[F1 ANNEX I

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

SCOPE OF THE TEST SCHEME

Textual Amendments

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

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 10^3 to 10^4 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 10^6 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 10^3 to 10^4 pathogen cells per ml.

6.2. PCR

6.2.1. Prepare test and control templates for PCR according to the validated 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 optimised PCR programme (Appendix 6).

6.3. Analysis of the PCR product

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

6.3.2. Reveal DNA bands by staining in ethidium bromide (0,5 mg per L) for 30 to 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.**]**