L.N. 270 of 2004

PLANT QUARANTINE ACT, 2001
(ACT NO. XVIII OF 2001)

Control of *Ralstonia solanacearum* (Smith) Yabuuchi *et al* Regulations, 2004

IN exercise of the powers conferred by article 32 of the Plant Quarantine Act 2001, the Minister for Rural Affairs and the Environment has made the following regulations:-

Title and commencement

1. The title of these regulations is the Control of *Ralstonia solanacearum* (Smith) Yabuuchi *et al* Regulations, 2004 and they shall come into force on 15th May, 2004.

Scope.

2. The scope of these regulations is to lay down the measures to be taken against *Ralstonia solanacearum (Smith) Yabuuchi et al.*, previously known as *Pseudomonas solanacearum (Smith) Smith*, (hereinafter referred to as 'the organism'), in order to, with respect to the host plants of the organism listed in Schedule I Section I, to these regulations (hereinafter referred to as 'the listed plant material'):(a) locate it and determine its distribution;(b) prevent its occurrence and spread; and(c) if found, to prevent its spread and to control it with the aim of eradication.

Official surveys.

3. The Plant Health Department shall conduct annual systematic official surveys for the organism on the listed plant material originating in Malta. In order to identify other possible sources of contamination threatening the production of the listed plant material, the Plant Health Department shall carry out a risk assessment and, unless no risk of spread of the organism has been identified during that assessment, it shall, in production areas of the listed plant material, conduct targeted official surveys for the organism on plants other than the listed plant material, including wild solanaceous host plants, as well as on surface water which is used for irrigation or spraying of listed plant material and on liquid waste discharged from industrial processing or packaging premises handling listed plant material and used for irrigation or spraying of listed plant material. The extent of these targeted surveys shall be determined according to the risk identified. The Plant Health Department may also conduct official surveys for the organism on the material, such as

growing medium, soil and solid waste from industrial processing or packaging premises.

Methods to carry out surveys

4. The official surveys provided for in regulation 3 of these regulations shall be carried out:

(a) for the listed plant material, according to the details set down in point 1 of Section II, Schedule I to these regulations; and,

(b) for host plants other than the listed plant material, and for water including liquid waste, in accordance with appropriate methods and, where appropriate, samples shall be taken and subjected to official or officially supervised laboratory testing;

(c) where appropriate for other material, in accordance with appropriate methods.For these surveys, further details of the inspection procedures and the number, origin, stratification and timing of collection of samples shall be decided by the Plant Health Department based on sound scientific and statistical principles and the biology of the organism and taking into account the particular production systems of the listed plant material and, as appropriate, of other host plants of the organism.

Notification of results of surveys.

5. The details and results of the official surveys provided for in regulation 3 of these regulations shall be notified each year to the other Member States and to the Commission in accordance with the provisions of point 2 of Section II, Schedule I to these regulations. These notifications shall be submitted by the 1st June except for potatoes use as farm-saved seed for which the notification shall be submitted by the 1st September. The details and results for crops shall be related to the previous year's production.

Suspected cases.

6. (1) The suspected occurrence or confirmed presence of the organism in Malta shall be reported to the Plant Health Department.

(2) In each case of suspected occurrence, the Plant Health Department shall ensure completion of official or officially supervised laboratory testing using, for the listed plant material, the relevant method set out in Schedule II to these regulations and in accordance with the conditions specified in point 1 of Schedule III, or, in all other cases, any other officially approved method, in order to confirm or refute the suspected occurrence. In the case of confirmation, the requirements laid down in point 2 of ScheduleIII shall apply.

(3) Pending the confirmation or refutation of a suspected occurrence under regulation 6(2) of these regulations, in each case of suspected occurrence where, either:

(i) diagnostic symptoms of the disease caused by the organism have been seen and a positive result in the rapid screening test(s), as specified in Schedule II section I, point 1 and section II has been obtained; or,

(ii) a positive result in the screening test(s) as specified in Schedule II section I, point 2 and section III has been obtained,the Plant Health Department shall:

(a) prohibit the movement of plants and tubers from all crops, lots or consignments from which the samples have been taken, except under their control and provided that it has been established that there is no identifiable risk of the organism spreading;

(b) take steps to trace the origin of the suspected occurrence;

(c) introduce appropriate additional precautionary measures based on the level of estimated risk, particularly in relation to production of the listed plant material and the movement of seed potato lots other than those referred to in point (a), produced on the place of production from which the samples referred to in point (a) were taken, in order to prevent any spread of the organism.

(4) In those cases of suspected occurrence where there is a risk of contamination of the listed plant material or surface water from or into another Member State(s), where the suspected occurrence has been reported in Malta the Plant Health Department shall immediately notify, according to the risk identified, the details of the said suspected occurrence to any other Member Stateconcerned, and shall cooperate with the said Member States. Where any other Member State so notifies the Plant Health Department, the Plant Health Department shall introduce precautionary measures in accordance with regulation 3(ii)(c) of these regulations and take any further action, as appropriate.

Where the presence of organism is confirmed

7. (1) lf official or officially supervised laboratory testing, using, for the listed plant material, the relevant method set out in Schedule II to these regulations or, in all other cases, any other officially approved method, confirms the presence of the organism in a sample taken pursuant to these regulations, the Plant Health Department, having

regard to sound scientific principles, the biology of the organism and the particular production, marketing and processing systems of the host plants of the organism in Malta, shall:

(a) for the listed plant material;

(i) establish an investigation to determine the extent and primary source(s) of the contamination in accordance with the provisions of Schedule IV to these regulations, with further testing in accordance with regulation 6(2) of these regulations, on, at least, all clonally related seed potato stocks, and,

(ii) designate as contaminated the listed plant material, consignment and/or lot from which the sample was taken, and the machinery, vehicle, vessel, store, or units thereof, and any other objects including packaging material which have been in contact with the listed plant material from which the sample was taken; also designate as contaminated, where appropriate, any field, unit of protected crop production and place of production from which the listed plant material was harvested and from which the sample was taken; and for those samples taken in the growing season, designate as contaminated any field, place of production, and, where appropriate, unit of protected crop production from which the sample was taken, and,

