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

[F1 ANNEX II

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

Textual Amendments

F1 Substituted by Commission Directive 2006/63/CE of 14 July 2006 amending Annexes II to VII to Council Directive 98/57/EC on the control of Ralstonia solanacearum (Smith) Yabuuchi et al..

SECTION VI

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

A. DIAGNOSTIC AND DETECTION TESTS

1. Stem streaming test

The presence of *R. solanacearum* in stems of wilting potato, tomato or other host plants can be indicated by the following simple presumptive test: Cut the stem just above the soil level. Suspend the cut surface in a tube of clean water. Observe for characteristic spontaneous streaming of threads of bacterial slime from the cut vascular bundles after a few minutes.

2. Detection of poly-ß-hydroxybutyrate granules

1. Prepare a smear of bacterial ooze from infected tissue or from a 48-hour culture on YPGA or SPA medium (Appendix 2) on a microscope slide.

2. Prepare positive control smears of a biovar 2 strain of *R. solanacearum* and, if considered useful, a negative control smear of a known PHB negative sp.

3. Allow to air dry and pass the lower surface of each slide rapidly above a flame to fix the smears.

4. Stain preparation with either Nile Blue or Sudan Black and observe microscopically as described below:

Nile blue test:

(a) Flood each slide with 1 % aquous solution of Nile Blue A and incubate for 10 minutes at 55 °C.

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

(c) Flood the smear with 8 % aqueous acetic acid and incubate for one minute at ambient temperature.

(d) Wash briefly in gently running tap water. Remove excess water with tissue paper.

(e) Re-moisten with a drop of water and apply a coverslip.

(f) Examine the stained smear with an epifluorescence microscope at 450 nm under oil immersion at a magnification of 600 to 1 000 using an oil- or water-immersion objective.

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

Sudan Black test:

(a) Flood each slide with 0,3 % Sudan Black B solution in 70 % ethanol and incubate for 10 minutes at ambient temperature.

(b) Drain off the staining solution and wash briefly in tap water, removing excess water with tissue paper.

(c) Dip the slides briefly in xylol and blot dry on tissue paper. *Caution: Xylol is harmful, take necessary safety precautions and work in a fume cupboard*.

(d) Flood the slides with 0,5 % (w/v) aqueous safranin and leave for 10 seconds at ambient temperature. *Caution: Safranin is harmful, take necessary safety precautions and work in a fume cupboard*.

(e) Wash in gently running tap water, blot dry on tissue paper and apply a coverslip.

(f) Examine stained smears with a light microscope using transmitted light under oil immersion at a magnification of 1 000 using an oil-immersion objective.

(g) Observe for blue-black staining of PHB granules in cells of *R. solanacearum* with pink-stained cell walls.

3. Serological agglutination tests

Agglutination of *R. solanacearum* cells in bacterial ooze or symptomatic tissue extracts is best observed using validated antibodies (see Appendix 3) labelled with appropriate coloured markers such as red *Staphylococcus aureus* cells or coloured latex particles. If using a commercially available kit (see Appendix 3), follow the manufacturers instructions. Otherwise perform the following procedure:

(a) Mix drops of a suspension of labelled antibody and bacterial ooze (approximately 5 µl each) on windows of multiwell test slides.

(b) Prepare positive and negative controls using suspensions of *R. solanacearum* biovar 2 and a heterologous strain.

(c) Observe for agglutination in positive samples after gentle mixing for 15 seconds.

4. Selective isolation

4.1. Selective plating

Note: Before using this method for the first time, perform preliminary tests to ensure reproducible detection of 10^3 to 10^4 colony-forming units of *R. solanacearum* per ml added to extracts from samples which previously tested negative.

Use an appropriately validated selective medium such as SMSA (as modified by Elphinstone *et al.*, 1996; see Appendix 2).

Care is required to differentiate *R. solanacearum* from other bacteria able to develop colonies on the medium. Furthermore, colonies of *R. solanacearum* may show atypical morphology if plates are overcrowded or antagonistic bacteria are also present. Where effects of competition or antagonism are suspected, the sample should be re-tested using a different test.

Highest sensitivity of detection by this method can be expected when using freshly prepared sample extracts. However, the method is also applicable for use with extracts which have been stored under glycerol at -68 to -86 °C.

As positive controls, prepare decimal dilutions from a suspension of 10^6 cfu per ml of a virulent biovar 2 strain of *R. solanacearum* (e.g. NCPPB 4156 = PD 2762 = CFBP 3857). To avoid any possibility of contamination, prepare positive controls totally separately from samples to be tested.

For each newly prepared batch of a selective medium its suitability for growth of the pathogen should be tested before it is used to test routine samples.

Test control material in an identical manner as the sample(s).

4.1.1. Perform an appropriate dilution plating technique aiming to ensure that any background saprophytic colony-forming populations are diluted out. Spread 50 - 100 µl per plate of sample extract and each dilution.

4.1.2. Incubate plates at 28 °C. Read plates after 48 hours and daily thereafter up to six days. Typical *R. solanacearum* colonies on SMSA medium are milky white, flat, irregular and fluidal and after three days incubation develop pink to blood-red coloration in the centre with internal streaking or whorling. (see website http://forum.europa.eu.int/Public/irc/sanco/Home/main).

Note: Atypical colonies of *R. solanacearum* sometimes form on this medium. These may be small, round, entirely red in colour and non-fluidal or only partially fluidal and therefore difficult to distinguish from saprophytic colony-forming bacteria.

4.1.3. Purify presumptive *R. solanacearum* colonies after streaking or dilution plating onto a general nutrient medium to obtain isolated colonies (see Appendix 2).

4.1.4. Store cultures short-term in sterile water (pH 6 to 8, chlorine free) at room temperature in the dark, or long term in a suitable cryoprotectant medium at -68 to -86 °C or lyophilised.

4.1.5. Identify presumptive cultures (see Section VI.B.) and perform a pathogenicity test (see Section VI. C).

Interpretation of selective plating test results

The selective plating test is negative if no bacterial colonies are observed after six days or if no presumptive colonies typical of *R. solanacearum* are found, provided that no inhibition is suspected due to competition or antagonism by other bacteria and that typical *R. solanacearum* colonies are found in the positive controls.

