L.N. 90 of 2007

PLANT QUARANTINE ACT
(CAP. 433)

Control of Potato Ring Rot (Amendment) Regulations, 2007

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

Title, commencement and scope.
L.N. 290 of 2004.

1. (1) The title of these regulations is the Control of Potato Ring Rot (Amendment) Regulations, 2007 and they shall be read and construed as one with the Control of Potato Ring Rot Regulations, 2004, hereinafter in these regulations referred to as "the principal regulations".

(2) These regulations shall come into force on the 1st April, 2007.

(3) The scope of these regulations is to transpose Council Directive 2006/56/EC amending the Annexes to Council Directive 93/85/EEC on the control of potato ring rot.

Substitutes Schedules I, II, III and IV of the principal regulations.

2. Schedules I, II, III and IV of the principal regulations shall be substituted by Schedules I, II, III and IV respectively, set out in Schedule I to these regulations.

Adds Schedule V to the principal regulations.

3. Schedule V, as set out in Schedule II to these regulations, shall be added immediately after Schedule IV of the principal regulations.

SCHEDULE I

(Regulation 2)

"SCHEDULE 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

The presented scheme describes the various procedures involved in:

(i) Diagnosis of ring rot in potato tubers and plants;

(ii) Detection of *Clavibacter michiganensis* ssp. *sepedonicus* in samples of potato tubers and plants;

(iii) Identification of *Clavibacter michiganensis* ssp. *sepedonicus* (*C. m*. subsp. *sepedonicus*).

GENERAL PRINCIPLES

Optimized protocols for the various methods, validated reagents and details for the preparation of test and control materials are provided in the Appendices. A list of the laboratories that were included in optimization and validation of protocols is provided in Appendix 1.

Since the protocols involve detection of a quarantine organism and will include the use of viable cultures of *C. m*. subsp. *sepedonicus* as control materials, it will be necessary to perform the procedures under suitable quarantined conditions with adequate waste disposal facilities and under the conditions of appropriate licences as issued by the official plant quarantine authorities.

Testing parameters must assure consistent and reproducible detection of levels of *C. m*. subsp. *sepedonicus* at the set thresholds of the selected methods.

Precise preparation of positive controls is imperative.

Testing according to the required thresholds also implies correct settings, maintenance and calibration of equipment, careful handling and preservation of reagents and all measures to prevent contamination between samples, e.g. separation of positive controls from test samples. Quality control standards must be applied to avoid administrative and other errors, especially concerning labelling and documentation.

A suspected occurrence, as referred to in regulation 4(3) implies a positive

result in diagnostic or screening tests performed on a sample as specified in flow charts.

If the first screening test (IF or PCR/FISH) is positive, then contamination with Cms is suspected and a second screening test must be done. If the second screening test is positive, then the suspicion is confirmed (suspected occurrence) and the testing according to the scheme must be continued. If the second screening test is negative, then the sample is considered not contaminated with Cms.

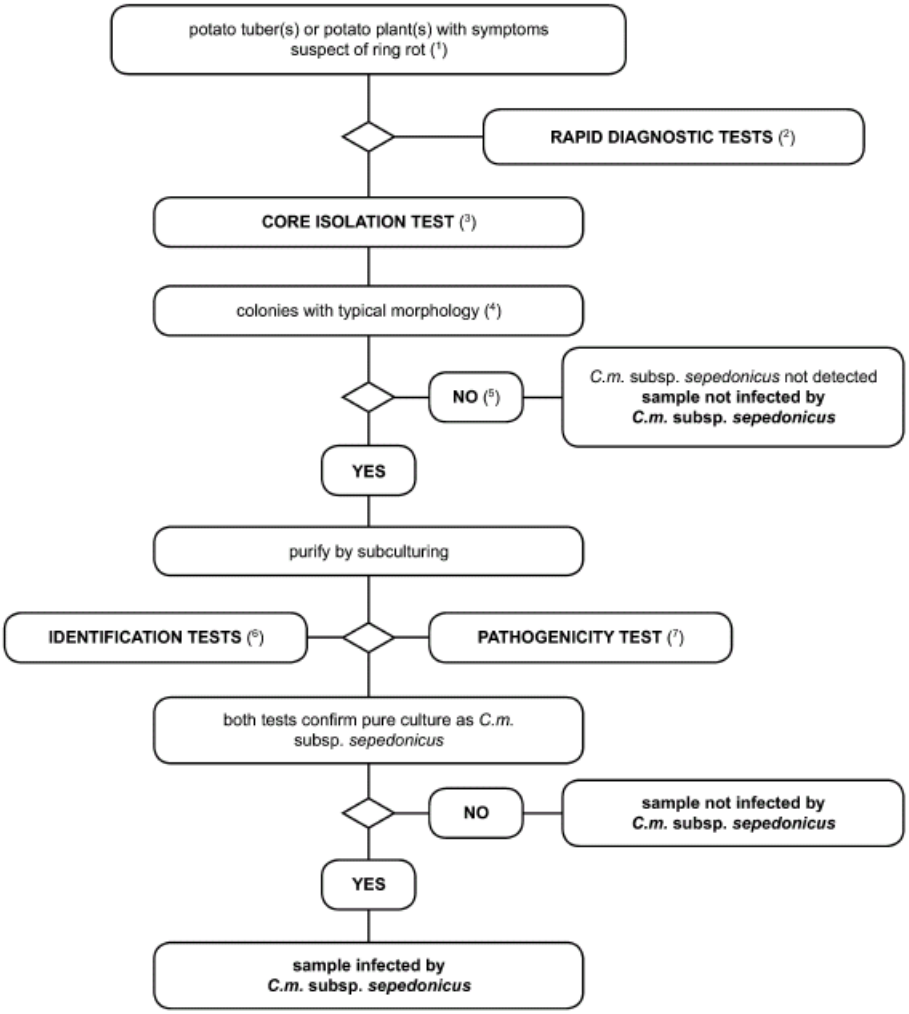
Therefore a positive IF test as referred to in regulation 4(3) is defined by a positive IF reading confirmed by a second screening test (PCR/FISH).

Confirmed presence as referred to in regulation 5(1) implies the isolation and identification of a pure culture of C. m. subsp. sepedonicus with confirmation of pathogenicity.

1. FLOW CHART DIAGRAM PRESENTATION

1.1. Detection scheme for the diagnosis of Ring Rot in potato tubers and potato plants with symptoms of ring rot

The testing procedure is intended for potato tubers and plants with symptoms typical or suspect of ring rot. 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 *C. m*. subsp. *sepedonicus*.



(1) Description of symptoms is provided in section 2.

(2) Appropriate tests are:

– IF-test (section 4).

– PCR test (section 6).

– FISH test (section 5).

(3) Although isolation of the pathogen from plant material with typical symptoms by dilution plating is straightforward, culturing may fail from advance stages of infection. Saprophytic bacteria which grow on diseased tissue may outgrow or inhibit the pathogen on the isolation medium. Therefore it is recommended to use both non selective and selective media, preferably MTNA (section 8) or Bioassay test (section 7).

(4) Description of typical colony morphology is provided in section 8.

(5) If the isolation test is negative, but diseases symptoms are typical, then isolation must be repeated.

(6) Reliable identification of a pure culture of *C. m.* subsp. *Sepedonicus* is achieved by using the tests listed in section 9.

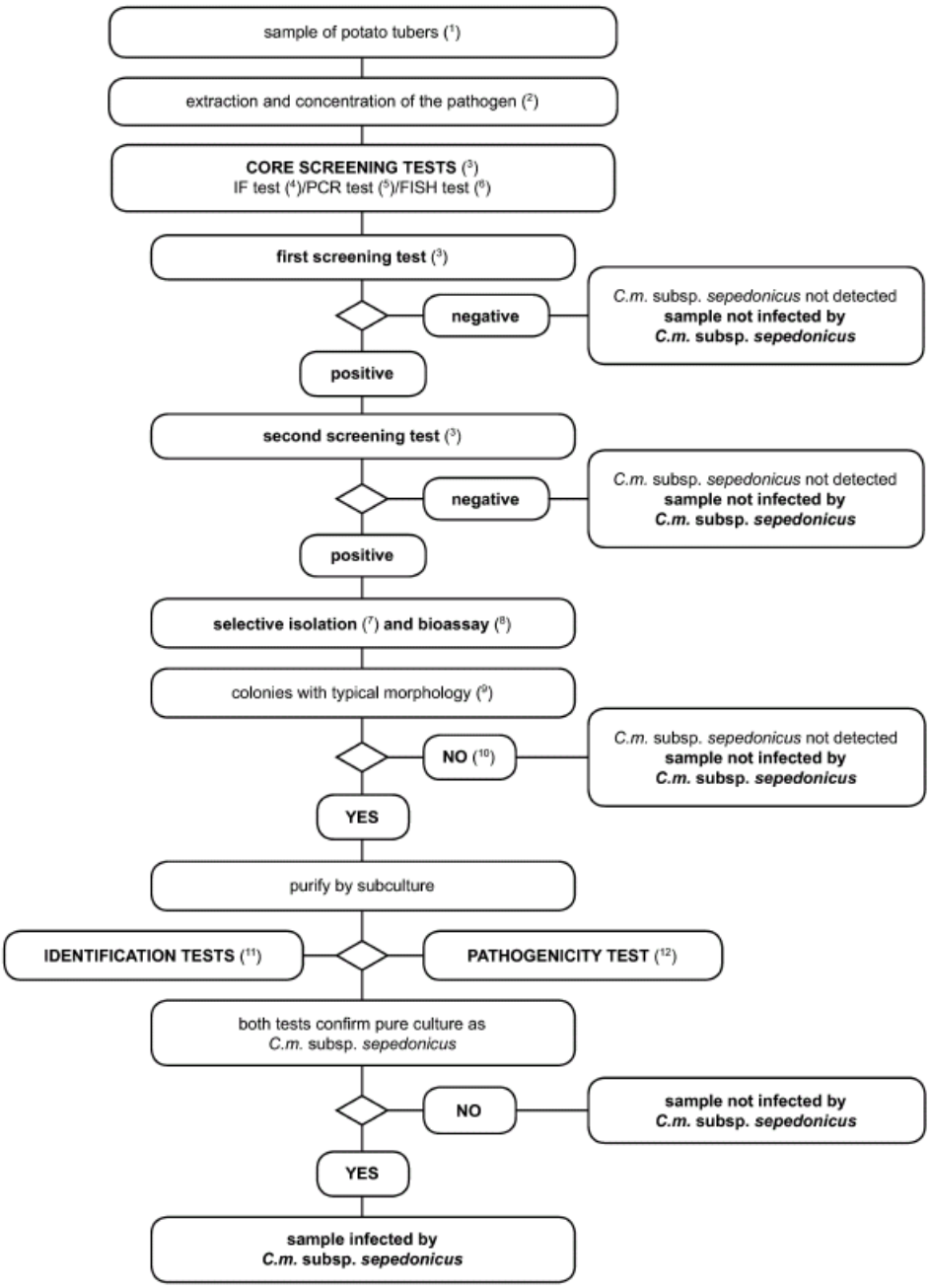
(7) The pathogenicity is described in section 10.