(iii) determine, in accordance with the provisions of point 1 of Schedule V to these regulations, the extent of probable contamination through pre- or post-harvest contact, through production, irrigation or spraying links or through clonal relationship with the designated contamination, and,

(iv) demarcate a zone on the basis of the designation of contamination under point (a)-(ii) of this sub-regulation, the determination of the extent of probable contamination under point (a)-(iii) of this sub-regulation, and the possible spread of the organism, in accordance with the provisions of point 2(i) of Schedule V to these regulations;

(b) for crops of host plants other than those mentioned under point (a) where production of the listed plant material is identified at risk,

(i) establish an investigation in accordance with point (a)(i) of this sub-regulation;and

(ii) designate as contaminated the host plants of the organism from which the sample was taken; and

(iii) determine the probable contamination and demarcate a zone in accordance with points (a)(iii) and (a)(iv) of this sub-regulation, respectively, in relation to production of the listed plant material;

(c) for surface water (including liquid waste discharges from industrial processing or packaging premises handling listed plant material) and associated wild solanaceous host plants, where production of the listed plant material is identified at risk through irrigation, spraying, or flooding of the surface water,

(i) establish an investigation including an official survey at appropriate times on samples of surface water and if present wild solanaceous host plants to establish the extent of the contamination; and

(ii) designate as contaminated the surface water from which any sample was taken, to the extent appropriate and on the basis of the investigation under point (c)(i) of this sub-regulation; and

(iii) determine the probable contamination and demarcate a zone on the basis of the designation of contamination under point (c)(ii) of this sub-regulation, and the possible spread of the organism taking into account the provisions of points 1 and 2 (ii) of Schedule V to these regulations.

(2) The Plant Health Department shall immediately notify the other Member States and the Commission, in accordance with the provisions of point 3 of Schedule V, of any contamination designated under paragraphs l(a)(ii) and l(c)(ii) of this regulation and the details of the zone demarcation under paragraph 1 (a) (iv) of this regulation and, where applicable, under paragraph l(c)(iii) of this regulation. The Plant Health Department shall at the same time submit to the Commission the additional notification set out at point 4 of Schedule V to these regulations.

(3) Where the Plant Health Department receives a notification from other Member States in terms of paragraph 2 of Article 5 of the European Council Directive 98/57, it establish an investigation in accordance with paragraph l(a)(i) of this regulation and, where applicable, paragraph l(c)(i) of this regulation and take further action,

as appropriate, in accordance with regulations (7)1 and (7)2 of these regulations.

Contaminated material.

8. (1) The listed plant material designated to be contaminated under sub-regulation 7(1)(a)(ii) of these regulations may not be planted and, under the control and approval of the Plant Health Department, it shall be subjected to one of the provisions of point 1 of Schedule VI to these regulations, such that it is established that there is no identifiable risk of the organism spreading.

(2) The listed plant material determined as probably contaminated under sub-regulation 7(1)(a) (iii) and (c)(iii) of these regulations including listed plant material for which a risk has been identified, produced on places of production determined as probably contaminated under sub-regualtion 7(1)(a)(iii) of these regulations may not be planted and shall, under the control of the Plant Health Department, be put to appropriate use or disposal as specified in point 2 of Schedule VI to these regulations, such that it is established that there is no identifiable risk of the organism spreading.

(3) Any machinery, vehicle, vessel, store, or units thereof, and any other objects including packaging material, designated as contaminated under sub-regulation 7(1)(a)(ii) of these regulations or determined as probably contaminated under sub-regulations 7(1)(a)(iii) and (c)(iii) of these regulations, shall either be destroyed or decontaminated using appropriate methods as specified in point 3 of Schedule Vl to these regulations. After decontamination, any such objects shall no longer be considered contaminated.

(4) Without prejudice to the measures implemented under sub-regulations 8(1), 8(2) and 8(3)-above, in the zone demarcated under regulation 7(1)(a)(iv) and (c)(iii) of these regualtions, a series of measures, as specified in points 4.1 and 4.2 of Schedule VI to these regulations, shall be implemented. Details of these measures shall be notified annually to the other Member States and to the Commission.

Seed poatatoesAct XVIII of 2001.

9. Seed potatoes shall meet the requirements of the Plant Quarantine Act, 2001- and shall derive in direct line from potato material obtained under an officially approved programme which has been found free of the organism in official or officially supervised testing using the relevant method set out in Schedule II to these regulations. The testing shall be carried out by the Plant Health Department:

(a) in cases where there has been confirmed any finding of the organism in local seed potato production,

(i) by testing of the earlier propagations, including the initial clonal selection and systematic testing of basic seed potato clones; or

(ii) in cases where it has been established that there is no clonal relationship, by testing of all basic seed potato clones or earlier propagations including the initial clonal selection, and

(b) in other cases, either on each plant of the initial clonal selection or on representative samples of the basic seed potatoes or earlier propagations.

Prohibition of holding and handling of organism.

10. The holding and handling of the organism is hereby banned.

Derogations for experimental and scientific purposesAct XVIII of 2001L.N. 97 of 2004.

11. Without prejudice to the provisions of the Plant Quarantine Act, 2001, the Plant Health Department may authorise derogations from the measures referred to in regulations 8 and 10 of these regulations in accordance with the provisions laid down in the Plant Quarantine (Harmful Organisms) Regulations, 2004 for trial or scientific purposes, and for work on varietal selections.

Additional measuresAct XVIII of 2001.

12. (1) The Plant Health may adopt such additional or stricter measures as may be required to combat the organism or to prevent it from spreading, insofar as they are in compliance with the provisions of the Plant Quarantine Act, 2001.(2) The details of these measures shall be notified to the other Member States and to the Commission.

