# [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.

7. BIOASSAY TEST

Note:

Preliminary testing with this method should permit reproducible detection of $10^3$ to $10^4$ 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 $10^5$ to $10^6$ 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*.**]**