicles for cross-contamination are gloves and micropipetters. Ensure that clean gloves are used to set up PCR, and that care is taken to avoid contact of the glove finger tips with the samples to be tested. Micropipetters are commonly contaminated by aerosols, which are generated during pipetting of positive samples. These end up in other tubes, on gloves, and in the barrel of the pipetter, from where they can be released into subsequent PCR tubes. Without adequate precautions, this will become a frequent occurrence due to the fact that a single 50-µL amplified PCR reaction mixture contains billions of copies of the target DNA, of which only one in a subsequent PCR reaction is enough to generate a false positive result. Suitable precautions to limit such false positives (in addition to careful working) are either to use filtered micropipette tips that prevent contamination of the barrel or to use one set of micropipetters solely to pipette PCR reagents (and not the samples to be

tested). Moreover, it is advisable to use a separate work area for setting up PCR, and never use it for gel electrophoresis of PCR products or preparation of samples to be tested. The inclusion of multiple negative controls is essential in order to monitor and reveal cross-contamination.

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Infectivity Titrations for Plant Disease Evaluation

K Y Lum

Principles

Infectivity titration is a technique that gives a measure of the relative capacity of an organism to infect its host. The basic principles of infectivity titration have been derived from earlier work in the field of medical bacteriology, but have evolved with time to be applicable to almost any infective system (Meynell and Meynell 1979), including bacteria-plant systems (Boelema 1973, 1977, Ercolani 1973, 1976, Goto 1978, Hartman and Kelman 1973, Lum and Kelman 1981, Perombelon 1972, Victoria and Kelman 1977). The essential steps in an infectivity assay are given below.

Essential steps

1 Preparation of serial dilutions of known concentrations of the bacterial pathogen.

2 Inoculation of each serial dilution into a separate group of the host.

3 Recording the response of each individual host after a specific incubation period.

4 Expression of the recorded data as an estimate of the magnitude of the response.

Infectivity titrations can be used to estimate the relative resistance of two or more cultivars to a given bacterial species or strain, or the relative virulence of two or more bacterial strains on a specific host. Such estimates could be expressed either in terms of differences in the doses required to elicit the same level of response from the host, or differences in the response of the host to the same dose of the bacterial strain.

Any indicating effect can be used, as long as it is related to disease severity (wilting, death, etc.). Both host and pathogen effects can be taken as quantal or quantitative responses. Although infectivity titrations with quantitative responses can offer greater precision than with quantal responses (Meynell 1957, Meynell and Meynell 1979), they have limited applications, especially with plant pathogenic bacteria. Quantal effects are all-or-none responses (alive or dead, healthy or diseased, turgid or wilted).

The frequency of response in infectivity titrations with microorganisms in living hosts is often related to the logarithm of the inoculum dose in the form of an integrated log-normal distribution (Meynell and Meynell 1979, Finney 1971). Linearity of the log-normal dose-response curve can be obtained by transforming the proportion of the response values into probits, and plotting the probit values against the logarithm of the inoculum dose. Responses outside the 0.05–0.95 levels are disregarded, since these values correspond to very extreme points which carry little weight (Finney 1971).

In infectivity titrations based on quantal responses that are transformed into probits, the parameters of the log-dose/probit-response curves are:

- the median effective dose (ED_{50})—the dose that causes a response in 50% of the inoculated plants;
- the slope (B) of the probit regression line.

These parameters describe the relationship between dose and response for any host-pathogen combination, and are derived directly from the linear equation $y = a + Bx$, following probit analysis.

Methodology

Cultures

1 Maintain cultures as suspensions in 5 mL sterile distilled water in plastic-capped glass culture tubes stored at approximately 20°C.

2 Before using for inoculations, streak cultures onto triphenyl tetrazolium chloride (TZC) agar plates (Kelman 1954) to check for purity and virulent colony type.

3 Pick up typical fluidal colonies, and streak onto CPG (basal TZC medium) agar plates. Incubate the plates for 48 h at 30°C before use in the preparation of inoculum.