SCHEDULE I
SECTION I

List of host plants of *Ralstonia solanacearum (Smith) Yabuuchi et al.* referred to in regulation 2 of these regulations

Plants (including tubers), other than true seed, of *Solanum tuberosum L.* Potato

Plants, other than fruits and seeds, of *Lycopersicon lycopersicum (L.) Karsten ex Farw.* Tomato

SECTION II

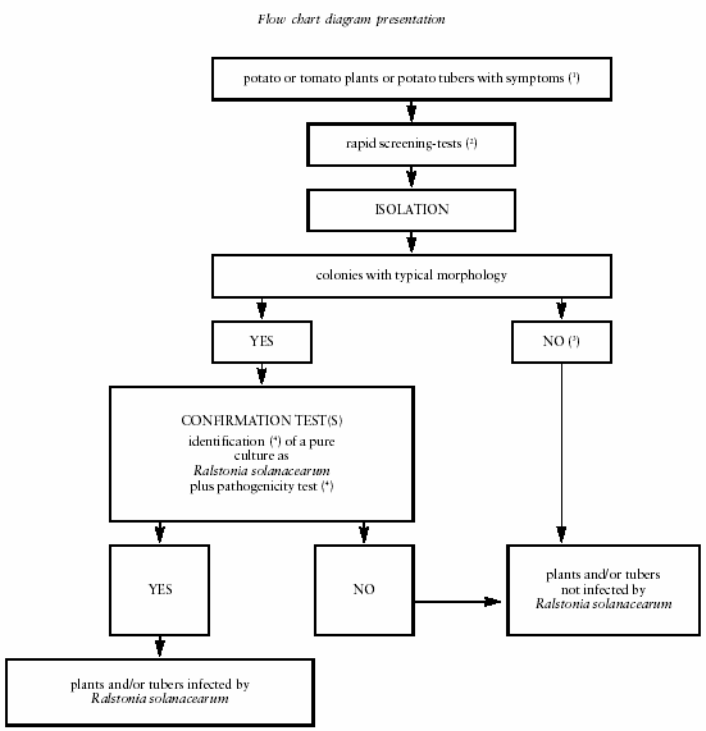
	Surveys
	1. The official surveys referred to in regulation 4(a) shall be based on the biology of the organism and the particular production systems in Malta and shall comprise: (i) in the case of potato, - at appropriate times, visual inspection of the growing crop, and/or sampling of both seed and other potatoes in the growing season or in store. These samples shall be subjected to official or officially supervised visual inspection by cutting of tubers, and - in the case of seed potatoes, and where appropriate for other potatoes, official or officially supervised laboratory testing using the method set out in Schedule II, (ii) in the case of tomato, - visual inspection, at appropriate times, of at least the growing crop of plants intended for replanting for professional use.
	2. The notification of the official surveys referred to in regulation 5 shall include: (i) in the case of surveys on potatoes, - estimated total area grown, in hectares, of seed and other potatoes, - stratification by seed category and ware, and where appropriate, by region, - number and timing of samples taken for testing, - number of visual inspections in the field, - number (and size of sample) of visual inspections on tubers; (ii) in the case of surveys on, at least, the growing crop of plants of tomato intended for replanting for professional use, - estimated total number of plants, - number of visual inspections; (iii) in the case of surveys on host plants other than potatoes and tomatoes, including wild solanaceous host plants,

	- species, - number and timing of samples taken, - area sampled, as appropriate, - method of analysis; (iv) in the case of surveys on water and liquid waste discharges from industrial processing or packaging premises, - number and timing of samples, - area/ location of premises sampled, as appropriate, - method of analysis.

SCHEDULE II

Test scheme for diagnosis, detection and identification of *Ralstonia solanacearum (Smith)* Yabuuchi ***et al.***

	SCOPE OF THE TEST SCHEME
	The presented scheme describes the various procedures involved in: (i) diagnosis of brown rot in potato tubers and of bacterial wilt in potato and tomato plants; (ii) detection of *Ralstonia solanacearum* in samples of potato tubers; (iii) identification of *Ralstonia solanacearum*.
	SECTION I **APPLICATION OF THE TEST SCHEME**
	1. Diagnosis of brown rot in potato tubers and of bacterial wilt in potato and tomato plants The testing procedure is intended for potato tubers with symptoms typical or suspect of brown rot and for potato and tomato plants with symptoms typical or suspect of bacterial wilt. 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 *Ralstonia solanacearum*.



Flowchart references:

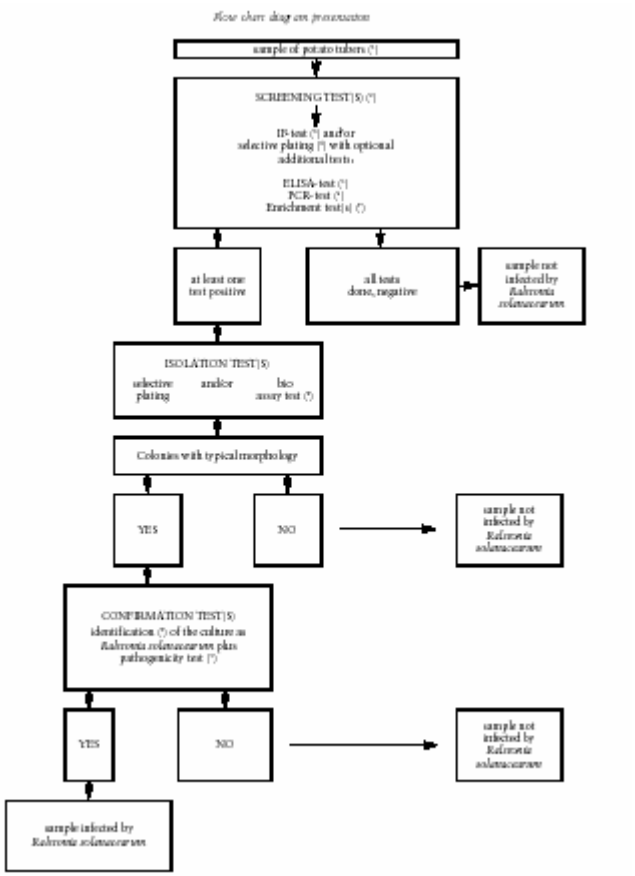
(1) Description of symptoms is provided in section II.1.

(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 *Ralstonia 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 characterisation is optional but recommended for each new case.
	2. Detection and identification of *Ralstonia 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 *Ralstonia solanacearum*. 

