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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/42 (3)

Diagnostics
Diagnostic

PM 7/42 (3) *Clavibacter michiganensis* subsp. *michiganensis*

Specific scope

This Standard describes a diagnostic protocol for *Clavibacter michiganensis* subsp. *michiganensis*.[1,2]

Specific approval and amendment

Approved in 2004-09.
Revision adopted in 2012-09.
Second revision adopted in 2016-04.

1. Introduction

Clavibacter michiganensis subsp. *michiganensis* was originally described in 1910 as the cause of bacterial canker of tomato in North America. The pathogen is now present in all main areas of production of tomato and is quite widely distributed in the EPPO region (EPPO/CABI, 1998). Occurrence is usually erratic; epidemics can follow years of absence or limited appearance.

Tomato is the most important host, but in some cases natural infections have also been recorded on *Capsicum*, aubergine (*Solanum dulcamara*) and several *Solanum* weeds (e.g. *Solanum nigrum*, *Solanum douglasii*, *Solanum trifolium*). Other solanaceous plants are susceptible upon artificial inoculation (Thyr *et al.*, 1975). Several solanaceous and non-solanaceous plants, e.g. *Datura stramonium*, *Chenopodium album* and *Amaranthus retroflexus*, have been identified as reservoirs for epiphytic survival and spread (Chang *et al.*, 1992). The significance of these epiphytic populations is not fully understood, although they seem to contribute to infections through pruning wounds (Carlton *et al.*, 1994).

The pathogen is frequently seed transmitted, both internally in seed and on the seed surface. The quantity of bacteria in infected seed usually averages between 10^2 and 10^4 cfu per seed. Latently infected young plants are also considered important for spread (De León *et al.*, 2011). Further information can be found in the EPPO data sheet on *C. michiganensis* subsp. *michiganensis* (EPPO/CABI, 1997).

The diagnostic procedure for symptomatic plants (Fig. 1) comprises isolation from infected tissue on non-selective and/or semi-selective media, followed by identification of presumptive isolates including determination of pathogenicity. This procedure includes tests which have been validated (for which available validation data is presented with the description of the relevant test) and tests which are currently in use in some laboratories, but for which full validation data is not yet available. Two different procedures for testing tomato seed are presented (Fig. 2). In addition, a detection protocol for screening for symptomless, latently infected tomato plantlets is presented in Appendix 1, although this method has yet to be fully validated.

2. Identity

Name: *Clavibacter michiganensis* subsp. *michiganensis* (Smith, 1910; Davis *et al.*, 1984).
Synonyms: *Corynebacterium michiganense* subsp. *michiganense* (Smith, 1910; Carlson & Vidaver, 1982), *Corynebacterium michiganense* pv. *michiganense* (Smith, 1910; Dye & Kemp, 1977), *Corynebacterium michiganense* (Smith, 1910; Jensen, 1934).
Taxonomic position: Procaryotae Kingdom, Division II Firmicutes Gibbons & Murray 1978, Class I Firmibacteria. The genus *Clavibacter* was designed to accommodate the plant pathogenic coryneform bacteria, the cell wall peptidoglycan of which contains 2,4-diaminobutyric acid as a dibasic amino acid (Davis *et al.*, 1984). Strictly aerobic; Gram-positive rods which do not produce endospores. V, Y and palisade arrangements of cells are usually present.
EPPO code: CORBMI.
Phytosanitary categorization: EPPO A2 List no. 50, EU Annex designation II/A2.

[1]This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols.*
[2]The use of names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

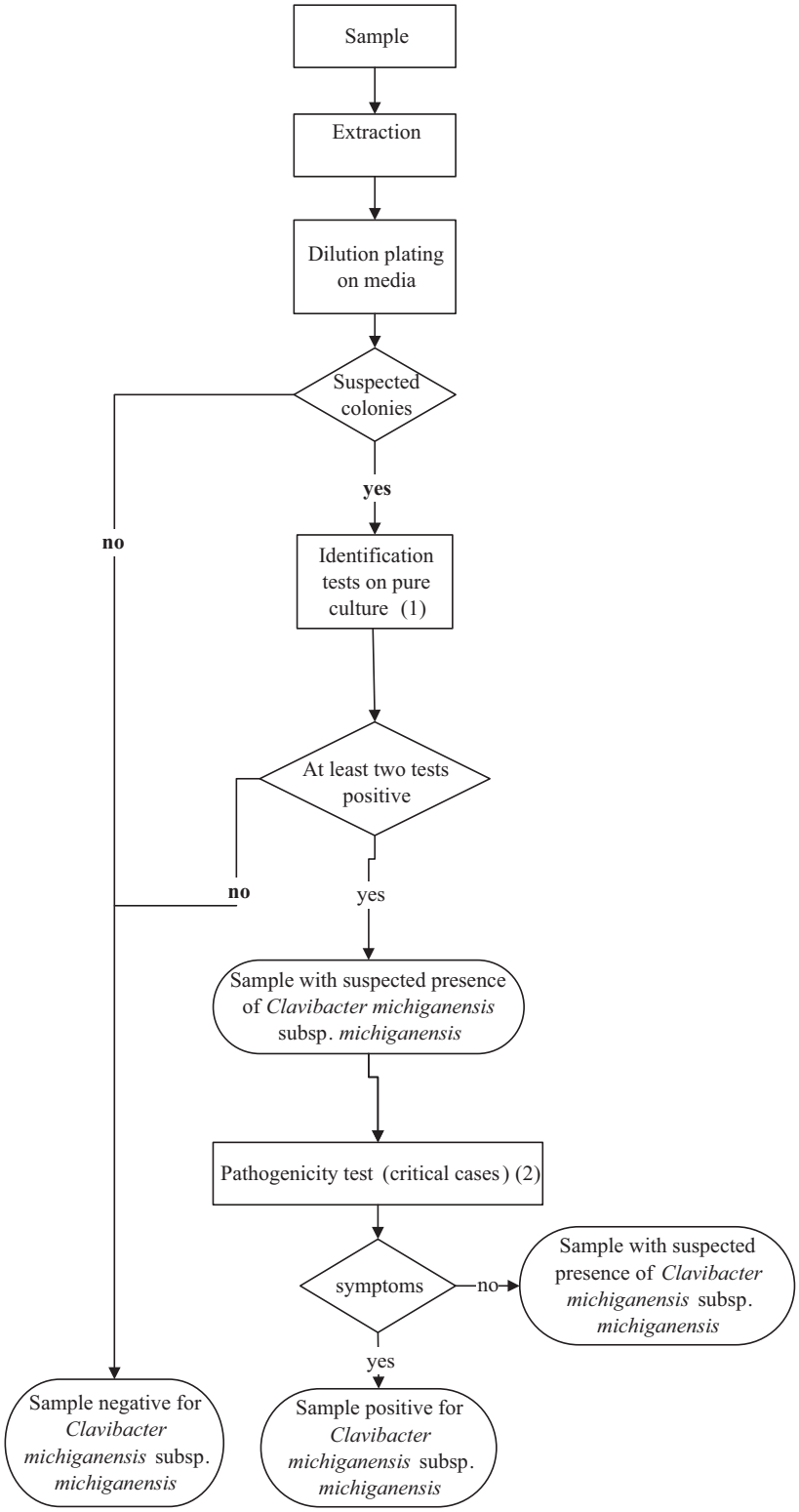

Fig. 1 Flow diagram for the detection and identification of *C. michiganensis* subsp. *michiganensis* in symptomatic samples. (1) At least two tests based on different characteristics of the pathogen (e.g. combinations of biochemical, serological or molecular tests) or two molecular tests based on different DNA sequence targets in the genome. (2) Information on critical cases is available in PM 7/76 *Use of EPPO diagnostic protocols*.