1.2. Scheme for detection and identification of *Clavibacter michiganensis* ssp. *sepedonicus* in samples of asymptomatic potato tubers

Principle

The testing procedure is intended for detection of latent infections in potato tubers. A positive result from at least two screening tests, based on different biological principles, must be complemented by the isolation of the pathogen; followed by, in case of isolation of typical colonies, confirmation of a pure culture as *C. m.* subsp. *sepedonicus*. A positive result from only one of the screening tests is not sufficient to consider the sample suspect.

Screening tests and isolation tests must permit a detection threshold of 103 to 104 cells/ml resuspended pellet, included as positive controls in each series of tests.



(1) The standard sample size is 200 tubers although the procedure can be used with smaller samples if 200 tubers are not available.

(2) Pathogen extraction and concentration methods are described in section 3.1.

(3) If at least two tests based on different biological principles are positive, isolation and confirmation have to be done. Perform at least one screening test. When this test is negative, the sample is considered to be negative in case this test is positive a second or more screening tests based on different biological principles are required to verify the first positive result. If the second ore other tests are negative the sample is considered negative. Further tests are not necessary.

(4) Immunofluorescence (IF) test.

Always use a polyclonal antibody for IF screening, additional monoclonal antibodies may provide more specifity (see section 4).

(5) PCR test.

Use appropriately validated PCR reagents and protocols (see section 6).

(6) Fish test.

Use validated reagents and protocols (see section 5).

(7) Selective isolation.

With the MTNA medium of NCP-88 medium and a 1/100 dilution of the resuspended pellet, this is in many cases a suitable method for direct isolation of *C. m*. subsp *sepedonicus*. Typical colonies can be obtained 3 to 10 days after planting. The pathogen then can be purified and identified. For full exploitation of its potential, the test requires careful preparation of heel end cores to avoid secondary bacteria associated with the potato tuber which are competitors with *C. m*. subsp *sepedonicus* on the medium and may overgrow the pathogen. If the plate test fails isolation must be done from plants used for the bio assay (see section 8).

(8) The bioassay test is used for isolation of C. m. subsp sepedonicus from potato extract pellets by selective enrichment in eggplants (*Solanum melongena*). The test requires optimal incubation conditions as specified in this method. Bacteria inhibitory to *C. m*. subsp *sepedonicus* on the MTNA or NCP-88 medium will most likely not interfere in this test (see section 7).

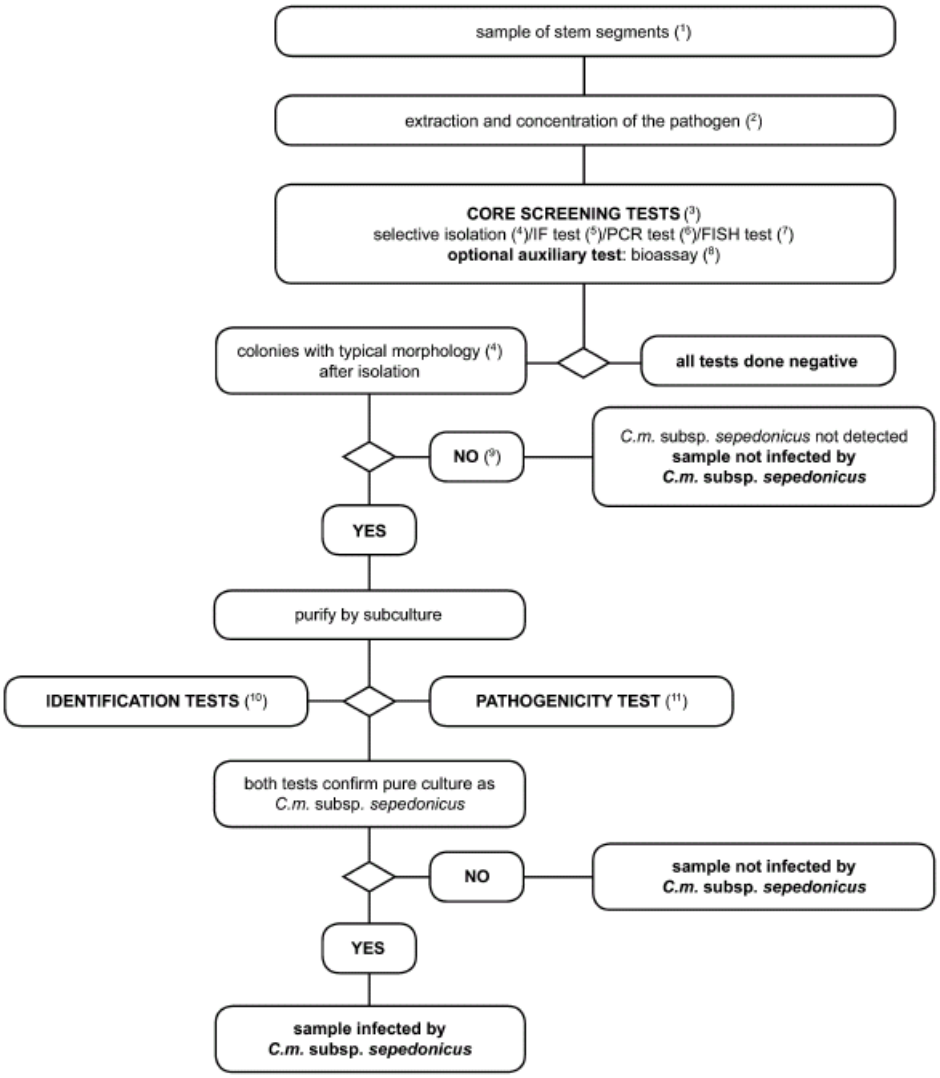
(9) Typical colony morphology is described in section 8.

(10) Culturing or bioassays can fail due to competition or inhibition by saprophytic bacteria. If positive results are obtained in screening tests, but the isolation tests are negative, then repeat the isolation tests from the same pellet or by taking additional vascular tissue near the heel end from cut tubers of the same sample and, if necessary, test additional samples.

(11) Reliable identification of pure presumptive *C. m*. subsp *sepedonicus* cultures is achieved using the tests described in section 9.

(12) The pathogenicity test is described in section 10.

1.3. Scheme for detection and identification of *Clavibacter michiganensis* ssp. *sepedonicus* in samples of asymptomatic potato plants



(1) See section 3.2 for recommended sample sizes.

(2) Pathogen extraction and concentration methods are described in section 3.2.

(3) If at least two tests based on different biological principles are positive, isolation and confirmation have to be done.

Perform at least one screening test. When this test is negative the sample is considered to be negative. In case this test is positive a second or more screening tests based on different biological principles are required to verify the first positive result. If the second or other tests are negative the sample is considered negative. Further tests are not necessary.

(4) The selective isolation test and typical colony morphology are described in section 8.

(5) The IF test is described in section 4.

(6) PCR tests are described in section 6.

(7) The FISH test is described in section 5.

(8) The bioassay is described in section 7.

(9) Culturing or bioassays can fail due to competition of inhibition by saprophytic bacteria. If positive results are obtained in screening tests but the isolation tests are negative, then repeat the isolation tests, and if necessary, test additional samples.

(10) Reliable identification of pure presumptive *C. m.* subsp *sepedonicus* cultures is achieved using the tests described in section 9.

(11) The pathogenicity test is described in section 10.

2. VISUAL EXAMINATION FOR RING ROT SYMPTOMS

2.1. Potato plants

Under European climatic conditions symptoms are rarely found in the field and often only at the end of the season. Moreover the symptoms are frequently masked or confused by/with other diseases, senescence or mechanical damages. Therefore it may be easy to miss symptoms in field inspections. Wilting symptoms are very different from those of brown rot; wilting is usually slow and initially limited to the leaf margins. Young infected leaves often continue to expand although, less so in the infected zones. This creates odd shaped leaves. Leaves affected by blocking of the vascular tissues further down the stem often develop chlorotic, yellow to orange, intercostal areas. Infected leaflets, leaves and even stems may eventually die. Often leaves and tubers are simply reduced in size. Occasionally plants are stunted. Coloured pictures of a range of symptoms can be found on the web site http://forum.europa.eu.int/Public/irc/sanco/Home/main

2.2. Potato tubers

The earliest symptoms are a slight glassiness or translucence of the tissue without softening around the vascular system, particularly near the heel end. The vascular ring at the heel end may be slightly darker in colour than normal. The first readily identifiable symptom is one whereby the vascular ring has a yellowish coloration and when the tuber is gently squeezed, pillars of cheese-like material emerge from the vessels. This exudation contains millions of bacteria. Browning of the vascular tissue may develop and tuber symptoms at this stage are similar to those of brown rot caused by *Ralstonia solanacearum*. At first, these symptoms may be restricted to one part of the ring, not necessarily close to the heel end and may gradually extend to the whole ring. As the infection progresses, destruction of the vascular tissue occurs; the outer cortex may become separated from the inner cortex. In advanced stages of infection, cracks appear on the surface of the tuber, which are often reddish-brown at their margins. Recently in Europe several cases have occurred where the central cortex rots at the same time as the vascular ring resulting in secondary invasion with internal hollowing and necrosis. Secondary fungal or bacterial invasion may mask the symptoms and it may be difficult, if not impossible, to distinguish advanced ring rot symptoms from other tuber rots. Atypical symptoms may be possible. Coloured pictures of a range of symptoms can be found on the web site http://forum.europa.eu.int/Public/irc/sanco/Home/

3. SAMPLE PREPARATION

3.1. Potato tubers

Note:

- The standard sample size is 200 tubers per test. More intensive sampling requires more tests on samples of this size. Larger numbers of tubers in the sample will lead to inhibition or difficult interpretation of the results. However, the procedure can be conveniently applied for samples with less than 200 tubers where fewer tubers are available.