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 *Ralstonia 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 2 000 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 *Ralstonia 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 *Ralstonia 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 *Ralstonia 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 *Ralstonia 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 *Ralstonia 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 *Ralstonia 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 *Ralstonia*

	solanacearum. More important perhaps, it also dilutes potential inhibitors of the ELISA or PCR test. *Ralstonia 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 *Ralstonia 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 *Ralstonia solanacearum* on the SMSA medium will most likely not interfere in this test. (9) Confirmation test(s): Reliable identification of a pure culture of *Ralstonia 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 characterisation is optional but recommended for each new case.
	SECTION II **Diagnosis of brown rot in potato tubers and of bacterial wilt in potato and tomato plants**
	1. Symptoms 1.1 Symptoms in potato *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. 1.2 Symptoms in tomato *The tomato plant*. The first visible symptom is the flaccid appearance of the youngest leaves. Under favourable environmental conditions for the pathogen (soil temperature about 25 °C saturated humidity), epinasty and wilting of one side or of the whole plant follows within a few days which leads to total plant collapse. Under less favourable conditions (soil temperature below 21 °C) large numbers of adventitous roots may develop on the stem. It is possible to observe a greasy cord along the stem which is evidence of the necrosis in the vascular system. When the stem is cut crosswise discoloured brown vascular tissues of the stem exude drops of white or yellowish bacterial ooze.

2. Rapid screening tests

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

Stem streaming test

The presence of *Ralstonia solanacearum* in wilting potato or tomato 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 or tomato plants will not show this phenomenon.

Detection of poly-ß-hydroxybutyrate (PHB) granules

The PHB granules in the cells of *Ralstonia solanacearum* are visualised 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 1 000.
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 *Ralstonia 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 1 000.
PHB granules in cells of *Ralstonia solanacearum* stain blue-black. The cell wall stains pink.

Other tests

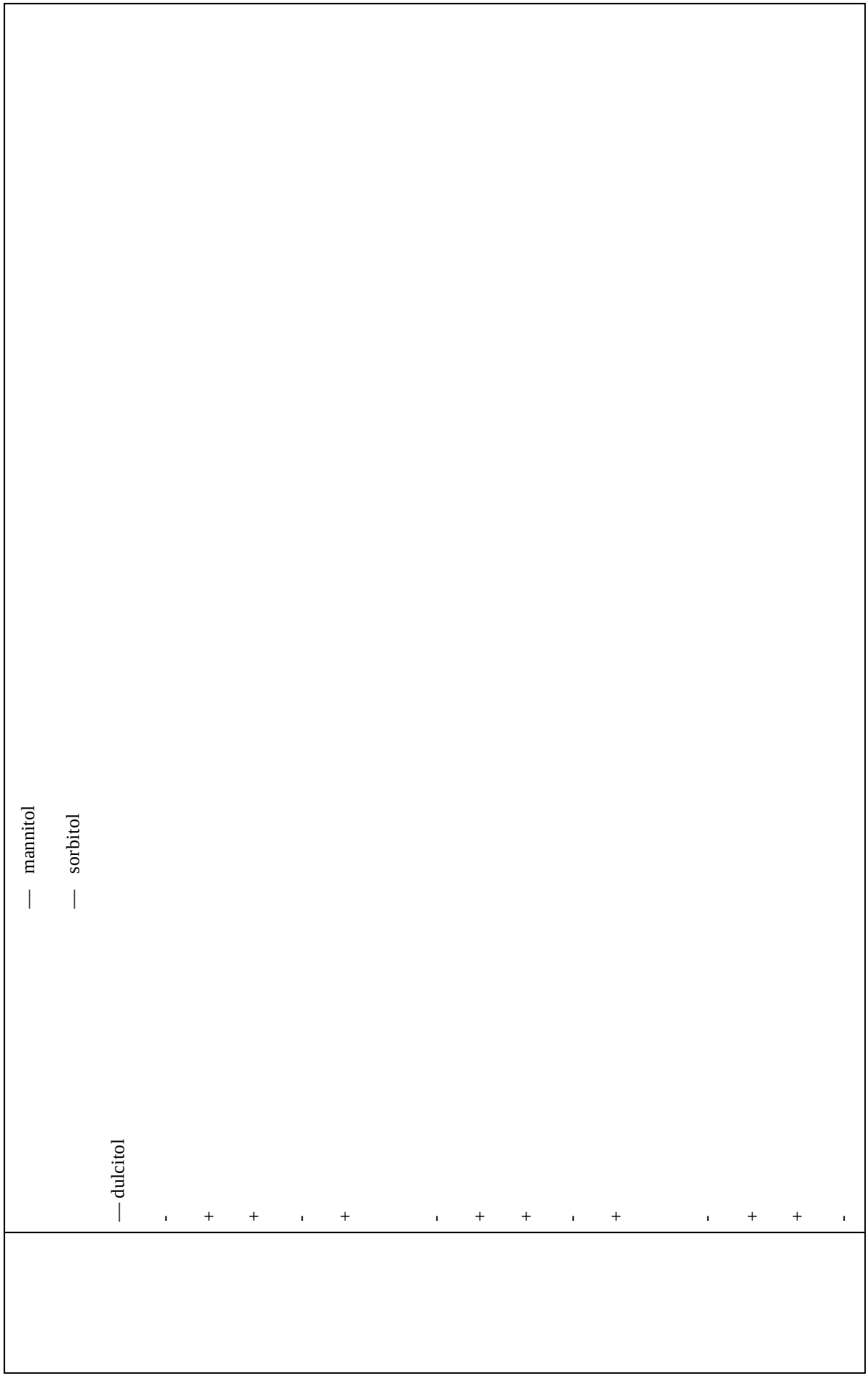
Other appropriate screening tests are the IF test (section III.2), the ELISA test (section III.3) and the PCR test (section III.4).

