Techniques for diagnosis of *Pseudomonas solanacearum* and for resistance screening against Groundnut Bacterial Wilt

Training Manual

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International Crops Research Institute for the Semi-Arid Tropics

Techniques for Diagnosis of *Pseudomonas solanacearum*, and for Resistance Screening against Groundnut Bacterial Wilt

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Edited by

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International Crops Research Institute for the Semi-Arid Tropics

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Objectives of the Training Course

The Groundnut Bacterial Wilt Working Group was established in 1990, to coordinate research on bacterial wilt of groundnut, and to ensure uniformity in some critical areas of detection, diagnosis, and resistance screening so that the benefits of research can readily be exchanged among Group members.

Drawing upon the expert knowledge of the Working Group members, we organized this Training Course to:

- update researchers on the methods for the detection and identification of *Pseudomonas solanacearum* and its biovars;
- consider various methods for the diagnosis of groundnut bacterial wilt;
- give comprehensive descriptions of resistance screening techniques;
- demonstrate integrated disease management practices in the field; and
- provide an opportunity for increased professional interaction among Group members.

Introduction

In this manual, we present a comprehensive range of methods for isolation, identification, and detection of the groundnut bacterial wilt pathogen, *Pseudomonas solanacearum*. Emphasis has been placed on the use of enzyme-linked immunosorbent assay (ELISA) to detect the bacterium in plant tissues, seed, and soil. Some of the techniques described have been in use for some time without undergoing any major improvements, while others are of relatively recent origin. The principles underlying the major forms of ELISA are outlined, as also the protocols for the production of polyclonal antibodies, and for ELISA. Some DNA-based diagnostics for *P. solanacearum* are also described. Infectivity titration technique to determine virulence of *P. solanacearum*, and several inoculation techniques to evaluate resistance to groundnut bacterial wilt are also described. The principles underlying each technique, and the technicalities are explained in detail to enable the nonspecialist to understand them.

Feed back from the users of this manual is most valuable, and suggestions for improvement in the clarity of presentation, for addition of new techniques, etc., are most welcome. Suggestions and comments can be sent to Dr V.K. Mehan, Coordinator, Groundnut Bacterial Wilt Working Group, ICRISAT Asia Center, Patancheru, 502 324, Andhra Pradesh, India.

V.K. Mehan

An Integrated System for the Identification of Bacteria

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The BACTID System: Introduction and Background

General

Bradbury (1970) commented on the general neglect of bacteriology in plant pathology laboratories. This applies, especially to laboratories functioning as part of Plant Clinics in less-developed 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 nonpathogens. Another difficulty is the wide range of media and reagents required for definitive identification of species of plant pathogens, in spite of there being a 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 may be hard to procure. Some reagents have to be bought 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 importance of good isolation techniques and of symptomology are stressed, with the need to avoid spending time on saprophytes incorrectly assumed to be significant. The concepts of **presumptive** and **confirmed** diagnosis are presented, it not always being necessary to proceed to full identification.

However, Lelliott and Stead emphasize that plant diseases in temperate regions require full resources. 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:

- resources likely to be available,
- level of identification required for pest management decisions, and

- the need to eliminate saprophytes while ending with isolates 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 may 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, and 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), serology or molecular methods (RFLP, probes, polymerase chain reaction).

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

BACTID Scheme for Preliminary Identification of Plant Pathogenic Bacteria

The scheme 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 standardised approach to **preliminary identification** is used. Some of the tests (or combinations of tests) may be looked upon as identifying certain genera. Other tests are used to approach presumptive diagnosis.

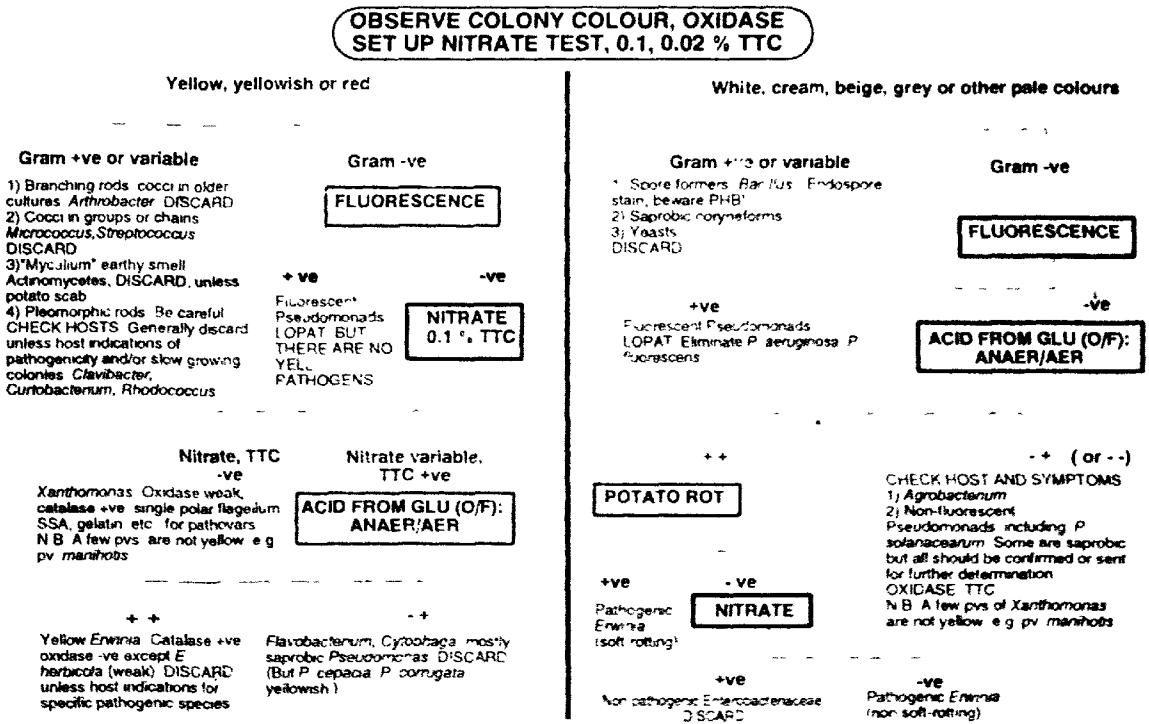

Fig. 1. Flow chart of scheme for preliminary identification of plant pathogenic bacteria in the tropics, emphasizing early elimination of non-pathogens. (Originally devised by senior author and J Kotkowsky of International Mycological Institute)