Preparation of inoculum

1 Suspend the bacterial growth on the CPG plates in 10 mL of sterile distilled water.

2 Mix the suspension thoroughly, and adjust to a concentration of approximately 5×10^9 cfu mL^{-1}, using a spectrophotometer and previously constructed calibration curve.

3 Prepare appropriate serial (10-fold) dilutions. Concentrations of viable cells in these dilutions can be verified by plating them out on tetrazolium chloride agar (TZCA) medium.

Propagation of test plants

Procedures for the propagation of test plants must be standardized as far as possible.

1 Raise plants in a sterile soil mix in individual pots or bags, and maintain them under standardized growth conditions.

2 Use 3- to 4-week-old plants. It is important to select plants which are homogenous in size and age for an experiment.

Inoculation procedures

1 Use the stem inoculation procedure of Winstead and Kelman (1952), modified to deliver a predetermined (fixed) number of bacterial cells to each test plant.

2 This can be achieved by using either a sterile, calibrated glass micropipette or a disposable micropipette tip.

3 Generally, about 50 µL of bacterial suspension of known concentration is taken up by the plant within 2–3 h, when inoculated into the stem at the third leaf axil.

4 The micropipette should be left in place until the bacterial suspension has been completely taken up by the plant.

5 A minimum of 20 plants is recommended for each treatment dose, with at least 6–8 serial dilutions per experiment. Generally, the range of concentrations should result in 10^8–10^1 cfu of the pathogen being delivered to the plant.

Evaluation of resistance/virulence

1 To determine and compare the virulence of different isolates of the pathogen, infectivity titrations must be carried out with each isolate using the same host cultivar, and preferably in a single experiment.

2 Similarly, to compare the resistance of different host cultivars, titrations must be carried out with the same isolate on different cultivars in a single experiment.

Data collection and analysis

1 Data on host response for each group of host plants are recorded as 'percentage quantal response', i.e., the percentages of the inoculated plants that wilted permanently or did not wilt permanently. These figures can then be converted into probit units from probit tables.

2 Inoculum doses are similarly converted to log-dose for use in plotting the probit response curve.

3 From the probit response curve and the regression line equation generated, obtain the ED_{50} value for each pathogen/host combination.

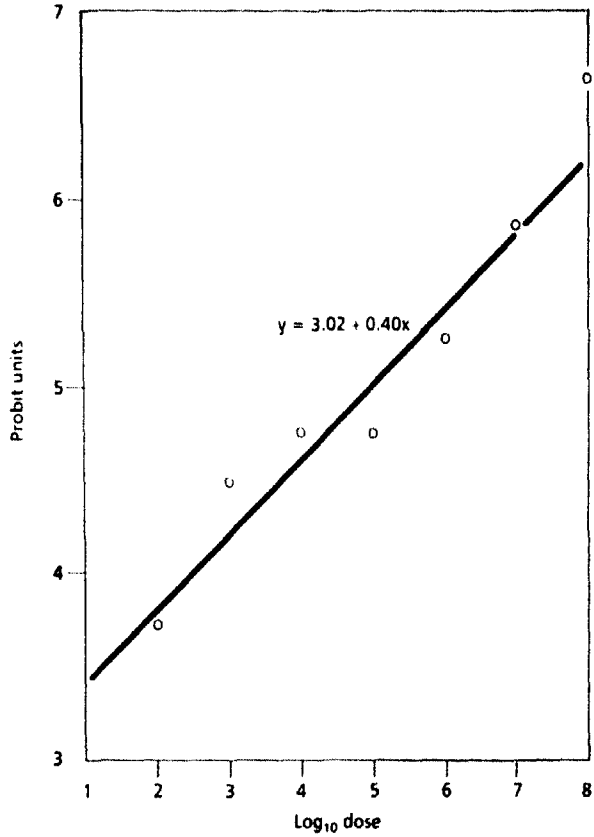

Figure 1—Log-dose/probit response relationship for tomato cultivar 'Bonny Best' and Pseudomonas solanacearum *strain 338.*

Interpretation of Results

Figure 1 shows a typical probit regression line describing the dose-response relationship between tomato cultivar 'Bonny Best' and *P. solanacearum* strain 338. The fitted regression line is described by the equation $y = 3.02 + 0.4x$. The ED_{50} value derived from the regression equation is 8.28×10^4 cfu mL^{-1}. ED_{50} values can be directly compared to give an indication of the relative resistance of a number of cultivars to a particular isolate of the pathogen (Table 1). On the other hand, a comparison of the ED_{50} values for different isolates on a single cultivar gives a relative measure of their virulence (Table 2).