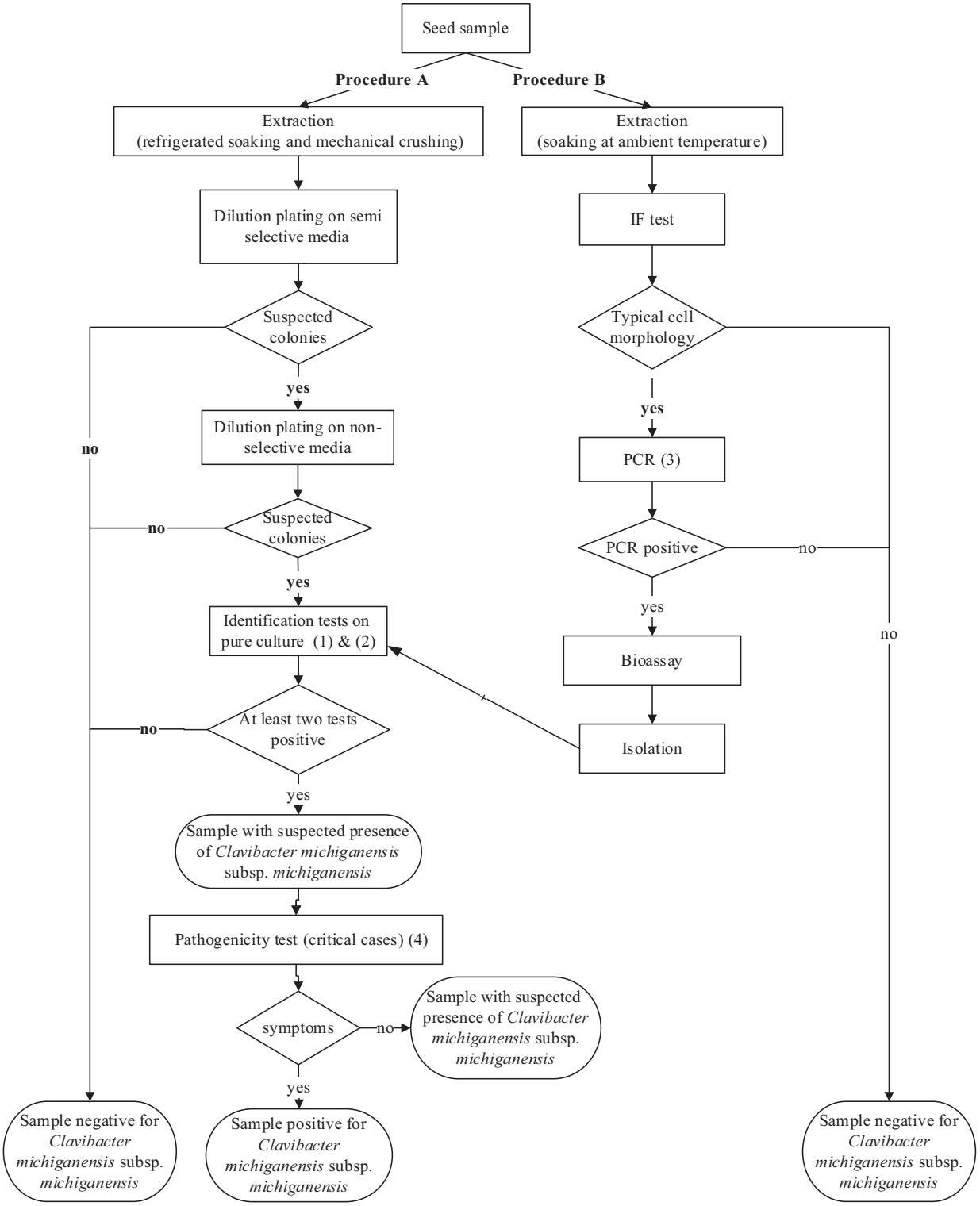

Fig. 2 Flow diagram for the detection and identification of *C. michiganensis* subsp. *michiganensis* in seed samples. (1) At least two tests based on different characteristics of the pathogen (e.g. combinations of biochemical, serological or molecular tests) or two molecular tests based on different DNA sequence targets in the genome. (2) Validation data provided in this protocol for Procedure A is based on a combination of dilution plating and two PCR tests. (3) Validation data provided in this protocol for Procedure B is based on the PCR test described in Appendix 5. (4) Information on critical cases is available in PM 7/76 *Use of EPPO diagnostic protocols*.

3. Detection

3.1 Disease symptoms

Generally, *C. michiganensis* subsp. *michiganensis* causes systemic infection of tomato plants. The pathogen can also cause spots on leaves, petioles, peduncles and fruits as a result of a local infection, usually under overhead irrigation. There are a wide range of symptoms which vary with place of production (glasshouse or field), age of the plant at the time of infection, cultural practices, cultivar, etc. Symptoms can be divided into two types, depending on whether infection is systemic within the vascular tissue or superficial. Additional pictures of symptoms are available on the EPPO Global Database (https://gd.eppo.int/).

3.1.1 Symptoms in systemic infections

In systemic infections, the disease can often be recognized at an early stage by dull green, oily areas between the leaf veins which rapidly desiccate. Subsequently, pale brown necrotic areas appear which are frequently marginal, giving the plant a scorched appearance. Small affected areas may coalesce and produce larger necrotic zones (Fig. 3). Downward turning of one or a few of the lower leaves occurs as the systemic infection progresses and often the leaflets along one side of a leaf become flaccid (Fig. 4), at least during periods of enhanced evapotranspiration. Under favourable conditions for symptom development (25–30°C and evapotranspiration stress), entire leaves wilt and shrivel within a few days (Fig. 5). Finally, the whole plant wilts and desiccates. Under less favourable conditions, irreversible wilting will be delayed and the plant may not show

Fig. 4 Flaccidity and wilting of leaflets.

Fig. 3 White desiccated leaf areas.

Fig. 5 Wilting and shrivelling of tomato plants.

any wilting when defoliation is done for crop management. Fruits of systemically infected plants may fail to develop, ripen unevenly or fall. They can appear marbled with longitudinal chlorotic streaks and internal bleaching of vascular and surrounding tissues. Typical leaf or fruit spot symptoms, arising from external infection, are less frequently found in glasshouse crops than in open field crops.

Following systemic infections, the vascular tissues of transversely cut stems of wilted plants usually appear dark yellow to brown, in particular at the nodes (Fig. 6). The vascular parenchyma in particular has a mealy appearance and is soft, resulting from bacterial degradation and ooze production (Fig. 7). The pith may collapse completely.

3.1.2 Symptoms in superficial infections

Superficial infections occur when bacteria multiply on the plant surface or within surface wounds and stomata. Leaves, stems and calyces may show a mealy appearance, as if dusted by coarse flour. Close examination reveals raised or sunken blisters which are usually white to pale orange. Mealy spots on stems are usually more discrete than those on leaves. A common leaf symptom is a dark brown spot surrounded by a yellow–orange area, usually at the edge of the leaf; this results from infection of a water-excreting gland (hydathode). The leaflets of the oldest infected leaves then curl and their margins yellow and become necrotic (Carlton *et al.*, 1998). Plants grow poorly and gradually entire plants desiccate. Yellow streaks may develop along the stem and occasionally these split open at the nodes to form cankers. Blister lesions on stems are sometimes observed.

3.1.3 Symptoms on fruits

Symptoms on affected fruits may begin as small, slightly raised lesions with a white margin or halo. These lesions may expand to a few millimetres with brown, roughened centres called 'bird's-eye' spots. These spots may occur in greenhouses under overhead irrigation conditions. Several lesions may be formed near the calyx where fruits touch in a cluster. The vascular tissues under the calyx scar and those leading to the seeds may be dark yellow to brown. Canker-like symptoms are actually rare; the disease's most common name, bacterial canker, is in reality a misnomer.

Disease symptoms are unlikely to appear before the setting of the fruits in the third or fourth cluster. Generally, symptoms caused by *C. michiganensis* subsp. *michiganensis* are not expressed on young plants. However, wilting may occur early on grafted plants, possibly even in the nursery, and leaf spots may develop on young plants as a result of local infections under saturated humidity (Fig. 8). Wilt symptoms caused by *C. michiganensis* subsp. *michiganensis* may be confused with other systemic tomato diseases caused by *Ralstonia solanacearum*, *Fusarium* spp. or *Verticillium* spp.

Fig. 7 Infected vascular stem tissues with mealy appearance of vascular parenchyma.

Fig. 6 Dark yellow-brown discoloration of vascular tissues on wilting plants from the field, in particular at the nodes,. Courtesy of A. Karahan, PPCRI, Ankara (TR).

Fig. 8 Local infection on sweet pepper leaf in a nursery.

3.2 Detection in symptomatic plants

3.2.1 Sample

Preferably all above-ground parts of a symptomatic plant should be included in a sample. Wilting plants are cut at the base of the stem and wilted leaves are removed and examined for discoloration of vascular tissues at the bases of broken petioles. The stem at each node is sectioned and similarly examined for dark yellow to brown discoloration of the vascular tissues at the cut surfaces. The epidermis and other tissues can then be carefully separated from the affected vessels. In the absence of vascular discoloration, stem sections should be taken at different levels of the plant and especially from the crown. In advanced stages of wilting, bacterial exudate in the vascular parenchyma tissues facilitates separation of the outer layers of stem tissue.

3.2.2 Extraction procedure

Small sections of affected vascular tissue are removed using a sterile scalpel and soaked in a small volume (approximately 5 mL) of sterile demineralized water or 0.01 M phosphate-buffered saline (PBS; Appendix 2) for up to 30 min to allow diffusion of bacteria out of the plant tissue. Usually, the suspension rapidly becomes milky. For extraction of the bacteria from peduncle or fruit spots, the surface can be washed and lightly surface-disinfected, for example by wiping with 70% ethanol. A few young spots can then be removed with a disinfected scalpel blade and soaked in a small volume of sterile 0.01 M PBS (Appendix 2) as above.

3.2.3 Dilution plating

Isolation of *C. michiganensis* subsp. *michiganensis* from infected plant tissue (vascular tissue, spots on leaves, peduncules or fruit or other decaying tissue) can usually be performed in 3–4 days on a suitable non-selective medium such as yeast peptone glucose agar (YPGA) or yeast extract–dextrose–calcium carbonate (YDC) (Appendix 2). If high numbers of saprophytes are suspected to be present or are present in the sample, a combination of semi-selective media (CMM1T, SCMF or SCM; Appendix 2) may be used. Typical colonies form within 5–10 days following incubation at 28°C (±3°C), depending on the isolation medium used. Descriptions of typical colonies on these media are presented in Table 1. Presumptive colonies are purified by subculturing on non-selective media, such as YPGA, YDC or nutrient agar. As positive controls, a serially diluted suspension of one or more reference strains of *C. michiganensis* subsp. *michiganensis* (Appendix 3) in sterile 0.01 M PBS (Appendix 2) can be inoculated onto the same media.