Starting point

It is essential to start with a pure culture of an isolate showing uniform and, preferably, recognizable characteristics of an organism by its symptoms (e.g., yellow domed, mucoid colonies for *Xanthomonas*). **Symptoms should definitely indicate bacterial disease**

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, TTC: 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 O/F test, then soft-rot (potato) as appropriate. *Alcaligenes* and some other nonpathogens (including *Pseudomonas*) may be nonacid-producing aerobically (in O/F test).

Additional tests: Additional tests could be done to confirm *Xanthomonas* and nonfluorescent white pseudomonads. *Agrobacterium* can be distinguished in the first instance from potentially pathogenic nonfluorescent *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 "saprophyte", but there are no other likely candidates, it should be investigated further; there is always the possibility of rare, obscure or new cases, as a Gram positive or Enterobacteriaceae.

Using the BACTID scheme

Using conventional media and commercial kits

These tests can be set up on conventional media and the scheme on the flow chart followed through. 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. However, these are expensive, hence the kit described below.

BACTID plate kit

A do-it-yourself kit, incorporating all the BACTID tests, has been devised. Most of the tests are done in the wells of a 96-well microtitre plate. Some media are prepared in disposable 7 mL culture tubes. The advantages of the kit are that (a) media can be prepared in advance and stored in the refrigerator ready for immediate use and (b) the kit is convenient for traveling. Further details are given under the heading BACTID Plate Kit below.

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 media and reagents used and instructions to prepare the BACTID plate kit. Further details are given under the heading BACTID Software in this paper.

BACTID Plate Kit

Introduction

The BACTID plate kit has been designed as a do-it-yourself kit, containing all necessary tests for preliminary identification. These are tests for using the program itself (Colony color, Gram, TTC, Oxidase, Fluorescence, Nitrate, O/F and Potato rot), and in addition, there are tests (Catalase, Gelatin, Starch) to help confirm the identification.

The kit consists of a 96-well microtitre plate containing the media for eight of these 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 may be stored in the fridge 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, catalase and oxidase tests are done with loopfuls of cultures grown on a nutrient agar (NA) slope. The

O/F test is included as paired tubes of the usual medium, one for aerobic and the other for anaerobic conditions. ELISA strips of 12 wells with a single row may also be used if single-isolate kits are preferred. At least one column should be reserved as noninoculated control.

Layout of BACTID tests on 96-well microtitre plate and separate tubes (See also Test Proforma, Fig. 2):

BACTID PLATE
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	NA (for oxidase)	Smear												
B	TTC 0.1%	Smear												
C	TTC 0.02%	Smear												
D	KMB	Smear												
E	SNA	Smear												
F	SSA	Smear												
G	NITRATE	Stab												
H	POTATO ROT	Stab												

Specific tests													
	NA slope for:	Streak											
	Colour/ Gram (EOM)												
	Oxidase (touch stroke)												
	Gelatin	Stab											
	O/F	Streak											

Fig. 2. Proforma for BACTID plate.

Microtitre plate

NA CATALASE test: Add a drop of 3% hydrogen peroxide. Bubbling indicates a positive reaction.

TTC (0.1, 0.2%): Bright red smear indicates tolerance to TTC.

KMB: There will be blue-green fluorescence under UV light (or in bright sunlight). With a UV viewer, it is not necessary to remove the lid from plastic microtitre plates.

SNA: Thick, creamy, mucilaginous growth indicates levan-positive strain. (Practice is required to recognize positive result.)

NITRATE: 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.

SSA: Add iodine for starch hydrolysis test. Clear zones in the blue iodine reaction indicate starch hydrolysis.

POTATO ROT: Positive strains will break down the disc.

Separate Tubes

Nutrient agar slope for Color/Gram: Observe color on the NA slope. Use a loop to remove culture from the slope for the Gram reaction. (The 3% KOH solubility test is recommended.)

Oxidase: Use oxidase touch sticks on NA slope. A deep purple color within 30 seconds indicates a positive result.

Gelatin: Gelatin-positive strains will liquefy the medium. Compare with noninoculated control and/or a known positive.

O/F: After stab inoculation, anaerobic tube is closed tight. Change from blue to yellow indicates acid production. Some bacteria may produce gas rather than acid in aerobic tube.

Preparing and inoculating BACTID plate.

Use sterile microtitre plates. Most of the media are prepared in the usual way. Molten media are poured into the well so that a space of 2 mm is left at the top when set. After preparation, the plates are sealed (using a bag sealer if available) and stored in the refrigerator. Slopes of Nutrient agar (NA) prepared in disposable plastic tubes are inoculated for observation of color and Gram, catalase and oxidase tests.

Inoculation: The wells for Nitrate, Gelatin, Potato rot and the O/F tubes are stab inoculated. The NA slope is streaked. The rest of the wells are inoculated by smearing the surface (see Fig. 2).

TTC agar wells

Use a clean loop to avoid carrying the TTC reagent on to other media.