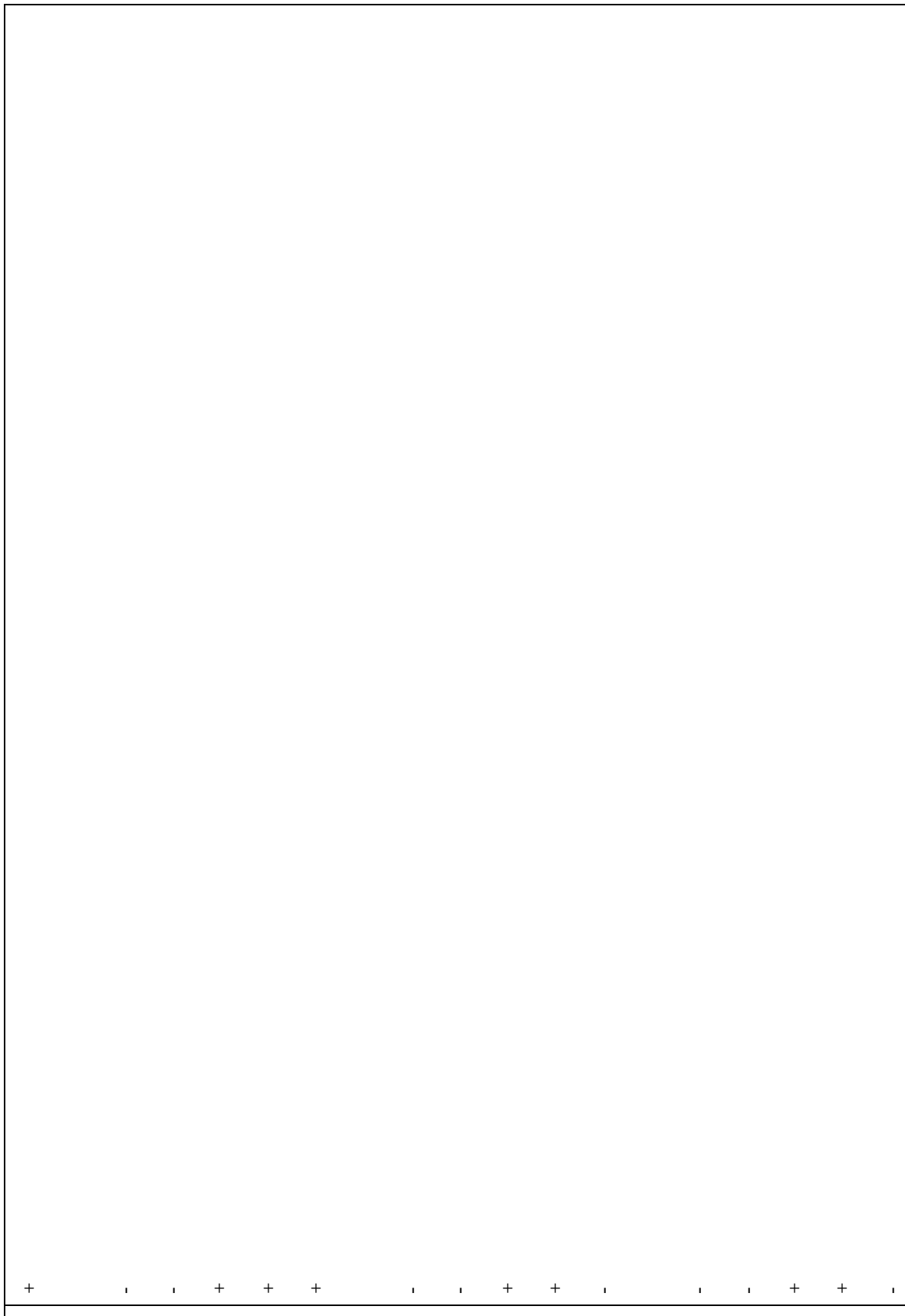
	3. Isolation procedure 3.1 Remove ooze or sections of discoloured tissue from the vascular ring in the potato tuber or from the vascular strands in the stem of potato or tomato plants. Suspend in a small volume of sterile distilled water or 50 mM phosphate buffer. Leave for 5—10 minutes on the bench. 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 *Ralstonia 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 *Ralstonia solanacearum* develop pearly-white, flat, irregular and fluidal colonies often with characteristic whorls. On Kelman's tetrazolium medium, typical colonies of virulent isolates of *Ralstonia solanacearum* are cream, flat, irregular and fluidal with blood red coloured whorls in the centre. Avirulent forms of *Ralstonia solanacearum* develop butyrous, deep-red colonies. On SMSA medium, typical colonies of virulent isolates of *Ralstonia solanacearum* are milky-white, flat, irregular and fluidal with blood red colouration in the centre. Avirulent forms of *Ralstonia 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 Ralstonia solanacearum Identify pure cultures of *Ralstonia 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 *Ralstonia 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. *Strain characterisation* Strain characterisation is optional but is recommended for each new case using at least one of the following: — Biovar determination

	Ralstonia 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 Utilisation of: — maltose — lactose — cellobiose


— mannitol
— sorbitol
— dulcitol
- + + - + - + + - + - + + -



	Additional tests differentiate biovar 2 in subphenotypes (Hayward, 1994):
	biovar 2
	biovar 2-A
	biovar 2-T
	utilisation of trehalose
	-
	+
	+
	utilisation 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] Reaction in 1 2 3 — tomato plants/eggplants wilting no reaction wilting — tobacco plants wilting no reaction

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

	no reaction — tobacco leaves necrosis (48 hrs) and wilting (7—8 days) HR (12—24 hrs) chlorosis (2—8 days) Race characterisation 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 *Ralstonia 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 *Ralstonia solanacearum* and for the assessment of the virulence of the cultures identified as *Ralstonia 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 *Ralstonia solanacearum*.
	SECTION III **DETECTION AND IDENTIFICATION OF *RALSTONIA 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 *Ralstoniasolanacearum* 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 homogenisation has just been achieved. Avoid excessive homogenisation. 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 homogenisation 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 *Ralstonia solanacearum*, preferably to race 3/biovar 2. Determine the titre on a suspension of 10^6 cells per ml from the homologeous

strain of *Ralstonia 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 1:2 000.

