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

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-ß-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 µl...
     - 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...

6.3.3. Observe stained gel under short wave UV transillumination (λ = 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 µl of each sample extract into an Eppendorf tube...
7.1.3. Remove the supernatant and dissolve the pellet in 200 µl of...
7.1.4. Centrifuge for 7 minutes at 7 000 g, remove the supernatant...
7.1.5. Spot 16 µl 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 µl of hybridisation solution (Appendix 7) to eight windows...
7.2.4. Apply coverslips (24 × 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 µl 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 6 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 8 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 6 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 6 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...

(**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).