The selective plating test is positive if presumptive *R. solanacearum* colonies are isolated.

4.2. Enrichment procedure

Use a validated enrichment medium such as modified Wilbrink broth (see Appendix 2).

This procedure can be used to selectively increase R. solanacearum populations in sample extracts and increase sensitivity of detection. The procedure also effectively dilutes inhibitors of the PCR reaction (1:100). It should be noted, however, that enrichment of R. solanacearum can fail due to competition or antagonism by saprophytic organisms which are often simultaneously enriched. For this reason, isolation of R.solanacearum from enriched broth cultures may be difficult. In addition, since populations of serologically related saprophytes can be increased,

the use of specific monoclonal antibodies rather than polyclonal antibodies is recommended where the ELISA test is to be used.

4.2.1. For enrichment-PCR, transfer 100 µl of sample extract into 10 ml of enrichment broth (Appendix 2) previously aliquoted into DNA-free tubes or flasks. For enrichment-ELISA, higher proportions of sample extract to broth can be used (e.g. 100 µl in 1,0 ml of enrichment broth).

4.2.2. Incubate for 72 hours at 27 to 30 °C in shaking culture or static culture with caps loosely- fitted to permit aeration.

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) in the above tests.

Note: If inhibition of enrichment of R. solanacearum is anticipated, due to high populations of certain competing saprophytic bacteria, enrichment of sample extracts before any centrifugation or other concentration steps may give better results.

5. IF Test
Principle

The use of the IF test as the principal screening test is recommended because of its proven robustness to achieve the required thresholds.

When the IF test is used as the principal screening test and the IF reading is positive, the Isolation, PCR or FISH test must be performed as a second screening test. When the IF test is used as the second screening test and the IF reading is positive, further testing according to the flow scheme is required to complete the analysis.

Note: Use a validated source of antibodies to *R. solanacearum* (see web site http://forum.europa.eu.int/Public/irc/sanco/Home/main). It is recommended that the titre is determined for each new batch of antibodies. The titre is defined as the highest dilution at which optimum reaction occurs when testing a suspension containing 10^5 to 10^6 cells per ml of the homologous strain of *R. solanacearum* and using an appropriate fluorescein isothiocyanate (FITC) conjugate according to the manufacturer's recommendations. Validated polyclonal antisera all had an IF titre of at least 1:2 000. During testing, the antibodies should be used at a working dilution(s) close to or at the titre.

The test should be performed on freshly-prepared sample extracts. If necessary, it can be successfully performed on extracts stored at -68 to -86 °C under glycerol. Glycerol can be removed from the sample by addition of 1 ml pellet buffer (Appendix 4), re-centrifugation for 15 minutes at 7 000 g and re-suspension in an equal volume of pellet buffer. This is often not necessary, especially if samples are fixed to the slides by flaming.

Prepare separate positive control slides of the homologous strain or any other reference strain of *R. solanacearum*, suspended in potato extract, as specified in Appendix 3 B, and optionally in buffer.

Naturally infected tissue (maintained by lyophilisation or freezing at -16 to -24 °C) should be used where possible as a similar control on the same slide.

As negative controls, aliquots of sample extract which previously tested negative for *R. solanacearum* can be used.

Standardised positive and negative control materials available for use with this test are listed in Appendix 3.

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

Test control material in an identical manner as the sample(s).

5.1. Prepare the test slides by one of the following procedures:

(i) For pellets with relatively little starch sediment:

Pipette a measured standard volume (15 µl is appropriate for 6 mm window diameter – scale up volume for larger windows) of a 1/100 dilution of the resuspended potato pellet onto the first window. Subsequently pipette a similar volume of undiluted pellet (1/1) onto the remaining windows on the row. The second row can be used as duplicate or for a second sample as presented in Figure 1.

(ii) For other pellets:

Prepare decimal dilutions (1/10, 1/100) 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 second row can be used as duplicate or for a second sample as presented in Figure 2.

5.2. Dry the droplets at ambient temperature or by warming to temperatures of 40 to 45 °C. Fix the bacterial cells to the slide either by heating (15 minutes at 60 °C), flaming, with 95 % ethanol or according to specific instructions from the suppliers of the antibodies.

If necessary, fixed slides may then be stored frozen in a desiccated box for as little time as necessary (up to a maximum of three months) prior to further testing.

5.3. IF procedure

(i) According to test slide preparation in 5.1(i):

Prepare a set of twofold dilutions The first well should have 1/2 of the titre (T/2), the others 1/4 of the titre (T/4), 1/2 of the titre (T/2), the titre (T) and twice the titre (2T).

(ii) According to test slide preparation in 5.1(ii):

Prepare the working dilution (WD) of the antibody in IF buffer. The working dilution affects the specificity.

Figure 1.

PREPARATION OF THE TEST SLIDE ACCORDING TO 5.1(I) AND 5.3(I)

	Dilutions of resuspended pellet						
	1/100	1/1	1/1	1/1	1/1	#	Dilution of resuspended pellet
(T = titre)	T/2	T/4	T/2	T	2T	#	Twofold dilutions of antiserum/ antibody

