II

(Acts whose publication is not obligatory)

COMMISSION

COMMISSION DECISION

of 9 September 1997

detailing an interim test scheme for the diagnosis, detection and identification of *Pseudomonas solanacearum* (Smith) Smith in potatoes

(97/647/EC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Directive 77/93/EEC of 21 December 1976 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community [1], as last amended by Directive 97/14/EC [2], and in particular Article 15 (3) thereof,

Whereas in Commission Decision 95/506/EC of 24 November 1995 authorizing Member States temporarily to take additional measures against the dissemination of *Pseudomonas solanacearum* (Smith) Smith as regards the Kingdom of the Netherlands [3], as last amended by Decision 96/599/EC [4], and in particular Article 1 paragraph 2 (a) (bb) thereof, when Member States carry out official or officially supervised testing of potatoes they are required to use quarantine procedure No 26 for *Pseudomonas solanacearum* (Smith) Smith as established by the European and Mediterranean Plant Protection Organization (EPPO) [5] or some other procedure approved in accordance with the procedure laid down in Article 16a of Directive 77/93/EEC;

Whereas the *ad hoc* Expert Committee on Bacterial Diseases of Plants set up by the services of the Commission of the European Communities under the auspices of the Standing Committee on Plant Health has determined the details of an interim test scheme which takes account of improved detection and testing procedures developed since the publication of EPPO quarantine procedure No 26; whereas this test scheme is interim since further research is anticipated especially on the sensitivity and specificity of individual tests with the aim of selecting and standardizing the optimum tests available for inclusion in an updated test method;

Whereas the interim test scheme provided for in this Decision is in accordance with the opinion of the Standing Committee on Plant Health,

[1] OJ L 26, 31. 1. 1977, p. 20.
[2] OJ L 87, 2. 4. 1997, p. 17.
[3] OJ L 291, 6. 12. 1995, p. 48.
[4] OJ L 265, 18. 10. 1996, p. 18.

[5] Bulletin EPPO/OEPP 20, 255—262 (1990).

HAS ADOPTED THIS DECISION:

Article 1

For the purpose of implementing the provision in Article 1 paragraph 2 (a) (bb) of Commission Decision 95/506/EC, as last amended, quarantine procedure No 26 for *Pseudomonas solanacearum* (Smith) Smith as established by the European and Mediterranean Plant Protection Organization (EPPO) shall be replaced by the interim test scheme for the diagnosis, detection and identification of *Pseudomonas solanacearum* (Smith) Smith laid down in the Annex to this Decision.

Article 2

This Decision is addressed to the Member States.

Done at Brussels, 9 September 1997.

For the Commission
Franz FISCHLER
Member of the Commission

ANNEX

INTERIM TEST SCHEME FOR DIAGNOSIS, DETECTION AND IDENTIFICATION OF *PSEUDOMONAS SOLANACEARUM* (SMITH) SMITH

SCOPE OF THE TEST SCHEME

The presented scheme describes the various procedures involved in:

i) diagnosis of brown rot in potato plants and tubers;

ii) detection of *Pseudomonas solanacearum* in samples of potato tubers;

iii) identification of *Pseudomonas solanacearum.*

In the Appendices, details are provided for the preparation of the test materials, i.e. growth media, buffers, solutions, reagents.

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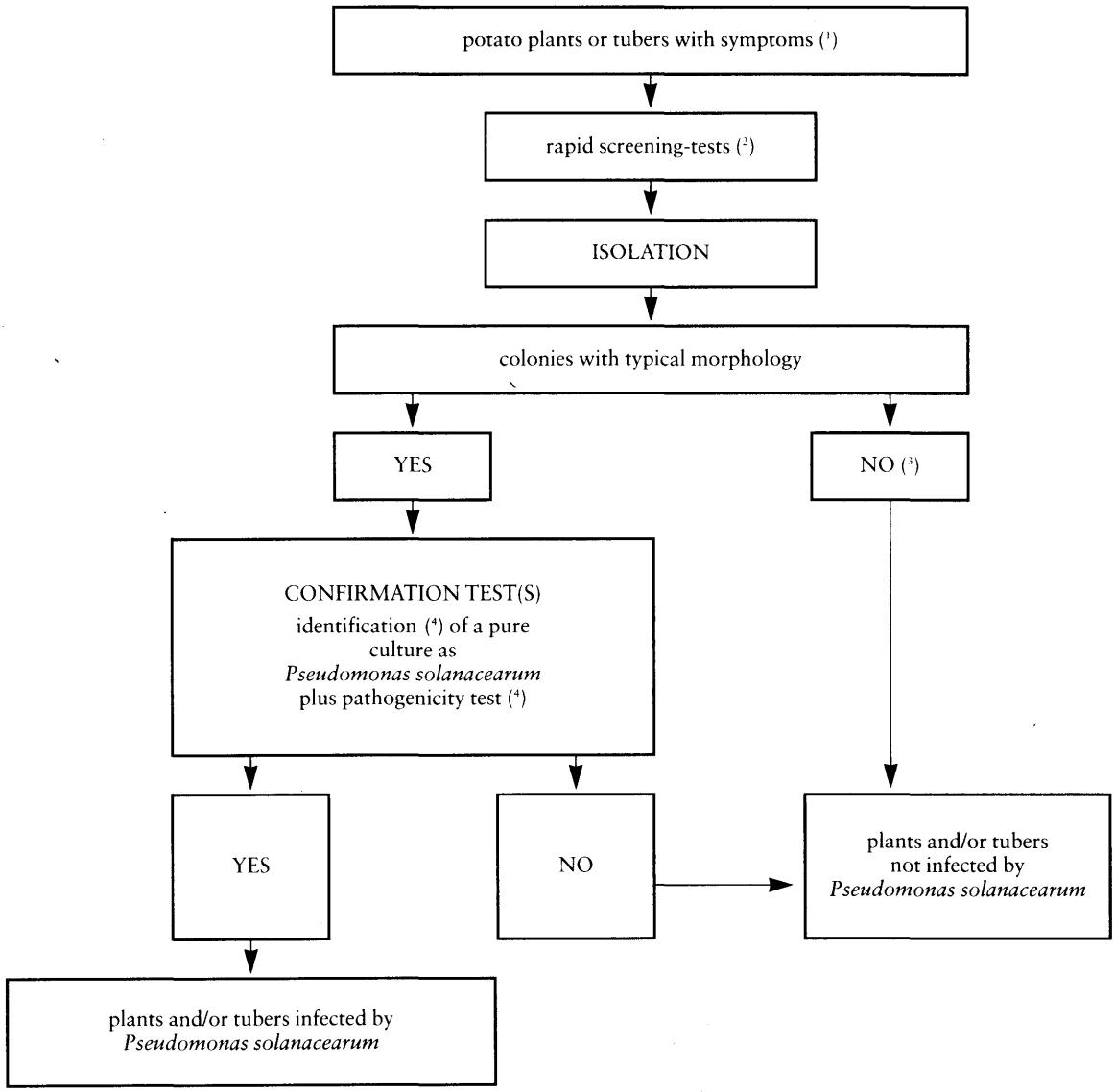
SECTION I

APPLICATION OF THE TEST SCHEME

1. **Diagnosis of brown rot in potato plants and tubers**

The testing procedure is intended for plants and tubers with symptoms typical or suspect of brown rot. It involves a rapid screening test, isolation of the pathogen from infected vascular tissue on diagnostic media and, in case of a positive result, identification of the culture as *Pseudomonas solanacearum*.