Table 1—Probit analyses of the quantal responses of five tomato cultivars to graded inoculum doses of Pseudomonas solanacearum strain 341 from Florida, USA.

Cultivar	Probit analysis[1]		
	Slope (B)[2]	$\log_{10}ED_{50}$	ED_{50} value
Bonny Best	0.205 ± 0.061	2.185 ± 0.745	1.53×10^2
Saturn	0.208 ± 0.061	3.077 ± 0.752	1.19×10^3
72-TR12-2	0.226 ± 0.052	3.456 ± 0.543	2.86×10^3
72-TR12-3	0.221 ± 0.054	3.765 ± 0.586	5.82×10^3
Venus	0.212 ± 0.061	3.882 ± 0.619	7.62×10^3

1 r values for the probit regression lines ranged from 0.94 to 0.99.
2 Slope (B) judged equal to $p = 0.86$.

Table 2—Probit analyses of quantal responses of tomato cultivar Bonny Best to graded inoculum doses of eight strains of Pseudomonas solanacearum.

Strain	Geographic origin	Probit analysis[1]			Virulence rank
		Slope (B)[2]	$\text{Log}_{10}ED_{50}$	ED_{50} value	
143	Australia	0.493 ± 0.088	3.440 ± 0.272	2.76×10^3	5
196	Philippines	0.510 ± 0.079	4.269 ± 0.264	1.86×10^4	8
221	Kenya	0.562 ± 0.100	4.032 ± 0.261	1.08×10^4	7
234	Brazil	0.555 ± 0.116	3.146 ± 0.273	1.40×10^3	3
270	Taiwan	0.439 ± 0.085	2.868 ± 0.309	7.38×10^3	6
338	Malaysia	0.506 ± 0.090	3.334 ± 0.273	2.16×10^3	4
341	Florida (USA)	0.625 ± 0.160	1.906 ± 0.290	8.00×10^1	1
342	North Carolina (USA)	0.514 ± 0.119	1.946 ± 0.340	8.80×10^1	2

1 r values for the probit regression lines ranged from 0.87 to 0.99.
2 Slope (B) for all eight regression lines judged equal to $p = 0.80$.

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Inoculation Techniques to Evaluate Resistance of Groundnut to Bacterial Wilt

V K Mehan, Y J Tan, and B S Liao

Background

Several inoculation techniques are used to evaluate resistance of groundnut to bacterial wilt caused by *Pseudomonas solanacearum* (Winstead and Kelman 1952, Kelman 1953, Li and Tan 1984, Tan et al. 1994).

When virulent cultures and succulent host plants are used, successful inoculations with pure cultures of *Pseudomonas solanacearum* can be made by several techniques. These include stem inoculation (stem puncture), hypodermic injection, and root inoculation (Kelman 1953). Soaking seed in bacterial suspension (6×10^8 cfu mL^{-1}) for 30 min is also a useful inoculation technique (Li and Tan 1984). Infested soil placed in pots or other containers can also be used as a source of inoculum.

The root inoculation technique, however, seems to be the best procedure to evaluate plants for resistance to *P. solanacearum*. Marked differences between resistant and susceptible genotypes can be identified by this technique. Inoculation by stem puncture usually produces higher wilt levels in resistant groundnut plants than does root inoculation. However, stem inoculation has an advantage in the differentiation of lines with very high levels of resistance.

Preparation of Inoculum

1. For inoculum preparation, use a highly virulent culture of *P. solanacearum*, maintained in sterile distilled water at room temperature.
2. Culture the bacterium on tetrazolium chloride agar (TZCA) to identify the virulent colonies (pink in the center and white on the edges).
3. Select the virulent colonies to multiply inoculum on sucrose peptone agar (SPA) or other suitable agar medium.

