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

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ANNEX I

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

GENERAL PRINCIPLES

3.1. Potato tubers

Note:

3.1.1. Remove with a clean and disinfected scalpel or vegetable knife the...

Note:

3.1.2. Collect the heel end cores in unused disposable containers which...

3.1.3. Process the heel end cores by one of the following...

Note:

3.1.4. Decant the supernatant. If excessively cloudy, clarify either by slow...

3.1.5. Concentrate the bacterial fraction by centrifugation at 7 000 g...

3.1.6. Resuspend the pellet in 1,5 ml pellet buffer (Appendix 3). Use 500 µl...

3.1.7. It is imperative that all C. m. subsp. sepedonicus positive...

3.2. Potato plants

Note:

3.2.1. With a clean disinfected knife or pruning shears, remove a...

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

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...

3.2.5. Divide the neat or concentrated sample extract into 2 equal parts....

4. IF TEST

Principle

Note:

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

4.2. Dry the droplets at ambient temperatures or by warming at...

4.3. IF procedure: According to test slide preparation in 4.1(i): Prepare a...

4.3.1. Arrange the slides on moist paper. Cover each test window...

4.3.2. Incubate the slides on moist paper under a cover for...

4.3.3. Shake the droplets off each slide and rinse carefully with...

4.3.4. Arrange the slides on moist paper. Cover the test windows...

4.3.5. Incubate the slides on moist paper under a cover for...

4.3.6. Shake the droplets of conjugate off the slide. Rinse and...

4.3.7. Pipette 5 to 10 µl of 0,1M phosphate-buffered glycerol (Appendix 3) or a commercially...

4.4. Reading the IF test:

4.4.1. Examine test slides on an epifluorescence microscope with filters suitable...

4.4.2. Observe for bright fluorescing cells with characteristic morphology of C....

4.4.3. There are several problems inherent to the specificity of the...

4.4.4. Consider only fluorescing cells with typical size and morphology at...

4.4.5. Interpretation of the IF reading:

5. FISH TEST

Principle

Note:

5.1. Potato extract fixation

5.1.1. Prepare fixative solution (see Appendix 7).

5.1.2. Pipette 100 µl of each sample extract into an Eppendorf tube...

5.1.3. Remove the supernatant and dissolve the pellet in 500 µl of...

5.1.4. Centrifuge for 8 min. at 7 000 g, remove the supernatant...

5.1.5. Spot 16 µl of the fixed suspensions onto a clean multitest...

5.1.6. Air-dry the slides (or on slide dryer at 37 °C) and...

5.2. Pre-hybridisation and hybridisation
- 5.2.1. Prepare a lysozyme solution containing 10 mg lysozyme (Sigma L–6876) in...
- 5.2.2. Dehydrate the cells in a graded ethanol series of 50 %,...
- 5.2.3. Prepare a moist incubation chamber by covering the bottom of...
- 5.2.4. Prepare the hybridisation solution (Appendix 7) allowing 45 µl per slide, and...
- 5.2.5. Place slides on a hot plate at 45 °C and apply...
- 5.2.6. Apply two coverslips (24 × 24 mm) to each slide without...
- 5.2.7. Prepare three beakers containing 1 l of Ultra pure water...
- 5.2.8. Remove the coverslips from the slides and place the slides...
- 5.2.9. Wash away excess probe by incubation for 15 mins. in...
- 5.2.10. Transfer the slide holder to 1/2 hybmix washing solution and incubate for a...
- 5.2.11. Dip the slides briefly in UPW and place them on...

5.3. Reading the FISH test
- 5.3.1. Observe the slides immediately with a microscope fitted for epifluorescence...
- 5.3.2. Observe for bright fluorescing cells with characteristic morphology of C....
- 5.3.3. If any contamination is suspected the test must be repeated....
- 5.3.4. There are several problems inherent to the specificity of the...
- 5.3.5. Consider only fluorescing cells with typical size and morphology, see...
- 5.3.6. Interpretation of the FISH test result:

6. PCR TEST

Principles

Note:

6.1. DNA purification methods
- 6.1.(a) Method according to Pastrik (2000)
- 6.1.(b) Other methods

6.2. PCR
- 6.2.1. Prepare test and control templates for PCR according to the validated...
- 6.2.2. Prepare the appropriate PCR reaction mix in a contamination-free environment...
- 6.2.3. Add 5 µl of DNA extract per 25 µl PCR reaction in...
- 6.2.4. Incorporate a negative control sample containing only PCR reaction mix...
- 6.2.5. Place tubes in the same thermal cycler which was used...