- Validation of all detection methods described below is based on testing of samples of 200 tubers.

- The potato extract described below can also be used for detection of the potato brown rot bacterium, *Ralstonia solanacearum*.

Optional pre-treatment in advance to sample preparation:

Wash the tubers. Use appropriate disinfectants (chlorine compounds when PCR-test is to be used in order to remove eventual pathogen DNA) and detergents between each sample. Air dry the tubers. This washing procedure is particularly useful (but not required) for samples with excess soil and if a PCR-test or direct isolation procedure is to be performed.

3.1.1. Remove with a clean and disinfected scalpel or vegetable knife the skin at the heel end of each tuber so that the vascular tissue becomes visible. Carefully cut out a small core of vascular tissue at the heel end and keep the amount of non-vascular tissue to a minimum (see web site: http://forum.europa.eu.int/Public/irc/sanco/Home/main)

Note:

Set aside any tubers with suspected ring rot symptoms and test separately.

If during removal of the heel end core suspect symptoms of ring rot are observed, the tuber should be visually inspected after cutting near the heel end. Any cut tuber with suspected symptoms should be suberised at room temperature for two days and stored under quarantine (at 4 to 10°C) until all tests have been completed. All tubers in the sample (including those with suspicious symptoms) should be kept according to Schedule II.

3.1.2. Collect the heel end cores in unused disposable containers which can be closed and/or sealed (in case containers are reused they should be thoroughly cleaned and disinfected using chlorine compounds). Preferably, the heel end cores

should be processed immediately. If this is not possible, store them in the container, without addition of buffer, refrigerated for not longer than 72 hours or for not longer than 24 hours at room temperature. Drying and suberisation of cores, and growth of saprophytes during storage may hinder detection of the ring rot bacterium.

3.1.3. Process the heel end cores by one of the following procedures: either,

(a) cover the cores with sufficient volume (approximately 40 ml) of extraction buffer (Appendix 3) and agitate on a rotary shaker (50 to 100 rpm) for four hours below 24°C or for 16 to 24 hours refrigerated, or

(b) homogenise the cores with sufficient volume (approximately 40 ml) of extraction buffer (Appendix 3), either in a blender (e.g. Waring or Ultra Thurax) or by crushing in a sealed disposable maceration bag (e.g. Stomacher or Bioreba strong guage polythene, 150 mm × 250 mm; radiation sterilised) using a rubber mallet or suitable grinding apparatus (e.g. Homex).

Note:

The risk of cross-contamination of samples is high when samples are homogenized using a blender. Take precautions to avoid aerosol generation or spillage during the extraction process. Ensure that freshly sterilised blender blades and vessels are used for each sample. If the PCR test is to be used, avoid carry-over of DNA on containers or grinding apparatus. Crushing in disposable bags and use of disposable tubes is recommended where PCR is to be used.

3.1.4. Decant the supernatant. If excessively cloudy, clarify either by slow speed centrifugation (at not more than 180 g for 10 minutes at a temperature between 4 to 10°C) or by vacuum filtration (40 to 100 µm), washing the filter with additional (10 ml) extraction buffer (Appendix 3).

3.1.5. Concentrate the bacterial fraction by centrifugation at 7 000 g for 15 minutes (or 10 000 g for 10 minutes) at a temperature between 4 to 10°C and discard the supernatant without disturbing the pellet.

3.1.6. Resuspend the pellet in 1,5 ml pellet buffer (Appendix 3). Use 500 µl to test for *C. m.* subsp. *sepedonicus*, 500 µl for *Ralstonia solanacearum* and 500 µl for reference purposes. Add sterile glycerol to final concentration of 10 to 25% (v/v) to the 500 µl of the reference aliquot and to the remaining test aliquot, vortex and store at -16 to -24°C (weeks) or at -68 to -86°C (months). Preserve the test aliquots at 4 to 10°C during testing.

Repeated freezing and thawing is not advisable.

If transport of the extract is required, ensure delivery in a cool box within 24 to 48 hours.

3.1.7. It is imperative that all *C. m.* subsp. *sepedonicus* positive controls and samples are treated separately to avoid contamination. This applies to IF slides and to all tests.

3.2. Potato plants

Note:

For detection of latent *C. m.* subsp. *sepedonicus* populations it is advised to test composite samples. The procedure can be conveniently applied for composite samples of up to 200 stem parts. (Where surveys are performed they should be based on a statistically representative sample of the plant population under investigation.)

3.2.1. With a clean disinfected knife or pruning shears, remove a 1 to 2 cm segment from the base of each stem, just above the soil level.

Disinfect stem segments briefly with ethanol 70% and immediately blot dry on tissue paper.

Collect stem segments in a closed sterile container according to the following sampling procedures:

3.2.2. Process the stem segments by one of the following procedures: either,

(a) cover the segments with sufficient volume (approximately 40 ml) of extraction buffer (Appendix 3) and agitate on a rotary shaker (50 to 100 rpm) for four hours below 24°C or for 16 to 24 hours refrigerated, or

(b) process immediately. By crushing the segments in a strong maceration bag (e.g. Stomacher or Bioreba) with an appropriate volume of extraction buffer (Appendix 3) using a rubber mallet or appropriate grinding apparatus (e.g. Homex). If this is not possible, store the stem segments refrigerated for not longer than 72 hours or for not longer than 24 hours at room temperature.

3.2.3. Decant the supernatant after settling for 15 minutes.

3.2.4. Further clarification of the extract or concentration of the bacterial fraction are not usually required but may be achieved by filtration and/or centrifugation as described in section 3.1.4 to 3.1.6.

3.2.5. Divide the neat or concentrated sample extract into 2 equal parts. Maintain one half at 4 to 10°C during testing and store the other half with 10 to 25% (v/v) sterile glycerol at -16 to -24°C (weeks) or at -68 to -86°C (months) in case further testing is required.

4. 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 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:

Always use a polyclonal antibody, when the IF test is used as the principal screening test. In case of a positive IF reading with a polyclonal antibody further screening of the sample with a monoclonal antibody may provide more specificity but can be less sensitive.

Use antibodies to a reference strain of *C. m.* subsp. *sepedonicus*. 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 105 to 106 cells per ml of the homologous strain of *C. m.* subsp. *sepedonicus* and using an appropriate fluorescein isothiocyanate (FITC) conjugate according to the manufacturer's recommendations. The crude polyclonal or monoclonal antibodies should have an IF titre of at least 1:2000. During testing, the antibodies should be used at a working dilution(s) (WD) close to or at the titre. Use validated antibodies (see web site http://forum.europa.eu.int/Public/irc/sanco/Home/main).

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 resuspension in an equal volume of pellet buffer. This is often not necessary, especially if slides samples are fixed to the slides by flaming (see 2.2).

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, and optionally in buffer.

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

As negative controls, use aliquots of sample extract which previously tested negative.

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).

4.1. 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 and 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.

4.2. Dry the droplets at ambient temperatures or by warming at temperature 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 3 months) prior to further testing.

4.3. IF procedure:

(i) According to test slide preparation in 4.1(i):

Prepare a set of twofold dilutions of the antibody in IF buffer. 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 4.1(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 4.1(i) and 4.3(i)

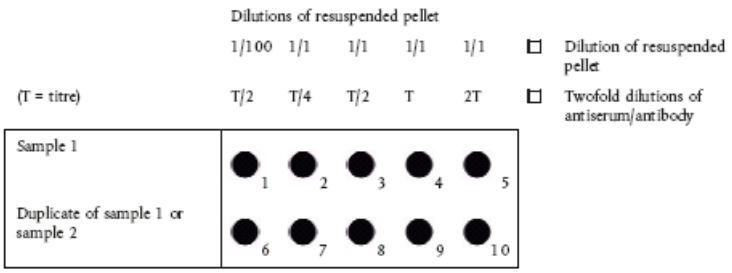
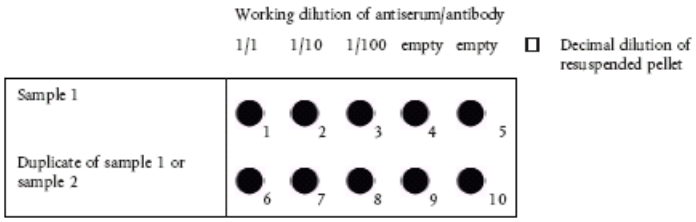


Figure 2. Preparation of the test slide according to 4.1(ii) and 4.3(ii)



4.3.1. Arrange the slides on moist 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:

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

4.3.3. Shake the droplets off each slide and rinse carefully with IF buffer. Wash by submerging for 5 minutes in IF buffer-Tween (Appendix 3) and subsequently for 5 minutes in IF buffer. Avoid causing aerosols or droplet transfer that could result in cross-contamination. Carefully remove excess moisture by blotting gently.

4.3.4. 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.