Inoculation Techniques

Stem inoculation

1. Prepare bacterial inoculum from 2- to 3-day-old cultures of highly virulent isolates grown on sucrose peptone agar (SPA) slants.
2. Wash the growth on a slant in 5 mL sterile distilled water, mix well, and dilute in sterile distilled water to obtain a bacterial population of 1×10^8 cfu mL^{-1}.
3. Inoculate 2-week-old plants by forcing a sharp needle into the stem through a droplet of bacterial suspension placed in the axil of the third fully expanded leaf below the stem apex. Alternatively, inject 100 µL of the suspension into the leaf axil with a hypodermic syringe.

4 Cover the inoculated plants with polythene for 24 h to prevent the droplet of suspension from drying out.

5 Incubate the plants in a greenhouse or growth chamber under the following conditions: temperature 30–32°C during the day and 25°C at night, RH > 85%, with 12 h light and 12 h dark periods. It is important to grow inoculated plants in high soil moisture conditions to ensure disease development.

6 Observe the wilt symptoms developing from 5 to 21 days after inoculation.

7 Record disease ratings using the following scale:

1 no symptoms

2 one leaf wilted at the inoculation point

3 two to three leaves wilted

4 four or more leaves wilted

5 whole plant wilted (dead plant).

8 Calculate the wilt intensity 21 days after inoculation, using the following formula (Winstead and Kelman 1952):

$$I = \frac{\Sigma(n_i \times v_i)}{V \times N} \times 100$$

where I = wilt intensity (%); n_i = number of plants with respective disease rating; v_i = disease rating (1, 2, 3, 4 or 5); V = the highest disease rating (5); and N = the number of plants observed.

Root inoculation Prepare bacterial inoculum as described above.

1 Uproot 2-week-old plants (grown in sterilized soil) from pots and wash off the soil from the root systems.

2 Trim the roots with scissors or a scalpel along one side of the plant, and pour 10 mL of bacterial suspension (1×10^8 cfu mL^{-1}) over the injured roots. Plant the inoculated plants in sterilized soil in suitable containers.

3 Grow the plants in a greenhouse or growth chamber as in Step 5 of the stem inoculation technique (see above).

4 Observe wilt symptoms from 5 to 21 days after inoculation. Calculate the wilt intensity as described above for the stem inoculation technique.

Infested soil Susceptible hosts can be planted in soil to which diseased plant debris has been added. Naturally infested soil placed in containers or in pots can also be used as a source of inoculum. However, results with these techniques are often erratic.

Seed inoculation Seed inoculation is a uniform and simple artificial inoculation technique suitable to screen large numbers of genotypes for wilt resistance.

1 Prepare bacterial inoculum from 2- to 3-day-old cultures of highly virulent isolates grown on SPA slants.

2 Wash the growth on a slant in 5 mL sterile distilled water, mix well, and dilute in sterile distilled water to obtain a bacterial population of 6×10^8 cfu mL^{-1}.

3 Soak seeds in the bacterial suspension for 25–30 min.

4 Sow the inoculated seeds in sterile soil in pots in the greenhouse or in the field. It is important to keep the soil wet to ensure disease development.

5 Incubate the pots with inoculated seed in a greenhouse or growth chamber under the following conditions: temperature 30–32°C during the day and 25°C at night, RH > 85%, with 12 h light and 12 h dark periods.

6 Observe wilt symptoms from 20 days after sowing. Calculate the wilt intensity as described above, for the stem inoculation technique. (Disease incidence in the field should be recorded).

Note—For successful resistance screening, it is important to maintain high temperature and high soil moisture levels after inoculation.

Field screening

Field screening under uniform, high disease pressure is useful to identify sources of resistance. It is desirable to use the same field each year to encourage buildup of the bacterial inoculum in the soil. Heavy clay or sandy soil fields are suitable for resistance screening. Genotypes to be screened are sown in replicated plots, with rows of a highly susceptible cultivar (Chico or J 11) arranged systematically throughout the trial. Good disease development is ensured by providing high soil moisture for up to 50 days after sowing.

