

Council Directive 98/57/EC of 20 July 1998 on the  
control of *Ralstonia solanacearum* (Smith) Yabuuchi et al.

Article 1	This Directive concerns the measures to be taken within the...
Article 2	(1) Member States shall conduct annual systematic official surveys for...
Article 3	Member States shall ensure that the suspected occurrence or confirmed...
Article 4	(1) In each case of suspected occurrence, the responsible official...
Article 5	(1) If official or officially supervised laboratory testing, using, for...
Article 6	(1) Member States shall prescribe that the listed plant material...
Article 7	(1) Member States shall prescribe that seed potatoes shall meet...
Article 8	Member States shall ban the holding and handling of the...
Article 9	Without prejudice to the provisions of Directive 77/93/EEC, Member States...
Article 10	Member States may adopt in relation to their own production...
Article 11	Amendments to the Annexes to this Directive, to be made...
Article 12	(1) Member States shall bring into force the laws, regulations...
Article 13	This Directive shall enter into force on the day of...
Article 14	This Directive is addressed to the Member States.

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ANNEX I

SECTION I

List of host plants of *Ralstonia solanacearum* (Smith) Yabuuchi et al. referred to in Article 1

SECTION II

Surveys

1. The official surveys referred to in Article 2(2)(a) shall be...
2. The notification of the official surveys referred to in Article...

ANNEX II

TEST SCHEME FOR DIAGNOSIS, DETECTION AND IDENTIFICATION  
OF *RALSTONIA SOLANACEARUM* (SMITH) YABUUCHI ET AL.

SCOPE OF THE TEST SCHEME

CONTENTS

## GENERAL PRINCIPLES

### SECTION I

#### APPLICATION OF THE TEST SCHEME

1. Detection scheme for the diagnosis of brown rot and bacterial...
2. Scheme for detection and identification of *Ralstonia solanacearum* in samples of...  
Principle:
3. Scheme for detection and identification of *Ralstonia solanacearum* in samples of...

### SECTION II

#### DETAILED METHODS FOR DETECTION OF *RALSTONIA SOLANACEARUM* IN POTATO TUBERS AND POTATO, TOMATO OR OTHER HOST PLANTS WITH SYMPTOMS OF BROWN ROT OR BACTERIAL WILT

1. Symptoms (see website <http://forum.europa.eu.int/Public/irc/sanco/Home/main>)
  - 1.1. Symptoms on potato
  - 1.2. Symptoms on tomato
  - 1.3. Symptoms on other hosts
2. Rapid screening tests
  - 2.1. Stem streaming test
  - 2.2. Detection of poly- $\beta$ -hydroxybutyrate (PHB) granules
  - 2.3. Serological agglutination tests
  - 2.4. Other tests
3. Isolation procedure
4. Identification tests for *R. solanacearum*

### SECTION III

1. Detailed methods for detection and identification of *Ralstonia solanacearum* in samples...
  - 1.1. Sample preparation  
Note:
    - 1.1.1. Remove with a clean and disinfected scalpel or vegetable knife the...
    - 1.1.2. Collect the heel end cores in unused disposable containers which...
    - 1.1.3. Decant the supernatant. If excessively cloudy, clarify either by slow...
    - 1.1.4. Concentrate the bacterial fraction by centrifugation at 7 000 g...
    - 1.1.5. Resuspend the pellet in 1.5 ml pellet buffer (Appendix 4). Use 500  $\mu$ l...
    - 1.1.6. It is imperative that all *R. solanacearum* positive controls and...
  - 1.2. Testing
2. Detailed methods for detection and identification of *R. solanacearum* in samples...
  - 2.1. Sample preparation
    - 2.1.1. Collect 1 to 2 cm stem segments in a closed sterile container...

- 2.1.2. Disinfect stem segments briefly with ethanol 70 % and immediately blot dry...
- 2.1.3. Decant the supernatant after settling for 15 minutes.
- 2.1.4. Further clarification of the extract or concentration of the bacterial fraction...
- 2.1.5. Divide the neat or concentrated sample extract into two equal parts....
- 2.2. Testing

#### SECTION IV

- 1. Scheme for detection and identification of *R. solanacearum* in water
- 2. Methods for detection and identification of *R. solanacearum* in water
  - Principle
  - 2.1. Sample preparation
    - Note:
    - 2.1.1. At selected sampling points, collect water samples by filling disposable...
    - 2.1.2. Transport samples in cool dark conditions (4 to 10 °C) and test...
    - 2.1.3. If required, the bacterial fraction may be concentrated using one...
  - 2.2. Testing