O/F test

Pairs of disposable plastic tubes of nutrient agar are used since the wells are too small to avoid diffusion of oxygen. The anaerobic tube is filled right to top, and the cap screwed tight. These tubes can be stored indefinitely. The aerobic tube is filled three-quarters full, and screwed tight during storage. After stab inoculation, the anaerobic tube is screwed tight again.

Potato rot test

Cores of potato tissue (5 mm diameter) are cut aseptically with a cork borer, and discs 8 mm deep are cut. The discs can be kept in bulk in the 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 the well. The potato discs are then stab inoculated. (The discs may discolor during storage or after adding water; this does not matter).

Freeze-dried potato discs will keep indefinitely when the prepared plates are stored in the refrigerator. If a freeze drier is not available, store potato plugs in the freezer until the plates are used. Otherwise, prepare potato plugs 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 catalase test and to use Kovac's reagent in conventional form)

Using microtitre strips instead of the full 96-well plates

BACTID Software

The BACTID program will be published soon (by the Natural Resources Institute, UK), as part of a package of integrated systems for bacterial identification. *BACTID* is an Expert System, which means it incorporates information on the bacterial groups and test methods as **HYPERTEXT**.

To identify bacteria, BACTID may be used in several ways:

1 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).

2 Performing selected tests in any desired sequence. This is useful when a particular group is suspected (e.g., *Xanthomonas*).

3. Performing all necessary tests at one time. The BACTID program should be used this way with the BACTID plate 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 (e.g., Press F4 key to see "flow chart") to obtain more information about the diagnostic tests and the characteristics of the different bacterial groups.

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

BACTID was written with the Knowledge Pro expert system shell (Knowledge Garden Inc.[1]). The interim release in use is a run time version in the public domain which cannot be edited.

Identification of Bacteria by Metabolic Profiles

Biolog identification system

[1] Knowledge Garden Inc., 473a Malden Bridge Road, Nassau, NY 12123, USA

Patterns of substrate use (metabolic profiles) are characteristic of species and some subspecific taxa. The *Biolog* system[2] presents 95 substrates (and a blank control) separately in the wells of a microtitre 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 microtitre plate reader. There may be manual data entry, entry of data files from the plate reader or the identification software may 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 tropics, concentrating on its suitability for small plant pathology laboratories (Black and Sweetmore, in press). For further discussion of adapting Biolog for use in these situations and suitability of databases, see Black and Sweetmore (1993; in press).

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 comfortable.

Preparation of inoculum. Washing off exogenous nutrients: Use 25 mL disposable sterile Universal bottles (graduated). Alternatively, autoclavable or disposable centrifuge tubes to hold the same amount are suitable. Centrifuge at 4000 rpm (2800 x g) for 30 min at 15°C. Pour off supernatant (to be autoclaved before disposal), resuspend with 20 mL sterile distilled water (water may be weighed in; tolerance ± 0.5 mL), homogenize (vortex mixer), and centrifuge again. Repeat this procedure so that the cultures are washed twice in water, and finally resuspend. If the suspension looks too thin, resuspend in about 16 mL.

Initial OD (turbidity). Suspensions should have OD_{590} 0.4-0.6. The requirement is 15 mL of final suspension (96 x 150 µL). Determine OD, and dilute accordingly using a turbidimeter, spectrophotometer, or determine OD by eye with turbidity standards provided by Biolog.

[2] Biolog Inc., 3938 Trust Way, Hayward, CA 94545, USA

Inoculation. Bring plates to room temperature. Inoculate each well with 150 µl of suspension; use either 8-channel pipette or repeating pipette and sterile pipette tips).

Incubation times

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

Reading plates

There are three general difficulties:

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

Reading by eye

1. Fully positive: whole well diameter filled with deep violet.
2. Borderline: incompletely filled wells with deep violet.
3. Fully negative (and control): clear, colorless.
4. Other readings: if necessary, record with special code to be dealt with later.

Using a plate reader. In a majority of cases, results as good as or better than reading by eye 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 covert OD_{590} readings to positive, negative and borderline:

Threshold borderline: A1 x 1.4

Threshold positive: A1 x 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 may 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* (Versions 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.

Bacterial Identifier program. Blackwell's *Bacterial Identifier* program has been adapted for use with *Biolog* metabolic profiling (Black and Sweetmore, 1993, in press). 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 Fig. 3.

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 data 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 analyse 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: (a) generation of a matrix of coefficients, and (b) 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.

