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EPPO STANDARD ON DIAGNOSTICS

PM 7/59 (2) *Clavibacter sepedonicus*

Specific scope: This Standard describes a diagnostic protocol for *Clavibacter sepedonicus*[1]

This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Specific approval and amendment: This Standard was initially developed under the EU DIAGPRO Project (SMT 4-CT98-2252). It was revised based on outcomes of Euphresco projects.

Approved as an EPPO Standard in 2005-09. Revision approved in 2021-03.

Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Clavibacter sepedonicus is the causal agent of potato ring rot. It was first described from Northern Europe and is now present in several potato-growing areas throughout the northern hemisphere. Traditionally, potato ring rot is a disease favoured by cool temperate climates (EPPO, 2020). 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 A2 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 areas. Direct losses are 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 and disposal costs, and effects on export trade. Certification based on visual inspection has generally not given good control of the disease because the pathogen can remain undetected at low incidence and asymptomatic latent infections can persist for long periods of time. Some potato cultivars rarely show symptoms although the pathogen multiplies within them.

This protocol allows detection and identification of latent infections of *C. sepedonicus* in concentrated potato extracts containing as few as 10^3–10^4 target cells per millilitre.

Flow diagrams describing the diagnostic procedure for *C. sepedonicus* in asymptomatic and symptomatic plant material are presented in Figures 1 and 2.

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

2 | IDENTITY

Name: *Clavibacter sepedonicus* (Spieckermann & Kotthoff 1914) Li et al., 2018.

Other scientific names: *Clavibacter michiganensis* subsp. *sepedonicus* (Spieckermann & Kotthof, 1914) Davis et al., 1984, Gillaspie, Vidaver & Harris 1984; *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, Actinobacteria, Actinomycetales, Microbacteriaceae.

EPPO Code: CORBSE.

Phytosanitary categorization: EPPO A2 list no. 51, EU Annex IIB.

3 | DETECTION

Clavibacter sepedonicus is difficult to detect due to the presence of low incidence, asymptomatic latent infections and the difficulty of obtaining cultures, even from symptomatic material, since it is slow growing on agar media and is easily overgrown by saprophytes.

3.1 | Disease symptoms

Ring rot is a vascular potato disease, affecting both stems and tubers.

3.1.1 | Potato tubers

Tuber infection occurs through the stolon. The initial symptom is a light-yellow glassy discoloration of the vascular ring of the tuber at the stolon end. This develops into a darker creamy yellow to light brown stain of the vascular ring with a crumbly to cheesy consistency (Figure 3), distinct from that of *Ralstonia solanacearum*. In the case of *R. solanacearum*, a creamy fluid exudate usually appears spontaneously on the vascular ring of the cut surface a few minutes after cutting. In the case of *C. michiganensis*, when pressure is applied

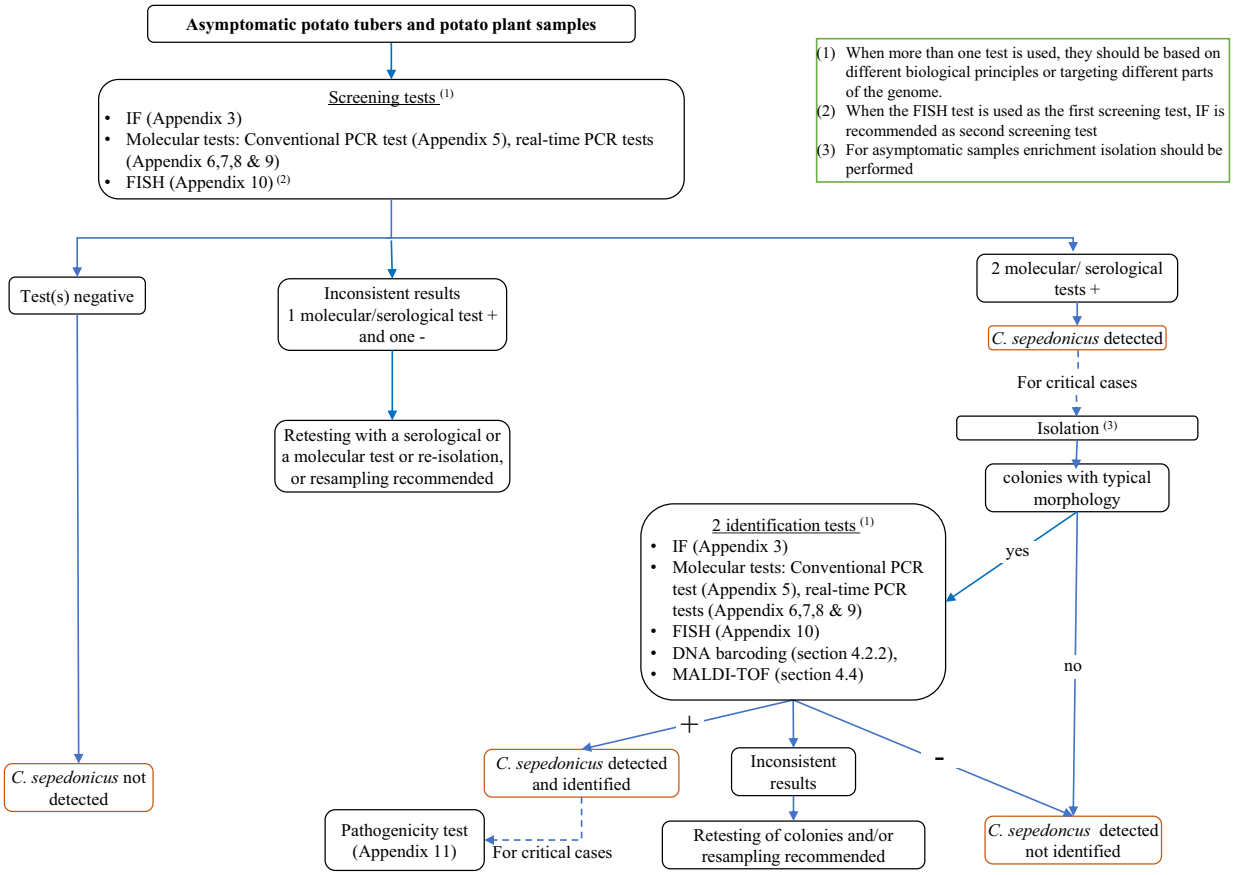

FIGURE 1 Flow diagram describing the diagnostic procedure for *C. sepedonicus* on asymptomatic potato tubers and potato plant samples

on the tuber, a creamy bacterial exudate with cheesy consistency is squeezed out of the vascular area. In severe cases, the vascular ring disintegrates, forming cavities and internal hollowing due to activity of cellulase enzymes (Figures 3 and 4). Such diseased tubers commonly show reddish brown blotches near the eyes and irregular shaped cracks on the skin. The affected tuber may then become crumbly and powdery, and is readily invaded by soft rot bacteria and secondary decay organisms. Infected tubers may mummify. Although not frequently mentioned in the literature, a distinctive feature is the progression of the disease from the heel end to the central cortex of the tuber. Secondary rotting may develop, extending from the vascular tissues into the central cortex.

Tuber symptoms may appear similar to those of brown rot caused by *R. solanacearum* in the initial stages of infection.

3.1.2 | Potato plants

Foliar symptoms are fairly typical of a vascular wilt and generally occur late in the season. They usually start as a wilt of the lower leaves which may involve some leaf rolling. Areas between the leaf veins eventually become chlorotic (Figure 5) and the leaf margins necrotic. Symptoms can be difficult to distinguish from those of other diseases and other crop damage; symptoms can also be masked by the natural senescence of the crop (Elphinstone, 2011). Wilt symptoms caused by *C. sepedonicus* may be confused with those caused by other systemic pathogens, e.g. *R. solanacearum*, *Dickeya* spp., *Pectobacterium* spp., *Rhizoctonia solani*, *Fusarium* spp. or *Verticillium* spp. Wilting caused by *Dickeya* spp. and *Pectobacterium* spp. is commonly associated with blackening of the stems and/or maceration of the mother tuber. Other wilts can be distinguished from those caused by *C. sepedonicus* since for the latter whole leaves or whole plants wilt rapidly. Other pictures of symptoms are available in the EPPO Global Database (EPPO, 2020).