Flow chart diagram presentation:



Flowchart references:

(1) Description of symptoms is provided in section II.1.

(2) Rapid screening tests facilitate presumptive diagnosis.

Appropriate tests are:
— Streaming test of vascular stem tissue (section II.2),
— Test for poly-ß-hydroxybutyrate granules (section II.2),
— IF test (section III.2),
— ELISA test (section III.3),
— PCR test (section III.4).

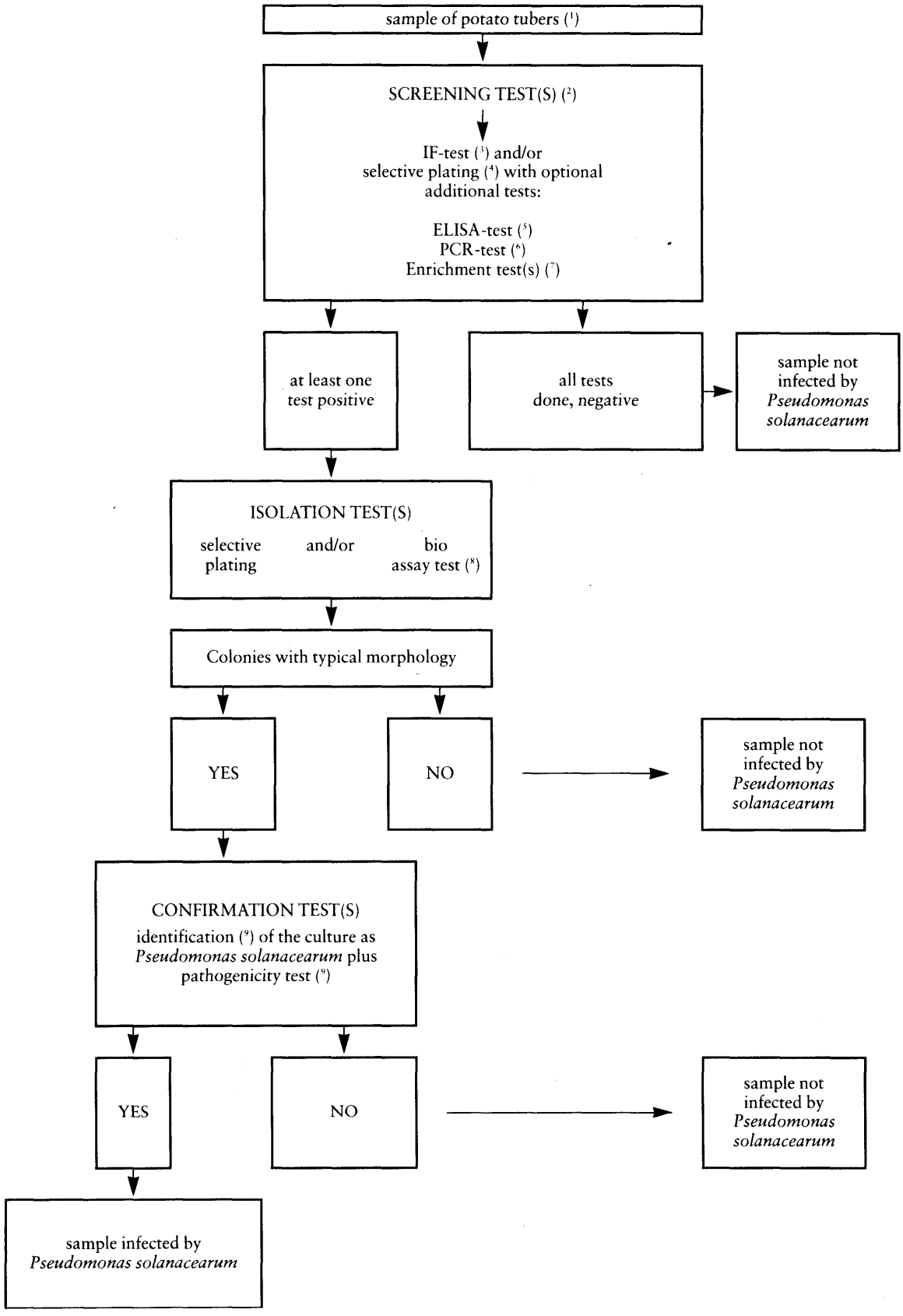
(3) Although isolation of the pathogen from plant material with typical symptoms by dilution plating is straightforward, culturing may fail from advanced stages of infection. Saprophytic bacteria which grow on diseased tissue may outgrow or inhibit the pathogen on the isolation medium. If the isolation test is negative, but disease symptoms are typical, then isolation must be repeated, preferably by a selective plating test.

(4) Reliable identification of a pure culture of *Pseudomonas solanacearum* is achieved by at least one of the tests listed in section II.4.1 in combination with a pathogenicity test (section II.4.3). Strain characterization is optional but recommended for each new case.

2. **Detection and identification of *Pseudomonas solanacearum* in samples of potato tubers**

The procedure is intended for detection of latent infections in potato tubers by one or, preferably, more screening test(s) which, if positive, are complemented by the isolation of the pathogen; followed by, in case of isolation of typical colonies, identification of a pure culture as *Pseudomonas solanacearum*.

Flow chart diagram presentation:



Flowchart references:

(1) Sample size

The standard size is 200 tubers. However, the procedure can be applied conveniently for samples with less than 200 tubers.

(2) Screening test(s)

A single test may not be sufficiently sensitive or reliable to detect *Pseudomonas solanacearum* in a sample. Therefore, more than one test is recommended and these tests should preferably be based on different biological principles.

(3) Immunofluorescence (IF) test

The IF test is a well established screening test. This is an advantage over other tests which are not yet fully developed or validated. The test is used for many other statutory bacteria, e.g. *Clavibacter michiganensis* subsp. *sepedonicus*. With the reading parameters specified in this method it is a sensitive test (sensitivity threshold of 10^3—10^4 cells per ml of potato extract pellet).

The critical factor for the reliability of the test result is the quality of the antiserum. Only antiserum with a high titre (minimum 2000 for the crude antiserum) is acceptable and all tests must be carried out at the antiserum titre or one dilution below the titre. The indirect method is preferred. The direct method can be applied if the test has a level of sensitivity and specificity equivalent to that of the indirect method.

The IF test has the advantage of subjective interpretation of cell staining morphology and fluorescence intensity which provide information on reaction specificity. Cross reactions by serologically related bacteria from soil or associated with potato tissues with cell morphology of *Pseudomonas solanacearum* are common. The IF test can be used as a sole screening test although in cases where cross-reactions are suspected an additional screening test based on a different biological principle should be done. In such cases selective plating is the most appropriate test.

(4) Selective plating

With the modified SMSA medium and testing methodology specified in this method, this is a sensitive and selective test for *Pseudomonas solanacearum*. The result is available 3—6 days after sample preparation. The pathogen is obtained directly in culture and can be readily identified. For full exploitation of its potential, the test requires careful preparation of heel ends to avoid secondary bacteria associated with the potato tuber which are competitors with *Pseudomonas solanacearum* on the medium and may affect the development of the pathogen. Some strains may grow poorly as the components of the medium may affect the target organism Care is also required to differentiate *Pseudomonas solanacearum* from other bacteria which may develop on the medium. Selective plating can be used as a sole screening test provided that in cases where inhibition of *Pseudomonas solanacearum* by other bacteria on the plates is suspected and a negative test result is obtained the sample is retested using a different test to confirm or refute the diagnosis. In such cases, the IF test is the most appropriate test.

