Diagnostics[1]
Diagnostic

Clavibacter michiganensis subsp. *sepedonicus*

Specific scope

This standard describes a diagnostic protocol for *Clavibacter michiganensis* subsp. *sepedonicus*.

Specific approval and amendment

This Standard was developed under the EU DIAGPRO Project (SMT 4-CT98-2252) by partnership of contractor laboratories and intercomparison laboratories in European countries.

Approved as an EPPO Standard in 2005-09.

Introduction

Potato ring rot caused by *Clavibacter michiganensis* subsp. *sepedonicus* is a damaging disease of potato. It was first described from Northern Europe and is prevalent in cool, northern latitudes. It is one of the few major plant pathogens which is not widely distributed in the area where the crop evolved. It is an EPPO-listed pest which is not yet established in many parts of the region and would cause serious economic damage if it were to become established in these pest-free areas. Direct losses are through losses due to wilting and tuber rotting in field and store. Even in the absence of symptoms, there can be significant reductions in yield. Indirect losses are through the statutory measures taken against any outbreaks and include restrictions on cropping, disinfection costs and effects on export trade. Control within the EU is governed by EC Council Directive 93/85/EEC (EU, 1993).

The disease is becoming increasingly widespread in the EPPO region. Traditionally, it has been seen as a disease largely restricted to the cool northern temperate countries of the world (OEPP/EPPO, 2005). Certification based on visual inspection has generally not given good control of the disease because the pathogen can remain as a latent infection for long periods of time. Some cultivars rarely show symptoms although the pathogen multiplies within them. These are referred to as tolerant cultivars and, in North America, several such cultivars have been removed from certification schemes because they are symptomless carriers. Relatively little is known of tolerance in the EPPO region, although cultivars such as 'Alpha' and 'Desirée' are considered to be tolerant.

[1]The figures in this Standard marked 'Web Fig.' are published on the EPPO website www.eppo.org.

Identity

Name: *Clavibacter michiganensis* subsp. *sepedonicus* (Spieckermann & Kotthof, 1914) Davis, Gillaspie, Vidaver & Harris 1984.
Synonyms: *Corynebacterium michiganensis* subsp. *sepedonicum* (Spieckermann & Kotthof, 1914) Carlson & Vidaver 1982; *Corynebacterium michiganensis* pv. *sepedonicum* (Spieckermann & Kotthof, 1914) Dye & Kemp 1977; *Corynebacterium sepedonicum* (Spieckermann & Kotthof, 1914) Skaptason & Burkholder 1942.
Taxonomic position: *Bacteria*, *Firmicutes*.
EPPO code: CORBSE.
Phytosanitary categorization: EPPO A2 list no. 51. EU Annex designation I/A2.

Detection

This protocol allows the detection and identification of latent infections of *C. m. sepedonicus* of 10^3–10^4 cells per mL of resuspended pellet. The diagnostic procedures comprise isolation from infected tissue (Fig. 1) and detection of latent infections (Fig. 2), including presumptive diagnosis with a rapid test, identification of presumptive isolates and determination of pathogenicity. *C. m. sepedonicus* is renowned for the difficulties encountered with its detection. The main problems are in detecting latent infection and in obtaining cultures even from symptomatic material, since it is slow growing on agar media and is easily overgrown by saprophytes.

Disease symptoms

Ring rot is a vascular disease in both aerial stems and tubers.