3.2 | Detection in symptomatic plant material

Detection is by isolation (3.2.1), possibly combined with an enrichment bioassay (3.2.2), immunofluorescence (IF) test (3.2.3.1) or molecular tests (3.2.3.2). A fluorescent in situ hybridization test (FISH, 3.2.3.3) may also be used. When the FISH test is used as the first screening test and found to be positive, the IF test should be performed as a second screening test (EU, 2006). *C. sepedonicus* can be considered not detected in a sample based on one

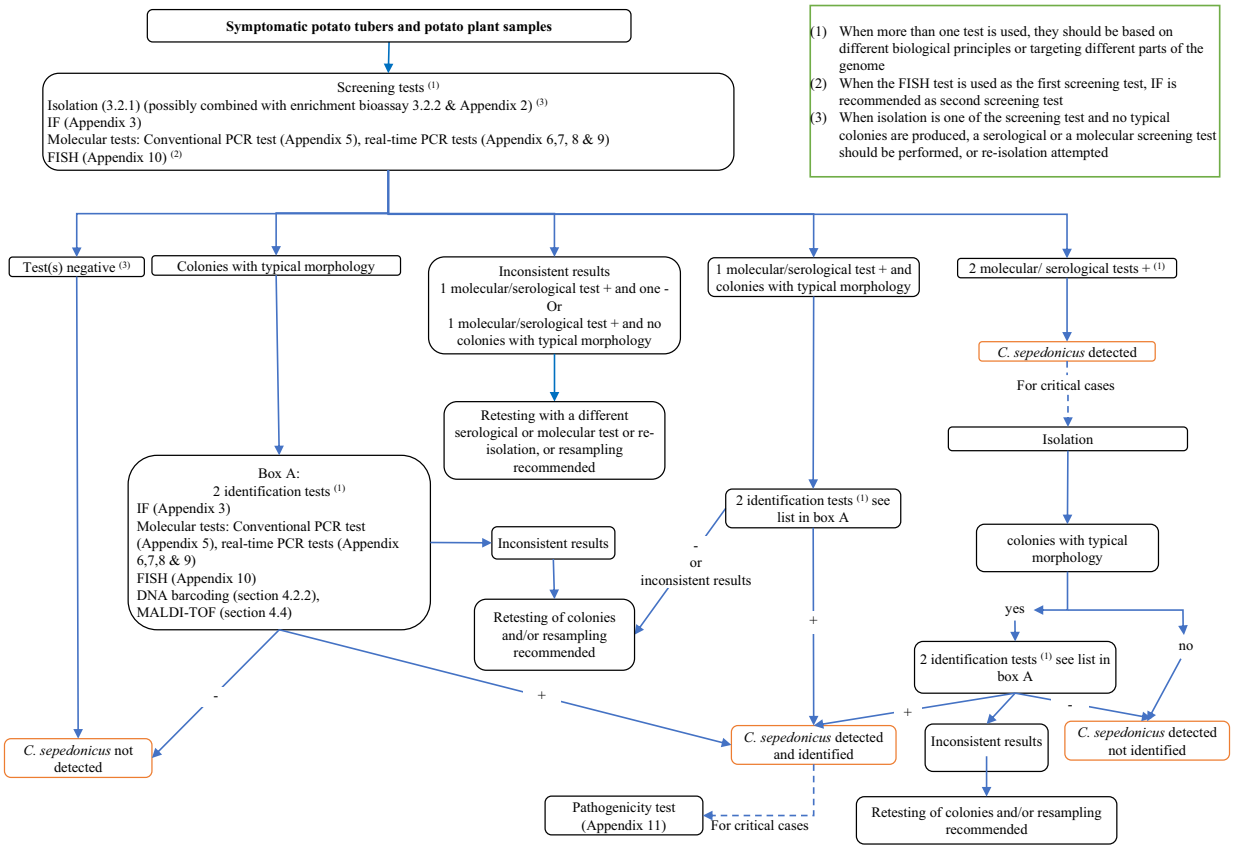

FIGURE 2 Flow diagram describing the diagnostic procedure for *C. sepedonicus* on symptomatic potato tubers and potato plant samples

FIGURE 3 Transverse section of a potato tuber infected by ring rot: discoloration of vascular ring and adjacent tissue, bacterial ooze. Courtesy Central Science Laboratory (now Fera) (GB)

screening test. In critical cases (EPPO, 2018), a positive result from two of these screening tests can be further supported by isolation (if not performed as a screening test) and subsequent identification of the isolated bacterium (see section 4).

3.2.1 | Isolation from symptomatic material

The methods described can be performed on tubers, stems or leaves, including those of test plants, e.g. *Solanum melongena* (aubergine). For isolation, the semiselective medium MTNA (Jansing & Rudolph, 1998) is recommended (Appendix 1). In addition to MTNA, a second non-selective medium such as Yeast Glucose Mineral (YGM) salts medium, Yeast Peptone Glucose Agar (YPGA) or Nutrient Dextrose Agar (NDA) (Lelliott & Stead, 1987) (Appendix 1) may also be used for isolation.

These non-selective media are useful in routine maintenance of pure cultures.

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 *S. melongena* from bioassay and pathogenicity tests. 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 symptomatic 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 1/10 000 dilutions, onto MTNA medium (Appendix 1) using a spread-plate technique. The initial 100 µL of undiluted potato aliquot may be spread out onto a first agar plate with a spreader, and the spreader then used, without flaming, on a second and then third agar plate.

FIGURE 4 Transverse section of a potato tuber infected by ring rot: showing cavities. Courtesy ANSES (FR)

FIGURE 5 Yellowing of plants caused by *C. sepedonicus*. Courtesy MA Kuznetsova (All-Russian Research Institute of Phytopathology)

A strain of *C. sepedonicus* should be plated as a positive control.

Isolation from plant tissues usually takes 3–10 days. The optimum incubation temperature for growth is approximately 21–23°C. Temperatures above 28°C can be deleterious to growth. Presumptive *C. sepedonicus* can then be identified and in critical cases their pathogenicity confirmed (see section 4).

If isolation on media fails, it is recommended to perform a bioassay in aubergine (see section 3.2.2).

3.2.2 | Bioassay in aubergine

The bioassay for *C. sepedonicus* is used as an enrichment method to facilitate isolation on agar media. Some cultivars of aubergine allow excellent selective enrichment of *C. sepedonicus* even in the absence of symptoms and can also be used in confirmatory pathogenicity tests. The bioassay procedure (including recommended cultivars) is described in Appendix 2.

3.2.3 | Other screening tests

3.2.3.1 | Immunofluorescence test

Instructions to perform an IF test are provided in the EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009). Sources of validated antibodies are given in Appendix 3. The IF test is usually performed on undiluted or concentrated plant extracts (see Potato tubers and Plant material other than tubers) and 10-fold dilutions of these in pellet buffer 10 mM PB pH 7.2 (Appendix 1). IF 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 ELISA monoclonal antibody 1H3 available from Agdia Inc.

Use of polyclonal antisera may give some false positive results. In the 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 Inc. is commonly used in the EPPO region.

3.2.3.2 | Molecular tests

DNA extraction procedures are described in Appendix 4.

The following molecular tests are recommended for screening

- Conventional PCR test Pastrik (2000), described in Appendix 5.
- Real-time PCR test Gudmestad et al. (2009) adapted by Vreeburg et al. (2018), described in Appendix 6.
- Real-time TaqMan PCR test Massart et al. (2014), described in Appendix 7.
- NYtor real-time TaqMan PCR test Vreeburg et al. (2018), described in Appendix 8.
- Real-time PCR adapted from Schaad et al. (1999), described in Appendix 9.

On-site tests have been developed by OptiGene (LAMP) and AGDIA (AmplifyRP XRT+) for *C. sepedonicus*. Unlike other tests included in this Standard, these tests have not been evaluated in interlaboratory comparison, but validation data are available from both companies.

3.2.3.3 | Fluorescent in situ hybridization test

The fluorescent in situ hybridization (FISH) test is described in Appendix 10.

3.3 | Detection in asymptomatic plant material

Detection is performed by IF (Immunofluorescence (IF) test) or molecular PCR tests (Molecular tests). A FISH test can also been used. *C. sepedonicus* can be considered not detected in a sample based on one screening test. In

critical cases (EPPO, 2018), a positive result from two different screening tests should be further supported by isolation following a bioassay (enrichment isolation) and subsequent identification of the isolated bacterium (see section 4).

3.3.1 | Test sample requirements

Screening methods have been validated based on a composite sample size of 200 potato tubers or stems from potato randomly collected from the population to be tested. More intensive sampling requires tests on additional subsamples of 200 tubers. The maximum number of tubers or stems that can be processed in one test is 200, as a higher number may lead to inhibition of the tests or difficulty in interpreting the results. The procedure can be conveniently applied to samples of fewer than 200 tubers or stems.

For *in vitro* plants, stem segments up to 2 cm from the base of the plant are collected.

3.3.1.1 | Potato tubers

Potato tubers can be first washed and air dried, if necessary, to remove any excess soil, which may contain saprophytic/opportunistic bacteria that may cause false-positive results in the IF test, and humic acids and other compounds, which can inhibit PCR tests. After removing a small area of peel with a sterile knife from the heel (stolon) end of each tuber, small cores (e.g. 0.2–0.5 g) of the exposed vascular tissue can be removed, keeping the amount of non-vascular tissue to a minimum.

After covering the 200 vascular tuber cores from each sample in sterile 50 mM PB, pH 7.0 (see Appendix 1), the bacteria can be extracted from the tissue by either:

a. rotary shaking (50–100 rpm) for 4 h below 24°C or for 16–24 h refrigerated, or
b. mechanical homogenization in a sealed bag using a suitable grinding apparatus (e.g. a Homex 6 homogenizer) or rubber mallet.

After decanting the supernatant, it can be clarified 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 PB (approximately 10 mL). The bacterial fraction can then be concentrated by centrifugation at 7000 *g* for 15 min (or 10 000 *g* for 10 min) at 4–10°C and discarding the supernatant without disturbing the pellet. After resuspending the pellet in 1.5 mL of 10 mM PB, pH 7.2 (Appendix 1), and in case further testing is required, a proportion of the extract (e.g. 500 µL) should be stored with 10–25% (v/v) sterile glycerol at –16 to –24°C (weeks) or at −68 to −86°C (months). The remainder of the resuspended pellet should be kept refrigerated (approximately 4°C) and used in the screening tests (see section 3.3.2), which should be optimized before use to enable detection of 10^3–10^4 cells/mL of a reference strain of *C. sepedonicus* added to a negative sample of resuspended pellet as a positive control.

3.3.1.2 | Plant material other than tubers

Plant material should preferably be processed immediately or within 72 h if kept refrigerated. Stored samples should be refreshed prior to testing by a cross-section at each end to expose freshly cut xylem vessels.