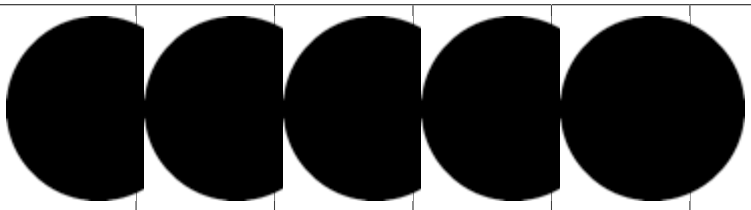
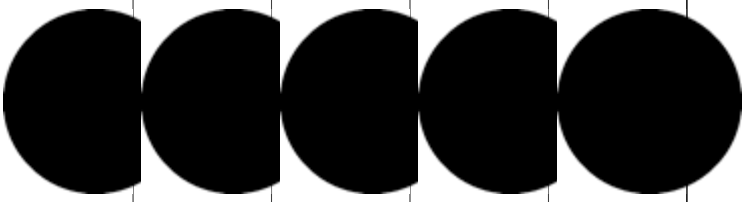
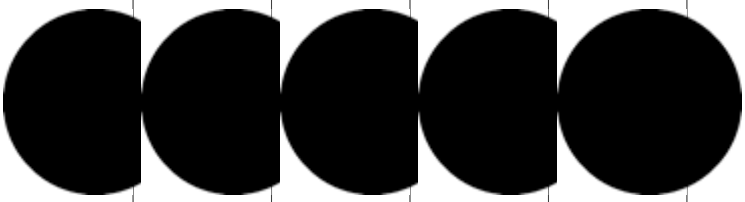
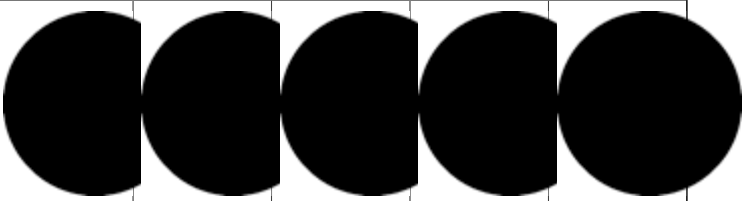
Sample 1					
	1	2	3	4	5
Duplicate of sample1 or sample 2					
	6	7	8	9	10

Figure 2.

PREPARATION OF THE TEST SLIDE ACCORDING TO 5.1(II) AND 5.3(II).

	Working dilution of antiserum/antibody						
	1/1	1/10	1/100	empty	empty	#	Decimal dilution of resuspended pellet
Sample 1							
	1	2	3	4	5		
Duplicate of sample 1 or sample 2							
	6	7	8	9	10		

5.3.1. Arrange the slides on moist tissue paper. Cover each test window completely with the antibody dilution(s). The volume of antibody applied on each window must be at least the volume of extract applied.

The following procedure should be carried out in the absence of specific instructions from the suppliers of the antibodies:

5.3.2. Incubate the slides on moist paper under a cover for 30 minutes at ambient temperature (18 to 25 °C).

5.3.3. Shake the droplets off each slide and rinse carefully with IF buffer. Wash by submerging for five minutes in IF buffer-Tween (Appendix 4) and subsequently in IF buffer. Avoid causing aerosols or droplet transfer that could result in cross-contamination. Carefully remove excess moisture by blotting gently.

5.3.4. Arrange the slides on moist paper. Cover the test windows 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 antibody applied.

5.3.5. Incubate the slides on moist paper under a cover for 30 minutes at ambient temperature (18 to 25 °C).

5.3.6. Shake the droplets of conjugate off the slide. Rinse and wash as before (5.3.3).

Carefully remove excess moisture.

5.3.7. Pipette 5 - 10 µl of 0,1M phosphate-buffered glycerol (Appendix 4) or a commercially antifading mountant on each window and apply a coverslip.

5.4. Reading the IF test:

5.4.1 Examine test slides on an epifluorescence microscope with filters suitable for excitation of FITC, under oil or water immersion at a magnification of 500-1 000. Scan windows across two diameters at right angles and around the perimeter. For samples showing no or low number of cells observe at least 40 microscope fields.

Check the positive control slide first. Cells must be bright fluorescent and completely stained at the determined antibody titre or working dilution. The IF test (Section VI.A.5.) must be repeated if the staining is aberrant.

5.4.2. Observe for bright fluorescing cells with characteristic morphology of *R. solanacearum* in the test windows of the test slides (see website http://forum.europa.eu.int/Public/irc/sanco/Home/main). The fluorescence intensity must be equivalent to the positive control strain at the same antibody dilution. Cells with incomplete staining or with weak fluorescence must be disregarded.

If any contamination is suspected the test must be repeated. This may be the case when all slides in a batch show positive cells due to the contamination of buffer or if positive cells are found (outside of the slide windows) on the slide coating.

5.4.3. There are several problems inherent to the specificity of the immunofluorescence test. Background populations of fluorescing cells with atypical morphology and cross reacting saprophytic bacteria with size and morphology similar to *R. solanacearum* are likely to occur in potato heel end core and stem segment pellets.

5.4.4. Consider only fluorescing cells with typical size and morphology at the titre or working dilution of the antibodies as in 5.3.

5.4.5. Interpretation of the IF reading:

(i) If bright fluorescing cells with characteristic morphology are found, estimate the average number of typical cells per microscope field and calculate the number of typical cells per ml of resuspended pellet (Appendix 5).

The IF reading is positive for samples with at least 5×10^3 typical cells per ml of resuspended pellet. The sample is considered potentially contaminated and further testing is required.

(ii) The IF reading is negative for samples with less than 5×10^3 cells per ml of resuspended pellet and the sample is considered negative. Further testing is not required.

6. PCR tests

Principles

When the PCR test is used as the principal screening test and found to be positive, isolation or IF must be performed as a second compulsory screening test. When PCR is used as the second screening test and found to be positive, further testing according to the flow scheme is required to complete the diagnosis.

Full exploitation of this method as principal screening test is only recommended when specialised expertise has been acquired.

Note: Preliminary testing with this method should permit reproducible detection of 10^3 to 10^4 cells of *R. solanacearum* per ml added to sample extracts which previously tested negative. Optimisation experiments may be required to achieve maximum levels of sensitivity and specificity in all laboratories.

Use validated PCR reagents and protocols (see Appendix 6). Preferably select a method with an internal control.

Use appropriate precautions to avoid contamination of sample with target DNA. The PCR test should be performed by experienced technicians, in dedicated molecular biology laboratories, in order to minimise the possibility of contamination with target DNA.

Negative controls (for DNA extraction and PCR procedures) should always be handled as final samples in the procedure, to make evident whether any carry over of DNA has occurred.

The following negative controls should be included in the PCR test:

— Sample extract that previously tested negative for *R. solanacearum*,

— Buffer controls used for extracting the bacterium and the DNA from the sample,

— PCR-reaction mix.

The following positive controls should be included:

— Aliquots of resuspended pellets to which *R. solanacearum* has been added (preparation see Appendix 3 B).