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 *Ralstonia 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 1/1 000 of the resuspended pellet in pellet buffer. 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): ¼ of the titre (T/4), ½ 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

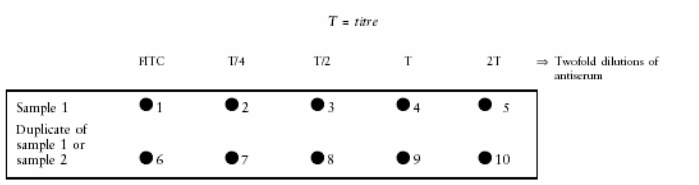
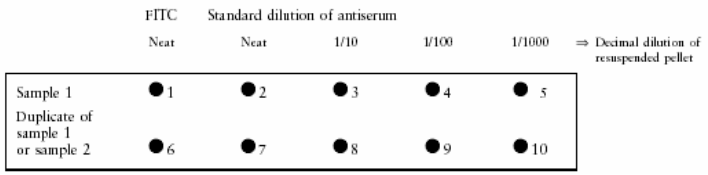


Figure 2
Preparation of the test slide according to 2.1 (ii) and 2.3 (ii)



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—1 000. 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 *Ralstonia 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 *Ralstonia solanacearum*, preferably to race 3/biovar 2. Determine the titre on a suspension of 10^6 cells per ml from the homologeous strain of *Ralstonia 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 *Ralstonia 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 *Ralstonia 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 × OD of the negative control. The ELISA test is positive if the optical density (OD) of the sample is > 2 × 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 *Ralstonia 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 µm Primer Y-2 (20 µM) 2,5 µl 1 µm Taq polymerase (5 U/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 samp le 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 (ii)

	20 seconds at 94 °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 *Ralstonia 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 *Ralstonia 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 *Ralstonia 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 *Ralstonia 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 *Ralstonia 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 *Ralstonia 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 *Ralstonia solanacearum* are isolated, provided that no inhibition is suspected by colonies of other bacteria and that colonies characteristic of *Ralstonia solanacearum* are found in the

	positive controls. The selective plating test is positive if colonies characteristic of *Ralstonia 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 *Ralstonia 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 *Ralstonia 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. Homogenise 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 *Ralstonia 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 *Ralstonia solanacearum*, and provided that *Ralstonia solanacearum* is detected in the positive controls. The bioassay test is positive if test plants are infected by *Ralstonia 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 *Ralstonia 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-sterilised aqueous solution of triphenyl tetrazolium chloride (Sigma) to obtain a final concentration of 50 mg per litre. Pour plates.

	<u>Appendix 2</u> **Materials for sample preparation**
	Maceration Buffer: 50 mM phophate buffer, pH 7,0 This buffer is used for tissue maceration. $Na_2 HPO_4$ 4,26 g $KH_2 PO_4$ 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 homogenisation 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.

$Na_2 HPO_4 \cdot 12 H_2O$

2,7 g

$Na H_2PO_4 \cdot 2 H_2O$

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 = $(p D^2)/4$ Where D = diameter of window (1) Surface area (s) of objective field = $(p d^2)/4$ Where d = diameter of field (2) Calculate d either by direct measurement or from the following formulae: $s = (p i^2)/(G^2K^2 \times 4)$ 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. (3) from (2) $d = v(4s)/p$ (4) from (3) $d = v((4 \times (p i^2)/(G^2K^2 \times 4))/p) = i/(GK)$ Count the number of typical fluorescent cells per field (c). Calculate the number of typical fluorescent cells per window (C). $C = c S/s$ Calculate the number of typical fluorescent cells per ml pellet (N) $N = C \times 1\,000/y \times F$ 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	
	$Na_2HPO_4 \times 12H_2O$
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 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 HCI. 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'- GGGGGTAG CTTGCTACC TGCC-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) but delete the agar and the tetrazolium salts. **Basal medium** Casamino acids (Difco) 1 g Bacto peptone (Difco) 10 g Glycerol

	5 ml Agar (Difco) 15 g Distilled water 1 litre Prepare ½ litre volumes of medium in one litre flasks. Dissolve the ingredients and check pH. Adjust pH, if necessary, to 6,5 before autoclaving. *Ralstonia 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 [2]

[2] If considered necessary, increasing bacitracin concentration to 300 ppm may reduce contaminating saprophytic bacteria without reducing the recovery of *Ralstonia solanacearum.*

	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.

	SCHEDULE III
	1. For each suspected occurrence for which a positive result in the screening test(s) has been identified according to, for the listed plant material, the relevant method set out in Schedule II or, in all other cases, any other officially approved method, and confirmation or refutation by completion of the said method is awaited, there should be retention and appropriate conservation of: - wherever possible, the lot, or part thereof (from which the sample has been taken) in its original packaging with label, - wherever possible, the remaining part of the samples, - any remaining extract and additional prepared material for the screening test(s) e.g. immunofluorescence slides, and, - all relevant documentation, until the completion of the said method.
	2. In the case of confirmation of the organism, there should be retention and appropriate conservation of: - the material specified in paragraph 1, and - a sample of the infected tomato or eggplant material inoculated with the tuber or plant extract, where appropriate, and - the isolated culture of the organism, until at least one month after the notification procedure under regulation 7(2) of these regulations.

	SCHEDULE IV
	The elements in the investigation referred to in sub-regulation 7(1)(a)(i) of these regulations shall include where relevant: (i) places of production, - growing or having grown, potatoes which are clonally related to potatoes found to be infected with the organism, - growing or having grown tomatoes which are from the same source as tomatoes found to be infected with the organism, - growing or having grown, potatoes or tomatoes which have been placed under official control because of the suspected occurrence of the organism, - growing or having grown, potatoes which are clonally related to potatoes that have been grown on places of production found to be infested with the organism, - growing potatoes or tomatoes and located in the neighbourhood of infested places of production, including such places of production sharing production equipment and facilities directly or through a common contractor, - using surface water for irrigation or spraying from any source confirmed or suspected to be infested with the organism, - using surface water for irrigation or spraying from a source used in common with places of production confirmed or suspected to be infested with the organism, - flooded or have been flooded with surface water confirmed or suspected to be infested with the organism; and, (ii) surface water used for irrigation or spraying of, or which has flooded field(s) or place(s) of production confirmed to be infested with the organism.