Stem segments can be disinfected briefly with 70% ethanol and immediately blotted dry on absorbent paper. After covering the stem segments from each sample in sterile 50 mM PB pH 7.0 (Appendix 1), the bacteria can be extracted from the tissue by either:

a. rotary shaking (50–100 rpm) for 4 h below 24°C or for 16–24 h refrigerated, or
b. mechanical homogenisation in a sealed bag using a suitable grinding apparatus (e.g. Homex 6 homogenizer) or rubber mallet.

Further clarification of the extract or concentration of the bacterial fraction is not usually required but may be achieved by filtration and/or centrifugation as described in section Potato tubers.

The neat or concentrated sample extract should then be tested immediately or within 2 h if kept at room temperature. If necessary, the remaining extract can be stored at 4–10°C during the testing period, although this may affect the reliability of pathogen isolation. The remaining extract should be stored with 10–25% (v/v) sterile glycerol at –16 to –24°C (weeks) or at −68 to −86°C(months) in case further testing is required.

3.3.2 | Screening tests

Screening is performed using an IF test (see Immunofluorescence test), a FISH test (see Appendix 10) and/or a molecular test (see Molecular tests). When the FISH test is used as the first screening test and found to be positive, the IF test should be performed as a second screening test (EU, 2006). A positive result from two different screening tests can be further supported by isolation following a bioassay (enrichment isolation see section 3.3.3) and subsequent identification of the isolated bacterium (see section 4). Confirmation of positive screening tests by isolation is recommended in critical cases (EPPO, 2018).

3.3.3 | Enrichment isolation

Isolation of *C. sepedonicus* directly from the heel end cores or stems of latently infected potatoes or aubergine

plants (from bioassay or pathogenicity tests) is possible, although the pathogen may be outgrown by rapidly growing saprophytic bacteria. The method described in section 3.2.1 can be used in parallel to the bioassay (see section 3.2.2).

During the Euphresco interlaboratory comparisons organized in 2009–2012 (van Vaerenbergh et al., 2017) the relative accuracy of semiselective isolation as a screening test was evaluated. Although it improved over the three interlaboratory comparisons (ILC) the relative accuracy remained lower than that for other screening tests (40.3 for ILC1, 70.8 for ILC2 and 87.3 for ILC3).

4 | IDENTIFICATION

When isolation is performed, identification of suspected *C. sepedonicus* isolates should be performed using at least two tests, based on different biological principles or targeting two different parts of the genome for molecular tests. Relevant tests are described below. For critical cases (EPPO, 2018), when a positive identification is made, it is recommended to perform a pathogenicity test to confirm infection in the sample.

4.1 | Immunofluorescence

Instructions to perform an IF test are provided in the EPPO Standard PM 7/ 97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009). Sources of validated antibodies are given in Appendix 3. A suspension of about 10^6 cells/mL is prepared in IF buffer (Appendix 1) and the IF procedure is applied.

4.2 | Molecular methods

4.2.1 | Molecular tests

The following molecular tests are recommended for identification.

- Conventional PCR test Pastrik (2000), described in Appendix 5.
- Real-time PCR test Gudmestad et al. (2009) adapted by Vreeburg et al. (2018), described in Appendix 6.
- Real-time TaqMan PCR test Massart et al. (2014), described in Appendix 7.
- NYtor real-time TaqMan PCR test Vreeburg et al. (2018), described in Appendix 8.
- Real-time PCR adapted from Schaad et al. (1999), described in Appendix 9.

DNA extraction procedures are described in Appendix 4.

4.2.2 | DNA barcoding

A protocol for routine barcoding using the *gyr*B sequences and general procedures for sequencing are described in Appendices 2, 7 and 8 of the EPPO Standard PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, *in press*). Reference sequences from strains are available at https://qbank.eppo.int/bacteria/.

4.3 | FISH test

The FISH test is described in Appendix 10.

4.4 | Matrix-assisted laser desorption/ionization-time of flight mass spectrometry

A matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) method for proteomic analysis has been described by Zaluga et al. (2011). This allows rapid, reliable and robust identification of *C. sepedonicus*. Database entries (mass spectra profiles, MSPs) specific for this species were created prior to the routine identification of isolates from plant samples. For their routine identification, all individual isolates were included in duplicate by directly depositing harvested 3-day-old bacterial cells from nutrient agar plates onto a stainless-steel plate, including a formic acid treatment. All spectra were obtained in linear positive-ion mode with an *m/z* range of 2000–20 000 Da. Validation of the database entries (MSPs) of reference isolates for this species is already available.

4.5 | Other tests

4.5.1 | Genomic fingerprinting tests

Isolates can be reliably identified by matching their unique BOX-PCR genomic fingerprints to those of reference strains of *C. sepedonicus* (see EPPO PM 7/100 *Rep-PCR tests for identification of bacteria*).

4.5.2 | Biochemical characteristics

Differential biochemical characteristics which can help to distinguish the plant pathogenic *Clavibacter* species are described in Li et al. (2018).

4.5.3 | Pathogenicity test

A pathogenicity test on aubergine plants may be performed in critical cases as final confirmation of a

diagnosis of *C. sepedonicus* and for assessment of virulence of cultures identified as *C. sepedonicus*. The test is described in Appendix 11.

5 | REFERENCE MATERIAL

Strains are available from:

	Name of strains from bacterial collections			
Strain description	**NCPPB**	**LMG**	**CFBP**	**DSMZ**
Type strain	2137	2889	2049	20744
Semifluidal colonies	2140	2899	3552	
Small dry, non-mucoid colonies	3898			
Fluidal colonies	4053			

6 | REPORTING AND DOCUMENTATION

Guidance on reporting and documentation is given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7 | PERFORMANCE CRITERIA

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

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Fornefeld, Julius Kühn-Institut, Braunschweig, Germany; eva.fornefeld@julius-kuehn.de

9 | FEEDBACK ON THIS DIAGNOSTIC PROTOCOL

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share, please contact diagnostics@eppo.int.

10 | PROTOCOL REVISION

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

ACKNOWLEDGEMENTS

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). The present revision was prepared by R. Vreeburg, NAK, Emmeloord (NL), J. Elphinstone and A. Aspin Fera Science Ltd, York (GB), J. Van Vaerenbergh and Bart Cottyn, ILVO, Merelbeke (BE), A. Kolodziejska, Central Laboratory, Piorin (PL), F. Poliakoff, Anses (FR) and E. Fornefeld, Julius Kühn-Institut, Braunschweig (DE).

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APPENDIX 1 - MEDIA AND BUFFERS

A MEDIA

All media are sterilized by autoclaving at 121°C for 15 min, except when stated otherwise.

MTNA medium (Jansing & Rudolph, 1998)
Unless otherwise stated all media components are from VWR Chemicals 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
Microbiological grade agar	16.0 g
Distilled water	To make up to 1.0 L

Dissolve ingredients, adjust pH to 7.2. After autoclaving 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 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.

This medium was evaluated during a Euphresco project. 92.9% diagnostic sensitivity was obtained after plating of vascular tissue from symptomatic tubers on MTNA.

Yeast extract glucose mineral salts (YGM) medium (Lelliott & Stead, 1987)

Bacto-Yeast-Extract (Difco)	2.0 g
D(+)-glucose (monohydrate)	2.5 g
K_2HPO_4	0.25 g
KH_2PO_4	0.25 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$MnSO_4 \cdot H_2O$	0.015 g
NaCl	0.05 g
$FeSO_4 \cdot 7H_2O$	0.005 g
Microbiological grade agar	18 g
Distilled water	To make up to 1.0 L

Dissolve ingredients and sterilize in aliquots of 0.5 L volumes of medium by autoclaving at 115°C for 20 min.

Nutrient dextrose agar (NDA)
Oxoid nutrient agar CM0003 (28 g of nutrient agar in 1 L of distilled water) containing 1% D(+)-glucose (monohydrate). Sterilize by autoclaving at 115°C for 20 min.

Yeast peptone glucose agar (YPGA) (Lelliott & Stead, 1987)

Yeast extract	5.0 g
Bacto peptone	5.0 g
Glucose	10.0 g
Microbiological grade agar	15.0 g
Distilled water	To make up to 1.0 L

Adjusted pH to 7.2.

B BUFFERS

IF buffer: 10 mM phosphate buffer saline (PBS), pH 7.2

$Na_2HPO_4 \cdot 12H_2O$	2.7 g
$NaH_2PO_4 \cdot 2H_2O$	0.4 g
NaCl	8.0 g
Distilled water	To make up to 1 L

Adjust pH to 7.2 before autoclaving.

With 0.1% Tween 20, this gives IF-buffer-Tween, used to wash slides.

Pellet buffer: 10 mM phosphate buffer (PB), pH 7.2

$Na_2HPO_4 \cdot 12H_2O$	2.7 g
$NaH_2PO_4 \cdot 2H_2O$	0.4 g
Distilled water	To make up to 1 L

Adjust pH to 7.2 before autoclaving.

Extraction buffer: 50 mM phosphate buffer (PB), pH 7.0

Na_2HPO_4 (anhydrous)	4.26 g
KH_2PO_4	2.72 g
Distilled water	To make up to 1 L

Adjust pH to 7.0 before autoclaving.

Phosphate-buffered glycerol, pH 7.6
This buffer is used as a mountant fluid on the windows of IF slides to enhance fluorescence.

$Na_2HPO_4 \cdot 12H_2O$	3.20 g
$NaH_2PO_4 \cdot 2H_2O$	0.15 g
Glycerol	50 mL
Distilled water	To make up to 100 mL

Adjust pH to 7.6 before autoclaving.