3.2.4 Rapid tests for presumptive diagnosis

Further testing in addition to isolation is not usually required for diagnosis from symptomatic material. However, in cases where more rapid results are required or initial isolation from samples with typical symptoms is unsuccessful, screening tests involving immunofluorescence (IF) and/or polymerase chain reaction (PCR) may be performed. Instructions for performing an IF test are provided in EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* and specific requirements for detection of *C. michiganensis* subsp. *michiganensis* by IF are given in Appendix 4. Instructions for performing a specific PCR test are given in Appendix 5. Care will be needed, as IF and PCR can produce false positive results due to lack of specificity of the antibodies or primers used. In addition, the PCR test has only been validated on seed extracts and inhibition may occur when performing this test on plant material resulting in false negative results. Samples testing positive in these screening tests will require further confirmatory testing by isolation. Appropriate use of control materials (including internal PCR controls) will also be essential to check for false negative results.

3.3 Detection in symptomless plantlets

Detection of latent infections of *C. michiganensis* subsp. *michiganensis* in nursery plants with no evident symptoms is erratic since the incidence of infections and the distribution of the pathogen (in roots, stem or leaves) can be variable depending on the culture conditions of the plantlets, for example whether or not they have been grafted or whether re-circulating water has been used for irrigation (van Vaerenbergh *et al.*, 1985; Huang & Tu, 2001; Kawaguchi *et al.*, 2010; Xu *et al.*, 2012). A method for testing nursery plants is presented in Appendix 1, although it has not yet been fully validated.

Table 1. Description of *Clavibacter michiganensis* subsp. *michiganensis* colonies on different isolation media

Medium	Mean development time	Colony description
CMM1T	5–10 days	Yellow, mucoid and convex (Fig. 9) becoming deeper yellow with longer incubation
SCM and SCMF	5–10 days	Translucent green–grey, mucoid, often irregularly shaped with a variable grey to black centre (Fig. 10)
YPGA	3–4 days	Light yellow, flat and semi-fluidal, round or irregular (Fig. 11) becoming deeper yellow, opaque and glistening upon longer incubation
YDC	2–3 days (at 27°C)	Yellow and mucoid (Fig. 12). Orange for pepper strains

Fig. 9 Appearance of cultures of three different *C. michiganensis* subsp. *michiganensis* isolates on CMM1 after 10 days of incubation at 28°C. Colonies of *C. michiganensis* subsp. *michiganensis* on CMM1T are yellow, mucoid and convex. They become deeper yellow with longer incubation. The shape of the colonies is often irregular as they become larger. The size and the colour of the colonies is rather variable. The *C. michiganensis* subsp. *michiganensis* colonies here are relatively small because the colonies are not in the presence of seed extract but in the presence of phosphate buffer. Photo courtesy of H. Koenraadt, Naktuinbouw (NL).

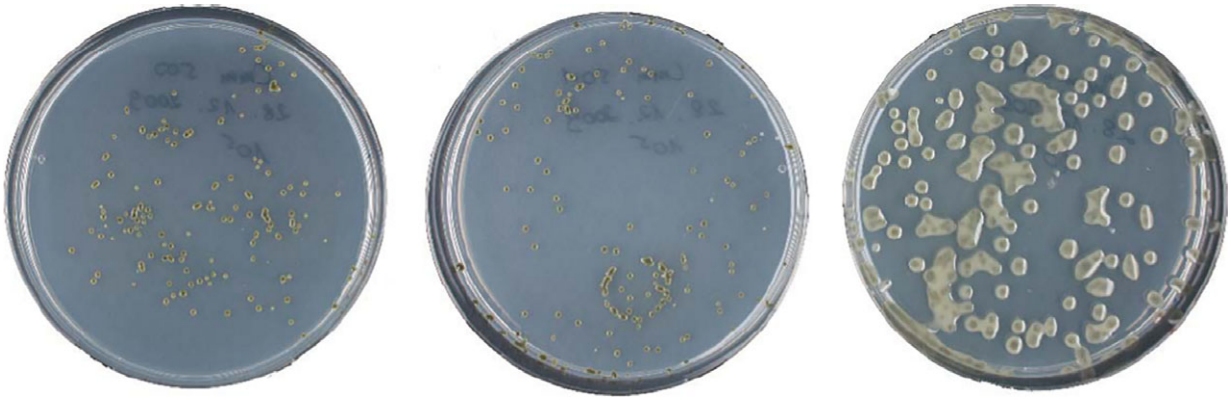

Fig. 10 Appearance of cultures of three different *C. michiganensis* subsp. *michiganensis* isolates on SCMF after 10 days of incubation at 28°C. Colonies are translucent grey, mucoid, often irregularly shaped with a variable grey to almost black centre. The shape of the colonies is often irregular as they become larger. Colonies of *C. michiganensis* subsp. *michiganensis* which grow very slowly and become intense grey to almost in black colour. Photo courtesy of H. Koenraadt, Naktuinbouw (NL).

3.4 Detection in seed samples

Two procedures are described for the detection of *C. michiganensis* subsp. *michiganensis* from seeds. The choice of procedure should be based on the experience of the user. A test performance study (ANSES, 2016) has shown that these two procedures are equivalent, except for isolation of strains (Procedure A is more sensitive for isolation than the bioassay of Procedure B). Procedures A and B, are described below in detail and are illustrated in the two columns of Fig. 2.

3.4.1 Sample and subsample size

The minimum recommended sample size is 10 000 seeds, providing a 95% probability of detecting a 0.03% incidence of contamination in the seed lot (as stated in the ISHI-Veg Seed Health Testing Methods Reference Manual). For Procedure A, at least two subsamples should be tested, with a maximum subsample size of 10 000 seeds. For Procedure B, the maximum subsample size is 2000 seeds. Sample and subsample sizes can be determined according to the thousand seed weight.

3.4.2 Treated seeds

Extraction of *C. michiganensis* subsp. *michiganensis* from seeds should result in optimal recovery of both externally and internally located bacterial cells. As seed treatment may affect the reliability of the tests described in procedures A and B (ISF, 2011), testing should preferably be performed on untreated seeds. The test method is suitable for seed that has been treated using physical or chemical [acid extraction (HCl), calcium or sodium hypochlorite, tri-sodium phosphate, etc.] processes with the aim of disinfestation/disinfection, provided that any residue, if present, does not influence the test. Sample spiking can be used to check for such inhibition. As the methods described have not been evaluated on primed, pelleted

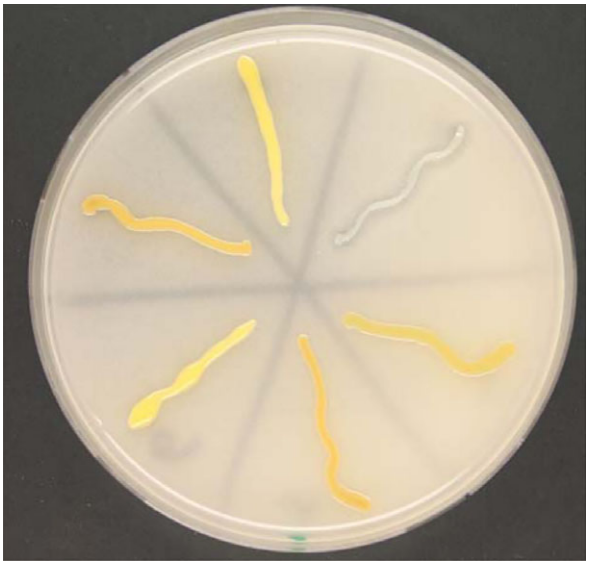

Fig. 11 Appearance of cultures of six representative colonies of *C. michiganensis* subsp. *michiganensis* showing variation in colony morphology after 3 days of incubation at 28°C on the generic nutrient medium YPGA. Colonies become deeper yellow, opaque and glistening with longer incubation. Photo H. Koenraadt, Naktuinbouw (NL).