A visual estimate of the percentage of wilted plants is an efficient evaluation method when large numbers of genotypes are to be tested.

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Identification of Pathotypes of *Pseudomonas solanacearum*

Y J Tan, B S Liao, and V K Mehan

Introduction

The term pathotype is used for strains of *Pseudomonas solanacearum* (Smith) Smith, specialized in pathogenicity/virulence on specific host cultivars. Strains of the wilt pathogen differ greatly in their virulence on groundnut. Pathotypes can be identified, based on their pathogenicity/virulence to indicator-cultivars with different levels of wilt resistance (Table 1).

***Table 1—Indicator host cultivars that can be used to identify pathotypes of the wilt pathogen,* Pseudomonas solanacearum.**

Indicator-cultivar	Resistance/ susceptibility level	Wilt incidence (range)
Jiankang	Highly resistant	<10%
Taishan Sanlirou	Highly resistant	<10%
Xiekangking	Highly resistant	<10%
Gouliaozhong	Moderately resistant	20-30%
Huangchuan Zhigan	Moderately resistant	20-30%
Zhonghua 117	Moderately susceptible	35-50%
E Hua 3	Susceptible	>50%
Zhao 18	Highly susceptible	>90%

Pathotype identification

1 Select several naturally infested bacterial wilt hot-spot locations, preferably with high populations of *P. solanacearum*.

2 Grow the indicator-cultivars in naturally infested fields in disease hot-spot locations, using randomized block designs. Each indicator-cultivar should be grown in replicated plots of 8 rows, 30 cm apart and 5 m long, with the seeds sown singly at 10-cm spacing; use of a seed protectant is recommended.

3 From 30 days after sowing, record the bacterial wilt incidence in replicated plots of each cultivar at 2-week intervals, up to 70 days after sowing.

4 Obtain isolates of the wilt pathogen from wilt-affected plants from each plot, and check for their identity on tetrazolium (TZC) agar medium.

5 Determine the biovars of the isolates of *P. solanacearum* obtained from wilt-affected plants from different locations. Biovars can be determined using the procedures described by Hayward (1964) and He et al. (1983).

6 Inoculate the indicator-cultivars with various isolates of *P. solanacearum*, collected from different locations, using a standard inoculation technique. The seed inoculation technique is useful for artificial inoculations; inoculated seeds should be sown in sterile soil in pots in the greenhouse at 30–32 °C during the day and 25°C at night, RH > 85%, with 12 h light and 12 h dark periods. It is important to keep the soil wet to ensure disease development.

7 Observe the reactions of the indicator-cultivars to different isolates of the wilt pathogen; record the wilt incidence and severity. Wilt symptoms can be observed 20 days after sowing.

Based on the reaction types of the indicator-cultivars, the isolates of *P. solanacearum* can be divided into different pathotypes. The reactions of the indicator-cultivars in naturally infested field plots can reveal the predominant pathotype in a location.

Table 2 shows how isolates of *P. solanacearum* can be identified as pathotypes using diagnostic cultivars.

Table 2—Identification of pathotypes of Pseudomonas solanacearum (isolated in China) using indicator-cultivars.

Groundnut cultivars[1]	Pathotypes						
	1	2	3	4	5	6	7
Xiekangking	R	R	R	M	M	M	S
Taishan Sanlirou	R	R	M	R	M	M	S
Huangchuan Zhigan	R	M	M	M	M	M	S
Lukangqing	R	M	M	M	M	M	S
Fuhuasheng	S	S	S	S	S	M	S
Ehua 1	S	S	S	S	S	M	S

1 R = highly resistant; M = moderately resistant; and S = susceptible.

References

Hayward, A.C. 1964. Characteristics of *Pseudomonas solanacearum*. Journal of Applied Bacteriology 27:265–277.

He, L.Y., Sequeira, L., and **Kelman, A.** 1983. Characteristics of strains of *Pseudomonas solanacearum* from China. Plant Disease 67:1357–1361.