(5) ELISA test

The ELISA test is generally less sensitive than the IF test (sensitivity threshold of 10^4—10^5 cells per ml of potato extract pellet). The test is cheap and fast but generally more vulnerable to false positive (cross reactions) and false negative results (inhibition by phenolic molecules in the potato extract). The requirements for antiserum specificity are extremely high. The ELISA test cannot be used as a single screening test.

(6) PCR test

PCR has the potential for very sensitive detection although the test is readily inhibited by plant or tuber extract components resulting in false negatives. Some potato cultivars contain more inhibitors than others. It is therefore necessary to remove these inhibitors. The inhibition can be reduced by dilution but the populations of *Pseudomonas solanacearum* are also diluted. Great care has to be taken in all steps of sample and test preparation to prevent contamination which would result in false positive tests. False positives could also arise from sequence homology of other organisms. For these reasons the direct PCR cannot be used as a sole screening test.

(7) Enrichment test

Incubating potato extract pellet samples in a semi-selective broth, such as modified SMSA broth, allows multiplication of *Pseudomonas solanacearum*. More important perhaps, it also dilutes potential inhibitors of the ELISA or PCR test. *Pseudomonas solanacearum* in enrichment broth can thus be detected by IF, ELISA and PCR. We do not recommend that direct plating is made from the enriched broths. These enrichment methods have not been thoroughly tried and tested. They are included here because they have good potential. However, because of the relative lack of experience with them, they cannot be used as sole detection methods.

(8) Bioassay test

The bioassay test is used for isolation of *Pseudomonas solanacearum* from potato extract pellets by selective enrichment in a host plant and can be done on tomato plants or eggplants. The test requires optimal incubation conditions as specified in this method. Bacteria inhibitory to *Pseudomonas solanacearum* on the SMSA medium will most likely not interfere in this test.

(9) Confirmation test(s)

Reliable identification of a pure culture of *Pseudomonas solanacearum* is achieved by at least one of the tests listed in section II.4.1. in combination with a pathogenicity test (section II.4.3.). Strain characterization is optional but recommended for each new case.

SECTION II

DIAGNOSIS OF BROWN ROT IN POTATO PLANTS AND TUBERS

1. Symptoms of brown rot

The potato plant

The early stage of infection is wilting of the leaves towards the top of the plant at high temperatures during the day with recovery at night. Wilting becomes rapidly irreversible and results in the death of the plant. The vascular tissue in transversely cut stems from wilted plants may become brown and a milky ooze exudes from the cut surface or can be easily expressed by squeezing. When a cut stem is placed vertically in water, threads of slime will stream from the vascular bundles.

The potato tuber

Potato tubers must be cut transversely close to the heel (= stolon) end. The early stage of infection is a glassy yellow to light brown discoloration of the vascular ring from which a pale cream ooze emerges spontaneously after a few minutes or when gentle pressure is applied with the thumbs on the skin near the cut surface. Later, the vascular discoloration becomes more distinct brown and the necrosis can extend into the parenchymatous tissue. In advanced stages, the infection breaks outwards from the heel end and the eyes which may result in reddish-brown, slightly sunken lesions on the skin from which bacteria may ooze, causing soil particles to adhere.

2. Rapid screening tests

Rapid screening tests facilitate presumptive diagnosis. Use one or more of the following tests:

Stem streaming test

The presence of *Pseudomonas solanacearum* in wilting potato stems can be assessed by the following simple presumptive test:

Cut the stem just above the soil level. Place the cut surface in a beaker with water. Shortly after, threads of bacterial slime will stream spontaneously out of the vascular bundles. Any other bacteria causing vascular infection in potato plants will not show this phenomenon

Detection of poly-ß-hydroxybutyrate (PHB) granules

The PHB granules in the cells of *Pseudomonas solanacearum* are visualized by staining with Nile blue A or with Sudan black B.

Either prepare a smear of the ooze or the suspended tissue on a microscope slide or prepare a smear of a 48 hour culture on YPGA or SPA (Appendix 1). Prepare positive control smears of a biovar2/race 3 strain and, if considered useful, a negative control smear of a heterologeous strain. Allow to dry. Pass the lower surface of the slide several times rapidly through the flame until the smear is fixed.

Nile blue test

(1) Flood the fixed smear with 1 % aqueous solution of Nile blue A.
Incubate for 10 minutes at 55 °C.

(2) Drain off the staining solution. Wash briefly in gently running tap water. Remove excess water with tissue paper.

(3) Flood the smear with 8 % aqueous acetic acid.
Incubate for one minute at ambient temperature.

(4) Wash in gently running tap water. Blot dry on tissue paper.

(5) Remoisten with a drop of water. Apply a coverslip.

(6) Examine the stained smear with an epifluorescence microscope at 450 nm under oil immersion at a magnification of 1000.

Observe for bright orange fluorescence of PHB granules. Also observe under normal light to ensure that the granules are intracellular and that cell morphology is typical of *Pseudomonas solanacearum*.

Sudan black test

(1) Flood the fixed smear with 0,3 % Sudan black B solution in 70 % ethanol. Incubate for 10 minutes at ambient temperature.

(2) Drain off the staining solution. Wash briefly in tap water. Remove excess water. Remove excess water with tissue paper.

(3) Dip the smear briefly in xylol. Blot dry on tissue paper.

Caution! Xylol is a harmful product. Work in a fume cabinet.

(4) Flood the smear with 0,5 % (w/v) aqueous safranin and leave for 10 seconds at ambient temperature.

Caution! Safranin is a harmful product. Work in a fume cabinet.

(5) Wash in gently running tap water. Blot dry on tissue paper. Apply a coverslip.

(6) Examine the stained smear with a light microscope using transmitted light under oil immersion at a magnification of 1000.

PHB granules in cells of *Pseudomonas solanacearum* stain blue-black. The cell wall stains pink.

Other tests

Other appropriate screening tests are the IF test (section III.2), the ELISA test (section III.3) and the PCR test (section III.4).

3. **Isolation procedure**

3.1 Remove ooze or sections of discoloured tissue from the vascular ring in the tuber or from the vascular strands in the stem. Suspend in a small volume of sterile distilled water or 50 mM phosphate buffer. Leave for 5—10 minutes on the bench.

3.2 Prepare a series of decimal dilutions of the suspension, e.g. 1/10 and 1/100 or more as considered appropriate.

3.3 Transfer a standard volume of the suspension and the dilutions on to a general nutrient medium (NA, YPGA and SPA, Appendix 1) and/or on to Kelman's tetrazolium medium (Appendix 1) and/or on to SMSA selective medium (Appendix 7). Spread or streak with an appropriate dilution plating technique. If considered useful, prepare separate plates of each medium used with a diluted cell suspension culture of a virulent biovar 2/race 3 strain of *Pseudomonas solanacearum* as a positive control.

3.4 Incubate the plates for three days at 28 °C. Incubation may be prolonged to six days if growth is slow, but colonies on SMSA plates often become atypical and die off.

On the general nutrient media, virulent isolates of *Pseudomonas solanacearum* develop pearly-white, flat, irregular and fluidal colonies often with characteristic whorls.