— A suspension of 10^6 cells per ml of *R. solanacearum* in water from a virulent isolate (e.g. NCPPB 4156 = PD 2762 = CFBP 3857; see Appendix 3 B).

— If possible use also DNA extracted from positive control samples in the PCR test.

To avoid potential contamination prepare positive controls in a separate environment from samples to be tested.

Sample extracts should be as free as possible from soil. It could therefore, in certain cases, be advisible to prepare extracts from washed potatoes if PCR protocols are to be used.

Standardized positive and negative control material available for use with this test are listed in Appendix 3).

6.1. DNA purification methods

Use positive and negative control samples as described above (see Appendix 3).

Test control material in an identical manner as the sample(s).

A variety of methods are available for purification of target DNA from complex sample substrates, thus removing inhibitors of PCR and other enzymatic reactions and concentrating target DNA in the sample extract. The following method has been optimised for use with the validated PCR methods shown in Appendix 6.

(a) Method according to Pastrik (2000)

1) Pipette 220 µl of lysis buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8,0], 1 mM EDTA [pH 8,0]) into a 1,5 ml Eppendorf tube.

2) Add 100 µl sample extract and place in a heating block or water bath at 95 °C for 10 min.

3) Put tube on ice for 5 min.

4) Add 80 µl Lysozyme stock solution (50 mg Lysozyme per ml in 10 mM Tris HCl, pH 8,0) and incubate at 37 °C for 30 min.

5) Add 220 µl of Easy DNA® solution A (Invitrogen), mix well by vortexing and incubate at 65 °C for 30 min.

6) Add 100 µl of Easy DNA® solution B (Invitrogen), vortex vigorously until the precipitate runs freely in the tube and the sample is uniformly viscous.

7) Add 500 µl of chloroform and vortex until the viscosity decreases and the mixture is homogeneous.

8) Centrifuge at 15 000 g for 20 min at 4 °C to separate phases and form the interphase.

9) Transfer the upper phase into a fresh Eppendorf tube.

10) Add 1 ml of 100 % ethanol (-20 °C) vortex briefly and incubate on ice for 10 min.

11) Centrifuge at 15 000 g for 20 min at 4 °C and remove ethanol from pellet.

12) Add 500 µl 80 % ethanol (-20 °C) and mix by inverting the tube.

13) Centrifuge at 15 000 g for 10 min at 4 °C, save the pellet and remove ethanol.

14) Allow the pellet to dry in air or in a DNA speed vac.

15) Resuspend the pellet in 100 µl sterile UPW and leave at room temperature for at least 20 minutes.

16) Store at -20 °C until required for PCR.

17) Spin down any white precipitate by centrifugation and use 5 µl of the supernatant containing DNA for the PCR.

(b) Other methods

Other DNA extraction methods, e.g. Qiagen DNeasy Plant Kit, could be applied providing that they are proven to be equally as effective in purifying DNA from control samples containing 10^3 to 10^4 pathogen cells per ml.

6.2. PCR

6.2.1. Prepare test and control templates for PCR according to the validated protocols (Section VI.A.6.). Prepare one decimal dilution of sample DNA extract (1:10 in UPW).

6.2.2. Prepare the appropriate PCR reaction mix in a contamination-free environment according to the published protocols (Appendix 6). Where possible, it is recommended to use a multiplex PCR protocol that also incorporates an internal PCR control.

6.2.3. Add 2-5 µl of DNA extract per 25 µl PCR reaction in sterile PCR tubes according to the PCR protocols, (see Appendix 6).

6.2.4. Incorporate a negative control sample containing only PCR reaction mix and add the same source of UPW as used in the PCR mix in place of sample.

6.2.5. Place tubes in the same thermal cycler which was used in preliminary testing and run the appropriately optimised PCR programme (Appendix 6).

6.3. Analysis of the PCR product

6.3.1. Resolve PCR amplicons by agarose gel electrophoresis. Run at least 12 µl of amplified DNA reaction mixture from each sample mixed with 3 µl loading buffer (Appendix 6) in 2,0 % (w/v) agarose gels in tris-acetate-EDTA (TAE) buffer (Appendix 6) at 5 to 8 V per cm. Use an appropriate DNA marker, e.g. 100 bp ladder.

6.3.2. Reveal DNA bands by staining in ethidium bromide (0,5 mg per L) for 30 to 60 minutes taking appropriate precautions for handling this mutagen.

6.3.3. Observe stained gel under short wave UV transillumination ($\lambda = 302$ nm) for amplified PCR products of the expected size (Appendix 6) and document.

6.3.4. For all new findings/cases verify authenticity of the PCR amplicon by performing restriction enzyme analysis on a sample of the remaining amplified DNA by incubating at the optimum temperature and time with an appropriate enzyme and buffer (see Appendix 6). Resolve the digested fragments by agarose gel electrophoresis as before and observe characteristic restriction fragment pattern under UV transillumination after ethidium bromide staining and compare with the undigested and digested positive control.

Interpretation of the PCR test result:

The PCR test is negative if the *R. solanacearum*-specific PCR amplicon of expected size is not detected for the sample in question but is detected for all positive control samples (in case of multiplex PCR with plant specific internal control primers: a second PCR-product of expected size must be amplified with the sample in question).

The PCR test is positive if the *R. solanacearum*-specific PCR amplicon of expected size and restriction pattern (when required) is detected, providing that it is not amplified from any of the negative control samples. Reliable confirmation of a positive result can also be obtained by repeating the test with a second set of PCR primers (Appendix 6).

Note: Inhibition of the PCR may be suspected if the expected amplicon is obtained from the positive control sample containing *R. solanacearum* in water but negative results are obtained

from positive controls with *R. solanacearum* in potato extract. In multiplex PCR protocols with internal PCR controls, inhibition of the reaction is indicated when neither of the two amplicons are obtained.

Contamination may be suspected if the expected amplicon is obtained from one or more of the negative controls.

7. FISH test

Principle

When the FISH test is used as the first screening test and found to be positive, Isolation or the IF test must be performed as a second compulsory screening test. When the FISH test is used as the second screening test and found to be positive, further testing according to the flow scheme is required to complete the diagnosis.