Glossary

Adjuvant Substance injected with an antigen that nonspecifically enhances or modifies the immune response to that antigen. Antibody production or the reactions of well-mediated immunity are more vigorous than would be the case if the antigen had been injected alone.

Affinity Strength of the antigen-antibody interaction. The strength of bonds formed by this reversible interaction determines the rate of association between antibody and antigen versus the rate of dissociation. High-affinity antibodies have a higher rate of association with an antigen, and bind more antigen than low-affinity antibodies.

Antibody Immunoglobulin molecule produced by an animal's B or T lymphocytes in response to an antigenic stimulus and capable of binding to an antigen.

Antigen Substance, usually protein or polysaccharide, capable of eliciting production of specific antibodies and capable of specifically binding to antigen-binding sites of antibody molecules. All immunogens are antigenic, but not all antigens are immunogenic.

Antiserum Serum from any animal containing antibodies to a specified antigen.

B cells or T cells (lymphocytes) Antibody-producing cells, found mainly in the blood, lymph nodes and spleen.

BACTID program Expert system (computer software) written around the BACTID system. Enables bacteria to be identified from test results while providing explanation (via hypertext) of the system, component tests (with ingredients and recipes for media and reagents), and the different bacterial groups.

BACTID scheme Scheme for identification of plant pathogenic bacteria based on elimination of saprophytes and nonpathogens at an early stage, and identification of pathogens to sufficient depth for pest-management decision-making purposes.

BACTID system System for identification of plant pathogenic bacteria appropriate to small, poorly equipped laboratories where full identification is not possible or cost-effective. Comprises BACTID scheme, BACTID kit, BACTID program. BACTID can be integrated with the BIOLOG® system.

BACTID Training Kit Kit for convenient demonstration of the tests included in the BACTID scheme. (Formerly BACTID plate kit). Eight media (for most of the tests) are arranged in rows of a 96-well microtiter plate; up to twelve isolates and controls can be run at one time.

BACTID Tube Kit All the tests in the BACTID scheme (and some additional tests for *Pseudomonas*) are done in microcentrifuge tubes; this gives more flexibility for routine identification.

BIOLOG® system System for identification of bacteria by matching the metabolic profile of an unknown isolate with standard profiles of known taxa.

Biovar Infrasubspecific term for strains of a species with distinctive biochemical or physiological properties. *Pseudomonas solanacearum* is divided into five biovars based on their ability to oxidize/utilize three disaccharides (cellobiose, lactose, and maltose) and three hexose alcohols (dulcitol, mannitol, and sorbitol).

Cross-reacting antigen Antigen capable of combining with antibody produced in response to a different antigen. Can cross-react due to sharing of determinants by the two antigens or because the antigenic determinants of each, although not identical, are closely related stereochemically to combine with antibody against one of them.

ELISA (enzyme-linked immunosorbent assay) Immunoassay system employing an enzyme bound to an antibody, as the immunologic probe determining the extent of antigen-antibody reaction.

Epitope Antigenic determinant of defined structure, e.g., an identified oligosaccharide, or a chemical hapten.

Expert system Computer program that assists with identification and analysis of problems and decision-making by processing a series of rules; it attempts to represent the knowledge and experience gained by an 'expert' in the field.

Freund's adjuvant Mixture of mineral oil and lanolin that enhances immune responses when emulsified with antigen for immunization. Freund's complete adjuvant includes killed mycobacteria, while Freund's incomplete adjuvant does not.

GN database Database of metabolic profiles of Gram-negative bacteria that can be used in the BIOLOG system.

Hapten Substance that can combine with antibody, but cannot initiate an immune response unless it is bound to a carrier before introduction into the body of an animal. Most haptens are small molecules and carry only one or two antigenic determinants, but some macromolecules are haptenic.

Hybridoma Cell or cell line formed from the fusion of a lymphocyte with a tumor cell.

Hypertext Explanatory text that can be called up onscreen in an expert system to assist decision making and interpretation of results.

Immunogen Substance that elicits an immune response when introduced into the tissues of an animal. To stimulate a response, immunogens must normally be foreign to the animal to which they are administered, of a molecular weight greater than 1000, and of protein or polysaccharide nature.