On Kelman's tetrazolium medium, typical colonies of virulent isolates of *Pseudomonas solanacearum* are cream, flat, irregular and fluidal with blood red coloured whorls in the centre. Avirulent forms of *Pseudomonas solanacearum* develop butyrous, deep-red colonies.

On SMSA medium, typical colonies of virulent isolates of *Pseudomonas solanacearum* are milky-white, flat, irregular and fluidal with blood red coloration in the centre.

Avirulent forms of *Pseudomonas solanacearum* develop less fluidal colonies which are completely pink to red on the SMSA medium.

3.5 Purify colonies with characteristic morphology by subculture on a general nutrient medium. Avoid regular subculturing which may induce loss of virulence.

4. **Confirmation test(s)**

4.1 *Identification of Pseudomonas solanacearum*

Identify pure cultures of *Pseudomonas solanacearum* by at least one of the following procedures:

Nutritional and enzymatic tests

Note: Include appropriate control strains in each test used.

The following phenotypic properties of *Pseudomonas solanacearum* are universally present or absent:

Fluorescent pigment	–
PHB inclusions	+
Oxidation/Fermentation (O/F) test	O+/F–
Catalase	+
Kovac's oxidase	+
Reduction of nitrate	+
Utilisation of citrate	+
Growth at 40 °C	–
Growth in 1 % NaCl	+
Growth in 2 % NaCl	–
Arginine dihydrolase	–
Gelatin liquefaction	–
Starch hydrolysis	–
Aesulin hydrolysis	–
Levan production	–

Media and methods are provided in Lelliott & Stead (1987)

IF-test

Prepare a suspension of 10^6 cells per ml from the culture and the control strain(s). Prepare a series of twofold dilutions of the antiserum. Apply the IF procedure (section III.2). The IF titre of the culture must be equivalent to that of the positive control.

ELISA test

Prepare a suspension of $>10^6$ cells per ml from the culture and the control strain(s). Apply the ELISA procedure (section III.3). The ELISA value of the culture must be equivalent to that of the positive control.

PCR test

Prepare a suspension of 10^6 cells per ml from the culture and the control strain(s). Apply the PCR procedure (section III.4). The PCR product of the culture must have the same size and restrictive enzyme analysis (REA) pattern as that of the positive control.

Fluorescent *in-situ* hybridisation (FISH)

Prepare a suspension of 10^6 cells per ml from the culture and the control strain(s). Apply the FISH procedure (van Beuningen *et al*, 1995) with the OLI-1 PCR primer (Seal *et al*, 1993) The culture must show the same reaction as the positive control.

Protein profiling

Denatured whole cell proteins are separated by polyacrylamide gel electrophoresis — PAGE (Stead, 1992a).

Fatty acid profiling (FAP)

Grow the culture and a positive control strain for 48 hours at 28 °C on trypticase soy agar and apply the FAP procedure (Janse, 1991; Stead, 1992a; Stead, 1992b). The profile of the culture must be identical to that of the positive control. Under the specified conditions, characteristic fatty acids are 14:0 3OH, 16:0 2OH, 16:1 2OH und 18:1 2OH.

4.2. *Strain characterization*

Strain characterization is optional but is recommended for each new case using at least one of the following: —

Biovar determination

Pseudomonas solanacearum is separated into biovars on the basis of the ability to produce acid from three hexose alcohols and three sugars (Hayward, 1964 & 1994):

	Biovar				
	1	2	3	4	5
Utilization of:					
— maltose	–	+	+	–	+
— lactose	–	+	+	–	+
— cellobiose	–	+	+	–	+
— mannitol	–	–	+	+	+
— sorbitol	–	–	+	+	–
— dulcitol	–	–	+	+	–

Additional tests differentiate biovar 2 in subphenotypes (Hayward, 1994):

	biovar 2	biovar 2-A	biovar 2-T
utilization of trehalose	–	+	+
utilization of inositol	+	–	+
utilisation of D-ribose	–	–	+
pectolytic activity	low	low	high

Race determination

The race (Buddenhagen *et al.*, 1962) can be determined on the basis of a pathogenicity test in tomato plants or eggplants and in tobacco plants and by a hypersensitivity reaction (HR) test in tobacco leaves (Lozano and Sequeira, 1970):

	Race(*)		
	1	2	3
Reaction in:			
— tomato plants/eggplants	wilting	no reaction	wilting
— tobacco plants	wilting	no reaction	no reaction
— tobacco leaves	necrosis (48 hrs) and wilting (7—8 days)	HR (12—24 hrs)	chlorosis (2—8 days)

(*) Race 4 (pathogenic on ginger and a few other hosts) and race 5 (pathogenic on mulberry only) are not included.

Race characterization by the pathogenicity test or tobacco hypersensitivity test may not be highly reliable and instead can be deduced from the biovar and the natural host of origin.

The culture can be further characterized by:

Genomic fingerprinting

Molecular differentiation of strains in the *Pseudomonas-solanacearum* complex can be done by:

RFLP analysis (Cook *et al*, 1989)

Repetitive sequence PCR [REP-, ERIC- & BOX-PCR (Louws *et al*, 1995; Smith *et al*, 1995)]

4.3. *Pathogenicity test*

This test is for confirmation of the diagnosis of *Pseudomonas solanacearum* and for the assessment of the virulence of the cultures identified as *Pseudomonas solanacearum.*

Prepare an inoculum of 10^6 cells per ml from the culture and a positive control strain. Inoculate 5—10 tomato plants or eggplants at, preferably, the third true leaf stage or older (section III, 6.). Incubate for up to two weeks at 22 °C—28 °C and high relative humidity with daily watering. Observe for wilting and/or epinasty, chlorosis, stunting.

Isolate from symptomatic plants as follows:

— Remove a section of tissue from the stem two cm above the inoculation point.

— Comminute and suspend in a small volume of sterile distilled water or 50 mM phosphate buffer. Then plate, incubate, and check for typical colonies of *Pseudomonas solanacearum.*

SECTION III

DETECTION AND IDENTIFICATION OF *PSEUDOMONAS SOLANACEARUM* IN SAMPLES OF POTATO TUBERS

Note: The standard sample size is 200 tubers. However, the procedure can be applied conveniently for samples with less than 200 tubers.

1. **Preparation of the sample for testing**

Note: The potato extract pellet obtained in this procedure can also be used for detection of *Clavibacter michiganensis* subsp. *sepedonicus*.

Pre-testing options if considered useful:

(i) Incubate the sample at 25—30 °C for up to two weeks to encourage multiplication of low *Pseudomonas-solanacearum* populations.

(ii) Wash the tubers under running water with appropriate disinfectants and detergents. Air dry the tubers.

1.1 Remove with a clean and disinfected scalpel or vegetable knife the skin at the heel end of the tuber so that the vascular tissues first become visible. Carefully cut out a small conical core (3—5 mm diameter) of vascular tissue at the heel end. Keep the amount of non-vascular tissue to a minimum. Process each of the tubers in the sample.

Note: Visual examination of the tubers (Section II.1) can be done at this stage. Set aside any tuber with symptoms or severe rotting and test separately (section II).

1.2 Collect the heel ends in a closed container. Preferably, the heel ends should be processed immediately. If this is not possible, store them for not more than 24 hours or, at 4 °C, for not longer than 72 hours.

1.3 Process the heel ends by one of the following procedures:

(i) Transfer the heel ends into an appropriate container.

Add a sufficient volume of maceration buffer (Appendix 2) to cover the heel ends.