Note: Use validated *R. solanacearum*-specific oligo-probes (see Appendix 7). Preliminary testing with this method should permit reproducible detection of at least 10^3 to 10^4 cells of *R. solanacearum* per ml added to sample extracts which previously tested negative.

The following procedure should preferably be performed on freshly prepared sample extract but can also be successfully performed on sample extract that has been stored under glycerol at -16 to -24 or -68 to -86 °C.

As negative controls, use aliquots of sample extract that previously tested negative for *R. solanacearum*.

As positive controls prepare suspensions containing 10^5 to 10^6 cells per ml of *R. solanacearum* biovar 2 (e.g. strain NCPPB 4156 = PD 2762 = CFBP 3857, see Appendix 3) in 0,01M phosphate buffer (PB) from a 3 to 5 day culture). Prepare separate positive control slides of the homologous strain or any other reference strain of *R. solanacearum*, suspended in potato extract, as specified in Appendix 3 B.

The use of the FITC-labelled eubacterial oligo-probe offers a control for the hybridisation process, since it will stain all eubacteria that are present in the sample.

Standardized positive and negative control material available for use with this test are listed in Appendix 3A).

Test control material in an identical manner as the sample(s).

7.1. Potato extract fixation

The following protocol is based upon Wullings *et al.* (1998):

7.1.1. Prepare fixative solution (see Appendix 7).

7.1.2. Pipette 100 µl of each sample extract into an Eppendorf tube and centrifuge for 7 minutes at 7 000 g.

7.1.3. Remove the supernatant and dissolve the pellet in 200 µl of fixative prepared < 24 hours previously. Vortex and incubate for one hour in the refrigerator.

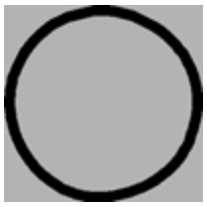
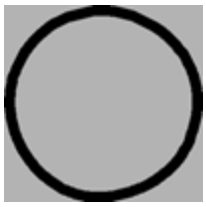
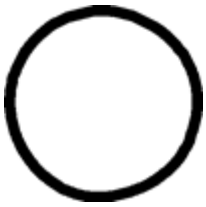
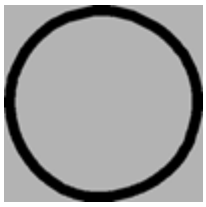
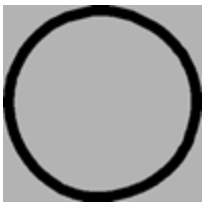
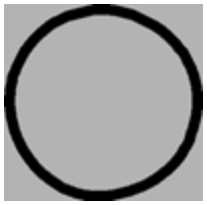
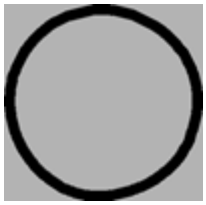
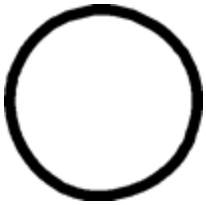
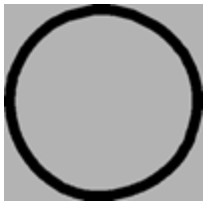
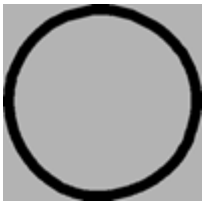
7.1.4. Centrifuge for 7 minutes at 7 000 g, remove the supernatant and resuspend the pellet in 75 µl 0,01M PB (see Appendix 7).

7.1.5. Spot 16 µl of the fixed suspensions onto a clean multitest slide as shown in Fig. 7.1. Applying two different samples per slide, undiluted and use 10 µl to make a 1:100 dilution (in 0,01 M PB). The remaining sample solution (49 µl) can be stored at

-20 °C after addition of one volume of 96 % ethanol. In case the FISH assay requires repeating, remove the ethanol by centrifugation and add an equal volume of 0,01 PB (mix by vortexing).

Fig. 7.1

LAYOUT FOR FISH SLIDE

Sample 1	Blank	Blank	Blank	Sample 2
				
window 1	window 2	window 3	window 4	window 5
Sample 1	Blank	Blank	Blank	Sample 2
				
window 6	window 7	window 8	window 9	window 10
Coverslip 1			Coverslip 2	

7.1.6. Air-dry the slides (or on slide dryer at 37 °C) and fix them by flaming.

At this stage the procedure may be interrupted and the hybridisation continued the following day. Slides should be stored dust-free and dry at room temperature.

7.2. Hybridisation

7.2.1. Dehydrate the cells in a graded ethanol series of 50 %, 80 % and 96 % for one minute each. Air dry the slides in a slide-holder.

7.2.2. Prepare a moist incubation chamber by covering the bottom of an air-tight box with tissue or filter paper soaked in 1x hybmix (Appendix 7). Pre-incubate the box in the hybridisation oven at 45 °C for at least 10 minutes.

7.2.3. Apply 10 µl of hybridisation solution (Appendix 7) to eight windows (windows 1, 2, 4, 5, 6, 7, 9 and 10; see Fig 7.1) of each slide leaving the two centre windows (3 and 8) empty.

7.2.4. Apply coverslips (24 × 24 mm) to the first and last four windows without trapping air. Place the slides in the pre-warmed moist chamber and hybridise for five hours in the oven at 45 °C in the dark.

7.2.5. Prepare three beakers containing 1 l of Milli Q (molecular grade) water, 1 l of 1x hybmix (334 ml 3x hybmix and 666 ml Milli Q water) and 1 l of 1/8x hybmix (42 ml 3x hybmix and 958 ml Milli Q water). Pre-incubate each in a waterbath at 45 °C.

7.2.6. Remove the coverslips from the slides and place the slides in a slide holder.

7.2.7. Wash away excess probe by incubation for 15 minutes in the beaker with 1x hybmix at 45 °C.

7.2.8. Transfer the slide holder to 1/8 hybmix washing solution and incubate for a further 15 minutes.

7.2.9. Dip the slides briefly in Milli Q water and place them on filter paper. Remove excess moisture by covering the surface gently with filter paper. Pipette 5 to 10 µl of anti-fading mountant solution (e.g. Vectashield, Vecta Laboratories, CA, USA or equivalent) on each window and apply a large coverslip (24 × 60 mm) over the whole slide.