#### SECTION V

- 1. Scheme for detection and identification of *R. solanacearum* in soil
- 2. Methods for detection and identification of *R. solanacearum* in soil
  - Principles
  - 2.1. Sample preparation
    - 2.1.1. Sampling of field soil should follow standard principals used for...
    - 2.1.2. Disperse sub-samples of 10 to 25 g of soil or sludge by...
    - 2.1.3. Maintain the suspension at 4 °C during testing.
  - 2.2. Testing

#### SECTION VI

##### OPTIMISED PROTOCOLS FOR DETECTION AND IDENTIFICATION OF *R. SOLANACEARUM*

- A. DIAGNOSTIC AND DETECTION TESTS
  - 1. Stem streaming test
  - 2. Detection of poly-β-hydroxybutyrate granules
    - Nile blue test:
    - Sudan Black test:
  - 3. Serological agglutination tests
  - 4. Selective isolation
    - 4.1. Selective plating
      - 4.1.1. Perform an appropriate dilution plating technique aiming to ensure that...
      - 4.1.2. Incubate plates at 28 °C. Read plates after 48 hours and daily...

- 4.1.3. Purify presumptive *R. solanacearum* colonies after streaking or dilution plating...
    - 4.1.4. Store cultures short-term in sterile water (pH 6 to 8, chlorine...
    - 4.1.5. Identify presumptive cultures (see Section VI.B.) and perform a pathogenicity test...  
Interpretation of selective plating test results
  - 4.2. Enrichment procedure
    - 4.2.1. For enrichment-PCR, transfer 100 µl of sample extract into 10 ml of...
    - 4.2.2. Incubate for 72 hours at 27 to 30 °C in shaking culture or...
    - 4.2.3. Mix well before using in ELISA or PCR tests.
    - 4.2.4. Treat enriched broth in an identical manner as the sample(s)...
5. IF Test
  - Principle
  - 5.1. Prepare the test slides by one of the following procedures:...
  - 5.2. Dry the droplets at ambient temperature or by warming to...
  - 5.3. IF procedure
    - 5.3.1. Arrange the slides on moist tissue paper. Cover each test...
    - 5.3.2. Incubate the slides on moist paper under a cover for...
    - 5.3.3. Shake the droplets off each slide and rinse carefully with...
    - 5.3.4. Arrange the slides on moist paper. Cover the test windows...
    - 5.3.5. Incubate the slides on moist paper under a cover for...
    - 5.3.6. Shake the droplets of conjugate off the slide. Rinse and...
    - 5.3.7. Pipette 5 - 10 µl of 0,1M phosphate-buffered glycerol (Appendix 4) or a...
  - 5.4. Reading the IF test:
    - 5.4.1. Examine test slides on an epifluorescence microscope with filters suitable...
    - 5.4.2. Observe for bright fluorescing cells with characteristic morphology of *R. solanacearum*...
    - 5.4.3. There are several problems inherent to the specificity of the...
    - 5.4.4. Consider only fluorescing cells with typical size and morphology at...
    - 5.4.5. Interpretation of the IF reading:
6. PCR tests
  - Principles
  - 6.1. DNA purification methods
    - (a) Method according to Pastrik (2000)
    - (b) Other methods
  - 6.2. PCR
    - 6.2.1. Prepare test and control templates for PCR according to the validated...
    - 6.2.2. Prepare the appropriate PCR reaction mix in a contamination-free environment...
    - 6.2.3. Add 2-5 µl of DNA extract per 25 µl PCR reaction in...
    - 6.2.4. Incorporate a negative control sample containing only PCR reaction mix...
    - 6.2.5. Place tubes in the same thermal cycler which was used...
  - 6.3. Analysis of the PCR product
    - 6.3.1. Resolve PCR amplicons by agarose gel electrophoresis. Run at least...
    - 6.3.2. Reveal DNA bands by staining in ethidium bromide (0,5 mg per L) for...