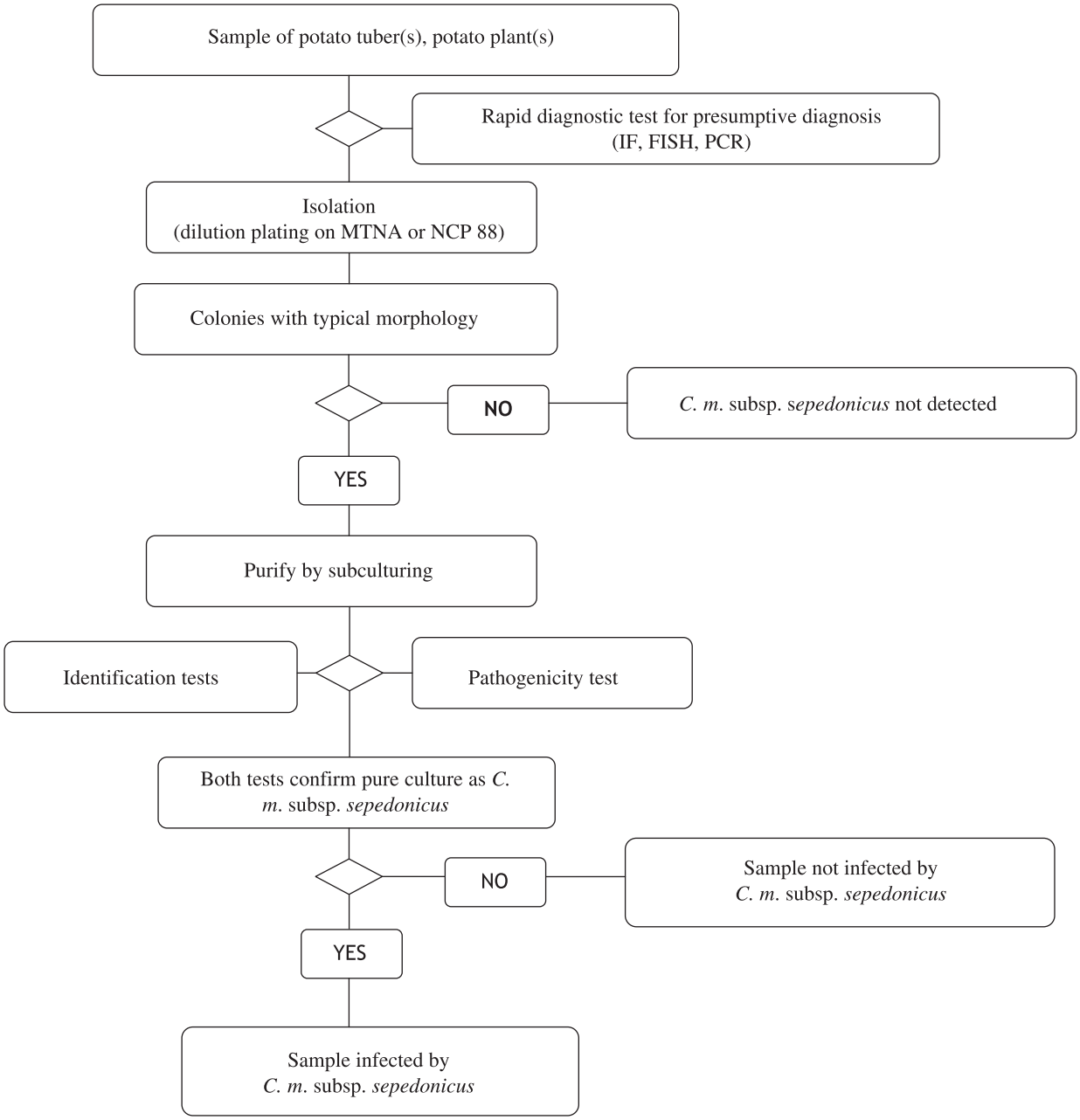

Fig. 1 Scheme for detection and identification of *Clavibacter michiganensis* subsp. s*epedonicus* in samples of symptomatic potato tubers and potato plants.

Potato tubers

Tuber symptoms are not unlike those of brown rot caused by *Ralstonia solanacearum*. The infected vascular bundles break down releasing bacteria into the adjacent cortical tissues which then rot partly due to activity of cellulase enzymes. This rot extends around the vascular ring as the rots from adjacent infected bundles merge. Although not frequently mentioned in the literature, a common feature in many of the specimens seen is the progression of the rot from the vascular tissues of the heel end to the central cortex of the tuber. In the early stages of disease, the rotted tissues usually remain creamy white and not brownish as in brown rot. Eventually however, rots do become discoloured as secondary invaders become established. Early rots have a cream cheese-like consistency and are distinct from the more slimy, ooze-like rots of brown rot.

Potato plants

Under European climatic conditions, symptoms are rarely found in the field and often only at the end of the season. Moreover the symptoms are frequently masked by or confused

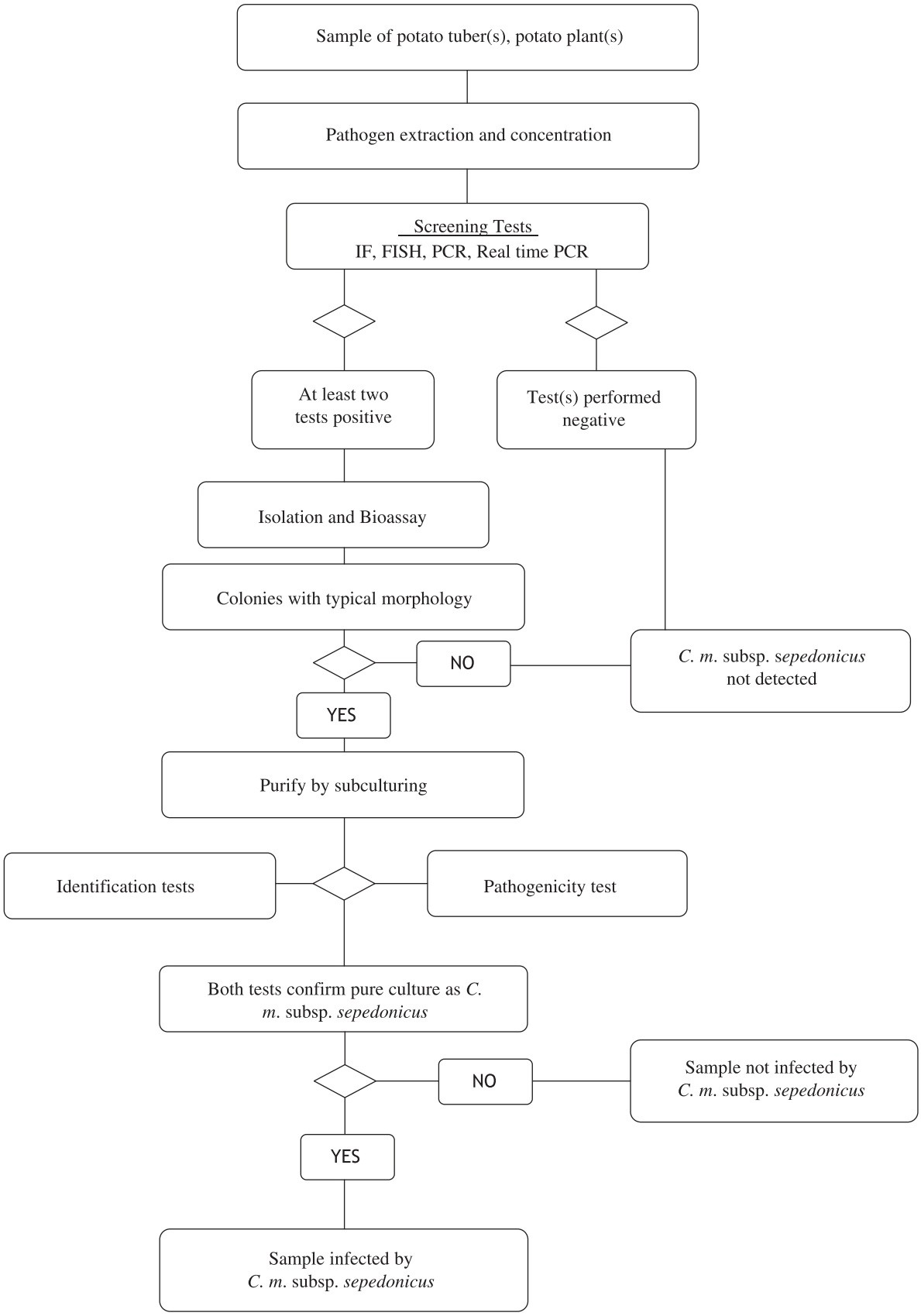

Fig. 2 Scheme for detection and identification of *Clavibacter michiganensis* subsp. s*epedonicus* in samples of asymptomatic potato tubers and potato plants.

with other diseases, senescence or mechanical damage, so symptoms may easily be missed in field inspections.

