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[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.

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

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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).**]**