	SCHEDULE V
	1. The elements in the determination of the extent of probable contamination under sub-regulations 7(1)(a)(iii) and 7(1)(c)(iii) of these regulations, shall include where relevant: - the listed plant material grown at a place of production designated as contaminated under sub-regulation 7(1)(a)(ii) of these regulations, - place(s) of production with a production link to the listed plant material designated as contaminated under sub-regulation 7(1)(a)(ii) to these regulations, including those sharing production equipment and facilities directly or through a common contractor, - the listed plant material produced in the place(s) of production referred to in the previous indent, or present in such place(s) of production during the period when the listed plant material designated as contaminated under sub-regulation 7(1)(a)(ii) of these regulations, was present on the places of production referred to in the first indent, - stores handling the listed plant material from the above places of production, - any machinery, vehicle, vessel, store, or units thereof, and any other objects including packaging material, that may have come into contact with the listed plant material designated as contaminated under sub-regulation 7(1)(a)(ii) of these regulations, - any of the listed plant material stored in, or in contact with, any of the structures or objects listed in the previous indent, prior to the cleansing and disinfection of such structures and objects, - as a result of the investigation and testing under sub-regulation 7(1)(a)(i) of these regulations, in the case of potato, those tubers or plants with a sister or parental clonal relationship to, and in the case of tomato, those plants with the same source as, the listed plant material designated to be contaminated under sub-regulation (1)(a)(ii) of these regulations and for which, although they may have tested negative for the organism, it appears that contamination is probable through a clonal link, - place(s) of production of the listed plant material referred to in the previous indent, - place(s) of production of the listed plant material using water for irrigation or spraying which has been designated as contaminated under sub-regulation 7(1)(c)(ii) of these regulations, - listed plant material produced on fields flooded with surface water confirmed to be infested with the organism.
	2. The determination of the possible spread under sub-regulations 7(1)(a)(iv) and 7(1)(c)(iii) of these regulations shall include: (i) in cases under sub-regulation 7(1)(a)(iv), a consideration of the following elements, - the proximity of other places of production growing the listed plant material, - the common production and use of seed potato stocks, - places of production using surface water for irrigation or spraying of listed plant material in cases where there is or has been risk of surface water run-off from, or flooding of, place(s) of production designated to be contaminated under sub-regulation (1)(a)(ii) of these regulations; (ii) in cases where surface water has been designated as contaminated under sub-reglation 7(1)(c)(ii) of these regulations:

	- place(s) of production producing listed plant material adjacent to, or at risk from flooding by, the surface water designated as contaminated, - any discrete irrigation basin associated with the surface water designated as contaminated.
	3. The details of the notification referred to in sub-regulation 7(2) to these regulations shall include: - date of reporting of suspected occurrence under regulation 6 to these regulations and dates of sampling and confirmation of the presence of the organism under regulation 7 to these regulations, as appropriate, - a description of the elements of the designated contamination and zone demarcation.
	4. The details of the additional notification referred to sub-regulation 7(2) shall include: - for any potato consignment or lot designated as contaminated, the certificates prescribed in The Plant Quarantine (Harmful Organisms) Regulations 2004 (L.N. 97 of 2004), the passport number or registration number of potato producers, collective warehouses and dispatching centres, as appropriate, - for any tomato plant consignment or lot designated as contaminated the crtificates prescribed in The Plant Quarantine (Harmful Organisms) Regulations 2004 (L.N. 97 of 2004), and passport number - the variety name and category for seed potato stocks, and where possible in all other cases, - such other information relating to the confirmed outbreak as the Commission may require.

	SCHEDULE VI 1. With refrence to sub-regulation 8(1) of these regulations the provisions shall be: - incineration, or - use as animal feed after heat treatment such that there is no risk of the organism surviving, or - deep burial, at a disposal site at which there is no risk of seepage to agricultural land or contact with water sources which could be used for irrigation of agricultral land, or - industrial processing through direct and immediate delivery to a processing plant with officially approved waste disposal facilities which conform to the provisions laid down in Schedule VII to these regulations, or - other measures, provided that it has been established that there is no identifiable risk of the organism spreading; such measures to be immediately notified to the Commission and to the other Member States.
	2. The appropriate use or disposal of the listed plant material referred to in sub-regulation 8 (2) of these regulations, under the control of the Plant Health Department, with appropriate communication between responsible official bodies to ensure such control at all times and approval by the responsible official body of the Member State where the potatoes are to be packed or processed in respect of the waste disposal facilities referred to in the first and second indents, shall be: (i) for potato tubers, - use as ware potatoes intended for consumption and packed at sites with appropriate waste disposal facilities, ready for direct delivery and use without repacking, and intended for such direct delivery and use, or - use as ware potatoes intended for industrial processing, and intended for direct and immediate delivery to a processing plant with appropriate waste disposal facilities, or - some other use or disposal, provided that it is established that there is no identifiable risk of the organism spreading, and subject to approval by the said responsible official bodies. Such measures to be immediately notified to the Commission and to the other Member States. (ii) for other plant parts including stem and foliage debris, - destruction, or - some other use or disposal, provided that it is established that there is no identifiable risk of the organism spreading; such measures shall be notified to the Commission and to other Member States.
	3. The appropriate methods for decontamination of the objects referred to in sub-regulation 8 (3) of these regulations shall be cleansing and, where appropriate, disinfection, such that there is no identifiable risk of the organism spreading and shall be employed under the supervision of the Plant Health Department.
	4. The series of measures to be implemented within the demarcated zone(s) established under sub-regulations 7 (1)(a)(iv) and (c)(iii) of these regulations and referred to in sub-regulation 8 (4) of these regulations shall include:
	4.1. In cases where places of production have been designated as contaminated under sub-regulation 7 (1)(a)(ii):

