[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.

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 Annex 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.**]**