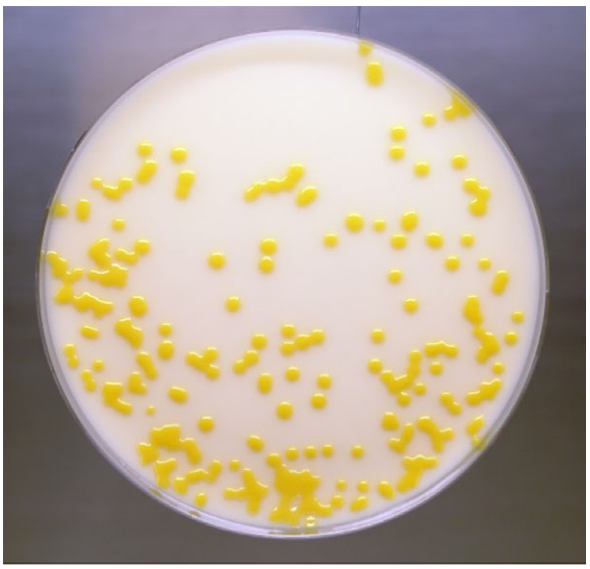

Fig. 12 Colonies of *C. michiganensis* subsp. *michiganensis* as they appear on YDC 3 days after plating.

and/or coated seeds the recovery of *C. michiganensis* subsp. *michiganensis* should be verified by spiking a subsample with a known suspension of a reference strain prior to seed extraction. The same procedure should be applied if it cannot be guaranteed that seed lots have not been treated, i.e. an extra subsample should be spiked. This will provide a control on inhibitory effects on growth of *C. michiganensis* subsp. *michiganensis* on isolation media (Procedure A), or on inhibition of its multiplication during enrichment (Procedure B). However, the user should be aware that often the spike cannot be retrieved in primed, pelleted and/or coated seeds.

Procedure A: dilution plating on semi-selective media

This dilution plating test on the semi-selective media CMM1T and SCMF or SCM has been validated according to EPPO Standard PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity.*

A1: Extraction from seeds

Each subsample should be transferred into a strong sterile polythene bag, for example Stomacher type or Bioreba with an internal filter. Then 4 mL of sterile 50 mM phosphate buffer (Appendix 2) is added per gram of seed and the subsamples are allowed to soak at 2–6°C for 16–24 h before extraction, for example using a Bagmixer (Interscience) or Stomacher (Seward). The time and intensity of extraction is critical because internally located bacteria need to be released from the seeds: 4 min of maceration at maximum speed are recommended for the Interscience Bagmixer and 8 min for the Seward Stomacher. The seed extraction buffer becomes milky after maceration. The subsamples are allowed to settle for 5 min at ambient temperature and 20–50 mL of the seed extract is then concentrated by centrifugation at low speed (180 *g* for 5 min to 1000 *g* for 1 min) to clear the subsample from most of the debris. The supernatant is decanted in a sterile tube and centrifuged at 9000 *g* for 20 min at approximately 4°C.[1] The supernatant is discarded and the pellet resuspended by vortexing with 2–5 mL of 50 mM PB to obtain the 10× concentrated seed extract. The resulting suspension is divided into 500-µL subsamples. Of these 1 × 500 µL is used for analysis and the second 1 × 500 µL for the spiked extract control in the isolation test. The remaining part is deep frozen for reference purposes.

A2: Dilution plating on semi-selective media

Aliquots of 100 µL of 10× concentrated seed extract and 10^{-1} and 10^{-2} dilutions prepared in 50 mM PB (Appendix 2) are spread on at least two of the semi-selective media (CMM1T and SCMF or SCM) and incubated at 28°C (±3°C).

[1]It should be noted that this additional centrifugation may not be possible for lots smaller than 2000 seeds.

A3: Controls included in the dilution plating test

Positive control

To ensure that *C. michiganensis* subsp. *michiganensis* is able to grow on media and form colonies of characteristic morphology, a suspension of a reference strain (Appendix 3) in 0.01 M PBS (Appendix 2) (approximately 10^3 cfu mL^{-1}) should be prepared. From this suspension, 100 µL should be spread on at least two of the semi-selective media CMM1T and SCMF or SCM and typical colonies should form within 10 days after incubation at 28°C (±3°C).

Spiked controls

As the ability to recover *C. michiganensis* subsp. *michiganensis* on semi-selective media can be strongly influenced by the presence of other microorganisms or inhibitory compounds present in seed extracts, additional controls should be added for each subsample.

Spiked extract control: The 10× concentrated seed extract for each subsample should be spiked with a rifampicin-resistant marked strain (see Appendix 3) at a recommended low level of 200–500 cfu mL^{-1}. This spiked extract is then spread (100 µL per plate) on each semi-selective medium (CMM1T and SCMF or SCM) and incubated for 10 days at 28°C (±3°C). If necessary, recovery of the spiked strain can then be distinguished from growth of any wild-type *C. michiganensis*, originating from an infected seed sample, by testing the ability of any isolates to grow on media (e.g. YPGA or YDC) containing 50 ppm rifampicin.

Spiked buffer control: To ensure that the antibiotic-resistant marked strain or the slow-growing strain (Appendix 3) can be effectively recovered from each isolation medium, a comparison of its recovery from the spiked 10× concentrated seed extract (as above) should be made with its recovery from 50 mM PB extraction buffer (Appendix 2) after spiking as above with 200–500 cfu mL^{-1}.

A4: Interpretation of results of the dilution plating test

After approximately 10 days of incubation at 28°C (±3°C) growth of *C. michiganensis* subsp. *michiganensis* can be recorded on the different semi-selective media. The colony features (colour and morphology) and recovery of *C. michiganensis* subsp. *michiganensis* on the control plates should be checked first. Colony features of *C. michiganensis* subsp. *michiganensis* may vary on different semi-selective media (see Table 1). Interpretation of the test is only possible if the results of the different controls are in the expected range (see below).

Interpretation of the positive control

Colonies on positive control plates should be in the expected concentration range (20–50 cfu per plate or higher if higher concentrations are used) and morphology.

Interpretation of the spiked controls

Spiked extract control: C. michiganensis subsp. *michiganensis* should be present on the plates of at least one of the semi-selective media for this control to be positive. No recovery of *C. michiganensis* subsp. *michiganensis* in this control is an indication of inhibition or loss of viability of the bacteria spiked into the extract due to toxicity or competing seed microflora, or failure of the media (e.g. due to incorrect preparation, age, physiological influences).

Spiked strain control: The colony numbers of the antibiotic-resistant marked strain should be in the expected concentration range. No recovery of *C. michiganensis* subsp. *michiganensis* in this control is an indication of failure of the media or loss of viability.

A5: Identification of presumptive isolates from seed extracts

Presumptive colonies (2–5 per isolation plate) should be transferred by streaking onto a non-selective medium (e.g. YPGA, YDC, or NA for further analysis (see Fig. 12 for colonies on YDC). Table 1 summarizes the colony morphology characteristics on the different media used (see also Figs 9–12).

Methods for identification of presumptive isolates of *C. michiganensis* subsp. *michiganensis* are described in the section Identification.

Procedure B: IF, PCR and bioassay

B1: Extraction from seeds

Seeds are soaked at room temperature. As with Procedure A, a spiked control should be included to allow the determination of possible inhibition of tests or effects of growth competition due to toxicity or biological activity in the seed extract.

Preparation of samples: Generally, five subsamples are prepared from one sample for testing. An extra 'reference subsample' to monitor inhibition should also be prepared. Transfer each subsample, including the reference subsample, into a suitable bag (e.g. Stomacher type or Bioreba with an internal filter). Add 7.5 mL of 0.1 M PBS (Appendix 2) per gram of seeds. Prepare a bag with 10 mL of PBS as a negative control. Soak the seeds with shaking at 120–150 rpm for 3 days at ambient temperature (minimum 18°C, maximum 28°C) to allow multiplication of *C. michiganensis* subsp. *michiganensis*. Seeds should be completely immersed in buffer to allow motion in the bags. Allow the seeds to settle after soaking. Concentration of the seed extract by centrifugation is not required because of the assumed multiplication of *C. michiganensis* subsp. *michiganensis* during soaking.

Preparation of spiked controls: To identify possible inhibitors of multiplication of *C. michiganensis* subsp. *michiganensis* during soaking, an early spike inhibition

control should be included before maceration for seeds. The early spike control is prepared by adding cells of a *C. michiganensis* subsp. *michiganensis* reference strain to a final concentration of 1000–5000 cells per mL of PBS to the reference subsample prior to shaking.

B2: Immunofluorescence test

Instructions for performing an IF test are provided in EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria*. Specific requirements for the IF test when testing tomato seeds for *C. michiganensis* subsp. *michiganensis* are given in Appendix 4. Seed extracts with a positive result in the IF test require further testing by PCR to confirm the IF-positive result. A further PCR test is also recommended if IF-negative results are obtained on the spiked seed sample macerates with two different antisera (see validation data in Appendix 4).

B3: PCR test on IF-positive seed extracts (adapted from Pastrik & Rainey, 1999)

This protocol has been developed to confirm IF-positive results. DNA is extracted from a seed extract using a commercial kit and detection is performed by PCR, as described in Appendix 5. Seed extracts testing positive by both IF and PCR screening tests can then be further analysed by a bioassay.

B4: Bioassay (enrichment) in tomato plantlets

To facilitate isolation of *C. michiganensis* subsp. *michiganensis* from seed samples testing positive in the IF and PCR screening tests, tomato plantlets can be used as a selective enrichment base. The bioassay is adapted from the method described by van Vaerenbergh & Chauveau (1987) and is described in Appendix 6, but has not yet been fully validated. When the bioassay test is positive, the bacterium should be isolated and identified as described in the section identification.