GuHCl buffer

Guanidine hydrochloride	50.95 g
Citric acid monohydrate	0.35 g
Citric acid trisodium (dihydrate)	0.02 g
Polyvinylpyrrolidone 40	2.00 g

EDTA	0.49 g
Triton X-100	0.33 mL
Molecular grade water	To make up to 100 mL

Guanidine hydrochloride is dissolved by first making a slurry of guanidine hydrochloride in 16.7 mL of 96% ethanol before adding the water; pH before autoclaving is 2–2.4.

3× Hybmix buffer for FISH

NaCl	78.9 g
Tris-HCl (pH 7.4)	3.63 g
EDTA (filter sterilized and autoclaved)	2.79 g
Distilled water	To make up to 500 mL

Dilute to 1× Hybmix buffer as required.

Hybridization solution for FISH

1x Hybmix	
Sodium dodecyl sulphate (SDS)	0.01%*
Probe EUB 338	5 ng/µL*
Probe *C. SEPEDONICUS* -CY3-01	5 ng/µL*

* Final concentration of the reagent in the hybridization mix (see Appendix 10).

Prepare quantities of hybridization solution according to the number of slides (Appendix 10).

Note: Store all solutions containing light-sensitive oligo-probes in the dark at −20°C. Protect from direct sunlight or electric light during use.

Fixative solution

Heat 9 mL of molecular grade water [e.g. ultra-pure water (UPW)] to about 60°C.

Add 400 mg of paraformaldehyde. Paraformaldehyde will dissolve after adding five drops of 1 N NaOH and stirring with a magnetic stirrer.

Adjust pH to 7.0 by addition of 1 mL of 0.1 M phosphate buffer (PB; pH 7.0) and five drops of 1 N HCl. Check pH with indicator strips and adjust if necessary, with HCl or NaOH.

Filter the solution through a 0.22 µm membrane filter and keep dust-free at 4°C until use.

Note: An alternative fixative is 96% ethanol. To use this dissolve the pellet in 50 µL of 0.01 M PB and 50 µL of 96% ethanol. Vortex mix and incubate at 4°C for 30–60 min.

Anti-fading mountant solutions

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

Additional components may be useful as follows:

Deflocculant for use with homogenization extraction method	
Lubrol flakes	0.5 g
Antifoam agent for use with homogenization extraction method	
DC silicone antifoam	1.0 mL
Antioxidant	
Tetrasodium pyrophosphate	1.0 g
Binding of PCR inhibitors	
Polyvinylpyrrolidone-40000 (PVP-40)	50.0 g

APPENDIX 2 - BIOASSAY

The bioassay may be performed prior to isolation or other screening tests. The bioassay is performed on an aliquot of the resuspended pellet after tuber extraction (Potato tubers). Only *S. melongena* (aubergine) plants should be used. The recommended cultivar is 'Black Beauty' but other cultivars with a similar susceptibility may be used (e.g. 'Long Tom', 'Balsas', 'Rima'). For inoculation, plant should be at leaf stage 2–3, up to full expansion of the third true leaf. Inoculate, by the slit or syringe method (see below), as many plants as possible with the available aliquot. 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 in the stems.

INOCULATION

- Slit inoculation

  Holding the stem of the plant between two fingers, pipette a drop (approximately 5–10 µL) of the suspended pellet onto the stem between the cotyledons and the first leaf. Using a sterile scalpel, make a diagonal slit, about 1 cm long and approximately 2/3 of the stem thickness deep, starting the cut from the pellet drop. Seal the cut with sterile Vaseline from a syringe.

- Syringe inoculation

  A 10 µL droplet of pellet suspension is pipetted in the stem at the cotyledons and is introduced by piercing with an entomological needle in the droplet through the stem. When test plants have low turgor, this volume is readily absorbed.

CONTROLS

As a positive control, inoculate five plants with a freshly prepared suspension of 10^5–10^6 cells/mL of a reference culture of *C. sepedonicus* in the sterile pellet buffer and, where possible, with naturally infected tuber tissue using the same inoculation method. As a negative control, inoculate five plants with sterile pellet buffer using the same inoculation method.

GROWTH CONDITIONS

Optimum growth temperature is approximately 21°C. Incubate plants for up to 4 weeks at 18–24°C, with sufficient light and high humidity (preferably higher than 70%) and conditions to prevent water logging or wilting through water deficiency. Appropriate precautions should be taken to avoid cross-contamination.

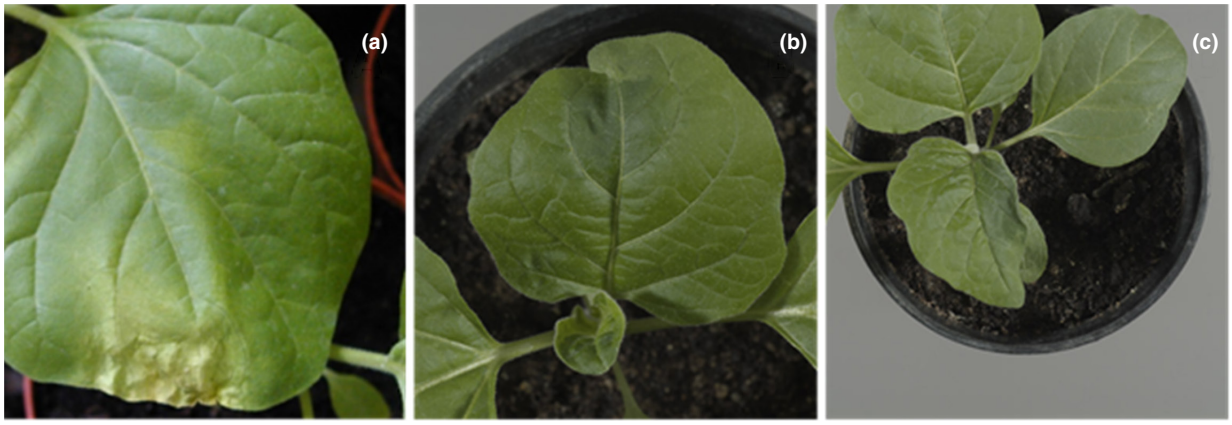

FIGURE A1 Symptoms on aubergine. A Courtesy ILVO (BE). B & C Courtesy Central Science Laboratory (now Fera) (GB)

INSPECTION OF PLANTS

Examine regularly for symptoms starting after 1 week.

- In case of symptoms

C. sepedonicus causes leaf wilting in *S. melongena*, which may commence as a marginal or interveinal flaccidity. Wilted tissue may initially appear dark green or mottled but turns paler before becoming necrotic (Figure A1). Interveinal wilts often have a greasy water-soaked appearance. Necrotic tissue often has a bright yellow margin. As soon as symptoms are observed, re-isolation should be performed, using sections of wilted leaf tissue, petiole of the affected leaf, or stem tissue (see section 3.2.1). Surface-disinfect the leaves and stems by wiping with 70% ethanol. Perform an IF test or PCR on the extract and isolate by dilution plating on suitable (selective) media (preferably MTNA) and/or non-selective media (e.g. NDA). A Gram stain may also be prepared. Identify purified cultures of presumptive *C. sepedonicus* and confirm pathogenicity (critical cases).

- When no symptoms are observed

Under certain circumstances, in particular where growing conditions are not optimal, it may be possible for *C. sepedonicus* to exist as a latent infection within *S. melongena* even after incubation periods up to 4 weeks.

If no symptoms are observed after 2 weeks, perform IF/molecular test or isolation from a composite sample of 1 cm stem sections of each test plant taken above the inoculation point.

INTERPRETATION OF THE BIOASSAY TEST

The bioassay test is valid when positive control plants show typical symptoms, *C. sepedonicus* can be detected and no symptoms are found on the negative controls.

The bioassay test is negative if *C. sepedonicus* is not detected in test plants and provided that *C. sepedonicus* is detected in positive controls.

The bioassay test is positive if *C. sepedonicus* is detected in test plants, regardless of whether symptoms develop or not.

APPENDIX 3 - IMMUNOFLUORESCENCE

Instructions to perform an IF test are provided in the EPPO Standard PM 7/ 97 *Indirect immunofluorescence test for plant pathogenic bacteria*.

C. sepedonicus is a typical, small Gram-positive 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.

The following commercially available antibodies have been tested in different interlaboratory comparisons:

Monoclonal antibody
Agdia (MAb 9A1)

Polyclonal antibodies
Loewe
Prime Diagnostics

Performance characteristics available

- Different antibodies: test results from the Euphresco interlaboratory comparison (2009-2012) are available in van Vaerenbergh et al. (2017).

Interlaboratory comparisons 1 and 2 were performed with antibodies from Prime Diagnostics and Agdia and Inter laboratory comparison 3 with antibodies used by the laboratories (Loewe, Prime Diagnostics, Agdia). The relative accuracy obtained was as follows (data for individual antibodies not available):

Interlaboratory comparison	Relative accuracy (%)[1]
Interlaboratory comparison 1	94
Interlaboratory comparison 2	96.8
Interlaboratory comparison 3	94.4

[1] As defined in Appendix 6 of PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* (EPPO, 2019).

- Antibody from Loewe
  - Vreeburg et al. (2016)

Analytical sensitivity 100% detection at 10^6 cfu/mL.

Diagnostic performance of IF compared to the infection status as determined by the EU directive testing scheme (EU, 2006).