Wilting symptoms in stems are not like those of other diseases, and are very different from those of brown rot. Wilting is usually slow, initially limited to the leaf margins. Young infected leaves often continue to expand, though less so in the infected zones. This creates odd-shaped leaves. Leaves affected by xylem blockages further down the stem often develop chlorotic, yellow to orange, interveinal areas. Infected leaflets, leaves and even stems may eventually die. Wilting symptoms are often absent, leaves and tubers being simply reduced in size. Occasionally plants are stunted.

Wilt symptoms caused by *C. m. sepedonicus* may be confused with those caused by other systemic pathogens, e.g. *R. solanacearum*, *Erwinia carotovora* subsp. *carotovora*, *Erwinia carotovora* subsp. *atroseptica*, *Erwinia chrysanthemi*, *Phoma exigua* var. *foveata*, as well as large populations of saprophytic bacteria. In particular, *E. chrysanthemi* can cause leaf symptoms and wilt that is very similar to the symptoms of *C. m. sepedonicus*. The only difference is blackening of the stems in *E. chrysanthemi* infections. Other wilts can be distinguished from those caused by *C. m. sepedonicus* since whole leaves or whole plants wilt rapidly.

Isolation from symptomatic material

The methods described can be applied to tubers, stems or leaves, including those of test plants, e.g. *Solanum melongena*. For isolation, the media MTNA (Jansing & Rudolph, 1998) and NCP 88 (de la Cruz *et al*., 1992) are preferred (Appendix 1). Useful elective media include Yeast glucose mineral salts medium (YGM) and Nutrient dextrose agar (NDA) (Lelliott & Stead, 1987) (Appendix 1). These media are useful in routine maintenance of pure cultures.

As positive controls, decimal dilutions should be prepared from a suspension of 10^6 cfu mL^{-1} of *C. m. sepedonicus* (e.g. NCPPB 4053). To avoid any possibility of contamination, positive controls should be totally separated from samples to be tested.

For testing samples, remove ooze or sections of discoloured tissue from the vascular ring in the potato tuber or from the vascular strands of stems or leaves of potato or aubergine. If necessary, surface-disinfect the material with 70% ethanol. Suspend or crush the material in a small volume of sterile distilled water or 50 mM phosphate buffer (PB) (Appendix 1) and leave for 5–10 min. Prepare a series of decimal dilutions of the suspension in 10 mM phosphate buffer (Appendix 1). As 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 sample, at 1/100 up to 1/10000 dilutions, onto MTNA medium or NCP-88 medium (Appendix 1), using spreaders and the spread-plate technique. Alternatively, the initial 100 µL potato aliquot can be spread out onto a first agar plate with a spreader, and the spreader then used on a second agar plate, streaking out any residue left on the spreader. This is finally repeated on a third plate, giving a dilution plating effect via the spreader. Plates are incubated in the dark at 21–23°C.

Isolation from plant tissues usually takes at least 10 days. The optimum temperature for growth is 21°C. Temperatures of 28°C can be deleterious to growth. The plates are initially examined, by comparison with positive controls, after 3 days, and then after 5, 7 and possibly 10 days. Presumptive colonies *C. m. sepedonicus* should be purified on YGM medium before the plates become too overgrown, i.e. preferably after 3–5 days. The purified cultures of presumptive *C. m. sepedonicus* can then be identified and their pathogenicity conformed (see *Identification* below).

Screening for latent infection

As a single test can provide a false positive result, a sample cannot be considered suspect on the basis of a positive result from only one of the screening tests. A positive result from at least two screening tests, based on different biological principles, should be complemented by the isolation of the pathogen, followed by the identification, including determination of pathogenicity.

Sampling and sample preparation

The standard sample size is 200 tubers. Larger number 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. The method in Appendix 2 has been validated and ring-tested.

Screening methods

Detection of latent infection is possible by performing an immuno-fluorescence test (IF test), fluorescent *in situ* hybridization test (FISH) or PCR. Details of these screening methods are given in Appendix 3. Further details of FISH or PCR can be found in the EU Amendment of Council Directive 93/85/EEC (EU 2006). Real time PCR (not yet included in the EU detection scheme for *C. m. sepedonicus*) offers a potentially robust, specific and sensitive technique.

Bioassay

The bioassay for *C. m. sepedonicus* is not so much a host test as a semi-selective enrichment which facilitates isolation on agar media. Some cultvars of aubergine provide an excellent selective enrichment medium for the growth of *C. m. sepedonicus* even in the absence of symptoms and also provide an excellent confirmatory host test. The recommended cultivar is 'Black Beauty' but, as this is now less easily available, other cultivars having similar susceptibility may be used, e.g. 'Long Tom', 'Balsa', 'Rima'. Growing conditions should be optimal, so as to reduce the risk of false negative test results. The bioassay procedure is described in Appendix 4.

Isolation

Isolation of *C. m. sepedonicus* directly from the heel end cores or stems of latently infected potatoes or aubergines is possible, though the pathogen may be outgrown by rapidly

growing saprophytic bacteria. The method described above (*Isolation from symptomatic tissue*) can be used in parallel to the bioassay.

Identification

C. m. sepedonicus is a typical, small Gram-positive (occasionally giving a weak or negative reaction) coryneform bacterium. Cells are club shaped, mostly single, often in pairs described as 'elbows' because they are in a 'V' formation, and occasionally in small irregular shaped groups which have been described as looking like Chinese characters. Cells from plant tissues tend to be more coccoid than those from agar plate cultures.

Pure cultures of putative *C. m. sepedonicus* should be identified using at least two tests based on different biological principles (biochemical, serology, molecular, FAP) from the following headings. Final confirmation can then be obtained by a pathogenicity test.

