Council Directive 93/85/EEC of 4 October 1993 on the control of potato ring rot

[F1 ANNEX I

TEST SCHEME FOR DIAGNOSIS, DETECTION AND IDENTIFICATION OF THE RING ROT BACTERIUM, *CLAVIBACTER MICHIGANENSIS* (Smith) Davis *et al.* ssp. *SEPEDONICUS* (Spieckermann et Kotthoff) Davis *et al.*

SCOPE OF THE TEST SCHEME

Textual Amendments

F1 Substituted by Commission Directive 2006/56/EC of 12 June 2006 amending the Annexes to Council Directive 93/85/EEC on the control of potato ring rot.

5. FISH TEST

Principle

When the FISH test is used as the first screening test and found to be positive, 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 *C. m.* subsp. *sepedonicus*-specific oligo-probes (Appendix 7). Preliminary testing with this method should permit reproducible detection of at least 10^3 to 10^4 cells of *C. m.* subsp. *sepedonicus* 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 °C or -68 to -86 °C.

As negative controls, use aliquots of sample extract that previously tested negative for *C. m.* subsp. *sepedonicus*.

As positive controls prepare suspensions containing 10^5 to 10^6 cells per ml of *C. m.* subsp. *sepedonicus* (e.g. strain NCPPB 4053, or PD 406) in 0,01M phosphate buffer (PB) from a three to five day culture (preparation see Appendix 2). Prepare separate positive control slides of the homologous strain or any other reference strain of *C. m.* subsp. *sepedonicus*, suspended in potato extract, as specified in Appendix 2.

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.

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

5.1. Potato extract fixation

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

5.1.1. Prepare fixative solution (see Appendix 7).

5.1.2. Pipette 100 µl of each sample extract into an Eppendorf tube and centrifuge for eight min at 7 000 g.

5.1.3. Remove the supernatant and dissolve the pellet in 500 µl of fixative prepared < 24 hours previously. Vortex and incubate overnight at 4 °C.

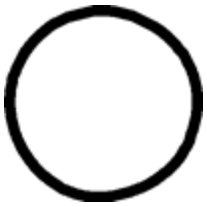
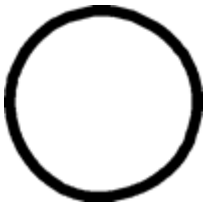
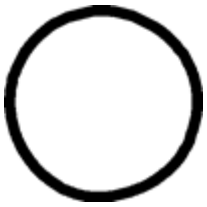
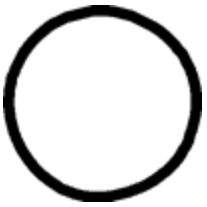
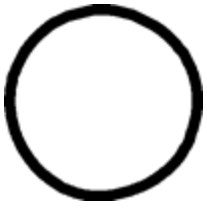
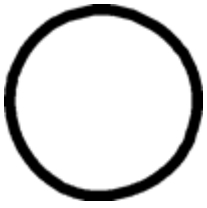
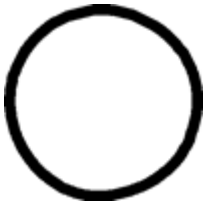
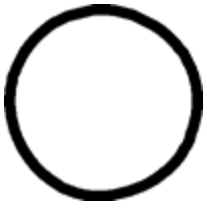
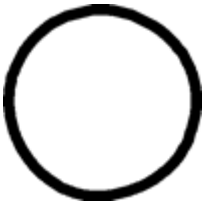
An alternative fixative is 96 % ethanol. To use this dissolve the pellet from step 5.1.2 in 50 µl 0,01M PB and 50 µl 96 % ethanol. Vortex mix and incubate at 4 °C for 30 to 60 minutes.

5.1.4. Centrifuge for 8 min. at 7 000 g, remove the supernatant and resuspend the pellet in 75 µl 0,01M PB (see Appendix 3).

5.1.5. Spot 16 µl of the fixed suspensions onto a clean multitest slide as shown in Fig. 3. Apply two different samples per slide, undiluted and use 10 µl to make a 1:100 dilution (in 0,01M PB). The remaining sample solution (49 µl) can be stored at -20 °C after addition of 1 volume of 96 % ethanol. In case the FISH assay requires repeating, remove the ethanol by centrifugation and add an equal volume of 0,01M PB (mix by vortexing).

Figure 3.

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	

5.1.6. 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.

5.2. Pre-hybridisation and hybridisation

5.2.1. Prepare a lysozyme solution containing 10 mg lysozyme (Sigma L–6876) in 10 ml buffer (100 mM Tris-HCl, 50 mM EDTA, pH8.0). This solution can be stored but it should only be freeze-thawed once. Cover each sample well with approximately 50 µl of lysozyme solution and incubate for 10 minutes at room temperature. Then dip the slides in demineralised water, once only and dry with filter paper.

Alternatively, instead of lysozyme add 50 µl of 40 to 400µg ml^{-1} proteinase K in buffer (20 mM Tris-HCl, 2 mM $CaCl_2$, pH 7,4) to each well and incubate at 37 °C for 30 minutes.

5.2.2. 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.

5.2.3. 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 55 °C for at least 10 minutes.

5.2.4. Prepare the hybridisation solution (Appendix 7) allowing 45 µl per slide, and preincubate for five minutes at 55 °C.

5.2.5. Place slides on a hot plate at 45 °C and apply 10 µl of hybridization solution to each of the four wells on the slide(s).

5.2.6. Apply two coverslips (24 × 24 mm) to each slide without trapping air. Place the slides in the pre-warmed moist chamber and hybridise overnight in the oven at 55 °C in the dark.

5.2.7. Prepare three beakers containing 1 l of Ultra pure water (UPW), 1 l of 1x hybmix (334 ml 3x hybmix and 666 ml UPW) and 1 l of 1/2x hybmix (167 ml 3x hybmix and 833 ml UPW). Pre-incubate each in a waterbath at 55 °C.

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

5.2.9. Wash away excess probe by incubation for 15 mins. in the beaker with 1x hybmix at 55 °C.

5.2.10. Transfer the slide holder to 1/2 hybmix washing solution and incubate for a further 15 mins.

5.2.11. Dip the slides briefly in UPW 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.

5.3. Reading the FISH test

5.3.1. Observe the slides immediately with a microscope fitted for epifluorescence microscopy at 630 or 1000x magnification under immersions 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 *C. m.* subsp. *sepedonicus* appear fluorescent red. Compare cell morphology with that of the positive controls. Cells must be bright fluorescent and completely stained The FISH test (section 9.4) 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.

5.3.2. Observe for bright fluorescing cells with characteristic morphology of *C. m.* subsp. *sepedonicus* 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.

5.3.3. 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 slide coating.

5.3.4. 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 *C. m.* subsp. *sepedonicus* may occur, although much less frequent than in the IF test, in potato heel end core and stem segment pellets.

5.3.5. Consider only fluorescing cells with typical size and morphology, see in 5.3.2.

5.3.6. Interpretation of the FISH test result:

(i) Valid FISH test results are obtained if bright green fluorescent cells of size and morphology typical of *C. m.* subsp. *sepedonicus* are observed using the FITC filter and if 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 *C. m.* subsp. *sepedonicus* 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.**]**