Comminute the heel ends in a Waring Blender or by Ultra Thurrax until complete homogenization has just been achieved. Avoid excessive homogenization.

Allow the macerate to soak for 15—30 minutes.

(ii) Transfer the heel ends into an appropriate container.

Add a sufficient volume of maceration buffer to cover the heel ends.

Place the container on a rotary shaker.

Incubate at 50—100 rpm for 4 hours at 20 °C—22 °C or for 16—24 hours at 4 °C.

(iii) Transfer the heel ends into a strong disposable maceration bag (e.g. Stomacher bag with dimensions 105 mm × 150 mm, radiation sterile).

Crush the heel ends carefully with an appropriate tool, e.g. a hammer, until complete homogenization has been achieved.

Add a sufficient volume of maceration buffer to cover the heel ends.

Allow the macerate to settle for 15—30 minutes.

1.4 Extract the bacteria from the processed heel ends by one of the following procedures:

(i) Decant the macerate gently in a centrifuge tube while leaving the debris in the container or bag. If the decanted macerate is cloudy, centrifuge at not more than 180 g for 10 minutes at a temperature below 10 °C.

Centrifuge the decanted macerate, or the supernatant from the first centrifugation step, at 7 000 g for 15 minutes or at 10 000 g for 10 minutes at a temperature below 10 °C.

Discard the supernatant without disturbing the pellet.

(ii) Filter the macerate through a filtration system with pore size of 40—100 μm. Enhance filtration using a vacuum pump.

Collect the filtrate in a centrifuge tube.

Wash the filter with maceration buffer.

Centrifuge filtrate at 7 000 g for 15 minutes or at 10 000 g for 10 minutes at a temperature below 10 °C.

Discard the supernatant without distrubing the pellet.

1.5 Resuspend the pellet in 1 ml pelletbuffer (Appendix 2).

Divide in two equal parts and transfer each part to a microvial.

Use one microvial for testing. Conserve the remainder of this extract at 4 °C during testing.

Add 10—25 % (v/v) of sterile glycerol to the other microvial. Vortex. Store at - 18 °C (weeks) or at - 70 °C (months).

2. **IF-test**

Use antiserum for *Pseudomonas solanacearum*, preferably to race 3/biovar 2. Determine the titre on a suspension of 10^6 cells per ml from the homologeous strain of *Pseudomonas solanacearum* with an appropriate dilution of the fluorescein isothiocyanate (FITC) conjugate, according to the manufacturer's recommendations. The crude antiserum should have an IF titre of at least 1:2000.

Use multiwell microscope slides with preferably 10 windows of at least 6 mm diameter.

Include a FITC conjugate control on each test slide. The test should be repeated with a PBS control included if any positive cell is observed in the FITC control.

Prepare separate positive control slides with a suspension of 10^6 cells per ml from a strain of the appropriate race/biovar of *Pseudomonas solanacearum*. Use one slide in each set of tests.

2.1 Prepare the test slides by one of the following procedures:

(i) For pellets with relative little starch:

Pipette a measured standard volume (15 µl is appropriate for 6 mm window diameter — scale up volume for larger windows) of the resuspended pellet on a row of windows. The remaining row can be used as duplicate or for a second sample as presented in Figure 1.

(ii) For other pellets:

Prepare decimal dilutions, viz. 1/10, 1/100 and 1/1000 of the resuspended pellet in pelletbuffer. Pipette a measured standard volume (15 µl is appropriate for 6 mm window diameter — scale up volume for larger windows) of the resuspended pellet and each dilution on a row of windows. The remaining row can be used as duplicate or for a second sample as presented in Figure 2.

2.2 Let the droplets dry. Fix the bacterial cells to the slide either by heating, flaming or with 95 % ethanol.

2.3 IF procedure

(i) According to test slide preparation in 2.1 (i):

Prepare a set of twofold dilutions of the antiserum in IF buffer (Appendix 3):

1/4 of the titre (T/4), 1/2 of the titre (T/2), the titre (T) and twice the titre (2T).

(ii) According to test slide preparation in 2.1 (ii):

Prepare the working dilution (WD) of the antiserum in IF buffer. The working dilution is the dilution of the antiserum with optimum specificity and is usually half of the titre.

Figure 1

Preparation of the test slide according to 2.1 (i) and 2.3 (i)

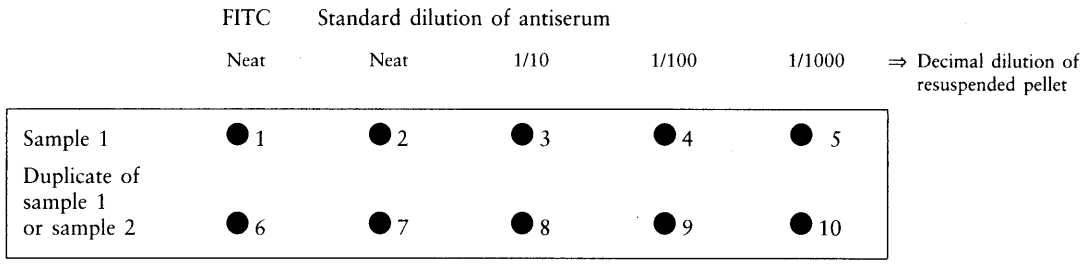
One standard dilution of resuspended pellet

T = titre

	FITC	T/4	T/2	T	2T	⇒ Twofold dilutions of antiserum
Sample 1	● 1	● 2	● 3	● 4	● 5	
Duplicate of sample 1 or sample 2	● 6	● 7	● 8	● 9	● 10	

Figure 2

Preparation of the test slide according to 2.1 (ii) and 2.3 (ii)



2.3.1 Arrange the slides on damp tissue paper.

Cover the test windows with the antiserum dilution(s). Apply PBS on the FITC windows. The volume of antiserum applied on the windows must be equivalent to the volume of extract applied.

2.3.2 Incubate under a cover for 30 minutes at ambient temperature.

2.3.3 Shake the droplets of antiserum off the slide and rinse the slides carefully with IF buffer. Wash for five minutes in IF buffer-Tween and subsequently for five minutes in IF buffer (Appendix 3). Carefully remove excess moisture.

2.3.4 Arrange the slides on damp tissue paper. Cover the test windows and the FITC window with the dilution of FITC conjugate used to determine the titre. The volume of conjugate applied on the windows must be identical to the volume of antiserum applied.

2.3.5 Incubate under a cover for 30 minutes at ambient temperature.

2.3.6 Shake the droplets of conjugate off the slide. Rinse and wash as before (2.3.3). Carefully remove excess moisture.

2.3.7 Pipette 5—10 μl 0,1 M phosphate-buffered glycerol (Appendix 3) or a similar mountant on each window and apply a coverslip.

2.4 Reading the IF test

Examine test slides on an epifluorescence microscope with filters suitable for excitation of FITC, under oil immersion at a magnification of 500—1000. Scan windows across two diameters at right angles and around the perimeter.

Check the positive control slide first. Cells must be bright fluorescent and completely stained. *Note: The test must be repeated if the staining is aberrant.*

Read the test slides. Observe first for absence of fluorescing cells in the FITC control windows. Fluorescing cells in the FITC control indicate non-specific binding of the conjugate, autofluorescence or contamination: *Note: Repeat the test if such is observed.*

Observe for bright fluorescing cells with characteristic morphology of *Pseudomonas solanacearum* in the test windows. The fluorescence intensity must be equivalent to the positive control strain at the same antiserum dilution. Cells with incomplete staining or with weak fluorescence must be disregarded, unless there are many such cells (see interpretation of the IF test result).