Biochemical characteristics

The main differential characters in traditional nutritional and physiological studies are given in Table 1. See also Schaad *et al*. (2001). API 50 CH strips from BioMérieux (FR) provide a convenient test procedure, as follows.

The basal medium should be modified YGM (Appendix 1). Strains are plated first on NBY (Appendix 1) and incubated at 24°C for 6 days. 2 mL of bacterial suspension (10^9 cells mL^{-1}) are then added to 25 mL of basal medium. The suspension is distributed into the microtubes under aseptic conditions following the instructions of the manufacturer, and read at 5, 8 and 12 days. Tests are positive when the colour turns from green to yellow, negative when there is no change in colour, and weak when a slight change in colour, but not yellow, is observed after 12 days. In general, *C. m. sepedonicus* strains utilize as carbon source: L-arabinose, D-xylose, galactose, D-glucose, D-fructose, D-mannose, mannitol, arbutin, aesculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, D-raffinose and D-turanose (positive results in more than 90% of strains). The β-gentiobiose, D-xyloside and amygdalin results are variable. The results are negative for other carbohydrates.

Table 1 Nutritional and physiological characters useful in identification of *C. m. sepedonicus*

Test	Reaction
O/F	**Inert or weakly oxidative**
Oxidase	–
Catalase	+
Nitrate reduction	–
Urease activity	–
H_2S production	–
Indole production	–
Citrate utilization	–
Starch hydrolysis	– or weak
Cellulase activity	+ or weak
Growth at 37°C	–
Growth in 7% NaCl	–
Gelatin hydrolysis	–
Aesculin hydrolysis	+
Acid from glycerol	–
Acid from lactose	– or weak
Acid from rhamnose	–
Acid from salicin	–

IF test

A suspension of about 10^6 cells mL^{-1} is prepared in IF buffer, and the IF procedure is applied (Appendix 3). A positive IF test is achieved if the IF titre of the culture is equivalent to that of the positive control.

PCR

A suspension of about 10^6 cells mL^{-1} is prepared in ultra pure water. 100 µL of the cell suspension in closed tubes is heated in a heating block or boiling waterbath at 100°C for 4 min. If required, addition of freshly prepared NaOH to a final concentration of 0.05 M may assist cell lysis. Appropriate PCR procedures are applied to amplify specific amplicons of *C. m. sepedonicus* (e.g. Pastrik, 2000; Li & De Boer, 1995; Schneider *et al*., 1993; Mills *et al*., 1997; Firrao & Locci, 1994; Slack *et al*., 1996). A positive identification of *C. m. sepedonicus* is achieved if the PCR amplicons are the same size and have the same restriction fragment length polymorphisms (if applicable) as for the positive control strain.

FISH test

A suspension of about 10^6 cells mL^{-1} is prepared in ultra pure water. The FISH procedure is applied (Appendix 3). A positive FISH test is achieved if the same reactions are achieved from the culture and the positive control.

BOX-PCR

Identification of *C. m. sepedonicus* can now also be done using one of several genetic fingerprinting techniques. BOX-PCR generated fingerprints are simple, reasonably robust and allow accurate identification of *C. m. sepedonicus* (Smith *et al*., 2001). Identification should be based on the comparison, on the same electrophoretic gel, of fingerprints of test strains against reference strains of *C. m. sepedonicus*, together with reference strains for all other subspecies of *C. michiganensis*. A suspension of about 10^6 cells mL^{-1} is prepared in ultra pure water. The test is applied according to the procedure described by Smith *et al*. (2001).

FAP

Fatty acid and protein profiles (FAP) are useful in identification and methods for each are given by Stead (1992a). Fatty acid profiles are of excellent diagnostic value. The profiles are fairly simple and despite the fart that the relative amounts of

the main acids can vary (which affects similarity indices in comparisons with libraries of profiles), there is an acid 15 : 1 anteiso A which is present in all *Clavibacter*, *Leifsonia* and most *Rathayibacter* strains but which is absent in *Curtobacterium* strains and is rare in other bacteria. On the basis of the methods described (Stead, 1992a; Stead, 1992b; Stead *et al*., 1992; Dickstein *et al*., 2001), this acid comprises > 4% of the profile for all *C. m. sepedonicus* strains but rarely if ever more than this for all other *Clavibacter* and *Rathayibacter* strains. Other acids occurring include 15 : 0 iso, 15 : 0 anteiso, 16 : 0 iso, 16 : 0 and 17 : 0 anteiso. Other acids may occur in trace amounts.

Pathogenicity test

The pathogenicity test should be performed as final confirmation of a diagnosis of *C. m. sepedonicus* and for assessment of virulence of cultures identified as *C. m. sepedonicus*. An inoculum of about 10^6 cells mL^{-1} is prepared from 3-day cultures of the test isolate and of an appropriate positive control strain of *C. m. sepedonicus*. These are inoculated into 5–10 stems of young aubergine seedlings and the plants incubated as described in Appendix 4.

With pure cultures, typical wilting should be obtained within 2 weeks. Plants not showing symptoms after this time should be incubated up to 4 weeks at temperatures conducive to growth of aubergine but not exceeding 25°C. If, after 4 weeks, symptoms are not present, the culture cannot be confirmed as being a pathogenic form of *C. m. sepedonicus*.

The pathogen should be re-isolated from symptomatic plants. Basically, the same method is followed as for *Isolation from symptomatic plants* above. A section of stem 2 cm above the inoculation point is removed, comminuted and suspended in a small volume of sterile distilled water or 50 mM phosphate buffer, which is then diluted and streaked onto MTNA and YPGA. After incubation for 3–5 days at 21–23°C, the plates are examined for formation of colonies typical of *C. m. sepedonicus*.

Hypersensitivity test

A tobacco hypersensitivity test is also useful for most strains. A suspension of about 10^8–10^9 cells mL^{-1} is prepared in ultra pure water. The test follows the procedure described by Nissinen *et al*. (1997). Of the reference strains, NCPPB 2137, NCPPB 2140 and NCPPB 4053 give fairly strong HR. NCPPB 3898 gives a negative or weak reaction.