	(a) in a field or unit of protected crop production designated to be contaminated under sub-regulation 7 (1)(a)(ii), either (i) during at least the four growing years following the designated contamination, - measures shall be taken to eliminate volunteer potato and tomato plants as well as other host plants of the organism including solanaceous weeds, and - the following shall not be planted: - potato tubers or plants, - tomato plants and seeds, - taking into account the biology of the organis m, - other host plants, - plants of species of *Brassica*, for which there is an identified risk of the organism surviving, - crops for which there is an identified risk of the organism spreading; - in the first potato or tomato cropping season following the period specified in the preceding indent, and on the condition that the field has been found free from volunteer potato and tomato plants and other host plants including solanaceous weeds for at least the two consecutive growing years prior to planting, - in the case of potatoes, officially certified seed potatoes shall be planted for ware production only and, - an official survey including testing, as detailed in regulation 3 of these regulations, shall be conducted, - in the potato or tomato cropping season succeeding that referred to in the previous indent and following an appropriate rotation cycle, in the case of potatoes officially certified seed potatoes shall be planted for either seed or ware production and in the case of potatoes and tomatoes an official survey as detailed in regulation 3 of these regulations, shall be conducted; or (ii) during the five growing years following that of the designated contamination, - measures shall be taken to eliminate volunteer potato and tomato plants as well as other host plants of the organism including solanaceous weeds, and - the field shall be established and maintained during the first three years either, in bare fallow or, in cereals according to the risk identified, or, in permanent pasture with frequent close cutting or intensive grazing or, as grass for seed production, followed by planting in the succeeding two years with non-host plants of the organism for which there is no identified risk of the organism surviving or spreading, - in the first potato or tomato cropping season following the period specified in the preceding indent, - in the case of potatoes, officially certified seed potatoes shall be planted for either seed or ware production, and an official survey including testing, as detailed in regulation 3 of these regulations, shall be conducted; (b) in other fields: - in the growing year following the designated contamination: - either no potato tubers or plants, or other host plants of the organism shall be planted, and measures shall be taken to eliminate volunteer potato and tomato plants and other host plants including solanaceous weeds as appropriate, or - in the case of potato tubers, officially certified seed potatoes may be planted for ware production only, on the condition that the responsible official bodies are satisfied that the risks of volunteer potato and tomato plants and other host plants of the

	organism including solanaceous weeds, have been eliminated. The growing crop shall be inspected at appropriate times and volunteer potato plants shall be tested for the organism; in addition, for potatoes, the harvested tubers shall be inspected; - for the first growing year following that specified in the first indent, - in the case of potatoes, only officially certified seed potatoes shall be planted for either seed or ware production, - for at least the second growing year following that in the first indent, - in the case of potatoes, only officially certified seed potatoes or seed potatoes grown under official control from officially certified seed potatoes shall be planted for either seed or ware production, - in each of the growing years referred to in the previous indents measures shall be taken to eliminate volunteer potato and tomato plants and other host plants of the organism including solanaceous weeds, and an official survey as detailed in regulation 3 of these regulations, shall be conducted, and in the cases where seed potatoes are planted for seed production testing of tubers shall be carried out; (c) immediately following the designation of contamination under sub-regulation 7(1)(a)(ii) of these regulations and in each of the subsequent growing years up to and including the first permissible potato or tomato cropping season on the field(s) designated as contaminated, as detailed in paragraph (a): - all machinery and storage facilities on the place of production and involved in potato or tomato production shall be cleansed and, where appropriate, disinfected using appropriate methods, as specified in point 3, - official controls on irrigation and spraying programmes, including a ban thereof, shall be introduced as appropriate in order to prevent the spread of the organism; (d) in a unit of protected crop production designated as contaminated under sub-regulation 7(1)(a)(ii) of these regulations where complete replacement of the growing medium is possible, - no potato tubers or plants, or other host plants of the organism including tomato plants and seeds shall be planted unless the said unit has been subjected to officially supervised measures to eliminate the organism and to remove all host plant material, including, at least, a complete change in growing medium and cleansing and, where appropriate, disinfection of the said unit and all equipment, and subsequently has been granted approval for potato or tomato production by the responsible official bodies, and - for potato production, this production shall be from officially certified seed potatoes, or from mini-tubers or micro-plants derived from tested sources, - official controls on irrigation and spraying programmes, including a ban thereof, shall be introduced as appropriate, in order to prevent the spread of the organism.
	4.2 Within the demarcated zone, without prejudice to the measures detailed under 4.1, the Plant Health Department shall: (a) immediately, and for at least three growing years, after the designated contamination: (aa) in cases where the demarcated zone has been determined under sub-regulation 7(1)(a)(iv) of these regulations, - ensure supervision by their responsible official bodies of premises growing, storing or handling potato tubers or tomatoes, together with premises which operate machinery for potato or tomato production under contract,

	- require cleansing and, where appropriate, disinfection of machinery and stores on such premises, using appropriate methods, as specified under point 3, - require the planting of only certified seed or seed grown under official control for all potato crops within that zone, and testing after harvest of seed potato crops grown in places of production determined as probably contaminated under sub-regulation 7(1)(a)(iii), - require the separate handling of harvested potato seed stocks to those of ware on all premises within the zone, - conduct an official survey as detailed in regulation 3 of these regulations, (ab) in cases where surface water has been designated as contaminated under sub-regulation 7(1)(c)(ii) of these regulations or included in the elements for the possible spread of the organism in accordance with Schedule V point 2, - conduct an annual survey at appropriate times including sampling of surface water and appropriate solanaceous host plants in the relevant water sources and testing in accordance with: i. for listed plant material, the relevant method set out in Schedule II, or ii. in other cases, any other officially approved method, - introduce official controls on irrigation and spraying programmes, including a ban on the use of the water designated as contaminated for the irrigation and spraying of listed plant material, and, where appropriate, other host plants in order to prevent the spread of the organism. This ban may be reviewed on the basis of the results obtained in the said annual survey, - in cases where liquid waste discharges are contaminated, introduce official controls on the disposal of waste from industrial processing or packaging premises handling listed plant material; (b) establish a programme, where appropriate, for the replacement of all seed potato stocks over an appropriate period of time.

	SCHEDULE VII
	The officially approved waste disposal facilities referred to in Schedule VI paragraph 1, fourth indent shall conform to the following provisions such that the risk of spreading the organism is obviated: (i) potato and tomato processing waste (including rejected potatoes and peelings and tomatoes) and any other solid waste associated with the potatoes and tomatoes shall be disposed by either, - deep burial at disposal site at which there is no risk of seepage to agricultural land or contact with water sources which could be used for irrigation of agricultural land. The waste shall be conveyed directly to the site under containment conditions such that there is no risk of loss of the waste, or - incineration; (ii) liquid processing waste: prior to disposal, liquid waste containing suspended solids shall be subjected to filtration or settlement processes to remove such solids. These solids shall be disposed of as set out in subparagraph (i). The liquid waste shall then be either: - heated to a minimum of 70 °C during at least 30 minutes, prior to disposal, or - otherwise disposed of subject to official approval and under official control such that there is no risk that the waste could come into contact with agricultural land or water sources which could be used for irrigation of agricultural land. The details thereof shall be notified to the other Member States and to the Commission.

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