7.3. Reading the FISH test

7.3.1. Observe the slides immediately with a microscope fitted for epifluorescence microscopy at 630 or 1 000 × magnification under immersion oil. With a filter suitable for fluorescein isothiocyanate (FITC) eubacterial cells (including most gram negative cells) in the sample are stained fluorescent green. Using a filter for tetramethylrhodamine-5-isothiocyanate, Cy3-stained cells of *R. solanacearum* appear fluorescent red. Compare cell morphology with that of the positive controls. Cells must be bright fluorescent and completely stained The FISH test (Section VI.A.7.) must be repeated if the staining is aberrant. Scan windows across two diameters at right angles and around the perimeter. For samples showing no or low number of cells observe at least 40 microscope fields.

7.3.2. Observe for bright fluorescing cells with characteristic morphology of *R. solanacearum* in the test windows of the test slides (see web site http://forum.europa.eu.int/Public/irc/sanco/Home/main). The fluorescence intensity must be equivalent or better than that of the positive control strain. Cells with incomplete staining or with weak fluorescence must be disregarded.

7.3.3. If any contamination is suspected the test must be repeated. This may be the case when all slides in a batch show positive cells due to the contamination of buffer or if positive cells are found (outside of the slide windows) on the the slide coating.

7.3.4. There are several problems inherent to the specificity of the FISH test. Background populations of fluorescing cells with atypical morphology and cross reacting saprophytic bacteria with size and morphology similar to *R. solanacearum* may occur, although much less frequent as in the IF test, in potato heel end core and stem segment pellets.

7.3.5. Consider only fluorescing cells with typical size and morphology.

7.3.6. Interpretation of the FISH test result:

(i) Valid FISH test results are obtained if bright green fluorescent cells of size and morphology typical of *R. solanacearum* are observed using the FITC filter and bright red fluorescent cells using the rhodamine filter in all positive controls and not in any of the negative controls. If bright fluorescing cells with characteristic morphology are found, estimate the average number of typical cells per microscope field and calculate

the number of typical cells per ml of resuspended pellet (Appendix 4). Samples with at least 5×10^3 typical cells per ml of resuspended pellet are considered potentially contaminated. Further testing is required. Samples with less than 5×10^3 typical cells per ml of resuspended pellet are considered negative.

(ii) The FISH test is negative if bright red fluorescent cells with size and morphology typical of *R. solanacearum* are not observed using the rhodamine filter, provided that typical bright red fluorescent cells are observed in the positive control preparations when using the rhodamine filter.

8. ELISA tests

Principle

ELISA can only be used as an optional test in addition to IF, PCR or FISH due to a relatively low sensitivity of this test. When DAS ELISA is used enrichment and the use of monoclonal antibodies are compulsory (see web site http://forum.europa.eu.int/Public/irc/sanco/Home/main). Enrichment of the samples before using ELISA may be useful in order to increase the sensitivity of the test, but it can fail due to competition by other organisms in the sample.

Note: Use an validated source of antibodies to *R. solanacearum* (see web site http://forum.europa.eu.int/Public/irc/sanco/Home/main) It is recommended that the titre is determined for each new batch of antibodies. The titre is defined as the highest dilution at which optimum reaction occurs when testing a suspension containing 10^5 to 10^6 cells per ml of the homologous strain of *R. solanacearum* and using appropriate secondary antibody conjugates according to the manufacturer's recommendations. During testing, the antibodies should be used at a working dilution close to or at the titre of the commercial formulation.

Determine the titre of the antibodies on a suspension of 10^5 to 10^6 cells per ml of the homologous strain of *R. solanacearum*.

Include a sample extract that previously tested negative for *R. solanacearum* and a suspension of a non-cross reacting bacterium in phosphate buffered saline (PBS) as negative controls.

As positive control use aliquots of sample extract, that previously tested negative, mixed with 10^3 to 10^4 cells per ml of *R. solanacearum* biovar 2 (e.g. strain NCPPB 4156 = PD 2762 = CFBP 3857, see Appendix 2 A and B). For comparison of results on each plate use a standard suspension of 10^5 to 10^6 cells per ml in PBS of *R. solanacearum*. Ensure positive controls are well separated on the microtitre plate from the sample(s) under test.

Standardised positive and negative control materials available for use with this test are listed in Appendix 3 A.

Test control material in an identical manner as the sample(s).

Two ELISA protocols have been validated.

(a) Indirect ELISA (Robinson Smith *et al.*, 1995)

1) Use 100 to 200 µl aliquots of sample extract. (Heating at 100 °C for four minutes in a waterbath or heating block may reduce non-specific results in some cases).

2) Add an equal volume of double strength coating buffer (Appendix 4) and vortex.

3) Apply 100 µl aliquots to each of at least two wells of a microtitre plate (e.g. Nunc-Polysorp or equivalent) and incubate for one hour at 37 °C or overnight at 4 °C.

4) Flick out the extracts from the wells. Wash the wells three times with PBS-Tween (Appendix 4), leaving the last washing solution in the wells for at least five minutes.

5) Prepare the appropriate dilution of antibodies against-*R. solanacearum* in blocking buffer (Appendix 4). For validated commercial antibodies, use the recommended dilutions (usually twice as concentrated as the titre).

6) Add 100 µl to each well and incubate for one hour at 37 °C.

7) Flick out the antibody solution from the wells and wash as before (4).

8) Prepare the appropriate dilution of secondary antibody-alkaline phosphatase conjugate in blocking buffer. Add 100 µl to each well and incubate for one hour at 37 °C.

9) Flick out conjugated antibody from wells and wash as before (4).

10) Add 100 µl alkaline phosphatase substrate solution (Appendix 4) to each well. Incubate in the dark at ambient temperature and read absorbance at 405 nm at regular intervals within 90 minutes.