Reference strains

NCPPB 2140 (semi fluidal colonies); NCPPB 3898 (small dry, non mucoid colonies); NCPPB 4053 (fluidal colonies); NCPPB 2137 (Type strain).

Reporting and documentation

Gidelines on reporting and documentation are given in EPPO Standard PM7/– (in preparation).

Further information

Further information on this organism can be obtained from:

D. Stead, Central Science Laboratory, Y041 1LZ York (UK). Tel. +44 1904462336; d.stead@csl.gov.uk

P. Müller, Biologische Bundesanstalt für Land- und Forstwirtschaft, Stahnsdorfer Damm 81, 14532 Kleinmachnow (Germany). Tel. +49 33203 48377; p.mueller@bba.de

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This protocol was originally drafted by D. Stead, Central Science Laboratory, York (GB) and revised by P. Müller, Biologische Bundesanstalt für Land- und Forstwirtschaft, Kleinmachnow (DE).

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Appendix 1. Media and buffers

MTNA medium

Unless otherwise stated all media components are from BDH. Yeast extract (Difco) 2.0 g; mannitol 2.5 g; K_2HPO_4 0.25 g; KH_2PO_4 0.25 g; NaCl 0.05 g; $MgSO_4{\cdot}7H_2O$ 0.1 g; $MnSO_4{\cdot}H_2O$ 0.015 g; $FeSO_4{\cdot}7H_2O$ 0.005 g; agar (Oxoid no. 1) 16.0 g; distilled water to 1.0 L. Dissolve ingredients, adjust pH to 7.2. After autoclaving (121°C for 15 min) and cooling down to 50°C, add the antibiotics trimethoprim 0.06 g, nalidixic acid 0.002 g, amphotericin B 0.01 g. Stock antibiotic solutions: trimethoprim (Sigma) and nalidixic acid (Sigma) (both at 5 mg/mL), in 96% methanol, amphotericin B (Sigma) 1 mg/mL in dimethyl sulfoxide. Stock solutions are filter-sterilized. Durability of basal medium is 3 months. After antibiotics are added durability is 1 month when stored refrigerated.

NCP-88 medium

Nutrient agar (Difco) 23 g; yeast extract (Difco) 2 g; D-mannitol 5 g; K_2HPO_4 2 g; KH_2PO_4 0.5 g; $MgSO_4{\cdot}7H_2O$ 0.25 g; distilled water to 1.0 L. Dissolve ingredients, adjust pH to 7.2. After autoclaving and cooling down to 50°C, add the following antibiotics: polymyxin B sulphate (Sigma) 0.003 g, nalidixic acid (Sigma) 0.008 g, cycloheximide (Sigma) 0.2 g. Dissolve antibiotics in stock solutions as follows: nalidixic acid in 0.01 M NaOH, cycloheximide in 50% ethanol, polymyxin B sulphate in distilled water. Stock solutions are filter-sterilized. Durability of basal medium is 3 months. After antibiotics are added, durability is 1 month when stored refrigerated.

Yeast extract mineral salts medium (YGM)

Bacto-Yeast-Extract (Difco) 2.0 g; D(+) glucose (monohydrate) 2.5 g; K_2HPO_4 40.25 g; KH_2PO_4 0.25 g; $MgSO_4{\cdot}7H_2O$ 0.1 g; $MnSO_4{\cdot}H_2O$ 0.015 g; NaCl 0.05 g; $FeSO_4{\cdot}7H_2O$ 0.005 g: Bacto-Agar (Difco) 18 g; distilled water to 1.0 L. Dissolve ingredients and sterilize 0.5 L volumes of medium by autoclaving at 115°C for 20 min.

Nutrient dextrose agar (NDA)

Difco bacto nutrient agar containing 1% D(+) glucose (monohydrate). Sterilize by autoclaving at 115°C for 20 min.

YGM-modified

Yeast extract 2.0 g; K_2HPO_4 0.25 g; KH_2PO_4 0.25 g; $MgSO_4{\cdot}7H_2O$ 0.1 g; $MnSO_4{\cdot}H_2O$ 0.15 g; NaCl 0.05 g; $FeSO_4{\cdot}7H_2O$ 0.005 g; bromothymol blue 0.05 g; distilled water to 1.0 L. Sterilize by autoclaving at 115°C for 20 min.

NBY medium

Nutrient agar 23 g; yeast extract 2.0 g; KH_2PO_4 0.5 g; K_2HPO_4 2.0 g; $MgSO_4{\cdot}7H_2O$ 0.25 g; D(–)mannitol 5.0 g; distilled water to 1.0 L. Sterilize by autoclaving at 115°C for 20 min.

50 mM phosphate buffer, pH 7.0

This buffer is used for extraction of the bacterium from plant tissues by homogenization or shaking. Na_2HPO_4 (anhydrous) 4.26 g; KH_2PO_4 2.72 g; distilled water to 1.00 L. Dissolve ingredients, check pH and sterilize by autoclaving at 121°C for 15 min. Additional components may be useful as follows: Lubrol flakes (deflocculant for use with homogenization extraction method) 0.5 g; DC silicone antifoam (antifoam agent for use with homogenization extraction method)

1.0 mL; tetrasodium pyrophosphate (antioxidant) 1.0 g; polyvinylpyrrolidone-40000 (PVP-40) (binding of PCR inhibitors) 50 g.

10 mM phosphate buffer, pH 7.2

This buffer is used for resuspension and dilution of potato tuber heel-end core extracts following concentration to a pellet by centrifugation. $Na_2HPO_4 \cdot 12H_2O$ 2.7 g; $NaH_2PO_4 \cdot 2H_2O$ 0.4 g; distilled water to 1.00 L. Dissolve ingredients, check pH and sterilize by autoclaving at 121°C for 15 min.