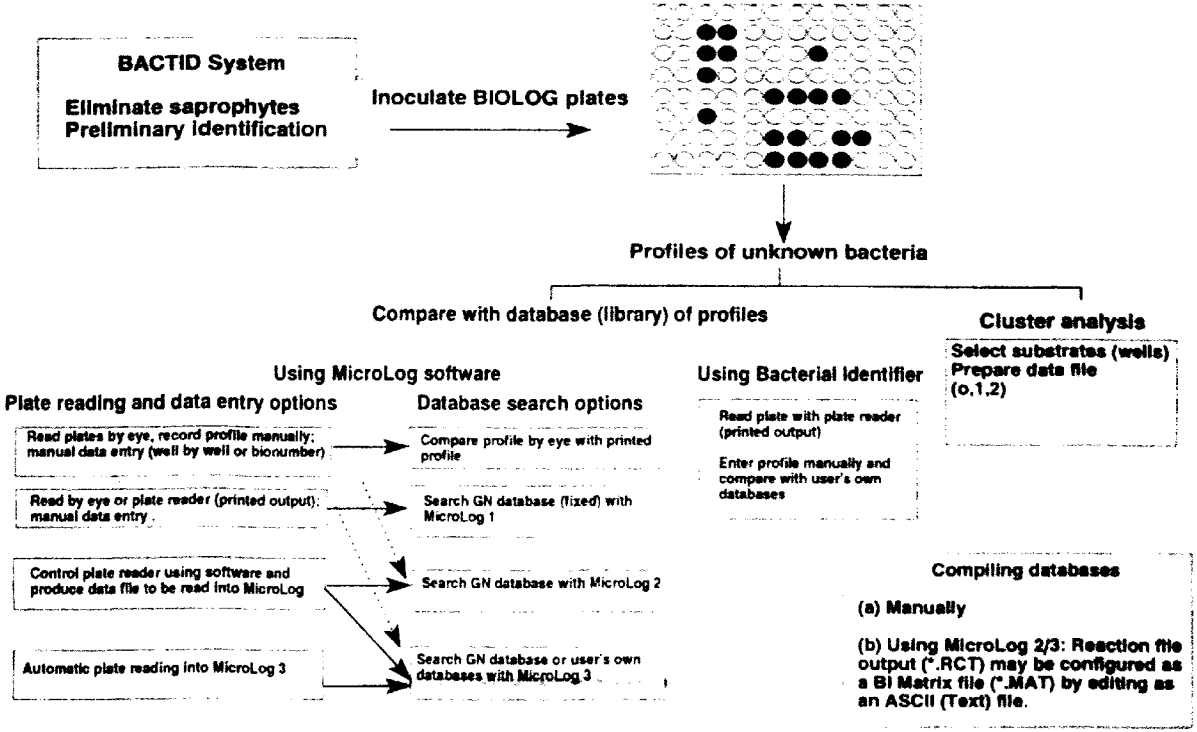

Fig. 3. Integration of BACTID system and metabolic profiling for identification of bacteria: Software options.

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Isolation and Identification of *Pseudomonas solanacearum*

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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 into 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 of 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 this onto an appropriate agar medium [e.g., sucrose peptone agar (SPA), tetrazolium chloride agar (TZCA)] in petri dishes, and incubate the plates at 28-30 °C.
5. Observe the colonies of the bacterium after 48-72 h of incubation.

   N.B.: Single colonies are most easily obtained by the 3-streak method. A small drop of bacterial suspension is placed on one side of the petri dish. The loop is re-sterilized, cooled down by touching the medium, and three lines are 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 TZCA, 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.

Detection of *Pseudomonas solanacearum*

Several conventional methods are suitable for the detection of *P. solanacearum* from soil samples; they are designed to enumerate either 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.

Detection methods have concentrated on detection in soil, using semiselective media.

Several nonselective media are suitable for isolation and growth of *P. solanacearum*. These are SPA, King's Medium B (KBA), and TZCA. The composition of these media is given below :

1. Sucrose peptone agar (SPA) (Hayward 1960)

Constituents		(in g L^{-1})
Sucrose		20
Peptone	:	5
K_2HPO_4	:	0.5
$MgSO_4.7\ H_2O$	:	0.25
Aagar	:	15

pH is adjusted to 7.2-7.4 using 40% NaOH.

2. King's Medium B agar (KBA) (King et al. 1954)

Constituents		(in g L^{-1})
Proteose peptone # 3	20	
K_2HPO_4	:	1.5
$MgSO_4.7\ H_2O$	:	1.5
Glycerol	:	10 mL
Agar		15

pH is adjusted to 7.2 using NaOH.

3. Tetrazolium chloride agar (TZCA) (Kelman 1954)

Constituents		(in gL^{-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.

Colony characteristics on the three culture media are described in Table 1.

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 agar (KBA)	White fluidal nonfluorescent colonies
Tetrazolium chloride agar (TZCA)	Fluidal white colonies with pink centers

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 which may 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, Kanganilla 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. ISEM requires access to an electron microscope, and there are problems in handling large numbers of samples.

ELISA is the most preferred among the serological tests. In developing countries, adoption of DIBA is more difficult than ELISA, because 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, using polyclonal and monoclonal antibodies against the bacterium. These detection methods do not require purification or culture of the bacterium. They are more rapid, more sensitive, and simpler to use than the 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 (RES), 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., *P. syzygii*, *P. pickettii*, *P. cepacia*). Some highly specific monoclonal antibodies produced at the University of Hawaii, and the RES 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 matrixes. It is different from the classical serological tests (e.g., Ochterlony double diffusion test) in which immunoprecipitin reactions are used. Immunospecificity is recognized through the action of the associated enzyme label on a suitable substrate rather than by observing the formation of an insoluble antigen-antibody complex.

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Serological Techniques for the Detection of *Pseudomonas solanacearum*

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Background

Antibodies are protein molecules produced naturally by biological organisms against external "non self" molecules. These "non-self" molecules are called antigens, and can take many different forms, such as the 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. Antibodies are then produced by the animal and released into its bloodstream, from which they may be harvested.

Polyclonal and Monoclonal Antibodies

Antibodies are produced in B-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 may have several distinct sites against which antibodies may 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. 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, i.e., the immune response is polyclonal. Thus, if an animal was immunized with an antigen and bled, the resulting blood would be polyclonal and would contain a mix of antibodies to the antigen.

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	Low	Initially high
Specificity	Variable with animal and breed. Partial cross reactions with common determinants. Seldom too specific	Standard. Unexpected cross-reactions may occur. May be too specific
Determinants recognised	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 may be extracellular, intracellular or structural; their biochemical nature may be protein, glycoprotein, polysaccharide, lipopolysaccharide or lipid. Immunogen preparations may 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 may be done by either placing the washed cells in a boiling waterbath 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 as 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 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: 48-h-old bacterial cultures are shaken in sterile distilled water for 30 min and the cells removed by centrifugation. 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 held overnight at 4°C. A precipitate forms, which 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 traces of ammonium sulphate.