Diagnostic sensitivity: 100%

Diagnostic specificity: 97.9%

Relative accuracy: 98.5 %

- Data from Loewe

The values have been determined according to the IF test procedure outlined in the manufacturer's instructions. All samples were at a concentration of 10^7 cells/mL and an antiserum dilution of 1:10 000.

	Visual evaluation (sec. Antibody Cy3-labelled)*
Inclusivity	
C. sepedonicus LMG 2889	++ to +++
C. sepedonicus LMG 2899	++
C. sepedonicus LMG 5870	++
C. sepedonicus LMG 5876	++
Exclusivity	
C. insidiosus DSMZ20157	++
C. michiganensis subsp. *michiganensis* LMG3680	Not detected
C. michiganensis subsp. *michiganensis* LMG5427	Not detected
C. michiganensis subsp. *michiganensis* DSMZ46364	+
Dickeya chrysanthemi DSMZ4610	Not detected
Dickeya chrysanthemi DSMZ30177	Not detected
Dickeya chrysanthemi DSMZ30178	Not detected
Pectobacterium atrosepticum St90BafZ	(+)
Pectobacterium atrosepticum DSMZ30168	(+)
Pectobacterium atrosepticum S12	(+)
Pseudomonas syringae LfL 06/215/1a	Not detected
Ralstonia solanacearum PD3275	Not detected
Ralstonia solanacearum PD3276	Not detected
Ralstonia solanacearum PD3278	Not detected
Ralstonia solanacearum 939-1	Not detected
Rhodococcus fascians GSPB369	Not detected

* Differentiation of the fluorescence signal: +++, very strong signal; ++, good signal; +, low signal.

- Monoclonal antibody AGDIA

Analytical sensitivity: data not available.

Analytical specificity

Inclusivity: positive results with 19 *C. sepedonicus* strains.

Exclusivity: did not react with representative strains of *Curtobacterium flaccumfaciens* pv. betae, *Rhodococcus fascians*, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, *Clavibacter insidiosus*, *Rathayibacter iranicus*, *Clavibacter michiganensis* subsp. *michiganensis*, *Rathayibacter tritici*, *Curtobacterium flaccumfaciens* pv. *oortii*, *Curtobacterium flaccumfaciens* pv. *poinsettiae*, *Rathayibacter rathayi*.

APPENDIX 4 - DNA EXTRACTION

Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out

1. DNA extraction from plant material

1.1. Procedure 1

DNA extraction from potato tuber tissue is performed using the Invitrogen Easy DNA^M kit, according to the manufacturer's instructions, and with the addition of 1 mg per 100 µL of lysozyme in the lysis buffer and heating to 37°C for 30 min, as validated by Pastrik and Maiss (2000).

If alternative extraction kits are used, they should first be shown to reliably extract amplifiable target DNA from a known negative plant extract to which has been added between 10^3 and 10^4 cfu/mL of a reference strain of *C. sepedonicus* as a positive control.

DNA should preferably be stored at approximately −20°C.

1.2. Procedure 2

An alternative DNA extraction protocol has been published in Vreeburg et al., 2018.

Allow the solid particles in the resuspended pellet to settle to the bottom and mix 100 µL of the supernatant with 11 µL of lysozyme solution (25 mg/mL lysozyme; Sigma-Aldrich Zwijndrecht, the Netherlands) in 100 mM Tris (Thermo Fisher Scientific, Waltham MA, USA) with 10 mM EDTA, pH 8.0.

Incubate by shaking at 37°C for 30 min.

Add 11 µL of 10% w/v SDS, 15 µL of 5 M NaCl and 20 µL of GuHCl buffer (Appendix 1). Incubate by shaking at 95°C for 15 min and then cool on ice. Next, add ice-cold MPC protein precipitation solution (EpiCentre, Madison, WI, USA), mix and keep on ice for 5 min, followed by centrifugation for 10 min at 3500 *g* at 4–20°C. Transfer the supernatant to a tube or deep-well block prefilled with 225 µL (per well) of isopropanol and 20 µL of SNAP bead solution (Stratec Molecular, Birkenfeld,

Germany). Wash the beads three times with 400 µL of 70% ethanol and elute with 100 µL of ultrapure water at 65°C. DNA should preferably be stored at approximately −20°C.

2. DNA extraction from colonies

For crude DNA extraction from presumptive *C. sepedonicus* colonies and from cultures of reference strains, suspend approximately 1 µL of cell material (e.g. using a 1-µL loop) or one colony in 100 µL of sterile distilled water. Heat in closed microvials at approximately 95°C or 100°C for a minimum of 10 min. A freezing step before the heating may be performed.

Alternatively, a cell suspension in 0.05 mM NaOH can be prepared. 100 µL of the cell suspension in closed tubes is heated at approximately 95°C for approximately 5 min.

The lysate can be stored at approximately −20°C.

APPENDIX 5 - CONVENTIONAL PCR (PASTRIK, 2000)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This conventional PCR is suitable for the detection and identification of *C. sepedonicus* in potato plant and tubers and on isolates.

1.2. For the detection of *C. sepedonicus*, the pathogen-specific primers set PSA-I/PSA-R is used, which is based on the intergenic spacer region of the 16S-23S rRNA genes of *C. sepedonicus.*

1.3. For the amplification of the internal PCR control, the plant-specific primer set NS-7-F/ NS-8-R is used, based on the sequences from 18S rRNA genes able to amplify a DNA fragment of plants (potato, aubergine, tomato).

1.4. Expected amplicon size from *C. sepedonicus* template DNA is 502 bp (PSA-primer set) and expected amplicon size from 18S rRNA internal PCR control is 377 bp (NS-primer set).

1.5. Oligonucleotides.

Forward primer	PSA-I	5′-CTC CTT GTG GGG TGG GAA AA-3′
Reverse primer	PSA-R	5′-TAC TGA GAT GTT TCA CTT CCC C-3′
Forward primer	NS-7-F	5′-GAG GCA ATA ACA GGT CTG TGA TGC-3′
Reverse primer	NS-8-R	5′-TCC GCA GGT TCA CCT ACG GA-3′

2. Methods

2.1. Nucleic acid extraction and purification.

2.1.1. Tissue source: plant, tubers, pure culture suspension.

2.1.2. DNA extraction procedures from plants, tubers and cultures are described in Appendix 4 (sections 1.1 and 2). The extraction procedure 1.2 (Vreeburg et al., 2018) has not been evaluated for this PCR.

2.1.3. Storage temperature and conditions: DNA should preferably be stored at approximately −20°C.

2.2. Conventional PCR.

2.2.1. Master Mix (Pastrik, 2000).

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	14.975	NA
PCR buffer (Invitrogen)	10×	2.5	1×
$MgCl_2$	50 mM	0.75	1.5 mM
BSA (fraction V)	10%	0.25	0.1%
dNTPs mix	20 mM	0.125	0.1 mM
Forward primer PSA-I	10 µM	0.5	0.2 µM
Reverse primer PSA-R	10 µM	0.5	0.2 µM
Forward primer NS-7-F	10 µM	0.1	0.04 µM
Reverse primer NS-8-R	10 µM	0.1	0.04 µM
Platinum Taq DNA polymerase (Invitrogen)	5 U/µL	0.2	1.0 U
Subtotal		20.0	
DNA extract		5.0	
Total		25.0	

2.2.2. PCR conditions

95°C for 3 min followed by 10 cycles of: 95°C for 1 min, 64°C for 1 min, 72°C for 1 min and 25 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 1 min and a final step of 72°C for 5 min.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: DNA of *C. sepedonicus* equivalent to a concentration of approximately 10^4 cfu/mL.

The PCR uses an internal positive control (IPC) to monitor each individual sample separately. The positive internal control target DNA is the 18S rRNA gene present in the matrix.

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract: same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: to assign results from PCR-based test the following criteria should be followed

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC and PAC (and IPC) should produce amplicons of the expected size.

When these conditions are met

- A test will be considered positive if amplicons of 502 bp are produced.
- A test will be considered negative if it produces no band or band(s) of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Data from Pastrik (2000).

4.1. Analytical sensitivity data

It was possible to detect artificially added *C. sepedonicus* in potato core fluid in the range of 2–20 cfu per PCR reaction mixture (20–200 cfu/mL potato core fluid).

4.2. Analytical specificity data

Inclusivity: 100% tested on seven target strains. All strains of *C. sepedonicus* were positive.

Exclusivity: 100% tested on 50 non-target strains (including 14 strains of four other species of *Clavibacter*).

APPENDIX 6 - REAL-TIME TAQMAN PCR TEST FOR DETECTION AND IDENTIFICATION OF *C. SEPEDONICUS* (GUDMESTAD ET AL., 2009 ADAPTED BY VREEBURG ET AL., 2018)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The test was published by Gudmestad et al. (2009), adapted by Vreeburg et al. (2018). It is used for the detection and identification of *C. sepedonicus*

1.2. The test was optimized for and validation data were obtained with potato tuber heel end cores. It may be used in other matrices, but a verification (see PM 7/98) should at least be conducted by the laboratories. Validation data were obtained using the DNA extraction method given in Appendix 4, section 1.2.

1.3. The target gene of this test is the Cellulase A gene, located on the native plasmid pCS1 (AY007311).

1.4. Oligonucleotides:

Forward primer	CelA_F	TCT CTC AGT CAT TGT AAG ATG AT
Reverse primer	CelA_R	ATT CGA CCG CTC TCA AA
Probe	CelA_probe	[FAM]-TTC GGG CTT CAG GAG TGC GTG T-[BHQ1]

1.5. The PCR was optimized for and the validation data were obtained with an ABI 7500 real-time PCR system.

1.6. Software and analysis setting should be validated in the laboratory to meet the requirements of the test.

2. Methods

2.1. Nucleic acid extraction and purification.

2.1.1. Tissue source: validated on tubers, may also be used for plant, pure culture suspension.

2.1.2. DNA extraction procedures from plants and potatoes are described in Appendix 4 (validation data were obtained with the procedure described in section 1.2).