IF-buffer

This buffer (10 mM phosphate buffered saline (PBS), pH 7.2) is used for dilution of antibodies. $Na_2HPO_4 \cdot 12H_2O$ 2.7 g; $NaH_2PO_4 \cdot 2H_2O$ 0.4 g; NaCl 8.0 g; distilled water to 1.0 L. Dissolve ingredients, check pH and sterilize by autoclaving at 121°C for 15 min. With 0.1% Tween 20, this gives IF-buffer-Tween, used to wash slides.

Phosphate-buffered glycerol, pH 7.6

This buffer is used as a mountant fluid on the windows of IF slides to enhance fluorescence. $Na_2HPO_4 \cdot 12H_2O$ 3.2 g; $NaH_2PO_4 \cdot 2H_2O$ 0.15 g; glycerol 50 mL; distilled water to 100 mL.

Anti-fading mountant solutions

Commercially available, e.g. Vectashield (Vector Laboratories) or Citifluor (Leica).

Appendix 2. Sample preparation from latently infected potato tubers

Preliminary washing is optional, but is particularly useful for samples with excess soil, or if a PCR test or direct isolation procedure is to be performed: wash the tubers; use appropriate disinfectants (chlorine compounds when PCR test is to be used, in order to remove any pathogen DNA) and detergents between each sample to clean the washing systems; air-dry the tubers.

Remove the epidermis around the heel end of each tuber using a regularly disinfected scalpel or potato peeler or adequate tool. Disinfection may be achieved by dipping the peeler in 70% ethanol and flaming. Remove conical tissue cores from the heel ends with a knife or potato peeler or other adequate tool, keeping any excess, nonvascular tissue to a minimum. Following removal, heel ends should preferably be processed immediately, or within 24 h. Otherwise they may be stored at –20°C for not more than 2 weeks.

Either, cover the cores with sufficient volume (about 40 mL) of 50 mM PB (Appendix I) and agitate on a rotary shaker (50–100 rev min^{-1}) for about 4 h below 24°C or for 16–24 h refrigerated (Dinesen & De Boer, 1995), or homogenize the cores with sufficient volume (about 40 mL) of 50 mM PB (Appendix I), 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 gauge polythene, 150 mm × 250 mm; radiation-sterilized) using a rubber mallet or suitable grinding apparatus (e.g. Homex)[2]. In both cases, decant the supernatant. If excessively cloudy, clarify either by slow speed centrifugation (at not more than 180 *g* for 10 min at 4–10°C) or by vacuum filtration (40–100 µm), washing the filter with additional (about 10 mL) 50 mM PB (Appendix I). Concentrate the bacterial fraction by centrifugation at 7,000 *g* for 15 min (or 10,000 *g* for 10 min) at 4–10°C and discard the supernatant without disturbing the pellet. Resuspend the pellet in sterile 10 mM PB pH 7.2 to give a total volume of approximately 1 mL. This should be divided equally into two parts for testing, and one part retained for reference purposes by freezing at –20°C after adding sterile glycerol to a final concentration of 10–25% (v/v).

Appendix 3. Screening methods

Immunofluorescence

IF is the serological method of choice. It is more sensitive than ELISA and is considered more specific (Baer & Gudmestad, 1993) since there are some non-mucoid strains, e.g. NCPPB 3898, which either do not react or give a weak reaction with the preferred monoclonal 1 H3 available from Agdia. Use of the IF test as the first screening test is recommended because of its proven robustness. It is recommended to use a polyclonal antibody although most polyclonal antibodies will give some false positive results. In case of a positive result with a polyclonal antibody, further screening of the sample with a monoclonal antibody may provide more specificity but can be less sensitive. Monoclonal antibody 9A1 from Agdia is well established. Follow a standardized procedure, e.g. the one described in EU directive 98/57/EC (EU, 1998) or by Janse (1988).

IF test

The following protocol has been ring-tested under the DIAGPRO project (SMT-4-CT98-2252). Use antibodies to a reference strain of *C. m. sepedonicus*. The crude polyclonal or monoclonal antibodies should have an IF titre of at least 1 : 2000. During testing, the antibodies should be used at a working dilution(s) close to or at the titre. Prepare separate positive control slides of the homologous strain or any other reference strain of *C. m. sepedonicus*, suspended in potato extract and optionally in buffer. As negative controls, use aliquots of sample extract which previously gave a negative test result. Test control material exactly like the sample(s).

Use undiluted preparations of the samples and their 1 : 10 and 1 : 100 dilutions in 10 mM PB (Appendix I). Apply a fixed

[2]A further method for extraction of C.M & sepedonicus has been described by Martin & Beaumanoir (2001) but this method has not been ring-tested under the DIAGPRO project (SMT-4-CT98-2252).

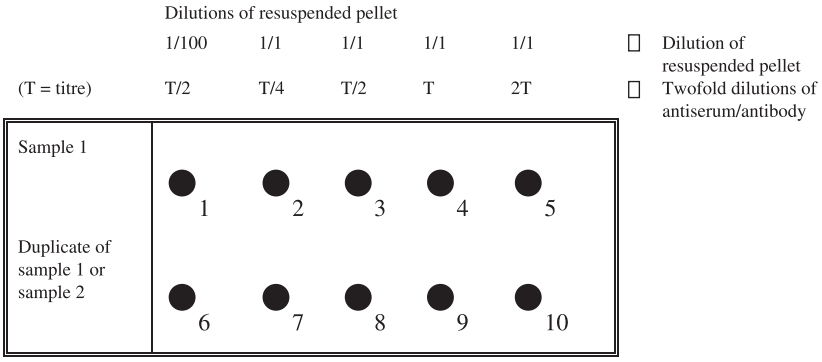

Fig. 3 Preparation of the IF test slide for samples with little starch.