3.4.3 Other seed tests

Other procedures have been suggested as suitable for use in seed testing but have not yet undergone systematic validation:

- Immunomagnetic separation followed by isolation (De León *et al*., 2006, 2008).
- Enrichment PCR. Enrichment of *C. michiganensis* subsp. *michiganensis* from tomato seed extracts in liquid or on solid (semi)-selective media and its subsequent detection by PCR can increase low numbers of target cells to a detectable level and allow distinction of viable pathogen populations from dead cells and/or free DNA (Schaad *et al.*, 1999; Ozakman & Schaad, 2003; Vanneste *et al.*, 2008). Enrichment of the bacterium from seed extracts has been demonstrated on solid nutrient yeast extract agar (NBY) and in liquid mSCM broth (Hadas *et al.*, 2005; De León *et al.*, 2008) but further optimization and validation using other recommended semi-selective media (Appendix 3) and specific PCR tests (Appendices 7 and 8) will be needed to establish a reliable procedure.

4. Identification

A culture is identified as *C. michiganensis* subsp. *michiganensis* when a positive result is obtained for at least two tests based on different characteristics of the pathogen (e.g. combinations of biochemical, serological or molecular tests) or two molecular tests based on different DNA sequence targets in the genome. The flow diagrams in Figs 1 and 2 show the combinations of tests for detection and identification of *C. michiganensis* subsp. *michiganensis* from symptomatic samples and seed samples, respectively.

Regarding the identification of suspected colonies after plating of seed extracts following Procedure A (described in the section Detection) it should be noted that the procedure validated is based on a combination of plating on semi selective media and two PCR tests for the identification of isolates with typical *C. michiganensis* subsp. *michiganensis* colony morphology.

In critical cases when a positive result is obtained, confirmation by pathogenicity test should be performed to confirm infection by *C. michiganensis* subsp. *michiganensis* in the sample.

4.1 Main identification tests

4.1.1 Molecular tests

4.1.1.1 PCR tests. Two PCR assays have been validated for identification.

4.1.1.1.1 Conventional PCR—This test is adapted and validated (according to EPPO Standard PM 7/98) from Pastrik & Rainey (1999). This PCR test is described in Appendix 7.

In a comparison of different PCR primers the adapted primers from Pastrik & Rainey (PSA-R/PSA-8) were more specific than those previously recommended (CMM5/CMM6 and PSA-4/PSA-R) from Dreier *et al.* (1995) and Pastrik & Rainey (1999), respectively. This confirms the observations of Kaneshiro & Alvarez (2001), Hadas *et al.* (2005) and Luo *et al.* (2008).

4.1.1.1.2 Real-time PCR—A real-time PCR test (Oosterhof & Berendsen, 2011) is described in Appendix 8. It was validated according to EPPO Standard PM 7/98.

4.1.1.2 Genomic fingerprinting. C. michiganensis subsp. *michiganensis* can be conveniently and reliably identified according to its unique BOX-PCR genomic fingerprint (see PM 7/100 *Rep-PCR tests for identification of bacteria*, EPPO (2010b)).

4.1.1.3 Barcoding. Comparisons of commercially sequenced PCR products amplified from selected housekeeping gene loci allow accurate differentiation of the *C. michiganensis* subspecies from their closest relatives. For example, assignment of an isolate to *C. michiganensis* can be achieved according to the unique 340–350 bp 16S rDNA sequence using the primers of Edwards *et al.* (1989) and internal primers of Coenye *et al.* (1999). The identification of *C. michiganensis* subsp. *michiganensis* is then determined by a partial sequence of approximately 500 bp of the *gyrB* gene, amplified using primers (*gyrB* 2F and *gyrB* 4R) described by Richert *et al.* (2005), as recently demonstrated by Zaluga *et al.* (2011). General procedures for sequencing are to be described in an EPPO Standard PM7/NEW *DNA barcoding as an identification tool for selected regulated pests* (in preparation).

4.1.2 Serological tests

4.1.2.1 Immunofluorescence test. Instructions for performing the IF test are provided in EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria*. Recommended polyclonal antisera from Prime Diagnostics (NL) and Loewe (DE) have been validated in the framework of the Clavitom project (see Appendix 4 for antisera serial numbers). Polyclonal antiserum IPO 69101 has also been validated in the Netherlands for the identification of pure cultures by IF.

4.1.2.2 Immunostrip rapid serological test. Suspected colonies can be rapidly presumptively tested with the Agdia Immunostrip which contains the specific monoclonal antibody Cmm1 (Kaneshiro *et al.*, 2006). A single colony is suspended by vortexing in 300 µL of Agdia SEB4 sample buffer. A strip with the end marked 'sample' is inserted vertically into the cell suspension. The strip should remain in the cell suspension until the control line is formed. The test line will appear if the cell suspension contains *C. michiganensis* subsp. *michiganensis*. The specificity of this test was verified at ILVO, Belgium with 102 isolates and strains of *C. michiganensis* subsp. *michiganensis* of different geographical origin. All produced a positive test result within 2 min of exposure. Some cross-reactions have been observed with other bacteria from tomato seeds and soil as well as some false negative results. For further confirmation, the test line can be removed from the strip and used in PCR.

4.1.3 Biochemical and physiological tests

4.1.3.1 Fatty acid profiling (FAP). Profiles of fatty acid methyl esters (FAMEs) of *C. michiganensis* subsp. *michiganensis* were found to be highly characteristic using the Microbial Identification System (Midi, Newark, US) (Gitaitis & Beaver, 1990). Presumptive isolates, and a reference culture of *C. michiganensis* subsp. *michiganensis,* are compared after growth for 48 h at 28°C on trypticase soy agar (TSA) and extraction and analysis of the cellular fatty acid methyl esters by gas–liquid chromatography.

4.1.3.2 MALDI/TOF mass spectrometry. Identification of *Clavibacter michiganensis* subspecies is possible by matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF) mass spectrometry (Zaluga *et al.*, 2011). A different and reproducible MALDI-TOF MS profile is produced for each of the *C. michiganensis* subspecies, allowing reliable identification.

4.1.3.3 Biolog system. C. michiganensis subsp. *michiganensis* has been differentiated from seed-borne saprophytes using the Biolog system to profile biochemical characteristics including sole carbon source utilization assays and chemical sensitivity assays, as described by Kaneshiro *et al.* (2006).

4.1.4 Other tests that can provide additional information

The following tests can provide indication on the presence of *C. michiganensis* subsp. *michiganensis*. They are not sufficient for a positive identification but may be useful in (rapidly) excluding non-pathogenic bacteria.

- *Biochemical characteristics.* The following set of phenotypic properties which are universally present or absent in *C. michiganensis* subsp. *michiganensis* should be determined: Gram-positive; slow oxidative metabolism of glucose; catalase-positive; oxidase-negative; aesculin hydrolysis-positive; acid produced aerobically from mannose, but not from mannitol; sodium acetate and sodium succinate used as carbon sources; growth in presence of 6% NaCl; potato starch hydrolysed; H_2S produced from peptone. The methods of Dye & Kemp (1977) may be used.
- Cell morphology. Cells of *C. michiganensis* subsp. *michiganensis* are Gram-positive, non-motile, non-spore-forming, short rods (about 0.4–0.75 × 0.8–2.5 µm) which may be straight to slightly curved or wedge shaped. Coccoid forms may also be observed. They predominantly appear as single cells but some V, Y and palisade arrangements are usually also present. Primary branching is uncommon.

4.2 Pathogenicity tests (confirmation)

4.2.1 Test in tomato plantlets (validated according to EPPO Standard PM 7/98)

Single colonies of putative *C. michiganensis* subsp. *michiganensis* isolates are individually suspended in 100 µL of sterile water or 0.01 M PBS (Appendix 2). Five tomato plantlets (e.g. 'Moneymaker'), with 2–3 fully expanded leaves, are inoculated with each isolate by injection into the stem at the cotyledons. Inoculations are additionally performed with a reference strain as a positive and sterile water as a negative control (Appendix 3). The plantlets are grown at 21–30°C and 70–80% relative humidity. The plantlets should not be watered 1 day before inoculation to facilitate inoculum uptake. Covering the plantlets

for 48 h after inoculation to maintain high humidity is not required in the aforementioned conditions of air humidity but is recommended in drier incubation climates. From the third day after inoculation onwards plantlets are observed for wilting. Wilting is usually evident 8 days post-inoculation but observations should be continued for up to 21 days post-inoculation. In France, a collection of 147 *C. michiganensis* subsp. *michiganensis* was tested for pathogenicity and 11 isolates did not show wilting symptoms after inoculation but canker formation was observed, due to stem splitting at the inoculation point (Fig. 13). The bacterium should then be re-isolated from plants showing symptoms of wilt or canker by removing a 1-cm section of stem from 2 cm above the inoculation point and suspending this section in sterile 0.01 M PBS (Appendix 2). Dilution plating on the media is then performed as described above (for symptomatic plants).

4.2.2 Cotyledon test (Lelliott & Stead, 1987)

This is a host test which can be used to confirm pathogenicity of occasional highly suspicious strains which do not cause wilting in young tomato plants. A single colony suspension is prepared in sterile physiological saline. Five freshly germinated tomato seedlings with expanding cotyledons are used. A droplet of suspension is added to the upper surface of each cotyledon and rubbed over the cotyledon surface, for example with a cotton-wool bud or a gloved finger. Some abrasion is necessary, but if abrasion is too heavy the results will be difficult to interpret. The pots should be covered with a polythene bag to maintain high humidity, and incubated for at least 48 h. Symptoms are white blisters or craters on the cotyledon surface, which can be examined with a hand lens. A reference strain as a positive control and sterile saline solution as a negative control should always be used. The bacterium should be re-isolated and identified.