2.1.3. Storage temperature and conditions: DNA should preferably be stored at approximately −20°C.

2.2. Real-time polymerase chain reaction.

2.2.1. Master Mix.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	5.4	NA
iTaq Universal Probes Supermix (Bio-Rad)	2×	10	1×
Forward primer (*CelA_F*)	10 µM	1	0.5 µM
Reverse primer (*CelA_R*)	10 µM	1	0.5 µM
Probe 1 (*CelA_probe*)	5 µM	0.6	0.15 µM
Subtotal		18	
DNA extract		2	
Total		20	

2.2.2. PCR conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of 15 s at 95°C and 60 s at 62°C.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). DNA of *C. sepedonicus* equivalent to a concentration of approximately 10^4 cfu/mL.

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: to assign results from PCR-based test the following criteria should be followed

Verification of the controls

- The PIC and PAC (as well as IC) amplification curves should be exponential, and the Ct value in the expected range.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation was carried out in accordance with PM7/98 and was published in Vreeburg et al. (2018).

4.1. Analytical sensitivity data

The test was developed and validated to classify 95% of samples with 5×10^3 cfu/mL positive, using a regression approach with a Ct cut-off value established in the laboratory.

4.2. Analytical specificity data

The test was 100% accurate, tested on seven *C. sepedonicus* isolates and 77 non-target strains, including potentially cross-reacting species and species that can be present on potato tubers.

4.3. Data on repeatability

100% for potato extracts spiked with 10^4 to 10^5 cfu/mL of different *C. sepedonicus* strains.

4.4. Data on reproducibility

100% for potato extracts spiked with 10^4 to 10^5 cfu/mL of different strains when performed in one laboratory. This test was part of a test performance study (TPS) in 2018. In this TPS, the Gudmestad et al., 2009 test detected 100% of the provided DNA samples isolated from extracts spiked with 1.2×10^4, 2.4×10^5 and 2.4×10^7 cfu/mL. Reproducibility including DNA extraction by the participating laboratories, using their own preferred extraction method, was 52% for 1.2×10^4, 83% for 2.4×10^5 and 89% for 2.4×10^7 cfu/mL.

APPENDIX 7 - REAL-TIME TAQMAN PCR TEST FOR DETECTION AND IDENTIFICATION OF *C. SEPEDONICUS* (MASSART ET AL., 2014)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. The test was published by Massart et al. (2014). It is used for the detection and identification of *C. sepedonicus*. This real-time PCR was developed and validated as a multiplex test targeting *C. sepedonicus* and *R. solanacearum* in asymptomatic potato tubers.

1.2. The real-time PCR was optimized for and the validation data were obtained with potato tuber heel end cores. It may be used in other matrices, but a verification (see PM 7/98) should at least be conducted by the laboratories. Validation data were obtained using DNA extraction methods given in Appendix 4, section 1.2.

1.3. The target gene for *C. sepedonicus* is the rRNA intergenic transcribed spacer region amplified by the primers and probe: MultiClav_F, MultiClav_R and MultiClav_P.

1.4. The positive internal control has been developed based on the sequence of a chloroplastic gene of ATP synthase beta-subunit present in potato, amplified by the primers and probe: MultiPot F, MultiPot_R and MultiPot_P.

1.5. Oligonucleotides:

Forward primer	MultiClav_F	TGG TTT CTT GTC GGA CCC TTT
Reverse primer	MultiClav_R	CGT CCA CTG TGT AGT TCT CAA TAT ACG
Probe	MultiClav_P	[FAM]- CGT CGT CCC TTG AGT GG -[mgb-NFQ]
Forward primer	MultiPot_F	GGT TTC GTA ATG TTC CTC ACC AA
Reverse primer	MultiPot_R	AAA GGT ATT TAT CCA GCA GTA GAT CCT T
Probe	MultiPot_P	[NED]-CAT GGT TGA CGT TGA AT-[mgb-NFQ]

This PCR was designed and validated as a multiplex with *R. solanacearum* (not shown here).

1.6. The real-time PCR was optimized for and the validation data were obtained with an ABI 7500 real-time PCR system.

1.7. Software and analysis setting should be validated in the laboratory to meet the requirements of the test.

2. Methods

2.1. Nucleic acid extraction and purification.

2.1.1. Tissue source: validated on tubers, may also be used for plant, pure culture suspension.

2.1.2. DNA extraction procedures from plants and potatoes are described in Appendix 4 (validation data were obtained with the procedure described in section 1.2).

2.1.3. Storage temperature and conditions: DNA should preferably be stored at approximately −20°C.

2.2. Real-time polymerase chain reaction.

2.2.1. Master Mix.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	1.1[1]	NA
QuantiTect Multiplex PCR Master Mix (Qiagen)	2×	5	1×
Forward primer (MultiClav_F)	10 µM	0.3	0.3 µM
Reverse primer (MultiClav_R)	10 µM	0.3	0.3 µM
Probe 1 (MultiClav_P)	5 µM	0.2	0.1 µM
Forward primer (MultiPot_F)	10 µM	0.3	0.3 µM
Reverse primer (MultiPot_R)	10 µM	0.3	0.3 µM
Probe 2 (MultiPot_P)	5 µM	0.5	0.25 µM
Subtotal		8	
DNA extract		2	
Total		10	

[1] This volume is changed to 0.3 µL per reaction when the Massart et al. (2014) real-time PCR is run with the *R. solanacearum* primers and probes. These are used in the same concentrations and volumes as *C. sepedonicus* primers and probes.

2.2.2. PCR conditions: initial denaturation at 95°C for 15 min, followed by 40 cycles of 20 s at 95°C and 60 s at 60°C.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid

extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). DNA of *C. sepedonicus* equivalent to a concentration of approximately 10^4 cfu/mL.

The Massart et al. (2014) real-time PCR uses an internal positive control (IPC) to monitor each individual sample separately. The positive internal control target is a chloroplastic gene of ATP synthase beta-subunit present in the potato DNA.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: to assign results from PCR-based test the following criteria should be followed

Verification of the controls
- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met
- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation was carried out in accordance with PM 7/98 and was published in Vreeburg et al. (2018).

4.1. Analytical sensitivity data

The test was developed and validated to classify 95% of samples with 5×10^3 cfu/mL positive, using a regression approach with a Ct cut-off value established in the laboratory.

4.2. Analytical specificity data

Inclusivity: 100 % evaluated on seven *C. sepedonicus* isolates.

Exclusivity: 99% the exclusivity was evaluated on 77 non-target strains, including potentially cross-reacting species and species that can be present on potato tubers. The test gave a positive signal for *C. tessellarius* strain LMG7292.

4.3. Data on repeatability

100% for potato extracts spiked with 10^4 to 10^5 cfu/mL of different *C. sepedonicus* strains.

4.4. Data on reproducibility

100% for potato extracts spiked with 10^4 to 10^5 cfu/mL of different strains when performed in one laboratory. This test was part of a test performance study (TPS) in 2018. In this TPS, the Massart et al. (2014) test detected 100% of provided DNA samples isolated from extracts spiked with 1.2×10^4, 2.4×10^5 and 2.4×10^7 cfu/mL. Reproducibility including DNA extraction by the participating laboratories, using their own preferred extraction method, was 88% for 1.2×10^4, 88% for 2.4×10^5 and 94% for 2.4×10^7 cfu/mL.

APPENDIX 8 - NYtor REAL-TIME TAQMAN PCR TEST FOR DETECTION AND IDENTIFICATION OF *C. SEPEDONICUS* (VREEBURG ET AL., 2018)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The test was published by Vreeburg et al. (2018). It is used for the detection and identification of *C. sepedonicus*. This real-time PCR is designed and

validated as a multiplex test targeting *C. sepedonicus*, *R. solanacearum* and *R. pseudosolanacearum* in asymptomatic potato tubers.

1.2. The PCR was optimized for and the validation data were obtained with potato tuber heel end cores. It may be used in other matrices, but at least a verification should be conducted by the laboratories. Validation data were obtained using the DNA extraction method given in Appendix 4, section 1.2.

1.3. The target gene in this test for *C. sepedonicus* is a genomic DNA fragment designated Cms-50, coding for a hypothetical protein, amplified by the primers and probe: Cms_F, Cms_R and Cms_P. This is the same region that is targeted by the real-time PCR Schaad et al. (1999) (Appendix 9). Cms-50 is from Mills et al., 1997.

1.4. The positive internal control target is a conserved region of the ATP synthase β gene from *Solanum tuberosum,* amplified by the primers and probe: Stub_F, Stub_R and Stub_P.

1.5. Oligonucleotides:

Forward primer	Cms_F	TGC TGA TAA CGT GAT CAA G
Reverse primer	Cms _R	CTG AGC AAC GAC AAG AAA
Probe	Cms _P	[ATTO647N]-ATG GCT CCT CGG TCC TTG AAT GTC-[BHQ3][1]
Forward primer	Stub_F	CGG ATA ATT CGT CCA ATC
Reverse primer	Stub_R	CCA GCA GTA GAT CCT TTA
Probe	Stub_P	[ATTO532]-CAA CCA TGC TTC AAC CTC GGA TC-[BHQ1][1]

[1] This PCR was designed and validated as a multiplex with *R. solanacearum* and *R. pseudosolanacearum* (not shown here).