Interpretation of the IF test result:

(i) If bright fluorescing cells with characteristic morphology are not found, then the IF test is negative.

(ii) If bright fluorescing cells with characteristic morphology are found, then determine the mean number of cells per microscope field and calculate the number of cells (N) per ml of resuspended pellet (Appendix 4).

A population of approximately 10^3 cells per ml of resuspended pellet is considered to be the limit of detection for the IF test,

— for samples with $N > 10^3$ cells per ml of resuspended pellet, the IF test is considered positive,

— for samples with $N > 10^3$ cells per ml of resuspended pellet, the IF test may be considered positive.

(iii) If large numbers ($> 10^5$ cells per ml) of incomplete or weakly fluorescing cells are seen at the titre of the antiserum, a second test should be carried out:

— either a test based on a different biological principle, or

— a repeat IF test, with either a second antiserum or a ten-fold dilution of the pellet.

3. **ELISA test**

Based upon Robinson-Smith *et al.*, 1995

Use antiserum for *Pseudomonas solanacearum*, preferably to race 3/biovar 2. Determine the titre on a suspension of 10^6 cells per ml from the homologeous strain of *Pseudomonas solanacearum*.

The use of *NUNC-Polysorb* microtitre plates is recommended.

Include a negative potato extract control and a phosphate buffered saline (PBS) control.

Use a suspension of $> 10^6$ cells per ml from a strain of the appropriate race/biovar of *Pseudomonas solanacearum* as the positive control. Test in an identical manner as the sample(s) but well-separated from the samples on the microtitre plate.

3.1 Pipette 100—200 µl of the resuspended pellet in a microvial.

Heat for 4 minutes at 100 °C. Remove the microvial on ice.

3.2 Add an equal volume of double strength carbonate coating buffer (Appendix 5). Vortex.

3.3 Apply 100 µl aliquots to each of at least two wells of the microtitre plate. Incubate for one hour at 37 °C or overnight at 4 °C.

3.4 Flick out the extracts from the wells. Wash the wells three times with PBS-Tween (Appendix 5), leaving the last washing solution in the wells for at least five minutes.

3.5 Prepare the appropriate dilution of *Pseudomonas solanacearum* antiserum in blocking buffer (Appendix 5). Apply 100 µl of antiserum dilution to the wells.

Incubate for one hour at 37 °C.

3.6 Flick out the antiserum from the wells. Wash the wells as before (3.4).

3.7 Prepare the appropriate dilution of alkaline phosphatase conjugate in blocking buffer. Apply 100 µl of conjugate dilution to the wells.

Incubate for one hour at 37 °C.

3.8 Flick out the conjugate from the wells. Wash the wells as before (3.4 and 3.6).

3.9 Prepare the alkaline phosphatase substrate solution (Appendix 5). Apply 100 µl to the wells. Incubate for 30 minutes to one hour in the dark at ambient temperature.

3.10 Read absorbance at 409 nm.

Interpretation of the ELISA test:

The ELISA test is negative if the optical density (OD) of the sample is $< 2 \times$ OD of the negative control.

The ELISA test is positive if the optical density (OD) of the sample is $> 2 \times$ OD of the negative control.

4. **PCR test**

Based on Seal *et al.*, 1993

Note: Filterplugged pipette tips must be used during all stages of sample preparation and other manipulations involving PCR.

Prepare a suspension of 10^6 cells per ml from a race 3/biovar 2 strain of *Pseudomonas solanacearum* as the positive control. Test in an identical manner as the sample(s).

4.1 Pipette 100 µl of the resuspended pellet into a microvial.

Alternatively, transfer 90 µl of the resuspended pellet to a microvial containing 10 µl of 0,5M NaOH. Mix by repeatedly inverting the microvial.

4.2 Heat for four minutes at 100 °C. Remove the microvial immediately on ice.

4.3 Prepare at least two decimal dilutions, e.g. 1/10 and 1/100 or more if considered useful, in sterile distilled or ultra pure water (UPW).

4.4 Prepare the PCR reaction mix (Appendix 6) in a sterile vial by adding the following components in the following order:

For a 50 µl reaction volume:

Component	Quantity	Final concentration
Sterile distilled or UPW	30,8 µl—33,8 µl	
10 × PCR Buffer	5,0 µl	1 ×
d-ATP	1,0 µl	0,2 mM
d-CTP	1,0 µl	0,2 mM
d-GTP	1,0 µl	0,2 mM
d-TTP	1,0 µl	0,2 mM
Primer OLI-1 (20 µM)	2,5 µl	1 mM
Primer Y-2 (20 µM)	2,5 µl	1 mM
Taq polymerase (5U/µl)	0,2 µl	1,0 U
Total volume	45 µl—48 µl	

For more reactions:

Calculate the quantity of each component for the required number of reactions.

Mix the components and transfer 45 µl—48 µl of the mix into sterile PCR vials.

Keep the vials with the PCR reaction mix on ice.

For 25 µl reaction volumes:

Scale down components accordingly.

4.5 PCR amplification

4.5.1 Optional: Pulse centrifuge the vials with the boiled sample and positive control.

Add, in the specified order, 2—5 µl of the sample(s), water control and positive control to the vials with the PCR reaction mix. Place the vials in the heating block of the DNA thermal cycler.

4.5.2 Run the following programme:

1 cycle of:
(i) 2 minutes at 96 °C: denaturation of template

50 cycles of
(ii) 20 seconds at 94 °C: denaturation
(iii) 20 seconds at 68 °C: annealing of primers
(iv) 30 seconds at 72 °C: extension of copy

1 cycle of:
(v) 10 minutes at 72 °C: further extension

1 cycle of:
(vi) hold at 4 °C

Note: These parameters are for a Perkin Elmer 9600. Other thermal cycles may require mineral oil overlay in the PCR reaction vials and/or modification of the duration of step (ii), (iii) and (iv) in the amplification profile.

4.5.3 Remove the vials from the thermal cycler. Analyse the PCR product. If not done immediately, store the vials at 4 °C for use in the same day or at -18 °C for longer.

4.6 Analysis of the PCR product

The PCR fragments are detected by agarose gelelectrophoresis and staining with ethidium bromide.

4.6.1 Prepare an appropriate agarose gel by gently bringing to the boil agarose in tris-acetate-electrophoresis (TAE) buffer.

4.6.2 Cool the molten agarose to 50—60 °C, pour into the mould of the electrophoresis unit and insert the comb. Let the solution solidify.

4.6.3 Remove the comb. Submerge the gel in TAE so that it is just covered (2—3 mm) with the buffer.

4.6.4 Place 3 µl droplets of loading buffer on parafilm. Add 12 µl of the PCR product from either the samples, the positive control or the water control and mix by gentle aspiration in the pipette tip before loading. The given volumes may be modified to fit with the capacity of the wells in the agarose gel.

4.6.5 Carefully load the wells of the gel. Include for reference an appropriate DNA marker in at least one well.

4.6.6 Connect the wires on the power supply and the electrophoresis equipment. Run the gel at 5—8 V/cm until the front of the tracking indicator is within 1 cm of the end of the gel.

4.6.7 Switch off the power supply. Disconnect the wires from the electrophoresis unit.

Carefully remove the gel. Soak it in ethidium bromide solution for 30—45 minutes.