Fig. 13 Canker due to stem splitting at the inoculation point. Photo Geves.

5. Reference strain

NCPPB 2979 (equivalent strain designations: LMG 7333, CFBP 4999, ICMP 2550). See also Appendix 3.

6. Reporting and documentation

Guidance on reporting and documentation is given in EPPO Standard PM7/77 (2).

7. Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data is also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended that this database is consulted 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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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.

When errata and corrigenda are in press, this will also be marked on the website.

11. Acknowledgements

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Appendix 1 – Sampling and screening of tomato nursery plantlets for latent infections

There are currently no validated protocols for detection of *C. michiganensis* subsp. *michiganensis* in populations of latently infected tomato plantlets. Moreover, testing of nursery plantlets is not regularly practised in control of this pathogen because of the large numbers of plants which need to be sampled for reasonable confidence of detection. The single published method for routine screening was performed on tomato seedlings before transplant and used PCR (Ghedini & Fiore, 1995). Samples consisted of 300 stem segments of 1 cm taken at the collar of the plantlets. The sensitivity threshold of the method was estimated at around 1.1×10^3 cells from 1 in 300 of latently infected stems.

The test procedure presented below is based on a procedure implemented at ILVO (BE) but it has not been validated. The test applies only to grafted tomato plantlets in nurseries. Grafting may transmit *C. michiganensis* subsp. *michiganensis* directly in the vascular tissue of the stem where it spreads systemically from the grafting union in both rootstock and scion and multiplies to detectable levels.[2] (Xu *et al.*, 2010). Assuming that only a few cells are initially present in the vascular tissue, sampling is delayed until 2 weeks after grafting to allow bacterial proliferation in infected plantlets. This is supported by inoculation experiments in the scion Growdena where 5-µL droplets of a suspension containing 10^3 cells per mL were introduced into the stem of 3-week old plantlets, after which the plantlets were incubated under nursery conditions. Incubation is performed by covering the plants to reduce light, retaining moisture at 100% relative humidity with some fogging during the day and keeping the plantlets at 21–23°C.

A sample consists of 100 plantlets and is taken randomly from plants of the same variety and deriving from the same seed lot. The objective is to have one sample per batch of approximately 20 000 plants. When a composite sample is taken from more than one variety, then the sample consists of a representative number of plantlets of each variety according to their relative number in the batch. However, the plantlets from each variety are separately packaged to be analysed separately. Care should be taken not to contaminate the plantlets with substrate or soil. Therefore, plantlets may be cut at the collar of the rootstock to avoid contamination. Each sample is processed and analysed in subsamples of 20 plants as described below.

Extraction procedure

A section of stem tissue a few millimetres in size is removed from each stem with a scalpel just above the grafting mark. Scalpel blades are disinfected between subsamples by dipping in ethanol and flaming. The pieces are transferred to an extraction bag and crushed with an appropriate tool, after which 4 mL of 10-mM phosphate buffer is added and the crushed tissues are allowed to soak for 15–30 min at room temperature. Subsequently, three decimal dilutions are prepared in 10 mM phosphate buffer and from each 50 µL are spread on duplicate plates of at least one of the recommended semi-selective media CMM1T, SCMF or SCM (Appendix 2).

[2]Laser grafting techniques are currently being developed which eliminate such risks

Appendix 2 – Preparation of buffers and media

1. Buffers

1.1 Phosphate buffer (PB) for the extraction and dilution of bacteria from seeds (Procedure A)

50 mM PB, pH 7.4

Component/process	Quantity
$Na_2HPO_4 \cdot 12H_2O$	19.57 g L^{-1}
KH_2PO_4	1.65 g L^{-1}
$Na_2S_2O_3$*	0.5 g L^{-1}
Distilled water	1000 mL
Autoclave for 15 min at 121°C and cool to room temperature	
Sterile Tween 20 (10% solution)	0.2 mL

*Recommended when seeds have been treated with hypochlorite.

1.2 Phosphate buffered saline (PBS) for extraction and dilution of bacteria from symptomatic plants and seeds (Procedure B)

0.01 M PBS, pH 7.2

Component/process	Quantity
$Na_2HPO_4 \cdot 12H_2O$	2.7 g L^{-1}
$NaH_2PO_4 \cdot 2H_2O$	0.4 g L^{-1}
NaCl	8.0 g L^{-1}
Distilled water	1000 mL
Autoclave for 15 min at 121°C and cool to room temperature	

2. Semi-selective media

2.1 CMM1T and SCMF

2.1.1 Description of the media

CMM1T (CMM1Tris100), pH 7.7

Component/process	Quantity
Sucrose	10.00 g L^{-1}
Trizma base (Tris base)	3.32 g L^{-1}
Tris-HCl	11.44 g L^{-1}
$MgSO_4 \cdot 7H_2O$	0.25 g L^{-1}
LiCl	5.00 g L^{-1}
Yeast extract	2.00 g L^{-1}
NH_4Cl	1.00 g L^{-1}
Casein acid hydrolysate (casamino acids)	4.00 g L^{-1}
Agar	15.00 g L^{-1}
Check pH 7.7* and autoclave at 121°C for 15 min	
Add after cooling to about 60°C:	
Polymyxin B sulfate, Sigma P4932 (stock solution: 10 mg mL^{-1} in distilled water)	10.00 mg L^{-1}
Nalidixic acid – sodium salt, Sigma N4382 (stock solution: 10 mg mL^{-1} in 0.1 M NaOH)	28.00 mg L^{-1}
Nystatin, Sigma N6261 (stock solution: 100 mg mL^{-1} in 50% DMSO/50% ethanol)	100.00 mg L^{-1}

*The buffering capacity of this medium is critical for its performance. The ratio of Trizma base and Tris-HCl has to be strictly followed in order to obtain the correct pH (Bert Woudt, Syngenta Seeds). NB: the pH should be measured but not adjusted.

SCMF (SCM Fast), pH 7.3

Component/process	Quantity
Agar	18.00 g L^{-1}
K_2HPO_4	2.00 g L^{-1}
KH_2PO_4	0.5 g L^{-1}
$MgSO_4$ (anhydrous)	0.122 g L^{-1}
H_3BO_3	1.50 g L^{-1}
Yeast extract	2.00 g L^{-1}
Sucrose	10.00 g L^{-1}
Check pH 7.3 and autoclave at 121°C for 15 min	
Add after cooling to about 60°C:	
Nalidixinic acid – sodium salt, Sigma N4382 (stock solution: 10 mg mL^{-1} in 0.1 M NaOH)	20.00 mg L^{-1}
Trimethoprim, Sigma T7883 (stock solution: 10 mg mL^{-1} in 100% methanol)	80.00 mg L^{-1}
100 mg nicotinic acid*/50 mL sterile distilled water	50.00 mL L^{-1}
Nystatin, Sigma N6261 (stock solution: 100 mg mL^{-1} in 50% DMSO/50% ethanol)	100.00 mg L^{-1}
Chapman's potassium tellurite (1% solution), Difco†	1.0 mL

*Add 100 mg nicotinic acid into 50 mL of sterile distilled water.
†The source of potassium tellurite is critical.

2.1.2 Performance criteria available for CMM1T and SCMF (source Naktuinbouw, 2010-02)

2.1.2.1 Analytical sensitivity. The detection limit was found to be 25 cfu mL^{-1} extract.

2.1.2.2 Analytical specificity. The specificity was determined by testing the ability of different isolates of *C. michiganensis* subsp. *michiganensis* and known antagonistic bacteria to grow on SCMF and CMM1T. With the exception of *C. michiganensis* subsp. *michiganensis* isolate number 60, all the *C. michiganensis* subsp. *michiganensis* isolates performed well (scored 3 or

higher) in terms of growth on the selective media. Further examination of *C. michiganensis* subsp. *michiganensis* isolate 60 revealed that morphology of *C. michiganensis* subsp. *michiganensis* 60 was atypical, but it was able to growth on conventional media (SCM and D2ANX).

2.1.2.3 Repeatibility. The repeatability was found to be 100 percent.

2.1.2.4 Reproducibility. For reproducibility one of the subsamples of the *C. michiganensis* subsp. *michiganensis* out of 8 at a low infection level was not detected. All 8 subsamples were detected at a high infection level. A second evaluation of the semi-selective media for this subsample showed that *C. michiganensis* subsp. *michiganensis* was present, but probably not detected because not enough suspected colonies were transferred to YDC. Thus, it is very important to select and transfer enough suspected *C. michiganensis* subsp. *michiganensis* colonies. In all other samples *C. michiganensis* subsp. *michiganensis* was detected, therefore in this case the reproducibility was found to be 94 percent.