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

4.3.6. Shake the droplets of conjugate off the slide. Rinse and wash as before (4.3.3).

Carefully remove excess moisture.

4.3.7. Pipette 5 to 10 µl of 0,1M phosphate-buffered glycerol (Appendix 3) or a commercially antifading mountant on each window and apply a coverslip.

4.4. Reading the IF test:

4.4.1. Examine test slides on an epifluorescence microscope with filters suitable for excitation of FITC, under oil or water immersion at a magnification of 500 to 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 4) must be repeated if the staining is aberrant.

4.4.2. 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 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.

4.4.3. 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 *C. m. sepedonicus* are likely to occur in potato heel end core and stem segment pellets.

4.4.4. Consider only fluorescing cells with typical size and morphology at the titre or working dilution of the antibodies as in 4.3.

4.4.5. 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 4).

The IF reading is positive for samples with at least 5 × 103 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 × 103 cells per ml resuspended pellet and the sample is considered negative. Further testing is not

required.

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 103 to 104 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 105 to 106 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

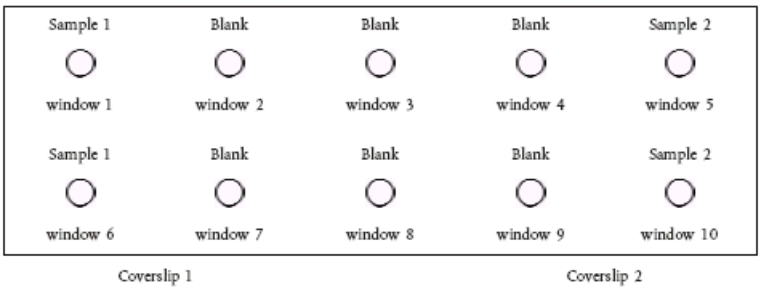
prepared < 24 hours previously. Vortex and incubate overnight at 4°C.

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



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 hybridization

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 400 µg 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 × 103 typical cells per ml of resuspended pellet are considered potentially contaminated. Further testing is required. Samples with less than 5 × 103 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.

6. PCR TEST

Principles

When the PCR test is used as the principal screening test and found to be positive, the IF test must be performed as a second compulsory screening test. When the PCR 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.

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 103 to 104 cells of *C. m*. subsp. *sepedonicus* 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. 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 *C. m*. subsp. *Sepedonicus*,

- 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 *C. m*. subsp. *sepedonicus* has been added (preparation see Appendix 2),

- a suspension of 106 cells per ml of *C. m*. subsp. *sepedonicus* in water from a virulent isolate (e.g. NCPPB 2140 or NCPPB 4053),

- if possible also use 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 advisible to prepare extractions from washed potatoes if PCR protocols are to be used.

6.1. DNA purification methods

Use positive and negative control samples as described above.

Prepare 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 method shown in Appendix 6.

6.1.(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 waterbath at 95°C for 10 minutes.

3. Put tube on ice for five minutes.

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 minutes.

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

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 minutes 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 minutes.

11. Centrifuge at 15 000 g for 20 minutes 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 minutes 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.

6.1.(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 103 to 104 pathogen cells per ml.

6.2. PCR

6.2.1. Prepare test and control templates for PCR according to the validated protocol (Appendix 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 protocol (Appendix 6). The validated PCR protocol is a multiplex reaction that also incorporates an internal PCR control.

6.2.3. Add 5 µl of DNA extract per 25 µl PCR reaction in sterile PCR tubes.

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 optimized 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 45 min taking appropriate precautions for handling this mutagen.

6.3.3. Observe stained gel under short wave UV transillumination (e.g. λ = 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 *C. m*. subsp. *sepedonicus*-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 *C. m*. subsp. *sepedonicus*-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 (section 9.3).

Note:

Inhibition of the PCR may be suspected if the expected amplicon is obtained from the positive control sample containing *C. m.* subsp. *sepedonicus* in water but negative results are obtained from positive controls with *C. m*. subsp. *sepedonicus* 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. BIOASSAY TEST

Note:

Preliminary testing with this method should permit reproducible detection of 103 to 104 colony-forming units of *C. m*. subsp. *sepedonicus* per ml added to sample extracts that previously tested negative (preparation see Appendix 2).

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.

Some varieties of eggplant provide an excellent selective enrichment medium for the growth of *C. m.* subsp. *Sepedonicus* even in the absence of symptoms and also provide an excellent confirmatory host test.

Growth conditions should be optimal to reduce the risk of false negative test results.

For cultural details, see Appendix 8.

7.1. Distribute the whole of the remaining test aliquot of the resuspended pellet from section 3.1.6 or 3.2.5 between eggplants by one of the methods given below (7.3 or 7.4). Use only plants at leaf stage two to three up to full expansion of the third true leaf. In order to ensure complete utilisation of the resuspended pellet as well as effective inoculation the procedures outlined below will require 15 to 25 eggplants per sample.

7.2. Do not water eggplants for one to two days prior to inoculation to reduce turgor pressure.

7.3. Slit inoculation

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

7.3.2. 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.

7.3.3. Seal the cut with sterile vaseline from a syringe.

7.4. Syringe inoculation

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

7.5. As the positive controls, inoculate 5 plants with an aqueous suspension of 105 to 106 cells per ml of a known culture of *C. m.* subsp. *sepedonicus* and, where possible, with naturally infected tuber tissue (see section 4) by the same inoculation method (7.3 or 7.4).

7.6. As the negative control, inoculate 5 plants with sterile pellet buffer by the same inoculation method (7.3 or 7.4).

7.7. Incubate plants in quarantine facilities for up to four weeks at 18 to

24°C. Incubate plants with sufficient light and high humidity (70 to 80%) and water to prevent water logging or wilting through water deficiency. *C. m. sepedonicus* cells are killed at temperatures above 30°C and the optimum temperature is 21°C. 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.

7.8. Examine regularly for symptoms starting after a week. Count the number of plants showing symptoms. *C. m*. subsp. *sepedonicus* causes leaf wilting in eggplants which may commence as a marginal or interveinal flaccidity. Wilted tissue may initially appear dark green or mottled but turns paler before becoming necrotic. Interveinal wilts often have a greasy water-soaked appearance. Necrotic tissue often has a bright yellow margin. Plants are not necessarily killed; the longer the period before symptoms develop, the greater the chance of survival. Plants may outgrow the infection. Young eggplants are much more susceptible to low populations of *C. m*. subsp. *Sepedonicus* than are older plants, hence the necessity to use plants at or just before leaf stage 3.

Wilts may also be induced by populations of other bacteria or fungi present in the tuber tissue pellet. These include *Ralstonia solanacearum*, *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica*, *Erwinia chrysanthemi*, *Phoma exigua* var. *foveata*, as well as large populations of saprophytic bacteria. In particular *Erwinia chrysanthemi* can cause leaf symptoms and wilt that is very similar to symptoms of *C. m. sepedonicus*. The only difference is blackening of the stems in case in *Erwinia chrysanthemi* infections. Other wilts can be distinguished from those caused by *C. m.* subsp. *sepedonicus* since whole leaves or whole plants wilt rapidly. Also a Gram stain can be prepared: this test will differentiate *C. m*. subsp. *sepedonicus* from *Erwinia* spp.

7.9. As soon as symptoms in eggplants are observed reisolation should be performed, using sections of wilted leaf tissue or stem tissue from plants (see 3.1.3 for tissue maceration). Surface disinfect the eggplant leaves and stems by wiping with 70% ethanol. Perform an IF test or PCR on the eggplant sap and isolate on to suitable (selective) media (see section 8). A Gram stain (Appendix 9) may also be prepared. Identify purified cultures of presumptive *C. m*. subsp. *sepedonicus* and confirm pathogenicity (see section 9 and 10).

7.10. Under certain circumstances, in particular where growing conditions are not optimal, it may be possible for *C. m*. subsp. *sepedonicus* to exist as a latent infection within eggplants even after incubation periods up to 4 weeks. If no symptoms are observed after 4 weeks perform IF/PCR on a composite sample of 1 cm stem sections of each test plant taken above the inoculation site. If the test is

positive reisolation on suitable (selective) media should be performed following the procedure under section 8. Identify purified cultures of presumptive *C. m*. subsp. *sepedonicus* and confirm pathogenicity (section 9 and 10).

Interpretation of the bioassay test result.

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 *C. m*. subsp. *sepedonicus*, and provided that *C. m*. subsp. *sepedonicus* is detected in positive controls.

The bioassay test is positive if the test plants are infected by *C. m*. subsp. *sepedonicus*.

8. ISOLATION OF *C. M*. SUBSP. *SEPEDONICUS*

Note:

Diagnosis is only completed if *C. m*. subsp. *sepedonicus* is isolated, subsequently identified (see section 9). and confirmed by a pathogenicity test (section 10). Although *C. m*. subsp. *sepedonicus* is a fastidious organism, it can be isolated from symptomatic tissue.

However, it may be outgrown by rapidly growing saprophytic bacteria and, therefore, isolations directly from the tuber or stem tissue pellet (section 3.1.6 or 3.2.5) is difficult. With selective medium and appropriate dilution of the resuspended pellet from the heel end cores or stems of potatoes direct isolation of *C. m*. subsp. *sepedonicus* may be possible.

Isolations shall be made from all symptomatic potato tubers or stem segments and from eggplants were no symptoms are observed but IF/PCR test from the composite sample was positive (see section 7.10). Maceration of eggplants stems when necessary should be carried out as described in section 3.1.3.

As positive controls, prepare decimal dilutions from a suspension of 10^6 cfu per ml of *C. m*. subsp. *sepedonicus* (e.g. NCPPB 4053 or PD 406). To avoid any possibility of contamination, prepare positive controls totally separated 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).

8.1. Selective plating

8.1.1. From a 100 µl aliquot from a resuspended potato pellet sample or eggplant sap make 10-fold dilutions in pellet buffer (Appendix 3).