Purified bacterial components

Purification of many antigens such as lipopolysaccharide, ribosome, 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 programme such as that detailed below can help produce polyclonal sera of a very high titre.

1. Immunize female inbred rabbits by injecting intramuscularly 0.5mL 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) into a centrifuge tube.

4. Allow the blood to clot at room temperature and then spin it at 2000 g for 15 min.

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

6. Test the antibody titre of the serum by the enzyme-linked immunosorbent assay (ELISA). If the titre is sufficiently high, continue bleeding at 3-weekly intervals, otherwise repeat step 2.

7. Continue bleeding up to 10 bleeds, or until the titre drops below that required.

Storage of antisera

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 either:

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

N.B. : 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 antisera

For several techniques, purification of the antibody is required and there are many methods that 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 10000 g for 10 min.
4. Discard supernatant, and drain tube by holding it upside down on a tissue.
5. Redissolve 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 DEAE-matrix with 0.5M HCl and then 0.5M NaOH, followed by 20 volumes of 10mM Tris (pH 8.5). Check 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 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 which fractions contain 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 liquid (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. A positive reaction is seen as the clumping together of antigen and antibody. Unfortunately, for a reaction to take place a high concentration of antigen is required. 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 good, 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 (A) ELISA.

ELISA assays take a variety of forms. All of them involve a plastic multi-welled 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 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, when a suitable chemical substrate is added, changes colour. So there will be a color change in those wells where the antigen is present.

(B) DIBA or Dot Blot. 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. Principally, immunofluorescence is used to locate antigens in tissues and cells. Instead of using an enzyme linked to an antibody to detect the antigen, it uses a flurochrome linked to an antibody.

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. The prime advantage of Western Blotting over Dot Blotting is that here antibody reactivity can be correlated with proteins/peptides of particular molecular weights.

Enzyme-Linked Immunosorbent Assays

In most cases, ELISA is the assay of choice for the 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 microtitre plate, a large number of samples can be screened at once.

Variations

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 may be less sensitive than the indirect ELISA.

Indirect ELISA: The antigen is coated onto the plate and the enzyme used is conjugated to an anti-species antibody. This increases the sensitivity of the test

DAS-or Sandwich ELISA: (Double Antibody Sandwich ELISA): The plate is first coated with a specific antibody to capture the antigen. This increases the sensitivity of the test. It can be direct or indirect

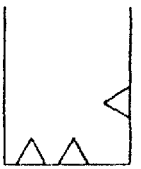
$F(ab')_2$ ELISA - As in the DAS ELISA, the plate is first coated with the antibody, but instead of using the whole antibody, only the $F(ab')_2$ portion is used. The second antibody used is, however, whole. Unlike the other ELISAs, the enzyme used here is coupled to protein A. This binds to the Fc portion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

Competitive ELISA: Carried out in a way similar to the above ELISAs but an inhibitor antigen is included so that the amount of antigen present can be determined.

Principles of the Indirect ELISA

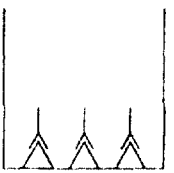
For the detection of *P. solanacearum* in infected plant and soil samples, the indirect ELISA has been found to be the most suitable.

1. Plant extract adsorbed onto plate; incubate.



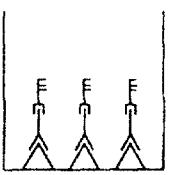
2. Wash plate.

3. Add specific antibody; incubate.



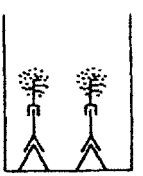
4. Wash plate.

5. Add enzyme conjugate.



6. Wash plate.

7. Add enzyme substrate; observe colour change; intensity proprtional to amount of bacteria in plant extract.



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 coating buffer.
3. Coat the wells of a suitable microtitre plate with this supernatant, using 100 µL well^{-1}. Include positive and negative controls.
4. Incubate in a moist chamber at 37°C for 1 h, or overnight at 4°C.
5. Wash three times in washing buffer (flick-out, hang-dry method), leaving in last change for 5 min.
6. Add 100 µL of a polyclonal antibody, diluted to working concentration in blocking buffer, to each well. Alternatively, use monoclonal antibody and continue as above but with anti-mouse conjugate.
7. Incubate and wash as in steps 4 and 5.
8. Add 100 µL of second antibody (peroxidase conjugated anti-rabbit (Sigma Chemical Co.)], diluted 1:5000 in blocking buffer, 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 colour change is observed (5-30 min). A yellow color develops indicating the termination of the reaction.
11. Stop the reaction by adding 25 µL of 3 M HCl to each well.
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 x 10^6 cfu mL^{-1} bacterial cells in either plant or soil samples

Materials for indirect ELISA

Carbonate Coating Buffer (pH 9.6)

6.36g Na_2CO_3
11.72g $NaHCO_3$
Make up to 1L with dH_2O

10 x PBS

80g NaCl
2g KH_2PO_4
14.2g $Na_2HPO_4.12H_2O$
2g KCl
Make up to 1L with distilled water

Washing Buffer - Phosphate Buffered Saline plus Tween (PBS-T)[1]

100mL 10 x PBS
5mL 10% Tween 20
Make up to 1L with distilled water

Blocking Buffer[1]

10mL 10 x PBS
2g Polyvinyl pyrrolidine (mw 44000)
0.5mL 10% Tween 20
0.5g Full Cream milk powder (e.g., NIDO, Nestle)
Make up to 100 mL with distilled water

Peroxidase Substrate (must be made up daily, as required)

100µL 3,3',5,5'-Tetramethyl-benzidine (Sigma) @ 10mg/ml in DMSO
2µL 30% H_2O_2
1mL 1M sodium acetate, pH 5.8
Make up to 10 mL with distilled water

[1] must be made up daily, as required

Appendix

Important Definitions

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

Affinity: The 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 lymphocytes in response to an antigenic stimulus and capable of binding to an antigen.