2.2 SCM

SCM pH 7.3

Component/process	Quantity
Yeast extract	0.1 g L^{-1}
Sucrose	10.0 g L^{-1}
H_3BO_3	1.5 g L^{-1}
$MgSO_4$ (anhydrous)	0.122 g L^{-1}
K_2HPO_4	2.0 g L^{-1}
KH_2PO_4	0.5 g L^{-1}
Agar	18 g L^{-1}
Check pH 7.3 and autoclave at 121°C for 15 min	
Add after cooling to about 60°C:	
Nalidixinic acid – sodium salt, Sigma N4382 (stock solution: 10 mg mL^{-1} in 0.1 M NaOH)	30.00 mg L^{-1}
100 mg nicotinic acid*/50 mL distilled water	50.00 mL L^{-1}
Nystatin, Sigma N6261 (stock solution: 100 mg mL^{-1} in 50% DMSO/50% ethanol)	100.00 mg L^{-1}
Chapman's potassium tellurite solution (1% solution), Difco†	1.0 mL L^{-1}

*Add 100 mg nicotinic acid into 50 mL of sterile distilled water.
†The source of potassium tellurite is critical.

3. Non-selective medium

Yeast peptone glucose agar (YPGA)

Component/process	Quantity
Difco yeast extract	5 g L^{-1}
Difco Bacto peptone	5 g L^{-1}
D(+) glucose	10 g L^{-1}
Difco Bacto agar	15 g L^{-1}
Distilled water to 1 L	
Autoclave for 15 min	

To prepare the spiked extract control (for detection in seed samples) add 50 ppm rifampicin after cooling to approximately 60°C.

YDC (yeast extract–dextrose–calcium carbonate) medium

Component/process	Quantity
Yeast extract (Sigma)	10 g L^{-1}
$CaCO_3$	20 g L^{-1}
Distilled water	870 mL L^{-1}
Agar (Sigma)	15 g L^{-1}
Check pH 6.9 and autoclave for 15 min	
Then add glucose solution (20 g glucose in 100 mL distilled water)	100 mL

To prepare the spiked extract control (for detection in seed samples), add 50 ppm rifampicin after cooling to approximately 60°C.

Appendix 3 – Recommended reference strains of *C. michiganensis* subsp. *michiganensis* used as positive controls

Strain number	Origin	Isolated from	Isolation year	Growth on medium
NCPPB 2979*, CFBP 4999*, LMG 7333*, PD223*	Hungary	*Solanum lycopersicum*	1957	On SCMF and CMM1T (5–9 days)
NCPPB 1468, LMG 3690, PD520	UK Channel Islands	*Solanum lycopersicum*	1962	Rapid on SCMF and CMM1T (7–9 days)
NAK Cmm 96, ZUM 3059,	The Netherlands	*Solanum lycopersicum*	Data not available	Rapid on SCMF and CMM1T (7–9 days)

(continued)

Table *(continued)*

Strain number	Origin	Isolated from	Isolation year	Growth on medium
ALV 4000 rifampicin resistant†, PD5709				
CFBP 2496	Algeria	*Solanum lycopersicum*	1978	Growth variable depending on medium (see Fig. 14)
CFBP 2492	Algeria	*Solanum lycopersicum*	1985	Growth variable depending on medium (see Fig. 14)

*Species type strain.
†Allows distinction between the endogenous *C. michiganensis* subsp. *michiganensis* and the spike.

Appendix 4 – Immunofluorescence test

For general instructions on how to perform the IF test see EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria*. Specific features are presented below for seed samples. Always use validated antisera.

1. Preparation of positive and negative controls

The positive control (Fig. 15) for the IF test is a multi-well slide with decimal dilutions (10^3, 10^4, 10^5, 10^6 cells per mL) of a *C. michiganensis* subsp. *michiganensis* reference strain in PBS (Appendix 2), sterile water or seed extract. The bacterial suspension used for the serial dilutions is prepared from a 3–7 day culture on YPGA This slide can be prepared in advance. PBS used for soaking seeds is used for the negative control.

2. IF procedure

Preparation of test slides

- Pipette 40 µL of each undiluted seed extract and controls onto individual windows (at least 8 mm diameter) of a multispot microscope slide. All five subsamples except the 'spiked control' are deposited on the same slide (one

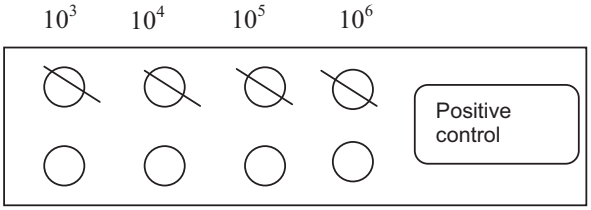

Fig. 15 Positive control slide.

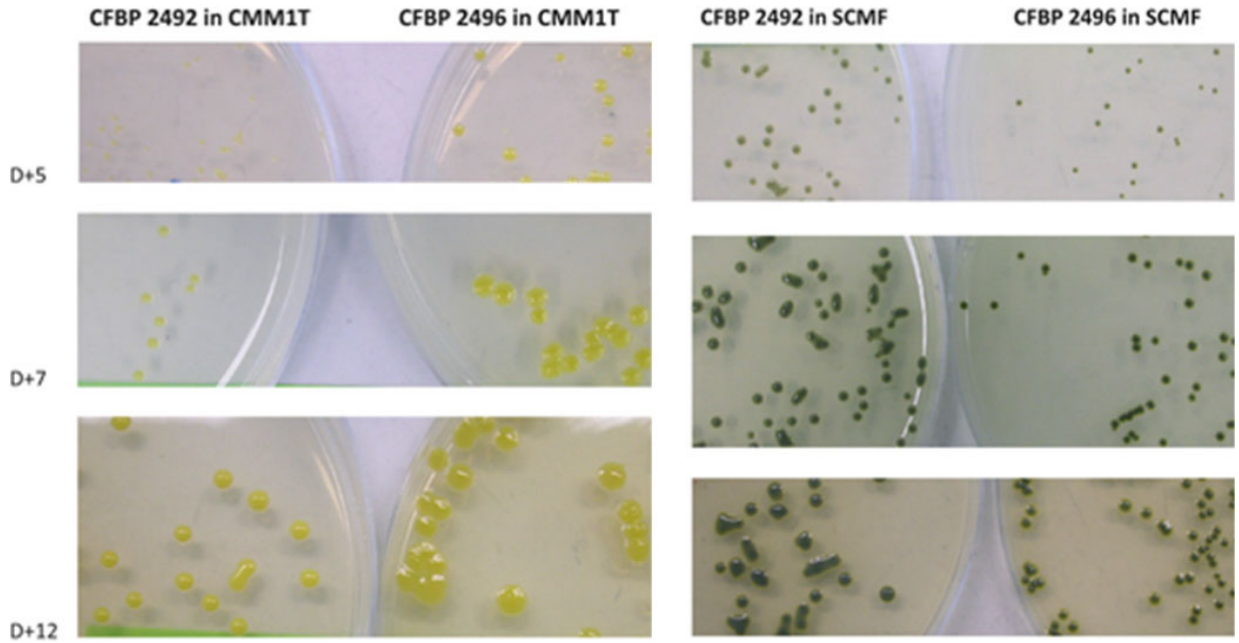

Fig. 14 Comparison of growth of CFBP 2496 and CFBP 2492 on two media.

sample per slide, Fig. 16). The 'spike control' and negative and positive controls are deposited on separate slides.
- Dilution of seed extracts is not usually required for these types of samples. However, in cases with concentrated debris or sediment, prepare a second slide with 1:10 dilution of sample extracts.
- Store seed extracts at 2–6°C in case the IF test needs to be repeated or further analysis is required. Optimal storage is possible for 2 weeks or longer under these conditions. The storage method described in PM 7/97 [below −68°C under glycerol (10–25% v/v)] is not recommended for tomato seed extracts due to the risk of losing cells during centrifugation.
- It is recommended to use a validated polyclonal rabbit antiserum. Re-testing of IF-positive extracts with a second antiserum is strongly recommended to provide more confidence in the result. Prime Diagnostics (NL) has a validated polyclonal rabbit antiserum and Loewe Biochimika (DE) have a validated polyclonal goat antiserum.

To reduce background staining it is recommended to use Evans blue (diluted at 1:300 in the conjugate solution).

Reading the IF test

Instructions for reading the IF test are given in Appendix 1, Section 4, of PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria*. Always refer to the positive control and to the 'early spike control' slides to evaluate the staining and the typical cell shape (note that bacteria from a fresh culture are usually bigger than those extracted directly from seeds). Cells of *C. michiganensis* subsp. *michiganensis* are small (1.0–0.5 µm), slightly club-shaped (coryneform) (Fig. 17), brightly fluorescent, with a dark core and should have a completely stained cell wall. Dividing cells have a typical V-shape (Fig. 18). If morphologically typical cells are detected, the cell concentration per mL of seed extract should be calculated as described in Appendix 1, Section 6, of PM 7/97.

As cross-reactions with tomato seed saprophytes can occur, seed extracts with a positive result in an IF test require further testing to confirm the IF-positive result. This testing is performed by direct PCR with PSA R/8 primers (Appendix 5).

If the result of the 'early spike control' is negative, a conventional PCR test should be performed (Appendix 7).