8.1.2. Isolation from undiluted potato pellet usually fails due to the fastidious growth habit of Cms and competition by saprophytes. Since the bacterium is usually present in high populations in infected tissues, the saprophytes can usually be diluted out, whilst the pathogen remains. It is therefore recommended to spread 100 µl from each of the samples, 1/100 up to 1/10 000 dilutions onto MTNA medium or NCP-88 medium (Appendix 5) (if using 90 mm diameter petri dishes- adjust volume for alternative dish sizes), using spreaders (hockey sticks) and the spread plate technique.

Note:

An alternative strategy is to spread out the initial 100 µl potato pellet aliquot onto a first agar plate with a spreader and then remove the spreader to a second agar plate, streaking out any residue left on the spreader; finally repeat this with a third plate, thus giving a dilution plating effect via the spreader.

8.1.3. Incubate plates in the dark at 21 to 23°C.

8.1.4. Initial examinations of the plates including, by reference to the control plates, counts of any *C. m.* subsp. *sepedonicus* like colonies are made after 3 days, with further counts after 5, 7, eventually 10 days.

8.2. Purification of suspicious colonies

Note:

Subculturing of *C. m.* subsp. *sepedonicus*-like colonies should be carried out onto YGM media for eggplant inoculation and/or subsequent identification; this should be done before the plates become too overgrown i.e. preferably after three to five days.

8.2.1. Streak *C. m.* subsp. *sepedonicus*-like colonies on to one of the following media: (formulae are given in Appendix 5):

nutrient dextrose agar (for use in subculturing only),

yeast peptone glucose agar,

yeast extract mineral salts agar.

Incubate at 21°C to 24°C for up to 10 days.

C. m. subsp. *sepedonicus* is slow-growing, usually producing pin-point,

cream, domed colonies within 10 days. Photos of typical colonies of *C. m.* subsp. *sepedonicus* (see web site http://forum.europa.eu.int/Public/irc/sanco/Home/main).

8.2.2. Re-streak to establish purity.

Growth rates are improved with subculture. Typical colonies are creamy-white or ivory, occasionally yellow, rounded, smooth, raised, convex-domed, mucoid-fluidal, with entire edges and usually 1 to 3 mm in diameter.

A simple Gram stain (Appendix 9) may help to select colonies for further testing.

8.2.3. Identify presumptive cultures (see section 9) and perform a pathogenicity test (see section 10).

9. IDENTIFICATION

Identify pure cultures of presumptive *C. m.* subsp. *sepedonicus* isolates using at least two of the following tests based on different biological principles.

Include known reference strains where appropriate for each test performed.

9.1. Nutritional and enzymatic identification tests

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

All media should be incubated at 21°C and examined after six days. If no growth has occurred, incubate for up to 20 days.

All tests must include a known *C. m.* subsp. *sepedonicus* control. Nutritional and physiological tests must be made using inocula from nutrient agar subcultures. Morphological comparisons must be made from nutrient dextrose agar cultures.

Tests	Expected result
Oxidation/Fermentation (O/F) test	Inert or weakly oxidative
Oxidase activity	-
Growth at 37°C	-
Urease activity	-
Aesculin hydrolysis	+
Starch hydrolysis	- or weak

Tolerance of 7% NaCl	-
Indole production	-
Catalase activity	+
H_2S production	-
Citrate utilization	-
Gelatin liquefaction	-
Acid glycerol	-
Acid from lactose	- or weak
Acid from rhamnose	-
Acid from salicin	-
Gram stain (Appendix 9)	+

9.2. IF-test

(a) Prepare a suspension of approximately 106 cells per ml in IF buffer (Appendix 3).

(b) Prepare a 2-fold dilution series of an appropriate antiserum.

(c) Apply the IF procedure (section 4).

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

9.3. PCR test

(a) Prepare a suspension of approximately 106 cells per ml in ultra pure water (UPW).

(b) Heat 100 µl of the cell suspension in closed tubes in a heating block or boiling waterbath at 100°C for four minutes. If required, addition of freshly-prepared NaOH to a final concentration of 0,05M may assist cell lysis. The samples may then be stored at -16 to -24°C until required.

(c) Apply appropriate PCR procedures to amplify *C. m.* subsp. *sepedonicus* specific amplicons (e. g. Pastrik, 2000; see Appendix 4; Li and de Boer, 1995; Mills et al., 1997; Pastrik and Rainey, 1999; Schaad et al., 1999.

(d) A positive identification of *C. m*. subsp. *sepedonicus* is achieved if the PCR amplicons are the same size and have the same restriction fragment length polymorphisms as for the positive control strain.

9.4. FISH test

(a) Prepare a suspension of approximately 106 cells per ml in UPW.

(b) Apply the FISH procedure (section 5).

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

9.5. Fatty acid profiling (FAP)

(a) Grow the culture on trypticase soy agar (Oxoid) for 72 hours at 21°C (+/– 1°).

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

(c) 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 15:1 Anteiso A, 15:0 Iso, 15:0 Anteiso, 16:0 Iso, 16:0 and 17:0 Anteiso is highly indicative of *C. m. sepedonicus*. Other genera such as *Curtobacterium*, *Arthrobacter* and *Micrococcus* also have some of these acids but 15:1 Anteiso A is a rare acid in these bacteria but occurs in all *Clavibacter* spp. at between 1 to 5%. In *C. m. sepedonicus* the value is usually around 5%.

9.6. BOX-PCR

(a) Prepare a suspension of approximately 106 cells per ml in UPW.

(b) Apply the test according the procedure (Smith et al., 2001).

10. CONFIRMATION TEST

The pathogenicity test must be performed as final confirmation of a diagnosis of *C. m.* subsp. *sepedonicus* and for assessment of virulence of cultures identified as *C. m*. subsp. *sepedonicus*:

10.1. Prepare an inoculum of approximately 106 cells per ml from three day cultures of the isolate to be tested and an appropriate positive control strain of *C. m*. subsp. *sepedonicus*.

10.2. Inoculate 5 to 10 eggplant stems of young seedlings at leaf stage 3 (section 7.3 or 7.4).

10.3. Incubate at 18 to 24°C with sufficient light and high relative humidity with appropriate watering to avoid waterlogging or drought stress (section 7.7). With pure cultures, typical wilting should be obtained within two weeks but plants not showing symptoms (see section 7.8) after this time should be incubated up to three weeks at temperatures conducive to eggplant growth but not exceeding 25°C (Appendix 8). If after three weeks symptoms are not present, the culture cannot be confirmed as being a pathogenic form of *C. m.* subsp. *sepedonicus*.

10.4. Isolate from symptomatic plants by removing a section of stem 2 cm above the inoculation point. Comminute and suspend in a small volume of sterile distilled water or 50 mM phosphate buffer (Appendix 3). Isolate from the suspension by dilution spreading or streaking onto MTNA and YPGA (Appendix 5), incubate for three to five days at 21 to 23°C and observe the formation of colonies typical of *C. m*. subsp. *sepedonicus*.

Appendix 1

Laboratories involved in optimisation and validation of protocols

Laboratory ([1])	Location	Country
Agentur für Gesundheit und Ernährungssicherheit	Vienna and Linz	Austria
Departement Gewasbescherming	Merelbeke	Belgium
Plantedirektoratet	Lyngby	Denmark
Central Science Laboratory	York	England
Scottish Agricultural Science Agency	Edinburgh	Scotland
Laboratoire National de la Protection des Végétaux, Unité de Bactériologie	Angers	France
Laboratoire National de la Protection des Végétaux, Station de Quarantaine de la Pomme de Terre	Le Rheu	France
Biologische Bundesanstalt	Kleinmachnow	Germany
Pflanzenschutzamt Hannover	Hannover	Germany
State Laboratory	Dublin	Ireland
Plantenziektenkundige Dienst	Wageningen	Netherlands
Norwegian Crop Research Institute, Plant Protection Centre	Aas	Norway
Direcção-Geral de Protecção das Culturas	Lisbon	Portugal
Nacionalni institut za biologijo	Ljubljana	Slovenia
Centro de Diagnostico de Aldearrubia	Salamanca	Spain

([1]) Contact scientists: see website http://forum.europa.eu.int/Public/irc/sanco/Home/main

---

Appendix 2

Preparation of positive and negative controls for the core screening tests PCR/IF and FISH

Produce a 72 hour culture of a virulent strain of *C. m.* subsp. *sepedonicus* (NCPPB 4053 or PD 406) on MTNA basal medium and suspend in 10 mM phosphate buffer to obtain a cell density of approximately 1 to 2 × 108 cfu per ml. This is usually obtained by a faintly turbid suspension equivalent to an optical density of 0,20 at 600 nm.

Remove the heel end cores of 200 tubers taken from a white skin variety production known to be free from *C. m.* subsp. *sepedonicus*.

Process the heel ends as usual and resuspend the pellet in 10 ml.

Prepare 10 sterile 1,5 ml microvials with 900 µl of the resuspended pellet.

Transfer 100 µl of the suspension of *C. m.* subsp. *sepedonicus* to the first microvial. Vortex.

Establish decimal levels of contamination by further diluting in the next five microvials.

The six contaminated microvials will be used as positive controls. The four non-contaminated microvials will be used as negative controls. Label the microvials accordingly.

Prepare aliquots of 100 µl in sterile 1,5 ml microvials thus obtaining nine replicas of each control sample. Store at -16 to -24°C until use.

The presence and quantification of *C. m.* subsp. *sepedonicus* in the control samples should be first confirmed by IF.

For the PCR test perform DNA extraction from positive and negative control samples with each series of test samples.

For IF and FISH tests perform assays on positive and negative control samples with each series of test samples.

For IF, FISH and PCR assays *C. m.* subsp. *sepedonicus* must be detected in at least the 106 and 104 cells/ml of the positive controls and not in any of the negative controls.

Appendix 3

Buffers for test procedures

GENERAL: Unopened sterilized buffers can be stored for up to one year.