(b) DASI ELISA

1) Prepare the appropriate dilution of anti-*R. solanacearum* polyclonal immunoglobulins in coating buffer pH 9.6 (Appendix 4). Add 200 µl to each well. Incubate at 37 °C for four to five hours or at 4 °C for 16 hours.

2) Wash the wells three times with PBS-Tween (Appendix 4).

Add 190 µl of sample extract to at least two wells. Also add positive and negative controls in two wells each per plate. Incubate for 16 hr at 4 °C.

3) Wash the wells three times with PBS-Tween (Appendix 4).

4) Prepare an appropriate dilution of *R. solanacearum*-specific monoclonal antibodies in PBS (Appendix 4) also containing 0,5 % bovine serum albumin (BSA) and add 190 µl to each well. Incubate at 37 °C for two hours.

5) Wash the wells three times with PBS-Tween (Appendix 4).

6) Prepare an appropriate dilution of anti-mouse immunoglobulins conjugated with alkaline phosphatase in PBS. Add 190 µl to each well. Incubate at 37 °C for two hours.

7) Wash the wells three times with PBS-Tween (Appendix 4).

8) Prepare an alkaline phosphatase substrate solution containing 1 mg p-nitrophenyl phosphate per ml of substrate buffer (Appendix 4). Add 200 µl to each well. Incubate in the dark at ambient temperature and read absorbance at 40 nm at regular intervals within 90 minutes.

Interpretation of ELISA test results:

The ELISA test is negative if the average optical density (OD) reading from duplicate sample wells is < 2x OD of that in the negative sample extract control well, providing the OD for the positive controls are all above 1,0 (after 90 minutes incubation with the substrate) and are greater than twice the OD obtained for negative sample extracts.

The ELISA test is positive if the average OD readings from duplicate sample wells is > 2x OD in the negative sample extract well provided that OD readings in all negative control wells are < 2x those in the positive control wells.

Negative ELISA readings in positive control wells indicate that the test has not been performed correctly or that it has been inhibited. Positive ELISA readings in negative control wells indicate that cross-contamination or non-specific antibody binding has occurred.

9. Bioassay test

Note: Preliminary testing with this method should permit reproducible detection of 10^3 to 10^4 colony-forming units of *R. solanacearum* per ml added to sample extracts that previously tested negative (preparation see Appendix 3).

Highest sensitivity of detection can be expected when using freshly prepared sample extract and optimal growth conditions. However, the method can be successfully applied to extracts that have been stored under glycerol at -68 to -86 °C.

The following protocol is based upon Janse (1988):

9.1. Use 10 test plants of a susceptible tomato cultivar (e.g. Moneymaker or cultivar with equivalent susceptibility as determined by the testing laboratory) at the third true leaf stage for each sample. For cultural details, see Appendix 8. Alternatively, use eggplants (e.g. cultivar Black Beauty or cultivars with equivalent susceptibility), use only plants at leaf stage two to three up to full expansion of the third true leaf. Symptoms have been show to be less severe and to develop more slowly in eggplant. Where possible, it is therefore recommended to use tomato seedlings.

9.2. Distribute 100 µl of sample extract between the test plants.

9.2.1. Syringe inoculation

Inoculate the plant stems just above the cotyledons using a syringe fitted with a hypodermic needle (not less than 23G). Distribute the sample between the test plants.

9.2.2. Slit inoculation

Holding the plant between two fingers, pipette a drop (approximately 5 - 10 µl) of the suspended pellet on the stem between the cotyledons and the first leaf.

Using a sterile scalpel, make a diagonal slit, about 1.0 cm long and approximately 2/3 of the stem thickness deep, starting the cut from the pellet drop.

Seal the cut with sterile vaseline from a syringe.

9.3. Inoculate by the same technique, five seedlings with an aqueous suspension of 10^5 to 10^6 cells per ml prepared from a 48 hr culture of a virulent biovar 2 strain of *R. solanacearum* as a positive control and with pellet buffer (Appendix 4) as negative control. Separate positive and negative control plants from the other plants to avoid cross-contamination.

9.4. Grow the test plants in quarantine facilities for up to four weeks at 25 to 30 °C and high relative humidity with appropriate watering to prevent waterlogging or wilting through water deficiency. To avoid contamination incubate positive control and negative control plants on clearly separated benches in a glasshouse or growth chamber or, in case space is limited, ensure strict separation between treatments. If plants for different samples must be incubated close together, separate them with appropriate screens. When fertilising, watering, inspecting and any other manipulations take great care to avoid cross-contamination. It is essential to keep glasshouses and growth chambers free of all insect pests since they may transmit the bacterium from sample to sample.

Observe for symptoms of wilting, epinasty, chlorosis and/or stunting.

9.5. Isolate from infected plants (Section II.3.) and identify purified cultures of presumptive *R. solanacearum* (Section VI.B.).

9.6. If no symptoms are observed after three weeks perform IF/PCR/Isolation on a composite sample of 1 cm stem sections of each test plant taken above the inoculation site. If the test is positive perform dilution plating (section 4.1).

9.7. Identify any purified cultures of presumptive *R. solanacearum* (Section VI.B.).

Interpretation of the bioassay test results

Valid Bioassay test results are obtained when plants of the positive control show typical symptoms, the bacteria can be reisolated from these plants and no symptoms are found on the negative controls.

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

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

B. IDENTIFICATION TESTS

Identify pure cultures of presumptive *R. solanacearum* isolates using at least two of the following tests based on different biological principles.

Include known reference strains where appropriate for each test performed (see Appendix 3).

1. Nutritional and enzymatic identification tests

Determine the following phenotypic properties, which are universally present or absent in *R. solanacearum*, according to the methods of Lelliott and Stead (1987), Klement *et al.* (1990), Schaad (2001).

Test	**Expected result**
Fluorescent pigment production	–
Poly-ß-hydroxybutyrate inclusions	+
Oxidation/fermentation (O/F) test	O+/F–
Catalase activity	+
Kovac's oxidase test	+
Reduction of nitrate	+
Utilisation of citrate	+
Growth at 40 °C	–
Growth in 1 % NaCl	+
Growth in 2 % NaCl	–
Arginine dihydrolase activity	–
Gelatine liquefaction	–
Starch hydrolysis	–
Aesculin hydrolysis	–

Levan production	–

2. IF test

2.1. Prepare a suspension of approximately 10^6 cells per ml in IF buffer (Appendix 4).

2.2. Prepare a twofold dilution series of an appropriate antiserum (see website http://forum.europa.eu.int/Public/irc/sanco/Home/main).