- 6.3.3. Observe stained gel under short wave UV transillumination ( $\lambda = 302$  nm) for...
- 6.3.4. For all new findings/cases verify authenticity of the PCR amplicon...
- Interpretation of the PCR test result:
7. FISH test
  - Principle
  - 7.1. Potato extract fixation
    - 7.1.1. Prepare fixative solution (see Appendix 7).
    - 7.1.2. Pipette 100  $\mu$ l of each sample extract into an Eppendorf tube...
    - 7.1.3. Remove the supernatant and dissolve the pellet in 200  $\mu$ l of...
    - 7.1.4. Centrifuge for 7 minutes at 7 000 g, remove the supernatant...
    - 7.1.5. Spot 16  $\mu$ l of the fixed suspensions onto a clean multitest...
    - 7.1.6. Air-dry the slides (or on slide dryer at 37 °C) and...
  - 7.2. Hybridisation
    - 7.2.1. Dehydrate the cells in a graded ethanol series of 50 %,...
    - 7.2.2. Prepare a moist incubation chamber by covering the bottom of...
    - 7.2.3. Apply 10  $\mu$ l of hybridisation solution (Appendix 7) to eight windows...
    - 7.2.4. Apply coverslips (24  $\times$  24 mm) to the first and last...
    - 7.2.5. Prepare three beakers containing 1 l of Milli Q (molecular grade)...
    - 7.2.6. Remove the coverslips from the slides and place the slides...
    - 7.2.7. Wash away excess probe by incubation for 15 minutes in the...
    - 7.2.8. Transfer the slide holder to 1/8 hybmix washing solution and incubate for...
    - 7.2.9. Dip the slides briefly in Milli Q water and place...
  - 7.3. Reading the FISH test
    - 7.3.1. Observe the slides immediately with a microscope fitted for epifluorescence...
    - 7.3.2. Observe for bright fluorescing cells with characteristic morphology of *R. solanacearum*...
    - 7.3.3. If any contamination is suspected the test must be repeated....
    - 7.3.4. There are several problems inherent to the specificity of the...
    - 7.3.5. Consider only fluorescing cells with typical size and morphology.
    - 7.3.6. Interpretation of the FISH test result:
8. ELISA tests
  - Principle
  - (a) Indirect ELISA (Robinson Smith et al. , 1995)
  - (b) DASi ELISA
  - Interpretation of ELISA test results:
9. Bioassay test
  - 9.1. Use 10 test plants of a susceptible tomato cultivar (e.g....
  - 9.2. Distribute 100  $\mu$ l of sample extract between the test plants.
    - 9.2.1. Syringe inoculation
    - 9.2.2. Slit inoculation
  - 9.3. Inoculate by the same technique, five seedlings with an aqueous...
  - 9.4. Grow the test plants in quarantine facilities for up to...
  - 9.5. Isolate from infected plants (Section II.3.) and identify purified cultures of presumptive...

- 9.6. If no symptoms are observed after three weeks perform IF/PCR/ Isolation...
- 9.7. Identify any purified cultures of presumptive *R. solanacearum* (Section VI.B.).  
Interpretation of the bioassay test results

## B. IDENTIFICATION TESTS

1. Nutritional and enzymatic identification tests
2. IF test
  - 2.1. Prepare a suspension of approximately 10<sup>6</sup> cells per ml...
  - 2.2. Prepare a twofold dilution series of an appropriate antiserum (see...
  - 2.3. Apply the IF procedure (Section VI.A.5.).
  - 2.4. A positive IF test is achieved if the IF titre...
3. ELISA test
  - 3.1. Prepare a suspension of approximately 10<sup>8</sup> cells per ml...
  - 3.2. Perform an appropriate ELISA procedure with a specific monoclonal antibody...
  - 3.3. A positive ELISA test is achieved if the ELISA reading...
4. PCR tests
  - 4.1. Prepare a suspension of approximately 10<sup>6</sup> cells per ml...
  - 4.2. Heat 100 µl of the cell suspension in closed tubes in...
  - 4.3. Apply appropriate PCR procedures to amplify *R. solanacearum* - specific amplicons...
  - 4.4. A positive identification of *R. solanacearum* is achieved if the...
5. FISH test
  - 5.1. Prepare a suspension of approximately 10<sup>6</sup> cells per ml...
  - 5.2. Apply the FISH procedure (Section VI.A.7.) with at least 2 R....
  - 5.3. A positive FISH test is achieved if the same reactions...
6. Fatty acid profiling (FAP)
  - 6.1. Grow the culture on trypticase soy agar (Oxoid) for 48 hours...
  - 6.2. Apply an appropriate FAP procedure (Janse, 1991; Stead, 1992).
  - 6.3. A positive FAP test is achieved if the profile of...
7. Strain characterisation methods
  - 7.1. Biovar determination
  - 7.2. Genomic fingerprinting
    - 7.2.1. Restriction fragment length polymorphism (RFLP) analysis (Cook et al. , 1989)....
    - 7.2.2. Repetitive sequence PCR using REP, BOX and ERIC primers (Louws...
    - 7.2.3. Amplified fragment length polymorphism (AFLP) analysis (Van der Wolf et al....
  - 7.3. PCR methods

## C. CONFIRMATION TEST

## Appendix 1

### Laboratories involved in optimisation and validation of protocols

## Appendix 2

### Media for isolation and culture of *R. solanacearum*

- (a) General growth media
  - Nutrient Agar (NA)
  - Yeast Peptone Glucose Agar (YPGA)
  - Sucrose Peptone Agar (SPA)
  - Kelman's Tetrazolium Medium
- (b) Validated selective growth media
  - SMSA medium (Englebrecht, 1994 as modified by Elphinstone et al. ,...
  - Note:
    - 1. Use of reagents other than those specified above may affect...
    - 2. Oxoid Agar #1 can be used in place of Bacto-Agar...
    - 3. Increasing bacitracin concentration to 2 500 U per l may...
- (c) Validated enrichment media
  - SMSA Broth (Elphinstone et al. , 1996)
  - Modified Wilbrink broth (Caruso et al. , 2002)

## Appendix 3

- A. Commercially available standardised control material
  - (a) Bacterial isolates
  - (b) Commercially available standardised control material
- B. Preparation of positive and negative controls for the core screening...