Antigen: A 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 (lymphocytes): Antibody producing cells, found mainly in the blood, lymph nodes and spleen.

Cross-reacting antigen: Antigen capable of combining with antibody produced in response to a different antigen. May 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): An immunoassay system employing an enzyme bound to an antibody, as the immunologic probe determining the extent of antigen-antibody reaction.

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

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

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. Most haptens are small molecules and carry only one or two antigenic determinants, but some macromolecules are haptenic.

Hybridoma: A cell or cell line formed from the fusion of a lymphocyte with a tumour cell.

Immunogen: A 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 upwards.

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 identifical polypeptide chains, of the four-chain immunoglobulin molecules.

L chain (light chain): A 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.

Monoclonal antibody: A homogenous antibody population produced by a clone of antibody-forming cells.

Myeloma: A neoplastic B-lymphocyte cell line which is immortal in cell culture.

Paratope: The antigen-combining site in an antibody.

Polyclonal antibody: A heterologous antibody population derived from many clones.

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.

Titre: 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 antigen is added. Serum titre is indicated as the reciprocal of the highest serum dilution, producing a discernible antigen-antibody reaction.

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

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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, put into PBS buffer (1:1 w/v), pressed, and soaked. The sample is centrifuged at 1000 rpm for 5 min to remove the pellet, and then at 10000 rpm for 10 min to remove the supernatant. The pellet is dissolved in 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

PBS buffer (pH 7.4)

NaCl	8.0 g
KH_2PO_4	0.2 g
$Na_2HPO4.\ 12H_2O$	2.9 g
KCl	0.2 g
$NaNO_3$	0.2 g

Dissolve these chemicals in distilled water to make up 1000 mL.

Coating buffer (pH 9.6)

Na_2CO_3	1.5 g
$NaHCO_3$	2.93 g
$NaNO_3$	0.2 g

Dissolve these chemicals in distilled water to make up 1000 mL.

Extraction buffer (pH 7.4)

Dissolve the following chemicals in 1000 mL PBS buffer:

Polyvinyl pyrolidon K-25	20.0 g
Tween 20	0.5 mL
1% egg albumin	10.0 g

Washing buffer (pH 7.4)

PBS containing 0.05% Tween 20

(1000 mL PBS + 0.5 mL Tween 20)

Enzyme conjugation buffer

Dissolve the following chemicals in 1000 mL PBS buffer:

Ovalbumin	2.0 g
Tween 20	0.5 mL
Polyvinyl pyrolidon K-25	20.0 g

Substrate buffer (pH 9.8)

Dissolve diethanolamine (97 mL) in about 800 mL distilled water, add 0.2 g $NaNO_3$, and adjust pH to 9.8 with concentrated HCl. Adjust the volume to 1000 mL 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 uL of the mixture to each well and cover the plates tightly

2. Incubate the plates at 37℃ for 2 h.

3. Washing after incubation

   Remove the solution from wells and wash the plate 4 times with washing buffer. Careful washing is extremely important

4. Addition of the sap diluted in extraction buffer

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

5. Incubate the plates at 4.6℃ for 18h (overnight)

6. Wash the plates as described above.

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 uL of mixture solution to each well, and cover plates tightly.

8. Incubate the plates at 37℃ for 1 h

9. Wash the plates as described above

10. Addition of enzyme substrate

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

    Add 100 uL of the enzyme substrate to each well

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

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

13. Measure the absorbance by 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 wells.

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

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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 plus 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 which copy in the 5' to 3' direction. Because of specific base pairing, if the sequence of one strand is known, then that 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. When temperatures are cooled down to below 70°C, the strands start to recombine, 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, by heating DNA of interest to separate the strands and by adding a short piece of DNA (termed an oligonucleotide or PCR primer) that is known to be a perfect match to the DNA, 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 2000 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 polymerase), add 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.

The first step in the PCR cycling procedure is to heat the reaction to 96°C for 2-3 min to separate the double-stranded DNA molecules. The second step is to 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. The third step is to increase the temperature to the optimum level for the activity of the DNA polymerase (for Taq polymerase, this is 72°C). Then the reaction should be incubated 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 seconds at 72°C is 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. Table 1 shows that, under optimum conditions, this will lead to the target sequence being amplified a million-fold, after 22 cycles of amplification.

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, Journal of General Microbiology 139: 1587-1594). The test can detect DNA of one bacterial cell. For the protocol described below, the maximum volume that can be tested is 31.4 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 potato tuber extract can generally be achieved. The corresponding sensitivity for groundnut extracts has not yet been determined.

Table 1. Generation of copies of double-stranded DNA-spanning sequences between two PCR primers.

Cycle number	Number of double-stranded amplified sequences
1	0
2	0
3	2
4	4
5	8
6	16
7	32
8	64
9	128
10	256
11	512
12	1024
13	2048
14	4096
15	8192
16	16384
17	32768
18	65536
19	131072
20	262144
21	524288
22	1048576
23	2097152
24	4194304
25	8388608
26	16777216
27	33544432
28	67108864
29	134217728
30	268435456

Preparation of samples to be tested by PCR

Materials required

1 small beaker of domestic bleach (containing 5-10% sodium hypochlorite).

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

1 beaker containing 95-100% ethanol

Two or more scalpels.

Bunsen burner

Microcentrifuge

Micropipetters and 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.