2.3. Apply the IF procedure (Section VI.A.5.).

2.4. A positive IF test is achieved if the IF titre of the culture is equivalent to that of the positive control.

3. ELISA test

Note: If performing only 2 identification tests, do not use another serological test in addition to this method.

3.1. Prepare a suspension of approximately 10^8 cells per ml in 1X PBS (Appendix 4).

3.2. Perform an appropriate ELISA procedure with a specific monoclonal antibody to *R. solanacearum*.

3.3. A positive ELISA test is achieved if the ELISA reading obtained from the culture is at least half that obtained for the positive control.

4. PCR tests

4.1. Prepare a suspension of approximately 10^6 cells per ml in molecular grade sterile water.

4.2. Heat 100 µl of the cell suspension in closed tubes in a heating block or boiling waterbath at 100 °C for four minutes. The samples may then be stored at -16 to -24 °C until required.

4.3. Apply appropriate PCR procedures to amplify *R. solanacearum*-specific amplicons (e.g. Seal *et al.* (1993); Pastrik and Maiss (2000); Pastrik *et al.* (2002); Boudazin *et al.* (1999); Opina *et al.* (1997), Weller *et al.* (1999).

4.4. A positive identification of *R. solanacearum* is achieved if the PCR amplicons are the same size and have the same restriction fragment length polymorphisms as for the positive control strain.

5. FISH test

5.1. Prepare a suspension of approximately 10^6 cells per ml in UPW.

5.2. Apply the FISH procedure (Section VI.A.7.) with at least 2 *R. solanacearum*-specific oligo-probes (Appendix 7).

5.3. A positive FISH test is achieved if the same reactions are achieved from the culture and the positive control.

6. Fatty acid profiling (FAP)

6.1. Grow the culture on trypticase soy agar (Oxoid) for 48 hours at 28 °C.

6.2. Apply an appropriate FAP procedure (Janse, 1991; Stead, 1992).

6.3. A positive FAP test is achieved if the profile of the presumptive culture is identical to that of the positive control. The presence of characteristic fatty acids are 14:0 3OH, 16:0 2OH, 16:1 2OH and 18:1 2OH and absence of 16:0 3OH is highly indicative of a *Ralstonia* sp.

7. Strain characterisation methods

Strain characterisation using one of the following methods is recommended for each new case of isolation of *R. solanacearum*.

Include known reference strains where appropriate for each test performed (see Appendix 3).

7.1. Biovar determination

R. solanacearum is separated into biovars on the basis of the ability to utilise and/or oxidise three disaccharides and three hexose alcohols (Hayward, 1964 and Hayward *et al.*, 1990). Growth media for the biovar test is described in Appendix 2. The test can be successfully performed by stab inoculating the media with pure cultures of *R. solanacearum* isolates and incubating at 28 °C. If the media are dispensed into sterile 96 well cell culture plates (200 µl per well) colour change from olive green to yellow can be observed within 72 hours, indicating a positive test result.

	Biovar				
	1	**2**	**3**	**4**	**5**
Utilisation of:					
Maltose	–	+	+	–	+
Lactose	–	+	+	–	+
D (+) Cellobiose	–	+	+	–	+
Mannitol	–	–	+	+	+
Sorbitol	–	–	+	+	–
Dulcitol	–	–	+	+	–

Additional tests differentiate biovar 2 sub-phenotypes

	Biovar 2A(Worldwide distribution)	**Biovar 2A(Found in Chile and Colombia)**	**Biovar 2T(Found in tropical areas)**
Utilisation of trehalose	–	+	+
Utilisation of *meso*-inositol	+	–	+
Utilisation of D ribose	–	–	+

a See Lelliott and Stead (1987)

Pectolytic activity[a]	low	low	high

a See Lelliott and Stead (1987)

7.2. Genomic fingerprinting

Molecular differentiation of strains in the *R. solanacearum* complex can be achieved using several techniques, including:

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 *et al.*, 1995; Smith *et al.*, 1995).

7.2.3. Amplified fragment length polymorphism (AFLP) analysis (Van der Wolf *et al.*, 1998).

7.3. PCR methods

Specific PCR primers (Pastrik *et al*, 2002; see Appendix 6) can be used to differentiate strains belonging to division 1 (biovars 3, 4 and 5) and division 2 (biovars 1, 2A and 2T) of *R. solanacearum*, as originally defined by RFLP analysis (Cook *et al.*, 1989) and 16S rDNA sequencing (Taghavi *et al.*, 1996).

C. CONFIRMATION TEST

The pathogenicity test must be performed as final confirmation of a diagnosis of *R. solanacearum* and for assessment of virulence of cultures identified as *R. solanacearum*.

1) Prepare an inoculum of approximately 10^6 cells per ml from a 24 to 48 hour culture of the isolate to be tested and an appropriate positive control strain of *R. solanacearum* (e.g. NCPPB 4156 = PD 2762 = CFBP 3857; see Appendix 3).

2) Inoculate 5 to 10 susceptible tomato or eggplant seedlings at the third true leaf stage (see Section VI.A.9).

3) Incubate for up to two weeks at 25 to 28 °C and high relative humidity with appropriate watering to avoid waterlogging or drought stress. With pure cultures typical wilting should be obtained within 14 days. If after this period symptoms are not present, the culture cannot be confirmed as being a pathogenic form of *R. solanacearum*.

4) Observe for symptoms of wilting and/or epinasty, chlorosis and stunting.

5) Isolate from symptomatic plants by removing a section of stem about 2 cm above the inoculation point. Comminute and suspend in a small volume of sterile distilled water or 50 mM phosphate buffer (Appendix 4). Isolate from the suspension by dilution spreading or streaking on a suitable medium, preferably onto a selective medium (Appendix 2), incubate for 48 to 72 hours at 28 °C and observe the formation of colonies typical of *R. solanacearum*.**]**