1. Buffers for extraction procedure

1.1. Extraction buffer (50 mM phosphate buffer, pH 7,0)

This buffer is used for extraction of the bacterium from plant tissues by homogenisation or shaking.

Na_2HPO_4 (anhydrous)	4,26 g
KH_2PO_4	2,72 g
Distilled water	1.00 L

Dissolve ingredients, check pH and sterilise by autoclaving at 121°C for 15 min.

Additional components may be useful as follows:

	Purpose	Quantity (per L)
Lubrol flakes	Deflocculant (*)	0,5 g
DC silicone antifoam	Anti-foam agent (*)	1,0 ml
Tetrasodium pyrophosphate	Anti-oxidant	1,0 g
Polyvinylpyrrolidone-40 000 (PVP-40)	Binding of PCR inhibitors	50 g

(*) For use with homogenisation extraction method.

1.2. Pellet buffer (10 mM phosphate buffer, pH 7,2)

This buffer is used for resuspension and dilution of potato tuber heel-end core extracts following concentration to a pellet by centrifugation.

$Na_2HPO_4.12H_2O$	2,7 g
$NaH_2PO_4.2H_2O$	0,4 g
Distilled water	1,00 L

Dissolve ingredients, check pH and sterilise by autoclaving at 121°C for 15 min.

2. Buffers for the IF test

2.1. IF-Buffer (10 mM phosphate buffered saline (PBS), pH 7,2)

This buffer is used for dilution of antibodies.

$Na_2HPO_4.12H_2O$	2,7 g
$NaH_2PO_4.2H_2O$	0,4 g
NaCl	8,0 g
Distilled water	1.0 L

Dissolve ingredients, check pH and sterilise by autoclaving at 121°C for 15 min.

2.2. IF-buffer-Tween

This buffer is used to wash slides.

Add 0,1% Tween 20 to the IF buffer.

2.3. Phosphate buffered glycerol, pH 7,6

This buffer is used as a mountant fluid on the windows of IF slides to enhance fluorescence.

$Na_2HPO_4.12H_2O$	3,2 g
$NaH_2PO_4.2H_2O$	0,15 g
Glycerol	50 ml
Distilled water	100 ml

Anti-fading mountant solutions are commercially available e.g. Vectashield® (Vector Laboratories) or Citifluor® (Leica).

Appendix 4

Determination of contamination level in IF and FISH tests

1. Count the mean number of typical fluorescent cells per field of view (c)

2. Calculate the number of typical fluorescent cells per microscope slide window (C)

$C = c \times S/s$

Where S = surface area of window of multispot slide, and

s = surface area of objective field.

$s = \pi i^2/4G^2K^2$ where i = field coefficient (varies from 8 to 24 depending upon ocular type)

K = tube coefficient (1 or 1,25)

G = magnification of objective (100x, 40x etc.).

3. Calculate the number of typical fluorescent cells per ml of resuspended pellet (N)

$N = C \times 1\,000/y \times F$

where y = volume of resuspended pellet on each window, and

F = dilution factor of resuspended pellet.

Appendix 5

Media for isolation and culture of *C. m.* subsp. *sepedonicus*

(a) General growth media

Nutrient agar (NA)

Nutrient Agar (Difco)	23,0 g
Distilled water	1,0 L

Dissolve ingredients and sterilise by autoclaving at 121°C for 15 min.

Nutrient dextrose agar (NDA)

Difco bacto nutrient agar containing 1% D(+) glucose (monohydrate). Sterilize by autoclaving at 115°C for 20 minutes.

Yeast peptone glucose agar (YPGA)

Yeast Extract (Difco)	5,0 g
Bacto-Peptone (Difco)	5,0 g
D(+) Glucose (monohydrate)	10,0 g

Bacto-Agar (Difco)	15,0 g
Distilled water	1,0 L

Dissolve ingredients and sterilise by autoclaving at 121°C for 15 min.

Yeast extract mineral salts medium (YGM)

Bacto-Yeast-Extract (Difco)	2,0 g
D(+) Glucose (monohydrate)	2,5 g
K_2HPO_4	0,25 g
KH_2PO_4	0,25 g
$MgSO_4.7H_2O$	0,1 g
$MnSO_4.H_2O$	0,015 g
NaCl	0,05 g
$FeSO_4.7H_2O$	0,005 g
Bacto-Agar (Difco)	18 g
Distilled water	1,0 L

Dissolve ingredients and sterilize 0,5 litre volumes of medium by autoclaving at 115°C for 20 minutes.

(b) Validated selective growth media

MTNA medium

Unless otherwise stated all media components are from BDH.

Yeast extract (Difco)	2,0 g
Mannitol	2,5 g
K_2HPO_4	0,25 g
KH_2PO_4	0,25 g
NaCl	0,05 g
$MgSO_4.7H_2O$	0,1 g
$MnSO_4.H_2O$	0,015 g

$FeSO_4.7H_2O$	0,005 g
Agar (Oxoid no. 1)	16,0 g
Distilled water	1,0 L

Dissolve ingredients, adjust pH to 7,2. After autoclaving (at 121°C for 15 minutes) and cooling down to 50°C, add the antibiotics: trimethoprim 0,06 g, nalidixic acid 0,002 g, amphotericin B 0,01 g.

Stock antibiotic solutions: trimethoprim (Sigma) and nalidixic acid (Sigma) (both at 5 mg/ml), in 96 % methanol, amphotericin B (Sigma) (1 mg/ml) in dimethyl sulfoxide. Stock solutions are filter-sterilized.

Note:

Durability of basal medium is three months. After antibiotics are added durability is one month when stored refrigerated.

NCP-88 medium

Nutrient agar (Difco)	23 g
Yeast extract (Difco)	2 g
D-mannitol	5 g
K_2HPO_4	2 g
KH_2PO_4	0,5 g
$MgSO_4.7H_2O$	0,25 g
Distilled water	1,0 L

Dissolve ingredients, adjust pH to 7,2. After autoclaving and cooling down to 50°C, add the following antibiotics:

Polymyxin B sulphate (Sigma) 0,003 g, nalidixic acid (Sigma) 0,008 g, Cycloheximide (Sigma) 0,2 g.

Dissolve antibiotics in stock solutions as follows: nalidixic acid in 0,01 M NaOH, cycloheximide in 50% ethanol, polymyxin B sulphate in distilled water. Stock solutions are filter-sterilized.

Note:

Durability of basal medium is three months. After antibiotics are added durability is one month when stored refrigerated.

Appendix 6

Validated PCR protocol and reagents

Note:

Preliminary testing should permit reproducible detection of at least 103 to 104 cells of *C. m. sepedonicus* per ml of sample extract.

Preliminary testing should also show no false positive results with a panel of selected bacterial strains.

1. Multiplex PCR protocol with internal PCR control (Pastrik, 2000)

1.1. Oligonucleotide primers

Forward primer PSA-1	5'- ctc ctt gtg ggg tgg gaa aa -3'
Reverse primer PSA-R	5'- tac tga gat gtt tca ctt ccc c -3'
Forward primer NS-7-F	5'- gag gca ata aca ggt ctg tga tgc -3'
Reverse Primer NS-8-R	5’- tcc gca ggt tca cct acg ga -3’

Expected amplicon size from C. m. subsp. sepedonicus template DNA = 502 bp (PSA-primer set).

Expected amplicon size from the 18S rRNA internal PCR control = 377 bp (NS-primer set).

1.2. PCR reaction mix

Reagent	Quantity per reaction	Final concentration
Sterile UPW	15,725 µl	
10x PCR buffer ([1]) (15 mM $MgCl_2$)	2,5 µl	1x (1,5 mM $MgCl_2$)
BSA (fraction V) (10%)	0,25 µl	0,1%
d-nTP mix (20 mM)	0,125 µl	0,1 mM
Primer PSA-1 (10 µM)	0,5 µl	0,2 µM
Primer PSA-R (10 µM)	0,5 µl	0,2 µM
Primer NS-7-F (10 µM) ([2])	0,1 µl	0,04 µM
Primer NS-8-R (10 µM) ([2])	0,1 µl	0,04 µM
Taq polymerase (5 U/µl) ([1])	0,2 µl	1,0 U
Sample volume	5,0 µl	
Total volume	25,0 µl	

(1) Methods were validated using Taq polymerase from Perkin Elmer (AmpliTaq or Gold) and Gibco BRL.

(2) Concentration of primers NS-7 F and NS-8-R were optimised for potato heel end core extraction using the homogenization method and DNA purification according to Pastrik (2000) (see section 6.1.a) and 6.2). Re-optimisation of reagent concentrations will be required if extraction by shaking or other DNA isolation methods are used.

1.3. PCR reaction conditions

Run the following programme:

1 cycle of:	(i)	3 minutes at 95°C (denaturation of template DNA)
10 cycles of:	(ii)	1 minute at 95°C (denaturation of template DNA)
	(iii)	1 minute at 64°C (annealing of primers)
	(iv)	1 minute at 72°C (extension of copy)
25 cycles of:	(v)	30 seconds at 95°C (denaturation of template DNA)
	(vi)	30 seconds at 62°C (annealing of primers)
	(vii)	1 minute at 72°C (extension of copy)
1 cycle of:	(viii)	5 minutes at 72°C (final extension)
	(ix)	hold at 4°C.

Note:

This programme is optimised for use with an MJ Research PTC 200 thermal cycler. Modification of the duration steps of cycles (ii), (iii) (iv), (v), (vi) and (vii) may be required for use with other models.

1.4. Restriction enzyme analysis of amplicon.

PCR products amplified from *C. m.* subsp. *sepedonicus* DNA produce a distinctive restriction fragment length polymorphism with enzyme Bgl II after incubation at 37°C for 30 minutes. The restriction fragments obtained from *C. m.* subsp. *sepedonicus*-specific fragment are 282 bp and 220 bp in size.