Sampling tubers or rhizomes:

1. Wash soil off the surface of tuber or rhizome. Wear gloves before starting isolation.
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 between 200 and 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 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 (see below for boiling method).

Sampling stems

1. Wash surface of stem to remove soil. Wear gloves.
2. Cut stem close to the bottom of plant with bleach-treated scalpel, and place 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 suspension by centrifugation as described above.

If the total volume of water is more than 1 mL, repeat centrifugations, carefully removing and discarding supernatant after each spin, 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 supernatant with a micropipetter. Boil sample as described below.

Testing cultures

1. Pour about 1 mL of 12-36-h-old TYGB culture into a screw-capped Eppendorf, and spin at full speed (12 000 g) in a microcentrifuge for 5 min. You should be able to see a bacterial pellet after centrifugation. If you can not, 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, each time spinning and decanting supernatant.
3. Resuspend cells in 50-100 µL water, and boil.

N.B.: 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 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 (121°C for 15 min).

Soil samples

Soil extracts cannot be processed by this test directly, owing to the presence of PCR-inhibitory substances. Soil extracts should, therefore, be plated on selective media. Confirmation that particular colonies are *P. solanacearum* can be made by growth in TYGB and treatment as described above.

Boiling samples

1. Use boiling water, rather than a dry heating block, to provide good heat transfer.
2. Concentrate samples, if necessary, **before** boiling by centrifugation (12 000 g, 5 min), followed by removal of some of the upper liquid using a pipette. Do this carefully, and resuspend the pellet by vortexing.
3. Boil samples for 10 min as quickly 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 down to room temperature, spin tubes for a few seconds to remove condensation on 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 950.0 µL

Total volume 1 mL

10x 'TBE' Buffer

89 mM Tris

2.5 mM Na_2EDTA

8.9 mM boric acid

Adjust to pH 8.3 with NaOH.

1.5% Agarose gel in 1x TBE buffer

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

5x Orange G Loading dye

15% FICOLL 400

40 mM EDTA pH 8.0

0.25% Orange G

for ~5 mL loading dye:

0.75 g FICOLL 400

0.0125 g Orange G

400 µL 0.5M EDTA pH 8.0

4.5 mL Sterile distilled water

100 bp ladder (1 µg 10 µL^{-1})

Mix the following:

150 µL sterile distilled water

40 µL 5x Orange G loading dye

10 µL 100 bp ladder stock (1 µg µL^{-1}, supplier: Pharmacia)

Ethidium bromide stock (10 mg mL^{-1})

Wearing gloves, dissolve one 100 mg Ethidium Bromide tablet (Sigma) in 10 mL sterile water.

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 the following:

dNTP mix (1.25 mM each dNTP) 8 µL
10 x PCR buffer5 µL
25 mM $MgCl_2$4 µL
Primer OL11 (20 µM) 1.25µL
Primer Y2 (20 µM)1.25µL
Taq polymerase (5U µL⁻¹)0.2µL

DNA sample 1-31.5µL

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

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 (recipe given above).

Making a mix for more than one sample:

It is advisable to include two positive and two negative controls in each PCR run. Hence, the minimum number of PCR tubes to set up will be five. 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. As pipetting is often not 100% accurate, the mix is made for the total number of samples, plus two positive and two negative controls, plus one extra to ensure that there is sufficient mix.

For example, if we have 2 plant samples, we make a mix for 2 + 4 controls + 1 extra = 7 samples. We generally test 5 µL of the sample, and add 26.3 µL sterile distilled water for each sample. Thus a mix for 7 would be as follows:

Sterile distilled water 184.1 µL

dNTP mix 56 µL

10 x PCR buffer 35µL

25 mM MgCl2 21 µL

Primer OLI1 (20 µM) 8.75 µL

(5'GGGGGTAGCTTGCTACCTGCC3')

Primer Y2 (20 µM) 8.75µL

(5'CCCACTGCTGCCTCCCGTAGGAGT3')

Taq polymerase (5U/µL) 1.4 µL

Total volume 315.0 µL (= 7 x 45 µL)

Mix all the ingredients well together using "PCR-only" micropipettors, then put 45 µL of the mix into the bottom of each of six tubes. Then add 5 µL of sample to each tube.

Always add 5 µL water to the negative controls first. Cover both with one drop of oil, and then close the tubes before moving onto the samples.

For pipetting the samples, find another (non-PCR) P20 Gilson micropipetter. This is necessary to remove the possibility of cross-contaminating the barrel of the Gilson used for the PCR set up.

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

Once oil has been added to the positive controls and the tubes have been closed, they are transferred to the PCR machine, programmed with the following heating profile:

Initial denaturation:96°C, 2 min 30 sec x 1 cycle

Denaturation 94°C, 15-30 sec

Annealling: 67-68°C, 15-30 sec[1] x 50 cycles

Extension: 72°C, 20 sec

[1] The exact time will depend on the PCR machine used.

Final extension: 72°C, 5 mins, 1 cycle,

Refrigerate till machine is switched off

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

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

Include 10 µl of 100 bp ladder (supplied by Pharmacia). Run the gel at about 100-120 V for about 1 h (till Orange dye has run about 5 cm down gel or more), and then stain in 0.5 µg/ml. Ethidium Bromide solution.

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

Cross-contamination during PCR

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 of samples are gloves and micropipetters. It is necessary to ensure that clean gloves are used to set up PCR, and that care is taken to avoid contact of the glove finger tips with 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 is needed in a subsequent PCR reaction 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 which prevent the barrel becoming contaminated, or to use one set of micropipetters solely to pipette PCR reagents (excluding samples to be tested). Moreover it is wise to use a work area for setting up PCR, which is never used 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.