## Appendix 4

### Buffers for test procedures

- 1. Buffers for extraction procedure
  - 1.1. Extraction buffer (50 mM phosphate buffer, pH 7,0)
  - 1.2. Pellet buffer (10 mM phosphate buffer, pH 7,2)
- 2. Buffers for the IF test
  - 2.1. IF-Buffer (10 mM phosphate buffered saline (PBS), pH 7.2)
  - 2.2. IF-buffer-Tween
  - 2.3. Phosphate buffered glycerol, pH 7,6
- 3. Buffers for the Indirect ELISA test
  - 3.1. Double strength coating buffer, pH 9,6.
  - 3.2. 10X Phosphate buffered saline (PBS), pH 7,4
  - 3.3. PBS-Tween

- 3.4. Blocking (antibody) buffer (must be freshly prepared).
- 3.5. Alkaline phosphatase substrate solution, pH 9,8
- 4. Buffers for DASi ELISA test
  - 4.1. Coating buffer, pH 9,6
  - 4.2. 10X Phosphate saline buffer(PBS) pH 7,2 to 7,4
  - 4.3. PBS-Tween
  - 4.4. Substrate buffer, pH 9,8

## Appendix 5

Determination of contamination level in IF and FISH tests

Count the mean number of typical fluorescent cells per field...

## Appendix 6

Validated PCR protocols and reagents

- 1. PCR protocol of Seal et al. (1993)
  - 1.1. Oligonucleotide primers
  - 1.2. PCR reaction mix
  - 1.3. PCR reaction conditions
  - 1.4. Restriction enzyme analysis of amplicon.
- 2. PCR protocol of Pastrik and Maiss (2000)
  - 2.1. Oligonucleotide primers
  - 2.2. PCR reaction mix
  - 2.3. PCR reaction conditions
  - 2.4. Restriction enzyme analysis of amplicon.
- 3. Multiplex PCR protocol with internal PCR control (Pastrik et al. , ...
  - 3.1. Oligonucleotide primers
  - 3.2. PCR reaction mix
  - 3.3. PCR reaction conditions
  - 3.4. Restriction enzyme analysis of amplicon.
- 4. *R. solanacearum* biovar-specific PCR protocol (Pastrik et al. , 2001)
  - 4.1. Oligonucleotide primers
  - 4.2. PCR reaction mix
  - 4.3. PCR reaction conditions
  - 4.4. Restriction enzyme analysis of amplicon.
- 5. Preparation of the loading buffer
  - 5.1. Bromphenol blue (10 %- stock solution )
  - 5.2. Loading buffer
- 6. 10X Tris Acetate EDTA (TAE) buffer, pH 8.0



## Appendix 7

### Validated reagents for FISH test

1. Oligo-probes
2. Fixative solution
3. 3X Hybmix
4. Hybridisation solution
5. 0,1M Phosphate buffer, pH 7,0

## Appendix 8

### Eggplant and tomato culture

Sow seeds of tomato ( *Lycopersicon esculentum* ) or eggplant...

Eggplants or tomatoes should be grown in a glasshouse with...

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### ANNEX III

For each suspected occurrence for which a positive result in...

### ANNEX IV

The elements in the investigation referred to in Article 5(1)(a)(i) shall include...  
places of production, growing or having grown, potatoes which are...

### ANNEX V

The elements to be considered in the determination of the...

### ANNEX VI

The provisions referred to in Article 6(1) shall be: use as animal...

### ANNEX VII

The officially approved waste disposal methods referred to in Annex VI paragraph 1,...  
potato and tomato waste (including rejected potatoes and peelings and tomatoes)...  
The options described in this Annex also apply to the...

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- (1) [OJ C 124, 21.4.1997, p. 12](#) and [OJ C 108, 7.4.1998, p. 85](#).
- (2) [OJ C 14, 19.1.1998, p. 34](#).
- (3) [OJ C 206, 7.7.1997, p. 57](#).
- (4) [OJ L 26, 31.1.1977, p. 20](#). Directive as last amended by Commission Directive 98/2/EC ([OJ L 15, 21.1.1998, p. 34](#)).