2. Preparation of the Loading buffer

2.1. Bromphenol blue (10%-stock solution)

Bromphenol blue	5 g
Distilled Water (bidest)	50 ml

2.2. Loading buffer

Glycerol (86%)	3,5 ml
Bromphenol blue (5.1)	300 µl
Distilled Water (bidest)	6,2 ml

3. 10x Tris Acetate EDTA (TAE) buffer, pH 8,0

Tris buffer	48,4 g
Glacial acetic acid	11,42 ml
EDTA (disodium salt)	3,72 g
Distilled water	1,00 L

Dilute to 1x before use.

Also commercially available (e.g. Invitrogen or equivalent).

Appendix 7

Validated reagents for FISH test

1. Oligo-probes

Cms-specific probe CMS-CY3-01: 5'- ttg cgg ggc gca cat ctc tgc acg -3'

Non-specific eubacterial probe EUB-338-FITC: 5'- gct gcc tcc cgt agg agt -3'

2. Fixative solution

[*WARNING! THE FIXATIVE CONTAINS PARAFORMALDEHYDE WHICH IS TOXIC. WEAR GLOVES AND DO NOT INHALE. IT IS ADVISABLE TO WORK IN A FUME CUPBOARD*]

(i) Heat nine ml molecular grade water (e.g. Ultra pure water (UPW)) to about 60°C and add 0,4 g paraformaldehyde. Paraformaldehyde dissolves after adding five drops of 1N NaOH and stirring with a magnetic stirrer.

(ii) Adjust pH to 7,0 by addition of 1ml of 0,1 M phosphate buffer (PB; pH 7,0) and five drops of 1 N HCl. Check pH with indicator strips and adjust if necessary with HCl or NaOH.

[*WARNING! DO NOT USE A PH METER IN SOLUTIONS WITH PARAFORMALEDHYDE*]

(iii) Filter the solution through a 0,22 µm membrane filter and keep dust-free at 4°C until further use.

(iv) Note:

Alternative fixative solution: 96% ethanol.

3. 3x Hybmix

NaCl	2,7 M
Tris-HCl	60 mM (pH 7,4)
EDTA (filter sterilised and autoclaved)	15 mM

Dilute to 1x as required.

4. Hybridisation solution

1x Hybmix

Sodium dodecyl sulphate (SDS)	0,01%
probe EUB 338	5 ng/µl
probe CMSCY301	5 ng/µl

Prepare quantities of hybridisation solution according to the calculations in Table. For each slide (containing two different samples in duplicate) 90 µl hybridisation solution is required.

Table: Suggested quantities for preparation of hybridisation mix

	2 slides	8 slides
Sterile UPW	50,1	200,4
3x hybmix	30,0	120,0
1% SDS	0,9	3,6
Probe EUB 338 (100 ng/µl)	4,5	18,0
Probe CMSCY301 (100 ng/µl)	4,5	18,0
Total volume (µl)	90,0	360,0

NB. Store all solutions containing light sensitive oligo-probes in the dark at -20°C. Protect from direct sunlight or electric light during use.

5. 0,1M Phosphate buffer, pH 7,0

Na_2HPO_4	8,52 g

KH_2PO_4	5,44 g
Distilled water	1,00 L

Dissolve ingredients, check pH and sterilise by autoclaving at 121°C for 15 min.

Appendix 8

Eggplant culture

Sow seeds of eggplant (*Solanum melongena*) in pasteurized seed compost. Transplant seedlings with fully expanded cotyledons (10 to 14 days) into pasteurized potting compost.

Eggplants should be grown in a glasshouse with the following environmental conditions:

Day length:		14 hours or natural day length if greater;
Temperature:	day:	21 to 24°C,
	night:	15°C.

Susceptible varieties of eggplant: ‘Black Beauty’,

‘Long Tom’,

‘Rima’,

‘Balsas’

Supplier: see website http://forum.europa.eu.int/Public/irc/sanco/Home/main

---

Appendix 9

Gram stain procedure (Hucker’s modification) (Doetsch, 1981) (*)

Crystal violet solution

Dissolve 2 g crystal violet in 20 ml 95% ethanol.

---

*Commercially available solutions and staining kits can also be used.

Dissolve 0,8 g ammonium oxalate in 80 ml distilled water.

Mix the two solutions.

Lugol's iodine

Iodine	1 g
Potassium iodide	2 g
Distilled water	300 ml

Grind the solids together in a pestle and mortar. Add to the water and stir to dissolve in a closed container.

Safranin counterstain solution

Stock solution:

Safranin O	2,5 g
95% ethanol	100 ml

Mix and store.

Dilute: 1:10 to obtain a working solution.

Staining procedure

1. Prepare smears, air dry and heat fix.
2. Flood slide with crystal violet solution for one minute.
3. Wash briefly with tap water.
4. Flood with Lugol's iodine for one minute.
5. Wash with tap water and blot dry.
6. Decolourise with 95% ethanol, added dropwise, until no further colour is removed or immerse with gentle agitation for 30 seconds.
7. Wash in tap water and blot dry.
8. Flood with safranin solution for 10 seconds.
9. Wash with tap water and blot dry.

Gram positive bacteria stain violet-blue; Gram negative bacteria stain pink-

red.

REFERENCES

1. Anonymous, 1987. Scheme of the detection and diagnosis of the ring rot bacterium Corynebacterium sepedonicum in batches of potato tubers. Commission of the European Communities, Luxembourg. Publ EUR 11288 EN, 21 pp.

2. Bradbury, J. F., 1970. Isolation and preliminary study of bacteria from plants. Rev. Pl. Path., 49, 213-218.

3. Dinesen, I. G., 1984. The extraction and diagnosis of *Corynebacterium sepedonicum* from diseased potato tubers. EPPO Bull. 14 (2), 147-152.

4. Doetsch, R. N., 1981. Determinative methods of light microscopy. In: Manual of methods for general bacteriology, American Society for Microbiology, Washington, 21-23.

5. Hugh, R. and Leifson, F., 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative bacteria. J. Bact., 66, 24-26.

6. Janse, J. D., 1991. Infra- and intra-specific classification of *Pseudomonas solanacearum* strains using whole cell fattyacid analysis. Systematic and Applied Microbiology 14; 335-345.

7. Janse, J. D. and J. Van Vaerenbergh. The interpretation of the EC method for the detection of latent ring rot infections (*Corynebacterium sepedonicum*) in potato. EPPO Bull., No 17, 1987, pp. 1-10.

8. Jansing, H. and K. Rudolph, 1998. Physiological capabilities of *Clavibacter michiganensis* ssp. *sepedonicus* and development of a semi-selective medium. Journal of Plant Diseases and Protection, 105, 590-601.

9. Kovacs, N., 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature, Lond., 178, 703.

10. Klement Z.; Rudolph, K and D. C. Sands, 1990. Methods in Phytobacteriology. Akadémiai Kiadó, Budapest, 568 pp.

11. Lelliott, R. A., 1966. The plant pathogenic coryneform bacteria. J. appl. Bact., 29, 114-118.

12. Lelliott, R. A., E. Billing and A. C. Hayward, 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads J. appl. Bact., 29, 470-

489.

13. Lelliott, R. A. and P. W., Sellar, 1976. The detection of latent ring rot (*Corynebacterium sepedonicum* (Spiek. et Kotth.) Skapt. et Burkh.) in potato stocks. EPPO Bull., 6 (2), 101-106.

14. Li, X. and S.H. de Boer, 1995. Selection of Polymerase Chain Reaction primers from RNA intergenic spacer region for specific detection of *Clavibacter michiganensis* ssp. *sepedonicus*. Phytopathology, 85, 837-842.

15. Mills, D., Russell, B., W. and J., W. Hanus, 1997. Specific detection of *Clavibacter michiganensis* ssp. *sepedonicus* by amplification of three unique DNA sequences isolated by subtraction hybridization. Phytopathology, 87, 8, 853-861.

16. Pastrik, K.-H. and R.A. Rainey. 1999. Identification and differentiation of *Clavibacter michiganensis* subspecies by polymerase chain reaction-based techniques. J. Phytopathology 147; 687-693.

17. Pastrik, K.-H., 2000. Detection of *Clavibacter michiganensis* ssp. *sepedonicus* in potato tubers by multiplex PCR with coamplification of host DNA. European Journal of Plant Pathology, 106, 155-165.

18. Ramamurthi, C. S., 1959. Comparative studies on some Gram-positive phytopathogenic bacteria and their relationship to the Corynebacteria. Mem. Cornell agric. Exp. Sta., 366, 52 pp.

19. Schaad, W., Berthier-Schaad, Y., Sechler, A. and Knorr, D. (1999) Detection of *Clavibacter michiganensis* subsp. *Sepedonicus* in potato tubers by BIO-PCR and an automated real-time fluorescence detection system. Plant Disease 83; 1095–1100.

20. Schaad, W. 2001. Laboratory guide for identification of plant pathogenic bacteria. Schaad [Hrsg.]. — 3. ed.; St. Paul, Minnesota:, 373 pp.

21. Skerman, V. B. D., 1967. A guide to the identification of the genera of bacteria. 2nd ed., William and Wilkins Company, Baltimore.

22. Smith, N. C.; Hennesy, J; Stead, D.E., 2001. Repetetive sequence-derived PCR profiling using the BOX-A1*Ralstonia solanacearum* primer for rapid identification of plant pathogen *Clavibacter michiganensis* ssp. *sepedonicus*. European Journal of Plant Pathology, 107 (7), 739-748.

23. Sneath, P. H. A. and V. G. Collins, 1974. A study in test reproductibility between laboratories: report of Pseudomonas working party. Antonie van Leeuwenhoek, 40, 481-527.

24. Stead, D.E. 1992. Grouping of plant pathogenic and some other *Pseudomonas* spp. using cellular fatty-acid profiles. International Journal of

Systematic Bacteriology 42; 281-295.

25. Wullings, B. A.; van Beuningen, A. R.; Janse, J. D. and A. D. L. Akkermans, 1998. Detection of *Ralstonia solanacearum*, which causes brown rot of potato, by fluorescent in situ hybridization with 23s rRNA-targeted probes. Appl. Environ. Microbiol. 64, 4546–4554.