Infectivity Titrations for Plant Disease Evaluations

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Principles

Infectivity titration is a technique which offers a measure of the relative capacity of an organism to infect its host. The basic principles of infectivity titration stem 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 infectivity titration method are:

1. prepare serial dilutions of known concentrations of the bacterial pathogen,
2. inoculate each serial dilution into a separate group of the host,
3. record the response of each individual host after a specific incubation period, and
4. express 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 may be expressed either in terms of differences in the doses required to elicit the same level of response from the host, or differences in 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 (e.g., 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 1970), they have limited applications, especially with plant pathogenic bacteria. Quantal effects are all-or-none responses, e.g., alive or dead, healthy or diseased, turgid or wilted.

The frequency of responses 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 1970, 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 (i) the median effective dose (ED_{50})--the dose that causes a response in 50% of the inoculated plants, and (ii) 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 5×10^8 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 TZC 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 six to eight 8 serial dilutions per experiment. Generally, the range of concentrations should result between 10^8 and 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 to different host cultivars, titrations must be carried out inactively 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 which 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.

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 $8.28 \times 10_4$ 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 2).

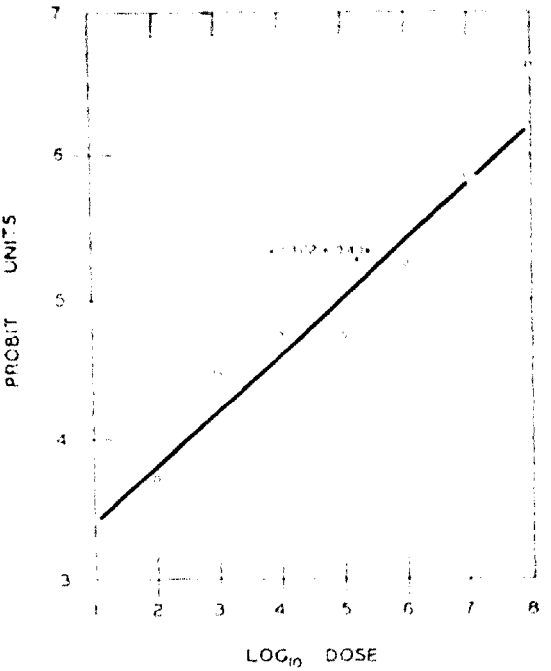

Figure 1. Log-dose/probit-response relationship for tomato cultivar Bonny Best and Pseudomonas solanacearum strain 338.

Table 1. Probit analysis of the quantal responses of the tomato line 72-TRI2-3 to graded inoculum doses of *P. solanacearum* strain 338.

Control group data	Sample size N = 20..	Responser = 0..	C Held Constant				
Transformed dose	8.00	7.00	6.00	5.00	4.00	3.00	2.00
Response	16.00	14.00	11.00	8.00	6.00	3.00	2.00
Sample size	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Sample probit	5.84	5.52	5.13	4.75	4.48	3.96	3.72
Sample probability	0.80	0.70	0.55	0.40	0.30	0.15	0.10

Initial values at start of interaction: a = 2.960, B = 0.362, C = 0.000

Final values of the parameters: For probit line A + Bx: a = 2.959, B = 0.363, C = 0.000

Table 2. Probit analyses of the quantal responses of five tomato cultivars to graded inoculum doses of *P. 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 3. Probit analyses of quantal responses of tomato cultivar Bonny Best to graded inoculum doses of eight strains of *P. solanacearum*.

Strain	Geographic origin	Probit analysis[1]		ED_{50} value	Virulence rank
		Slope (B)[2]	Log ED_{50}		
143	Australia	0.493 ± 0.088	3.440 ± 0.272	2.76 x 10	5
196	Philippines	0.510 ± 0.079	4.269 ± 0.264	1.86 x 10⁴	8
221	Kenya	0.562 ± 0.100	4.032 ± 0.261	1.08 x 10⁴	7
234	Brazil	0.555 ± 0.116	3.166 ± 0.273	1.46 x 10³	3
270	Taiwan	0.439 ± 0.085	2.868 ± 0.309	7.38 x 10	6
338	Malaysia	0.506 ± 0.090	3.334 ± 0.273	2.16 x 10	4
341	Florida (USA)	0.6.5 ± 0.160	1.906 ± 0.290	8.00 x 10	1
342	N. Carolina (USA)	0.514 ± 0.119	1.946 ± 0.440	8.80 x 10	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.

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 31.

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Inoculation Techniques to Evaluate Resistance to *Pseudomonas solanacearum*

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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 the preparation of inoculum, use a highly virulent culture of *P. solanacearum*, maintained in sterile distilled water at a 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 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 bacterial 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, and 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

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 min.
4. Sow the inoculated seeds in sterile soil in pots in the greenhouse or in soil 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).

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

N.B.: For successful resistance screening, it is important to maintain high temperature and high soil moisture levels following 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 build up 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*

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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.

Indicator levels can be used to identify pathotypes of the wilt pathogen, as shown in Table 1.

Table 1. Indicator-cultivars that can be used to identify pathotypes of the wilt pathogen

Indicator-cultivar	Resistance/susceptibility level	Wilt incidence
Jiankang	Highly resistant	<10%
Taishan Sanlirou	Highly resistant	<10%
Xiekangking	Highly resistant	<10%
Goulaozhong	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%

Use the following procedure to identify pathotypes:

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 seeds sown singly at 10 cm spacing; use of seed protectant is recommended.
3. From 30 days after sowing, record the bacterial wilt incidence in replicated plots of each cultivar at fortnightly 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. 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* using indicator-groundnut cultivars.

Groundnut cultivars	Pathotypes of *P. solanacearum*						
	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 ZhigangR	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

R = Highly resistant; M = Moderately resistant; and S = Susceptible.