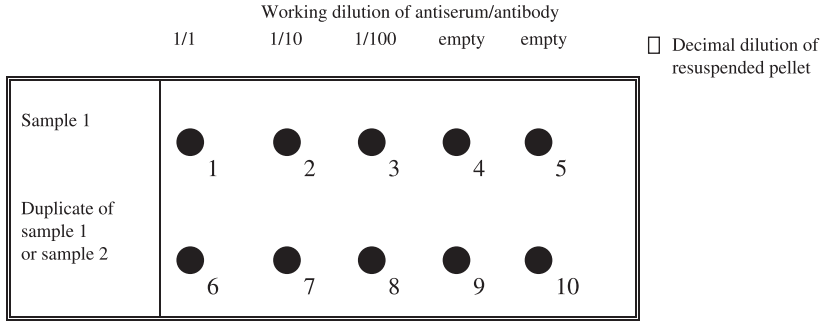

Fig. 4 Preparation of the IF test slide for other samples.

volume of these dilutions to the windows of a multispot test slide (e.g. 15 µL volume on 6 mm windows). For samples with relatively little starch sediment, it is possible to use only the undiluted and 1 : 100 diluted preparation. See Figs 3 and 4 for possible use of the windows. Dry the droplets at ambient temperatures or by warming at 40–45°C. Fix the bacterial cells to the slide either by heating (15 min at 60°C), flaming with 95% ethanol, or according to specific instructions from the suppliers of the antibodies. Cover each test window completely with the antibody dilution(s) made in IF buffer (Appendix I). The volume of antibody applied to each window should be at least the volume of extract applied. Incubate the slides, avoiding drying, on damp paper under a cover for 30 min at ambient temperature (18–25°C) (unless there are other specific instructions from the suppliers of the antibodies). Shake the droplets of antibody off each slide and rinse carefully with IF buffer. Wash by submerging for 5 min in IF buffer-Tween (Appendix I) and subsequently for 5 min in IF buffer. Arrange the slides on moist tissue paper. Cover the test windows with the dilution of FITC conjugate used to determine the titre. The volume of conjugate applied to the windows should be identical to the volume of antibody applied. Incubate the slides, avoiding drying, on damp paper under a cover away from direct light for 30 min at ambient temperature (18–25°C). Shake the droplets of conjugate off the slide. Rinse and wash as before. Carefully remove excess moisture. Pipette 5–10 µL of 0.1 M phosphate-buffered glycerol (Appendix I) or a commercial anti-fading mountant to each window and apply a coverslip.

Reading the IF test

Examine test slides on an epifluorescence microscope with filters suitable for excitation of FITC, under oil- or water-immersion at a magnification of × 500–1000. Scan windows across two diameters at right angles and around the perimeter. For samples showing no or low number of cells, observe at least 40 microscope fields. Check the positive control slide first: cells should be bright fluorescent and completely stained at the determined antibody titre or working dilution. The IF test should be repeated if the staining is aberrant. The fluorescence intensity of cells with the characteristic morphology of *C. m. sepedonicus* should be equivalent to or better than that of the positive control strain at the same antibody dilution. Consider only fluorescing cells with typical size and morphology at the titre or working dilution of the antibodies.

Interpretation of the IF reading

If bright fluorescing cells with characteristic morphology are found, estimate the average number of typical cells per microscope field and calculate the number of typical cells per mL of resuspended pellet (Appendix 5). The IF reading is positive for samples with at least 5×10^3 typical cells per mL of resuspended pellet: the sample is considered potentially contaminated. and further testing is required. The IF reading is negative for samples with less than 5×10^3 typical and/or atypical cells per mL resuspended pellet: the sample is considered negative and further testing is not required.

Fluorescent in situ hybridization (FISH)

FISH has proved to be a strong tool for the detection of bacteria in environmental samples (Amann *et al*., 1990). For the detection of *C. m. sepedonicus*, a FISH protocol based on that of van Beuningen *et al*., 1995) is available. This protocol has been evaluated in the routine testing of potato extract for some years now in the bacteriology laboratory of the Dutch NPPO at Wageningen, and has been found a reliable tool in confirmation of the IF test. The protocol should be performed as follows. As negative controls, use aliquots of sample extract that previously gave a negative test result for *C. m. sepedonicus*. As positive controls prepare suspensions containing 10^5–10^6 cells mL^{-1} of *C. m. sepedonicus* (e.g. strain NCPPB 4053) in 10 mM phosphate buffer (Appendix I) from a 3–5 day culture. Prepare separate positive control slides of the homologous strain or any other reference strain of *C. m. sepedonicus*, suspended in potato extract. Use validated *C. m. sepedonicus*-specific oligo-probes. Use of the FITC-labelled eubacterial oligo-probe offers a control for the hybridization process, since it will stain all eubacteria that are present in the sample. Follow the standard procedure described in EU (2006).

Interpretation of the FISH test result

Valid FISH test results are obtained if bright green fluorescent cells of size and morphology typical of *C. m. sepedonicus* are observed using the FITC filter, and bright red fluorescent cells using the rhodamine filter, in all positive controls and not in any of the negative controls. If bright fluorescing cells with characteristic morphology are found, estimate the average number of typical cells per microscope field and calculate the number of typical cells per mL of resuspended pellet (Appendix 5). Samples with at least 5×10^3 typical cells per mL of resuspended pellet are considered potentially contaminated, so that further testing is required. Samples with less than 5×10^3 typical cells per mL of resuspended pellet are considered negative (no further testing). The FISH test is negative if bright red fluorescent cells with size and morphology typical of *C. m. sepedonicus* are not observed using the rhodamine filter, provided that typical bright red fluorescent cells are observed in the positive control preparations when using the rhodamine filter.

PCR

For detection of *C. m. sepedonicus,* the PCR method of Pastrik (2000) is increasingly being used and appears to be quite robust with regard to specificity, sensitivity and reproducibility. This method was ring-tested in the DIAGPRO project (SMT-4-CT98-2252). The standard procedure is given in EU (2006).