Note: Wear disposable gloves at all times when handling ethidium bromide which is a powerful mutagen!

4.6.8 Destain in distilled water for 10—15 minutes.

4.6.9 Visualise the amplified DNA fragment(s) by UV transillumination. The PCR product of *Pseudomonas solanacearum* with primer set OLI-1 and Y-2 is 288 bp in length. Check against the DNA marker and against the positive control.

Note: The water control must be negative in each case. If positive, repeat the test.

4.6.10 Photograph the gel if a permanent record is required.

4.6.11 Confirm the authenticity of the amplified fragment by restriction enzyme analysis (REA).

4.7 Restriction Enzyme Analysis (REA)

4.7.1 Transfer 8,5 µl from the PCR product (4.5.3) to a new microvial. Add 1 µl of 10 × enzyme buffer and 0,5 µl of restriction enzyme *Ava* II.

4.7.2 Mix by gentle aspiration in the pipette tip. If drops remain on the walls of the vial, pulse spin in a microcentrifuge. Incubate for one hour at 37 °C.

4.7.3 Analyse the digested PCR fragment by agarose gelelectrophoresis as before (4.6).

Interpretation of the PCR test result:

The PCR test is negative if the characteristic 288 bp fragment is not detected and the fragment is detected for the positive control strain of *Pseudomonas solanacearum.*

The PCR test is positive if the 288 bp fragment is detected and REA-analysis of the amplified fragment is identical with the positive control strain of *Pseudomonas solanacearum.*

5. **Selective plating test**

Based upon Elphinstone *et al.*, 1996

5.1 Perform the test by an appropriate dilution plating technique, e.g.:

(i) Prepare at least two decimal dilutions, viz. 1/10 and 1/100 or more if considered useful, of the resuspended pellet in pelletbuffer. Pipette a measured standard volume (50—100 µl) of the resuspended pellet and each dilution onto modified SMSA selective medium (Appendix 7) and spread with a glass rod over the whole surface of the medium.

If considered useful, also perform a dilution streak of a 10 µl loopful of the resuspended pellet. Flame the loop between streaks.

(ii) Transfer a measured standard volume (50—100 µl) of the resuspended pellet onto modified SMSA selective medium and spread with a glass rod over the whole surface of the medium. Streak the rod without flaming on at least two other modified SMSA plates.

5.2 Apply, by the same dilution plating technique, a suspension of 10^6 cells per ml from a virulent race 3/biovar 2 strain of *Pseudomonas solanacearum* as the positive control on a set of separate modified SMSA plates.

5.3 Incubate the plates at 28 °C. Start reading the plates after three days. If negative, incubate further up to six days. Colonies of virulent isolates of *Pseudomonas solanacearum* are milky-white, flat, irregular and fluidal with blood red coloration in the centre and showing internal streaking or whorling.

5.4 Purify colonies with characteristic morphology by subculturing onto a general nutrient medium (Appendix 1).

5.5 Identify pure cultures (section II, 4.1) and confirm *Pseudomonas solanacearum* cultures by a pathogenicity test (section II, 4.3).

Interpretation of the selective plating test result:

The selective plating test is negative if no bacterial colonies are isolated after six days or if no colonies characteristic of *Pseudomonas solanacearum* are isolated, provided that no inhibition is suspected by colonies of other bacteria and that colonies characteristic of *Pseudomonas solanacearum* are found in the positive controls.

The selective plating test is positive if colonies characteristic of *Pseudomonas solanacearum* are isolated.

6. **Bioassay test**

Based upon Janse, 1988.

6.1 Use 10 test plants of susceptible tomato or eggplant seedlings at the third true leaf stage for each sample. Do not water the testplants for 24 hours before inoculation.

6.2 Distribute 100 µl of resuspended pellet between the testplants. Inoculate in the stem between the cotyledons and at one or more other places.

6.3 Inoculate, by the same technique, 10 seedlings with a suspension of 10^6 cells per ml from a virulent biovar 2/race 3 strain of *Pseudomonas solanacearum* as the positive control and with pelletbuffer as the negative control. Separate the positive control plants from the other plants to avoid cross-contamination.

6.4 Grow the test plants further for up to four weeks at 22 °C—28 °C and high relative humidity with daily watering. Observe for wilting, epinasty, chlorosis and/or stunting.

6.5 Isolate from infected plants (section II). Identify pure cultures with characteristic morphology (section II.4.1) and confirm *Pseudomonas solanacearum* cultures by a pathogenicity test (section II.4.3).

6.6 If considered useful, check absence of infection in batches of test plants not showing any indication of infection. Remove from each test plant a one cm section of stem from two cm above the inoculation point. Homogenize the tissues in maceration buffer. Perform dilution plating (section III.5.1). If positive, identify pure cultures with characteristic morphology (section II.4.1) and confirm *Pseudomonas solanacearum* cultures by a pathogenicity test (section II.4.3).

Interpretation of the bioassay test result:

The bioassay test is negative if test plants are not infected by *Pseudomonas solanacearum*, and provided that *Pseudomonas solanacearum* is detected in the positive controls.

The bioassay test is positive if test plants are infected by *Pseudomonas solanacearum*.

7. **Enrichment tests**

Based upon Elphinstone *et al.*, 1996

7.1 Transfer 100 µl of the resuspended pellet in three ml of modified SMSA broth (Appendix 7).

7.2 Incubate for 48 hours, and in any case not longer than 72 hours, at 28 °C with the cap of the tube loosely fitted for aeration.

7.3 Tighten cap and vortex. Aliquot for the IF test (this section, 2.), the ELISA test (this section, 3.) and/or the PCR test (this section, 4.).

8. **Pathogenicity test**

Refer to Section II.4.3.

Appendix 1

Nutrient media for isolation and culture of *Pseudomonas solanacearum*

Nutrient Agar (NA)

Nutrient Agar (Difco)	23 g
Distilled water	1 litre

Prepare ½ litre volumes of medium in one litre flasks.

Dissolve the ingredients.

Sterilise by autoclaving at 121 °C for 15 minutes.

Cool to 50 °C. Pour plates.

Yeast-Peptone-Glucose-Agar (YPGA)

Yeast Extract (Difco)	5 g
Bacto Peptone (Difco)	5 g
D(+) Glucose (monohydrate)	10 g
Bacto Agar (Difco)	15 g
Distilled water	1 litre

Prepare ½ litre volumes of medium in one litre flasks.

Dissolve the ingredients.

Sterilise by autoclaving at 121 °C for 15 minutes.

Cool to 50 °C. Pour plates.

Sucrose Peptone Agar (SPA)

Sucrose	20 g
Peptone	5 g
K_2HPO_4	0,5 g
$MgSO_4 \cdot 7H_2O$	0,25 g
Bacto Agar (Difco)	15 g
Distilled water	1 litre

Prepare ½ litre volumes of medium in one litre flasks.

Dissolve the ingredients. Adjust to pH 7,2—7,4 if necessary.

Sterilise by autoclaving at 121 °C for 15 minutes.

Cool to 50 °C. Pour plates.

Kelman's tetrazolium medium

Casamino acids (Difco)	1 g
Bacto Peptone (Difco)	10 g
Dextrose	5 g
Bacto Agar (Difco)	15 g
Distilled water	1 litre

Prepare ½ litre volumes of medium in one litre flasks.

Dissolve the ingredients. Sterilise by autoclaving at 121 °C for 15 minutes.

Cool to 50 °C.