1.6. The PCR was optimized for and the validation data were obtained with an ABI 7500 real-time PCR system.

1.7. Software and analysis setting should be validated in the laboratory to meet the requirements of the test.

2. Methods

2.1. Nucleic acid extraction and purification.

2.1.1. Tissue source: validated on tubers, may also be used for plant material or pure culture suspension.

2.1.2. DNA extraction procedures from plants and potatoes are described in Appendix 4 (validation data were obtained with the procedure described in section 1.2).

2.1.3. Storage temperature and conditions: DNA should preferably be stored at approximately −20°C.

2.2. Real-time polymerase chain reaction.

2.2.1. Master Mix.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	3.6[1]	NA
iTaq Universal Probes Supermix (Bio-Rad)	2×	10	1×
Forward primer (Cms_F)	10 µM	0.4	0.2 µM
Reverse primer (Cms_R)	10 µM	0.4	0.2 µM
Probe 1 (Cms_P)	2 µM	0.2	0.02 µM
Forward primer (*stub_f*)	10 µM	0.1	0.05 µM
Reverse primer (*stub_r*)	10 µM	0.1	0.05 µM
Probe 2 (*Stub_P*)	1 µM	0.2	0.01 µM
Subtotal		15	
DNA extract		5	
Total		20	

[1] This volume is changed to 0 µL per reaction when the NYtor PCR is run with the *R. solanacearum* and *R. pseudosolanacearum* primers and probes. See Vreeburg et al. (2018).

2.2.2. PCR conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic

acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). DNA of *C. sepedonicus* equivalent to a concentration of approximately 10^4 cfu/mL.

The NYtor real-time PCR uses an internal positive control (IPC) to monitor each individual sample separately. The positive internal control target is a chloroplastic gene of ATP synthase beta-subunit present in the potato DNA.

Alternative internal positive controls can include:

- specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: assign results from PCR-based test the following criteria should be followed

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation was carried out in accordance with PM 7/98 and validation data are published in Vreeburg et al. (2018).

4.1. Analytical sensitivity data

The test was developed and validated to classify 95% of samples with 5×10^3 cfu/mL positive, using a regression approach with a Ct cut-off value established in the laboratory.

4.2. Analytical specificity data

Inclusivity: 100%, evaluated on seven *C. sepedonicus* isolates.

Exclusivity: 100%. The exclusivity was evaluated on 77 non-target strains, including potentially cross-reacting species and species that can be present on potato tubers.

4.3. Data on repeatability

100% for potato extracts spiked with 10^4 to 10^5 cfu/mL of different *C. sepedonicus* strains.

4.4. Data on reproducibility

100% for potato extracts spiked with 10^4 to 10^5 cfu/mL of different strains when performed in one laboratory. This test was part of a test performance study (TPS) in 2018. In this TPS, the NYtor test detected >95% of provided DNA samples isolated from extracts spiked with 1.2×10^4, 2.4×10^5 and 2.4×10^7 cfu/mL. Reproducibility including DNA extraction by the participating laboratories, using their own preferred extraction method, was 58% for 1.2×10^4, 75% for 2.4×10^5 and 88% for 2.4×10^7 cfu/mL.

APPENDIX 9 - REAL-TIME PCR ADAPTED FROM SCHAAD ET AL. (1999)

The test below is described as it was carried out to generate the validation data from Vreeburg et al. (2016) *provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.*

1. General Information

1.1. The test was published by Schaad et al. (1999). It is used for the detection and identification of *C. sepedonicus*.

1.2. The target gene in this test for *C. sepedonicus* is a genomic DNA fragment designated Cms 50. This is the same region that is targeted by the real-time PCR Vreeburg et al. (2018) (Appendix 8). Cms-50 is from Mills et al., 1997.

1.3. COX-F, COX-R and COX-P real-time PCR primers and probe target the plant cytochrome oxidase gene sequences co-extracted from plant samples and are used as internal positive amplification control.

1.4. Oligonucleotides:

Forward primer	Cms 50-2-F	CGG AGC GCG ATA GAA GAG GA
Reverse primer	Cms 133-R	GGC AGA GCA TCG CTC AGT ACC
Probe	Cms 50-53T	[FAM]- AAG GAA GTC GTC GGA TGA AGA TGC G -[TAMRA]

Forward primer	COX-F	CGT CGC ATT CCA GAT TAT CCA
Reverse primer	COX-R	CAA CTA CGG ATA TAT AAG AGC CAA AAC TG
Probe	COX-P	[VIC]- TGC TTA CGC TGG ATG GAA TGC CCT -[TAMRA]

2. Methods

2.1. Nucleic acid extraction and purification.

2.1.2. Tissue source: validated on tubers, may also be used for plant, pure culture suspension.

2.1.3. DNA extraction procedures from plants and potato tubers: QuickPick Plant DNA kit (BioNobile) followed by DNA purification on PVPP columns (Vreeburg et al., 2016).

2.1.4. Storage temperature and conditions: DNA should preferably be stored at approximately −20°C.

2.2. Master Mix.

2.2.1. Real-time polymerase chain reaction.

Reagent	Working concentration	Quantity per reaction (µL)	Final concentration
Molecular-grade water	NA	1.5	NA
TaqMan Universal PCR Master Mix (Thermo Fisher)	2×	12.5	1×
Forward primer Cms 50-2-F	10 pmol/µL	0.75	300 nM
Reverse primer Cms 133-R	10 pmol/µL	0.75	300 nM
Probe Cms 50-53T	10 pmol/µL	0.75	300 nM
Forward primer COX-F	10 pmol/µL	1.5	600 nM
Reverse primer COX-R	10 pmol/µL	1.5	600 nM
Probe COX-P	10 pmol/µL	0.75	300 nM
Subtotal			20
DNA extract		5.0	
Total volume:		25.0	

2.2.2. PCR conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). DNA of *C. sepedonicus* equivalent to a concentration of approximately 10^4 cfu/mL.

The PCR uses an internal positive control (IPC) to monitor each individual sample separately. The positive internal control target DNA is a mitochondrial gene of cytochrome oxidase I present in the matrix.

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract: same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: to assign results from PCR-based test the following criteria should be followed

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential, and the Ct value in the expected range.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Data from Vreeburg et al. (2016)

Analytical sensitivity: 10^3 cfu/mL
Diagnostic sensitivity: 100%
Diagnostic specificity: 99.5%
Accuracy: 99.6%

Additional validation data

In 2019, a proficiency test was organized in Germany with nine of the participating laboratories using the real-time PCR adapted from Schaad et al. (1999) for blind testing of extracts from potato tubers spiked with *C. sepedonicus*. A set of 10 samples was tested of which two samples were spiked with *C. sepedonicus* at final concentrations of ≈10^6 and ≈10^5 cfu/mL, respectively, three samples were spiked with *R. solanacearum* and five samples were healthy.

Data on diagnostic sensitivity: 100%. The two known positive samples randomly distributed amongst the set of 10 samples of extracts from potato tubers were successfully detected by all nine laboratories. No false-negative results were obtained.

Data on diagnostic specificity: 100%. The eight known negative samples (three samples spiked with *R. solanacearum* and five samples of extracts from healthy potato tubers) were reported as negative by all nine laboratories. No false-positive results were obtained from healthy samples or from samples spiked with *R. solanacearum*.

Accuracy: 100%.

DNA-extraction methods used in the proficiency test: e.g. (1) Easy DNA Kit (Invitrogen), (2) InviMagUniversal Kit (Stratec), (3) Invitek Pathogen Kit with Lysozyme/Prot-K Lysis (30 min each) and extraction in King Fisher ml, (4) Agentcourt Genfind V2, semi-automated (Biomek 4000); Commercial Master Mixes used in the proficiency test: e.g. (1) TaqMan Universal PCR Master Mix (Thermo Fisher), (2) SensiFast Probe-No-Rox-Mix (Bioline), (3) Maxima Probe qPCR Master Mix (Thermo Fisher), (4) TaqMan Environmental Master Mix (Thermo Fisher), (5) QuantiTect Multiplex PCR Kit (Qiagen) or iQ™ Multiplex Powermix (Bio-Rad).

Data from Schaad et al. (1999).

Analytical sensitivity: ≈200 cfu/mL

Analytical specificity:

Inclusivity: 100% tested on 15 target strains. All strains of *C. sepedonicus*, including non-mucoid strains 80452 and INM, were positive.

Exclusivity: 100% tested on 18 non-target strains (11 strains of four other *C. michiganensis* subspecies, seven other closely related Gram-positive bacteria).

APPENDIX 10 - FLUORESCENT IN SITU HYBRIDIZATION

1. General information

Fluorescent in situ hybridization (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. sepedonicus*, a FISH protocol based on that of van Beuningen *et al.* (1995) is available. The results of this FISH procedure can be evaluated by epifluorescence microscopy. The FISH test has been found to be a reliable tool in confirmation of the IF test. When the FISH test is used as the first screening test and found to be positive, the IF test must be performed as a second compulsory screening test.

The procedure should preferably be performed on freshly prepared sample extract but can also be successfully performed on sample extracts that have been stored with glycerol at –16 to –24°C or – 68 to – 86°C.