SCHEDULE II

1. For each suspected occurrence for which a positive result in the screening test (s) has been identified according to the methods set out in Schedule I, and confirmation or refutation by completion of the said methods is awaited, there should be the retention and appropriate conservation of:

- all tubers sampled and, wherever possible, all plants sampled,
- 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 methods.

Retention of the tubers will enable variety testing to be undertaken where appropriate.

2. In the case of positive confirmation of the organism, there should be retention and appropriate conservation of:

- the material specified in paragraph 1, and
- a sample of the infected eggplant material inoculated with the tuber or plant extract, and
- the isolated culture of the organism,

until at least one month after the notification procedure under regulation 5(2).

SCHEDULE III

1. The elements to be considered in the determination of the extent of probable contamination under regulation 5(1)(b) shall include:

- tubers or plants grown at a place of production designated as contaminated under regulation 5(1)(a),

- place(s) of production with some production link to the tubers or plants, designated as contaminated under regulation 5(1)(a), including those sharing production equipment and facilities directly or through a common contractor,

- tubers or plants 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 tubers or plants designated as contaminated under regulation 5(1)(a) were present on the place of production referred to in the first indent,

- premises handling potatoes from the places of production referred to in the above indents,

- any machinery, vehicle, vessel, store, or units thereof, and any other objects including packaging material, that may have come into contact with the tubers or plants designated as contaminated under regulation 5(1)(a),

- any tubers or plants 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 testing under regulation 5(3), those tubers or plants with a sister or parental clonal relationship to the tubers or plants designated to be contaminated under regulation 5(1)(a) and for which, although they may have tested negative for the organism, it appears that contamination is probable through a clonal link. Variety testing may be undertaken to verify the identity of the contaminated and clonally related tubers or plants, and

- place(s) of production of the tubers or plants referred to in the previous indent.

2. The elements to be considered in the determination of the possible spread under regulation 5(1)(c) shall include:

- the proximity of other places of production growing potatoes or other host plants,

- the common production and use of seed potato stocks.

3. The notification referred to in the first subparagraph of regulation 5(2) shall be provided as follows:

- immediately after the presence of the organism has been confirmed by laboratory testing, using the methods set out in Schedule I, at least:

  - the variety name of the potato lot,

  - the type (ware, seed, etc.) and where applicable the seed category of potatoes,

- when there is a risk of contamination of potatoes from or into another Member States(s), the competent authority shall immediately notify the Member State(s) concerned of information needed to comply with regulation 5(3), such as:

  - the variety name of the potato lot,

  - the name and address of the consignor and the consignee,

  - the date of delivery of the potato lot,

  - the size of the potato lot delivered,

  - a copy of the plant passport or at least the plant passport number where appropriate, or where appropriate the registration number of the grower or merchant and a copy of the delivery notice.

The Commission shall be notified immediately when such information has been provided.

- After all investigations have been finalised, for each case:

  - the date that contamination was confirmed,

  - a brief description of the investigation carried out to identify the source and possible spread of the contamination, including the level of sampling undertaken,

  - information on the identified or presumed sources(s) of contamination,

  - details of the extent of the designated contamination, including the number of places of production and the number of lots with an indication of the variety and, if seed potatoes, category,

  - details of the zone demarcation, including the number of

places of production, not designated as contaminated, but included in the zone,

- such other information relating to the confirmed outbreak(s) as the Commission may require

---

SCHEDULE IV

1. The officially supervised measures referred to in regulation 5(4) of the principal regulations shall be:

- use as animal feed after heat treatment such that there is no risk of the organism surviving, or

- disposal at an officially approved dedicated waste disposal site at which there is no identifiable risk of escape of the pathogen into the environment e.g. through seepage to agricultural land, or

- incineration, or

- industrial processing through direct and immediate delivery to a processing plant with officially approved waste disposal facilities for which it has been established that there is no identifiable risk of the organism spreading, and with a system of cleansing and disinfection of at least the departing vehicles, or

- other measures, provided that it has been established that there is no identifiable risk of the organism spreading; such measures and their justification to be notified to the Commission and to the other Member States.

Any remaining waste associated with and arising from the above shall be disposed of by officially approved methods in accordance with Schedule V.

2. The appropriate use or disposal of tubers or plants determined as probably contaminated under regulation 5(1)(b) and referred to in regulation 5(5), under the control of the responsible official bodies of the Member States concerned, with appropriate communication between responsible official bodies to ensure such control at all times and approval by the responsible official bodies 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 indent, shall be:

- use as ware potatoes intended for consumption, packed ready for direct delivery and use without repacking, on a site with appropriate waste disposal facilities. Potatoes intended for planting may only be handled at the

same site, if this is done separately or after cleansing and disinfection, 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 and a system of cleansing and disinfection of at least the departing vehicles, 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.

3. The appropriate methods for cleansing and disinfecting of the objects referred to in regulation 5(6) shall be those for which it has been established that there is no identifiable risk of the organism spreading and shall be employed under the supervision of the responsible official bodies of the Member States.

4. The series of measures to be implemented within the demarcated zone established under regulation 5(1)(c) and referred to in regulation 5(7) shall include:

4.1. in places of production designated as contaminated under regulation 5(1)(a):

(a) in a field designated to be contaminated under regulation 5(1)(a), either

(i) - during at least the three growing years following the year of the designated contamination,

- measures shall be taken to eliminate volunteer potato plants and other naturally found host plants of the organism, and

- no potato tubers, plants or true seeds, or other naturally found host plants of the organism, or crops for which there is an identified risk of the organism spreading, shall be planted,

- in the first potato cropping season following the period specified in the preceding indent, and on the condition that the field has been found free from volunteer potato plants and other naturally found host plants of the organism during official inspections for at least the two consecutive growing years prior to planting, only ware potato production shall be allowed and the harvested tubers shall be tested according to the procedure detailed in Schedule I;

- in the potato cropping season succeeding that referred to in the previous indent and following an appropriate rotation cycle,

which shall be at least two years if seed potatoes are to be grown, potatoes may be planted for either seed or ware production, and an official survey as detailed in regulation 3(1) shall be conducted; or

(ii) - during the four growing years following that of the designated contamination,

- measures shall be taken to eliminate volunteer potato plants and other naturally found host plants of the organism, and

- the field shall be laid to, and maintained either, in bare fallow or in permanent pasture with frequent close cutting or intensive grazing,

- in the first potato cropping season following the period specified in the preceding indent, and on the condition that the field has been found free from volunteer potato plants and other naturally found host plants of the organism during official inspections for at least the two consecutive growing years prior to planting, seed or ware potato production shall be allowed and the harvested tubers shall be tested according to the procedure detailed in Schedule I;

(b) in all other fields of the contaminated place of production, and on the condition that the responsible official bodies are satisfied that the risk of volunteer potato plants and other naturally found host plants of the organism have been eliminated:

- in the growing year following that of the designated contamination either no potato tubers, plants or true seeds or other naturally found host plants of the organism shall be planted, or

- certified seed potatoes may be planted for ware production only,

- in the second growing year following that of the designated contamination, only certified seed or seed potatoes officially tested for the absence of ring rot and grown under official control on places of production other than those referred to in paragraph 4.1 shall be planted for either seed or ware production,

- for at least the third growing year following the designated contamination, only certified seed potatoes or seed potatoes grown under official control from 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 plants and

other naturally found host plants of the organism if present and in each potato field official testing of the harvested potatoes shall be conducted according to the procedure detailed in Schedule I,

(c) immediately following the designation of contamination under regulation 5(1)(a) and after the first subsequent growing year, all machinery and storage facilities on the place of production and involved in potato production shall be cleansed and disinfected as appropriate using appropriate methods, as specified in paragraph 3;

(d) in an unit of protected crop production where complete replacement of the growing medium is possible,

- no tubers, plants or true seeds shall be planted unless the production 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 disinfection of the production unit, and all equipment, and, subsequently has been granted approval for potato production by the responsible official bodies, and

- potato production shall be from certified seed potatoes, or from mini-tubers or micro-plants derived from tested sources;

4.2. within the demarcated zone, without prejudice to the measures detailed under paragraph 4.1, the competent authority shall:

(a) immediately following the designated contamination, ensure that all machinery and storage facilities on such premises and involved with potato production be cleansed and disinfected, as appropriate, and using appropriate methods, as specified in paragraph 3;

(b) immediately, and for at least three growing seasons, after the designated contamination:

- ensure supervision by their responsible official bodies of premises growing, storing or handling potato tubers, together with premises which operate potato machinery under contract,

- 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 designated as probably contaminated under regulation 5(1)(b),

- require the separate handling of harvested seed potato stocks to those of ware on all premises within the zone, or a system of cleansing and disinfection to be carried out between the handling of seed and ware stocks,

- conduct an official survey as detailed in regulation 3(1)(a);

(c) establish a programme, where appropriate, for the replacement of all seed potato stocks over an appropriate period of time.".

SCHEDULE II

(Regulation 3)

"SCHEDULE V

The officially approved waste disposal methods referred to in Schedule IV paragraph 1, shall conform to the following provisions such that any identifiable risk of spreading the organism is obviated:

(i) potato waste (including rejected potatoes and peelings) and any other solid waste associated with the potatoes (including soil, stones and other debris) shall be disposed by either,

- disposal at an officially approved dedicated waste disposal site at which there is no identifiable risk of escape of the organism into the environment e.g. through seepage to 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, or

- other measures, provided that it has been established that there is no identifiable risk of the organism spreading; such measures to be notified to the Commission and to the other Member States.

(ii) liquid 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 paragraph (i).

The liquid waste shall then be either:

- heated to a minimum of 60°C throughout the entire volume 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 identifiable risk that the waste could come into contact with agricultural land. The details thereof shall be notified to the other Member States and to the Commission.

The options described in this Schedule also apply to the waste associated with handling, disposal and processing of contaminated lots.".