Add a filter-sterilized aqueous solution of triphenyl tetrazolium chloride (Sigma) to obtain a final concentration of 50 mg per litre.

Pour plates.

Appendix 2

Materials for sample preparation

Maceration Buffer: 50 mM phophate buffer, pH 7,0

This buffer is used for tissue maceration.

Na_2HPO_4	4,26 g
KH_2PO_4	2,72 g
Distilled water	1 litre

Dissolve the ingredients and check pH. Aliquot as considered appropriate.

Sterilise by autoclaving at 121 °C for 15 minutes.

The addition of 5 % polyvinylpyrrolidone-40000 MWT (PVP-40) is recommended when performing the direct PCR test to reduce the incidence of amplification inhibition by aromatic molecules in the extract.

The addition of a deflocculant, an antifoam agent or an anti-oxidant is recommended using the Waring Blender or Ultra Turrax homogenization procedure for maceration of the potato tissue cores.

Lubrol flakes	0,5 g per litre
DC Silicone antifoam	1,0 ml per litre
Tetrasodiumpyrophosphate	1,0 g per litre

Autoclave separately. Add to the desired concentration.

Pelletbuffer: 10 mM phosphate buffer, pH 7,2

This buffer is used for resuspension and dilution of potato heel and pellets.

$Na_2HPO_4 \cdot 12H_2O$	2,7 g
$NaH_2PO_4 \cdot 2H_2O$	0,4 g
Distilled water	1 litre

Dissolve the ingredients and check pH. Aliquot as considered appropriate.

Sterilise by autoclaving at 121 °C for 15 minutes.

Appendix 3

Materials for the IF test

IF-buffer: 10 mM phosphate buffered saline (PBS) pH 7,2

This buffer is used for dilution of antisera.

$Na_2 HPO_4 \cdot 12 H_2O$	2,7 g
$NaH_2 PO_4 \cdot 2 H_2O$	0,4 g
NaCl	8,0 g
Distilled water	1 litre

Dissolve the ingredients and check pH. Aliquot as considered appropriate.

Sterilise by autoclaving at 121 °C for 15 minutes.

IF-buffer-Tween

This buffer is used for washing the slides. Add 0,1 % Tween 20 to the IF buffer.

0,1 M Phosphate buffered glycerol pH 7,6

This buffer is used as a mountant fluid on the windows of the IF slide to enhance fluorescence.

$Na_2 HPO_4 \cdot 12 H_2O$	3,2 g
$NaH_2 PO_4 \cdot 2 H_2O$	0,15 g
Glycerol	50 ml
Distilled water	100 ml

Appendix 4

Determination of contamination level in the IF test

Surface area (S) of window of multispot slide

$$= \frac{\pi D^2}{4}$$

Where D = diameter of window (1)

Surface area (s) of objective field

$$= \frac{\pi d^2}{4}$$

Where d = diameter of field (2)

Calculate d either by direct measurement or from the following formulae:

$$s = \frac{\pi i^2}{G^2 K^2 \times 4} \quad (3)$$

where i = field coefficient (depends upon ocular type and varies from 8 to 24),

K = tube coefficient (1 or 1,25),

G = magnification of 100x, 40x etc. objective.

from (2)

$$d = \sqrt{\frac{4s}{\pi}} \quad (4)$$

from (3)

$$d = \sqrt{\frac{4 \times \frac{\pi i^2}{G^2 K^2 \times 4}}{\pi}} = \frac{i}{GK}$$

Count the number of typical fluorescent cells per field (c).

Calculate the number of typical fluorescent cells per window (C).

$$C = c \frac{S}{s}$$

Calculate the number of typical fluorescent cells per ml pellet (N)

$$N = C \times \frac{1\,000}{y} \times F$$

where y = volume of pellet on window,

F = pellet dilution factor.

Appendix 5

Materials for the ELISA test

Double strength Carbonate Coating Buffer, pH 9,6

Na_2CO_3	6,36 g
$NaHCO_3$	11,72 g
Distilled water	1 litre

Dissolve the ingredients and check pH. Aliquot as considered appropriate.

Sterilise by autoclaving at 121 °C for 15 minutes.

Sodium sulphite at a final concentration of 0,2 % may be added as anti-oxidant if the extract contains a high fraction of aromatic molecules.

10 × Phosphate Buffered Saline (PBS), pH 7,4

NaCl	80 g
KH_2PO_4	2 g
$Na_2HPO_4 \cdot 12H_2O$	29 g
KCl	2 g
Distilled water	1 litre

Dissolve the ingredients and check pH. Aliquot as considered appropriate.

Sterilise by autoclaving at 121 °C for 15 minutes.

Phosphate Buffered Saline-Tween (PBS-T)

10 × PBS	100 ml
10 % Tween 20	5 ml
Distilled water	895 ml

Blocking (antibody) Buffer (must be freshly prepared)

10 × PBS	10 ml
Polyvinylpyrrolidone-44000 MWT (PVP-44)	2,0 g
10 % Tween 20	0,5 g
Milk powder	0,5 g
Distilled water	to 100 ml

Alkaline phosphatase substrate solution pH 9,8

Diethanolamine	97 ml
Distilled water	800 ml

Mix and adjust to pH 9,8 with concentrated HCl.

Make up to one litre with distilled water.

Add 0,2 g $MgCl_2$.

Dissolve two phosphatase substrate five mg tablets (Sigma) per 15 ml of solution.

Appendix 6

Materials for the PCR test

Oligonucleotide primer sequence

Primer OLI-1	5′-GGGGGTAGCTTGCTACCTGCC-3′
Primer Y-2	5′-CCCACTGCTGCCTCCCGTAGGAGT-3′

For materials see Seal *et al* (1993).

Appendix 7

Materials for selective plating and enrichment tests

SMSA Selective medium (Engelbrecht, 1994 as modified by Elphinstone *et al*, 1996).

Basal medium	
Casamino acids (Difco)	1 g
Bacto peptone (Difco)	10 g
Glycerol	5 ml
Agar (Difco)	15 g
Distilled water	1 litre

Prepare 1/2 litre volumes of medium in one litre flasks.

Dissolve the ingredients and check pH. Adjust pH, if necessary, to 6,5 before autoclaving. *Pseudomonas solanacearum* will not grow well on the medium at pH > 7,0.

Sterilise by autoclaving at 121 °C for 15 minutes.

Cool to 50 °C.

Add the following ingredients (all from Sigma) to obtain the specified final concentrations:

Crystal violet	5 mg per litre		
Polymixin B sulphate	100 mg per litre	(approximately 600 000 units)	Sigma P-1004
Bacitracin(*)	25 mg per litre	(approximately 1 250 units)	Sigma B-0125
Chloramphenicol	5 mg per litre		Sigma C-3175
Penicillin-G	0,5 mg per litre	(approximately 825 units)	Sigma P-3032
Tetrazolium salts	50 mg per litre		

Dissolve the ingredients in 70 % ethanol to the given concentrations for the volume of medium prepared. Some ingredients, *viz.* polymixin B and chloramphenicol require slight warming and shaking.

SMSA broth (Elphinstone *et al.*, 1996)

Prepare as for the SMSA selective medium but delete the agar.

Dispense in three ml aliquots into 30 ml disposable Universal tubes.

(*) If considered necessary, increasing bacitracin concentration to 300 ppm may reduce contaminating saprophytic bacteria without reducing the recovery of *Pseudomonas solanacearum*.

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