Oligo-probes:
C. sepedonicus-specific probe
C. SEPEDONICUS -CY3-01: 5′-TTG CGG GGC GCA CAT CTC TGC ACG-3’
Non-specific eubacterial probe
EUB-338-FITC: 5’-GCT GCC TCC CGT AGG AGT-3’

Use of the FITC-labelled eubacterial oligo-probe offers a control for the hybridization process, since it will stain all eubacteria present in the sample.

2. Methods

Preparation of hybridization mix: prepare quantities of hybridization solution according to the calculations in table below. For each slide (containing two different samples in duplicate), 90 µL of hybridization solution is required.

Suggested quantities for preparation of hybridization mix:

Reagent	Two slides (µL)	Eight slides (µL)
Sterile UPW	50.1	200.4
3× Hybmix	30.0	120.0
1% SDS	0.9	3.6
Probe EUB 338 (100 ng/µL)	4.5	18.0
Probe *C. SEPEDONICUS* CY301 (100 ng/µL)	4.5	18.0
Total volume (µL)	90.0	360.0

Tissue source: plant, tubers, pure culture suspension.

Fixative solution (see Appendix 1).

Potato extract fixation.

The following protocol is based on Wullings et al. (1998):

Prepare the fixative solution (see Appendix 1):

Pipette 100 µL of each sample extract into an Eppendorf tube and centrifuge for 8 min at 7000 *g*.
Remove the supernatant and dissolve the pellet in 500 µL of fixative prepared less than 24 h in advance.
Vortex and incubate overnight at 4°C.

An alternative fixative solution is 96% ethanol.

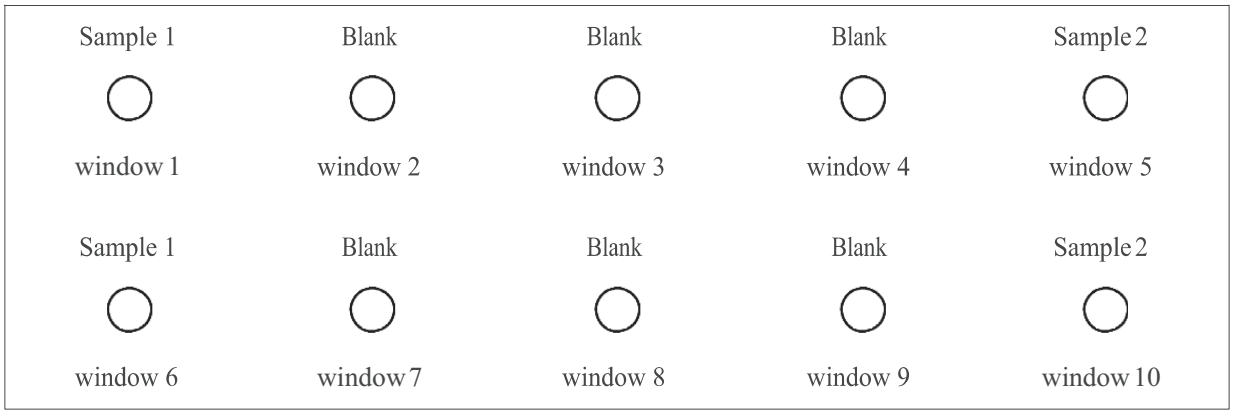

FIGURE A2 FISH slide

Dissolve the pellet in 50 µL of 0.01 M PB and 50 µL of 96 % ethanol.
Vortex mix and incubate at 4°C for 30–60 min.

Centrifuge for 8 min at 7000 *g*, remove the supernatant and resuspend the pellet in 75 µL of 0.1 M PB (see Appendix 1).

Spot 16 µL of the fixed suspensions onto a clean multitest slide as shown in Figure A2. Apply two different samples per slide, undiluted, and use 10 µL to make a 1:100 dilution (in 0.1 M PB).

The remaining sample solution (49 µL) can be stored at –20°C after addition of 1 volume of 96 % ethanol. In case the FISH test requires repeating, remove the ethanol by centrifugation and add an equal volume of 0.1 M PB (mix by vortexing).

Air-dry the slides (or use a slide dryer at 37°C) and fix them by flaming.

At this stage the procedure may be interrupted and the hybridization continued the following day. Slides should be stored dust-free and dry at room temperature.

3 Prehybridization and hybridization

Prepare a lysozyme solution containing 10 mg of lysozyme (Sigma L–6876) in 10 mL of buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0). This solution can be stored but it should only be freeze-thawed once. Cover each sample well with approximately 50 µL of lysozyme solution and incubate for 10 min at room temperature. Then dip the slides in demineralized water, once only and dry with filter paper.

Alternatively, instead of lysozyme add 50 µL of 40 to 400 µg/mL proteinase K in buffer (20 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.4) to each well and incubate at 37°C for 30 min.

Dehydrate the cells in a graded ethanol series of 50%, 80% and 96% for 1 min each. Air dry the slides in a slide-holder.

Prepare a moist incubation chamber by covering the bottom of an air-tight box with tissue or filter paper soaked in 1× Hybmix (Appendix 1). Pre-incubate the box in the hybridization oven at 55°C for at least 10 min.

Prepare the hybridization solution (Appendix 1), allowing 45 µL per slide, and preincubate for 5 min at 55°C.

Place the slides on a hot plate at 45°C and apply 10 µL of hybridization solution to each of the four wells on the slide(s).

Apply two coverslips (24 × 24 mm) to each slide without trapping air. Place the slides in the prewarmed moist chamber and hybridize overnight in the oven at 55°C in the dark.

Prepare three beakers containing 1 L of ultra-pure water (UPW), 1 L of 1× Hybmix (334 mL 3× Hybmix and 666 mL UPW) and 1 L of 1/2× Hybmix (167 mL 3× Hybmix and 833 mL UPW). Preincubate each in a water bath at 55°C.

Remove the coverslips from the slides and place the slides in a slide holder.

Wash away excess probe by incubation for 15 min in the beaker with 1× Hybmix at 55°C.

Transfer the slide holder to 1/2 Hybmix washing solution and incubate for a further 15 min.

Dip the slides briefly in UPW and place them on filter paper. Remove excess of moisture by covering the surface gently with filter paper. Pipette 5–10 µL of anti-fading mountant solution (e.g. Vectashield, Vecta Laboratories, CA, USA or equivalent) on each window and apply a large coverslip (24 × 60 mm) over the whole slide.

4. Reading the FISH test

Observe the slides immediately with a microscope fitted for epifluorescence microscopy at 630× or 1000× magnification under immersion oil. With a filter suitable for fluorescein isothiocyanate (FITC) eubacterial cells (including most Gram-negative cells) in the sample are stained fluorescent green. Using a filter for

tetramethylrhodamine- 5-isothiocyanate, Cy3-stained cells of *C. sepedonicus* appear fluorescent red. Compare the cell morphology with that of the positive controls. Cells must be bright fluorescent and completely stained. The FISH test should be repeated if the staining is aberrant. 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.

Observe for bright fluorescing cells with characteristic morphology of *C. sepedonicus* in the windows of the test slides. The fluorescence intensity must be equivalent or better than that of the positive control strain. Cells with incomplete staining or with weak fluorescence must be disregarded.

If any contamination is suspected the test should be repeated. This may be the case when all slides in a batch show positive cells due to the contamination of buffer or if positive cells are found (outside of the slide windows) on the slide coating.

There are several problems inherent to the specificity of the FISH test. Background populations of fluorescing cells with atypical morphology and cross-reacting saprophytic bacteria with size and morphology similar to *C. sepedonicus* may occur, although much less frequently than in the IF test, in potato heel end core and stem segment pellets.

Consider only fluorescing cells with typical size and morphology.

5. Essential procedural information

Controls

For a reliable test result to be obtained, the following controls should be included for each series.

As negative controls, use aliquots of sample extract that previously gave a negative test result for *C. sepedonicus*.

As positive controls prepare suspensions containing 10^5–10^6 cells/mL of *C. sepedonicus* (e.g. strain NCPPB 4053) in 10 mM PB (Appendix 1) from a 3–5-day culture. Prepare separate positive control slides of the homologous strain or any other reference strain of *C. sepedonicus* suspended in potato extract.

Interpretation of results: to assign results from FISH test the following criteria should be followed:

Verification of the controls

Valid FISH test results are obtained if bright-green fluorescent cells of size and morphology typical of *C. 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.

When these conditions are met

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 millilitre of resuspended pellet (see below).

Samples with at least 5×10^3 typical cells per millilitre of resuspended pellet are considered potentially positive and further testing is required.

Samples with less than 5×10^3 typical cells per millilitre of resuspended pellet are considered negative (no further testing required).

The FISH test is negative if bright-red fluorescent cells with size and morphology typical of *C. 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.

6. Determination of the number of cells per millilitre in a FISH test

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

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

where S is the surface area of the window of multispot slide and s is the surface area of the objective field. s is calculated as

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

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

3. Calculate the number of typical fluorescent cells per millilitre of resuspended pellet (N), as

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

where y is the volume of resuspended pellet on each window and F is the dilution factor of the resuspended pellet.

APPENDIX 11 - PATHOGENICITY TEST

An inoculum of about 10^6 cells/mL is prepared from fresh cultures with visible growth of the test isolate and of an appropriate reference strain of *C. sepedonicus*.

This is inoculated into 5–10 stems of young aubergine seedlings, one set of seedlings for each inoculum, and the plants incubated as described in Appendix 2.

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. sepedonicus*.

The pathogen should be re-isolated from symptomatic plants. The method is as for *Isolation from symptomatic plants* (see section 3.2.1.). 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 and/or NDA. After incubation for 3–5 days at 21–23°C, the plates are examined for formation of colonies typical of *C. sepedonicus*.