The following hints on PCR may usefully be noted:

- every published PCR protocol is based on its own particular method for extracting cells from tissue, and DNA from cells. These methods vary considerably, and it is therefore advisable always to use the specified extraction method
- early results obtained with a new method are often spurious but, once a method has been used regularly, it should produce reliable results. No PCR protocol should be expected to give reliable results if not used regularly by well-trained staff. This is especially true for detection of *C. m. sepedonicus* in latent infections. Sensitivity is possibly greater than by IF but the two methods have in any case a similar threshold of detection at approximately 10^3 cells per mL
- use validated PCR reagents and protocols, preferably 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 minimize the possibility of contamination with target DNA.

Interpretation of the PCR test result

The PCR test is positive if the *C. m. sepedonicus*-specific 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. The PCR test is negative if the *C. m. sepedonicus*-specific PCR amplicon of expected size is not detected for the sample in question but is detected for all positive control samples. In the case of multiplex PCR with plant-specific internal control primers, a second PCR product of expected size should be amplified with the sample in question.

Real-time PCR

Real-time PCR offers a potentially more robust, specific and sensitive technique. Early studies support this (Schaad *et al*., 1999; Simpkins (pers. comm.). Protocols use the TaqMan system and primers are based on the *C. m. sepedonicus* 50 primers of Mills *et al*. (1997), using an FAM-labelled probe. For the following protocol, ring tests were done under the DIAGPRO project (SMT-4-CT98-2252).

Sample preparation

Suitable methods for DNA purification from potato extracts, using InVitrogen Easy-DNA Extraction kits, are described by Pastrik (2000). A number of other commercially available kits (e.g. Qiagen DNeasy Plant Kit; Promega Wizard Magnetic DNA Purification kit) have similar efficiencies of DNA recovery and inhibitor removal. It is advisable to include a lysozyme step in order to facilitate cell lysis.

Real-time PCR procedure

The following procedure has been optimized for use in Applied Biosystems ABI Prism 7700 or 7900 Sequence Detection Systems. Primers and probe are as given in Schaad *et al*. (1999). The following reagent volumes are used: in a 25 µL reaction volume, 2.5 µL Taqman buffer, 3.5 µL Taqman $MgCl_2$, 0.5 µL of each dNTP (at 10 mM stock), 1 µL of each primer (cms50-2F and cms133R), 0.5 µL of probe (cms50-53T; 5′-FAM, 3′-TAMRA), 0.125 µL of AmpliTaq Gold, 12.375 µL

molecular grade H_2O, and 2 µL of template. Reaction conditions are: 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min (i.e. standard Taqman conditions). All samples are tested in duplicate.

Interpretation of the real-time PCR result

The real-time PCR test is positive if an increase in fluorescence of the reporter (FAM) is observed during the 40 PCR cycles for the sample in question (Ct < 40) but no increase is observed for all negative control samples. The real-time PCR test is negative if no increase in fluorescence of the reporter (FAM) is observed during the 40 PCR cycles for the sample in question but a corresponding increase is observed (critical threshold [Ct] < 40) for all positive control samples. The internal PCR control, e.g. COX assay (Weller *et al*., 2000), should also indicate that host (potato) DNA was freely amplified from the same sample (Ct < 40) as an indication that there was no inhibition or failure of the PCR reaction.

Appendix 4

Bioassay

The bioassay is performed on a sample of the resuspended pellet after tuber extraction (Appendix 2). Use only aubergine plants at leaf stage 2–3, up to full expansion of the third true leaf. Inoculate, by the slit or syringe method (EU, 2006), as many plants as possible with the available sample. This will normally require 15–25 aubergine plants per sample. Withhold water from plants for 1–2 days prior to inoculation to reduce turgor pressure.

As a positive control, inoculate 5 plants with an aqueous freshly prepared suspension of 10^5–10^6 cells mL^{-1} of a known culture of *C. m. sepedonicus* and, where possible, with naturally infected tuber tissue by the same inoculation method. As a negative control, inoculate 5 plants with sterile pellet buffer by the same inoculation method.

Incubate plants for up to 4 weeks at 18–24°C, with sufficient light and high humidity (preferably higher than 70%) 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. Appropriate precautions should be taken to avoid cross contamination.

Examine regularly for symptoms starting after one week. Count the number of plants showing symptoms. *C. m. sepedonicus* causes leaf wilting in aubergine, 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. As soon as symptoms are observed, reisolation should be performed, using sections of wilted leaf tissue or stem tissue from plants (See *Isolation from symptomatic tissue*). Surface-disinfect the leaves and stems by wiping with 70% ethanol. Perform an IF test or PCR on the aubergine sap and isolate on suitable (selective) media (preferably MTNA). A Gram stain may also be prepared. Identify purified cultures of presumptive *C. m. sepedonicus* and confirm pathogenicity.

Under certain circumstances, in particular where growing conditions are not optimal, it may be possible for *C. m. sepedonicus* to exist as a latent infection within aubergines 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, reisolate on suitable (selective) media (preferably MTNA) following the procedure under *Isolation*. Identify purified cultures of presumptive *C. m. sepedonicus* and confirm pathogenicity.

Interpretation of the bioassay test result

For the bioassay test results to be valid, plants of the positive control should show typical symptoms and *C. m. sepedonicus* should be re-isolated from these plants. No symptoms should be found on the negative controls. Given these conditions, the bioassay test result is negative if *C. m. sepedonicus* cannot be re-isolated from test plants, and the bioassay test is positive if *C. m. sepedonicus* can be reisolated from test plants.

Appendix 5. Determination of contamination level in IF and FISH tests

Count the mean number of typical fluorescent cells per field of view (c). Calculate the number of typical fluorescent cells per microscope slide window (C) as:

$$C = c \times S/s$$

Where S = surface area of window of multispot slide; s = surface area of objective field, calculated as:

$$s = \pi i^2/4G^2K^2$$

Where i = field coefficient (varies from 8 to 24 depending upon ocular type), K = tube coefficient (1 or 1.25), G = magnification of objective (100×, 40×, etc.).

Calculate the number of typical fluorescent cells per mL of resuspended pellet (N), as

$$N = C \times 1000/y \times F$$

Where y = volume of resuspended pellet on each window, and F = dilution factor of resuspended pellet.