6.3. Analysis of the PCR product
- 6.3.1. Resolve PCR amplicons by agarose gel electrophoresis. Run at least...
- 6.3.2. Reveal DNA bands by staining in ethidium bromide (0,5 mg per...
- 6.3.3. Observe stained gel under short wave UV transillumination (e.g. λ...
- 6.3.4. For all new findings/cases verify authenticity of the PCR amplicon...

Note:

7. BIOASSAY TEST

Note:

7.1. Distribute the whole of the remaining test aliquot of the...

7.2. Do not water eggplants for one to two days prior...

7.3. Slit inoculation
- 7.3.1. Holding the plant between two fingers, pipette a drop (approximately...
- 7.3.2. Using a sterile scalpel, make a diagonal slit, about 1,0 cm...

Appendix 1

Laboratories involved in optimisation and validation of protocols

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....
Remove the heel end cores of 200 tubers taken from...
Process the heel ends as usual and resuspend the pellet...
Prepare 10 sterile 1,5 ml microvials with 900 µl of the resuspended...
Transfer 100 µl of the suspension of C. m. subsp. sepedonicus...

Establish decimal levels of contamination by further diluting in the...
The six contaminated microvials will be used as positive controls....
Prepare aliquots of 100 µl in sterile 1,5 ml microvials thus obtaining...
The presence and quantification of C. m. subsp. sepedonicus in...
For the PCR test perform DNA extraction from positive and...
For IF and FISH tests perform assays on positive and...
For IF, FISH and PCR assays C. m. subsp. sepedonicus...

Appendix 3

Buffers for test procedures

1. Buffers for extraction procedure
   - 1.1. Extraction buffer (50 mM phosphate buffer, pH 7,0)
   - 1.2. Pellet buffer (10 mM phosphate buffer, pH 7,2)

2. Buffers for the IF test
   - 2.1. IF-Buffer (10 mM phosphate buffered saline (PBS), pH 7,2)
   - 2.2. IF-buffer-Tween
   - 2.3. Phosphate buffered glycerol, pH 7,6

Appendix 4

Determination of contamination level in IF and FISH tests

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

Appendix 5

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

(a) General growth media
- Nutrient agar (NA)
- Nutrient dextrose agar (NDA)
- Yeast peptone glucose agar (YPGA)
- Yeast extract mineral salts medium (YGM)

(b) Validated selective growth media
- MTNA medium
  - Note:
- NCP-88 medium
  - Note:

Appendix 6

Validated PCR protocol and reagents

Note:

1. Multiplex PCR protocol with internal PCR control (Pastrik, 2000)
   - 1.1. Oligonucleotide primers
   - 1.2. PCR reaction mix
   - 1.3. PCR reaction conditions
     Note:
   - 1.4. Restriction enzyme analysis of amplicon.

2. Preparation of the Loading buffer
   - 2.1. Bromphenol blue (10 %-stock solution)
   - 2.2. Loading buffer

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

Appendix 7

Validated reagents for FISH test

1. Oligo-probes

2. Fixative solution

3. 3x Hybmix

4. Hybridisation solution

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

Appendix 8

Eggplant culture

Sow seeds of eggplant (Solanum melongena) in pasteurized...
Eggplants should be grown in a glasshouse with the following...
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

Lugol's iodine

Safranin counterstain solution

Staining procedure

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ANNEX II

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

ANNEX III

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

ANNEX IV

The officially supervised measures referred to in Article 7(1) shall be: use...

ANNEX V

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

(**1**) OJ No C 93, 2. 4. 1993, p. 12.

(**2**) OJ No C 176, 28. 6. 1993, p. 210.

(**3**) OJ No C 161, 14. 6. 1993, p. 18.

(**4**) OJ No L 26, 31. 1. 1977, p. 20. Directive as last amended by Commission Directive 92/103/EEC (OJ No L 363, 11. 12. 1992, p. 1).

(**5**) OJ No L 180, 14. 7. 1980, p. 30.