Immunoglobulin (Ig) Serum globular glycoprotein. There are five classes of immunoglobulin: IgA, IgD, IgE, IgG, and IgM. IgG is the major immunoglobulin class in the serum of human beings and in most species from amphibians upward.

Immunoglobulin classes Subfamily of immunoglobulins, based on large differences in H-chain amino acid sequence: IgA, IgD, IgE, IgG, IgM.

Immunoglobulin subclasses Subpopulations of an Ig class based on more subtle differences in the H chains than are class differences. The H chain is a pair of identical polypeptide chains, of the four-chain immunoglobulin molecules.

L chain (light chain) Polypeptide chain present in all immunoglobulin molecules in two forms, κ or λ. Each four-chain Ig molecule has either two κ or two λ light chains.

Metabolic profile The usually unique metabolic characteristics (or fingerprint) of an organism determined by its ability to metabolize different substrates (usually recorded as positive or negative). In the BIOLOG system, 95 different substrates are presented to a bacterium in individual wells of a 96-well microtiter plate. (The remaining well is a substrate-free control).

MicroLog® software Software for making identifications with the BIOLOG system. It matches profiles from unknown isolates with standard profiles in a database. MicroLog software can be used with the GN database or with databases constructed by the user.

Monoclonal antibody Homogenous antibody population produced by a clone of antibody-forming cells.

Myeloma Neoplastic B-lymphocyte cell line (from mice) which is immortal in cell culture.

Paratope Antigen-combining site in an antibody.

Pathotype Strain specialized in pathogenicity/virulence on specific host cultivars.

Pathovar Strain or set of strains with the same or similar characteristics, differentiated at infrasubspecific level from other strains of the same species or subspecies on the basis of distinctive pathogenicity to one or most hosts.

Polyclonal antibody Heterologous antibody population derived from many clones.

Race Comprises isolates of *P. solanacearum* of a particular origin and host range. *Pseudomonas solanacearum* is divided into five races:

Race 1—affecting solanaceous and other plants including leguminous plants mainly in the lowlands of the tropics and subtropics; it includes biovars 1, 3, and 4.

Race 2—affecting banana, *Heliconia* spp, and plantain; it is indigenous to Central and South America; it includes biovar 1.

Race 3—mainly affecting potato and tomato in the higher latitudes worldwide and in the higher altitudes of the tropics; it includes biovar 2.

Race 4—mainly affecting ginger; it includes biovars 3 and 4.

Race 5—mainly affecting mulberry in China; it includes biovar 5.

Serum Blood plasma which does not contain fibrinogen. Serum is more commonly used than plasma in immunological procedures, because there is no danger of a clot forming when other materials are added to it.

Strains Isolates of the wilt pathogen that differ greatly in their virulence.

Titer In serological reactions, a relative measure of the amount of antibody in an antiserum per unit volume of original serum. The antibody is serially diluted and optimum concentration of antigen is added. Serum titer is indicated as the reciprocal of the highest serum dilution, producing a discernible antigen-antibody reaction.

Vaspar Mixture of white petroleum jelly (e.g., Vaseline® and liquid paraffin).

About ICRISAT

The semi-and tropics (SAT) encompasses parts of 48 developing countries including most of India, parts of southeast Asia, a swathe across sub-Saharan Africa, much of southern and eastern Africa, and parts of Latin America. Many of these countries are among the poorest in the world. Approximately one-sixth of the world's population lives in the SAT, which is typified by unpredictable weather, limited and erratic rainfall, and nutrient-poor soils.

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ICRISAT was established in 1972. It is one of 16 nonprofit, research and training centers funded through the Consultative Group on International Agricultural Research (CGIAR). The CGIAR is an informal association of approximately 50 public and private sector donors; it is co-sponsored by the Food and Agriculture Organization of the United Nations (FAO), the United Nations Development Programme (UNDP), the United Nations Environment Programme (UNEP), and the World Bank.

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International Crops Research Institute for the Semi-Arid Tropics
Patancheru 502 324, Andhra Pradesh, India

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