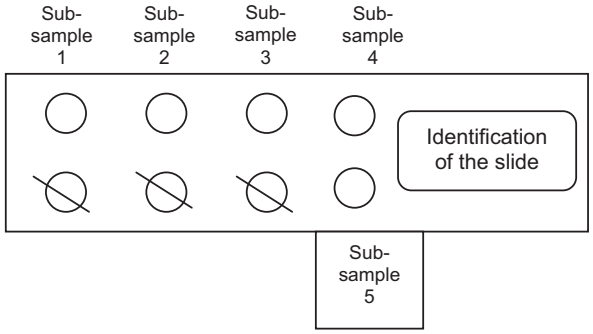

Fig. 16 Sample slide.

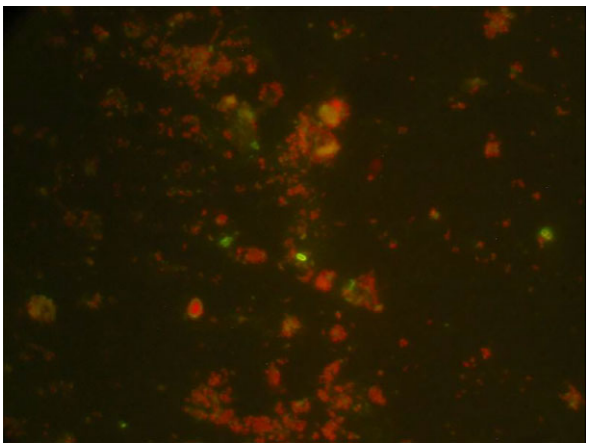

Fig. 17 Cells of *C. michiganensis* subsp. *michiganensis* (technique used: IF).

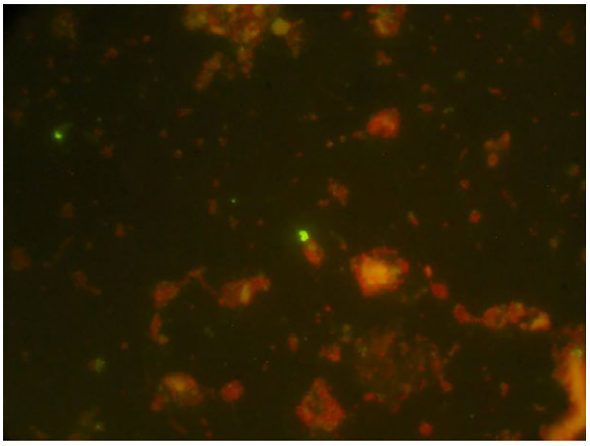

Fig. 18 Dividing cells of *C. michiganensis* subsp. *michiganensis* with a typical V-shape (technique used: IF).

3. Performance criteria available

The performance criteria were obtained during the CLAVITOM project (Convention C2008-12-Tomate/contrat de Branche 2008–2011).

3.1 IF on seed extracts

3.1.1 Analytical sensitivity (experimental infection) $10–10^2$ cfu mL^{-1}; repeatability 90–99%; varying with seed lot (Olivier *et al.*, 2010).

Diagnostic sensitivity: 88.2% [15 positive IF results out of 17 known positive samples; 10 artificially high-level contaminated samples (10^3 cfu mL^{-1} at day 0 and 10^5–10^6 at day 3) and 7 low-level positive treated samples (10^3 cfu mL^{-1})].

The reference method was a comparison with samples of known status.

3.1.2 Analytical specificity

IF with Prime Diagnostics (ref. 19845.08) then with Loewe (ref. Z01/5B-221194) antisera: 89.4% (388 positive IF results out of 434).

Specificity criteria:

Antisera (Prime Diagnostics, Loewe).

- Inclusivity: 100%.
- Exclusivity (Prime Diagnostics, Loewe): 84.3%.

Number of *C. michiganensis* subsp. *michiganensis* strains tested: 141.

Number of non-target organisms tested: 72 [2 pathogens of tomato; 12 other subspecies of *C. michiganensis*; 1 *Rathayibacter iranicus*; 57 tomato seed saprophytes (some chosen as they cross-react with antisera for IF: Prime Diagnostics 61.5% + Loewe 57%].

Complementary tomato seed saprophytes: 221 supplementary isolates – 28 were positive (exclusivity 87.3%). On the 28 Prime Diagnostics positive results, 20 were positive with Loewe (exclusivity of Prime Diagnostics + Loewe: 91%).

Cross-reactions observed with other *C. michiganensis* subspecies and some tomato seed saprophytes.

3.1.3 Diagnostic specificity: 100%

Comparison with the true status.

4 true negative IF results out of 4 true negative samples.

Data on 4 disinfected samples.

3.1.4 Other performance criteria

Relative accuracy: 90.5% (19 agreements out of 21 expected results on samples).

Standard test reference method: comparison with known status samples.

3.2 IF on plant extracts

3.2.1 Analytical sensitivity

Not performed.

3.2.2 Analytical specificity

Not performed.

3.2.3 Diagnostic sensitivity: 100%

Data from the routine plant analyses performed in 2010 and 2011 at the Plant Health Laboratory (LSV-ANSES, FR) on 126 samples tested:

- 60 were positive by IF and by plating (isolation of strains)
- no samples were positive by plating and negative by IF

Standard test: plating on YPGA and isolation/identification of isolates.

3.2.4 Diagnostic specificity: 68.2%

Data from the routine plant analyses performed in 2010 and 2011 at the Plant Health Laboratory (LSV-ANSES, FR) on 126 samples tested:

45 were negative by IF and by plating

21 samples were positive by IF and negative by plating

Standard test: plating on YPGA and isolation/identification of isolates.

3.2.5 Other performance criteria

Relative accuracy: 83.3% (on 126 samples tested by plating and IF).

Appendix 5 – Direct PCR test on IF-positive seed extracts (adapted from Pastrik & Rainey, 1999)

1. General information

1.1 This protocol was developed to confirm IF-positive results.

1.2 Specificity of the primer pair is provided by the forward primer PSA-4, located in the internal transcribed spacer (ITS) of the 16S and 23S rRNA genes. A single nucleotide substitution at the 3′-end of the primer provides specificity of PSA-4 compared with other *C. michiganensis* subspecies. Bacteria other than *C. michiganensis* subsp. *michiganensis* originating from samples regularly tested positive in the PCR with PSA-4 and PSA-R. In order to increase the specificity, the forward primer PSA-4 was therefore modified by extension by 3 bases at the 3′-end and removing 3 bases at the 5′-end (Woudt, unpublished information). This modified primer was proposed as PSA-8, as Pastrik and Rainey used numbers up to PSA-7 in their paper. PSA-8 combined with PSA-R did not react with DNA of non-target bacteria that were positive with PSA-4/PSA-R. It was concluded that PSA-8/PSA-R provides higher specificity than PSA-4/PSA-R. All the other characteristics related to this modified PCR are similar to the descriptions in Pastrik & Rainey (1999).

1.3 Primers:

PSA-8: 5′-TTG GTC AAT TCT GTC TCC CTT C-3′

PSA-R: 5′-TAC TGA GAT GTT TCA CTT CCC C-3′

1.4 An amplicon of 268 bp should be generated.

1.5 This PCR may be used in real-time PCR using SYBR Green master mix. Verification (or validation) according to PM 7/98 should be conducted.

2. Methods

2.1 Nucleic acid extraction

Before testing, an aliquot of 5 mL should be taken from each IF-positive seed subsample and stored at approximately 4°C to allow confirmation by bioassay if the PCR result is also positive. Glycerol should be added if the extract is to be stored at −20°C. However, if the macerate is used for PCR and bioassay immediately after IF, it can be stored at approximately 4°C without glycerol.

Transfer the total volume of the seed extract of each subsample into a type Falcon centrifuge tube. Centrifuge for 1 min at 1000 *g* and transfer the supernatant by pipetting. Separate the supernatant into 2 new clean tubes to avoid saturating the column kit.

Isolate DNA from seed extracts and DNA extraction controls using the Qiagen DNeasy® Blood and Tissue kit for Gram-positive bacteria according to the manufacturer's instructions.

Perform the PCR tests on undiluted and diluted DNA extracts. Testing diluted DNA (1/2 or 1/5) is recommended to avoid false negative PCR results due to inhibition.

Additional use of lysozyme to lyse Gram-positive bacterial cells is recommended.

2.2 Polymerase chain reaction

2.2.1 Master mix

Reagents	Working concentration	Volume (µL) in 20 µL	Final concentration
10× buffer* (Invitrogen)	1×	2.00	1×
$MgCl_2$ (Invitrogen)	50 mM	0.6	1.5 mM
dNTPs	2 mM	2.00	200 µM
Primer PSA-8	10 µM	1.00	0.50 µM
Primer PSA-R	10 µM	1.00	0.50 µM
Taq polymerase	5U μL^{-1}	0.16	0.8 U
DNA template		4.00	
Molecular-grade water		To make up to 20.00	
Total volume		20.00	

*10× buffer: Tris-HCL (pH 9.0) 750 mM; $(NH_4)_2$ SO_4 200 mM; Tween 20, 0.1% (v/v).

2.2.2 PCR conditions

An initial 5 min incubation at 94°C followed by 35 cycles of 30 s at 95°C, 20 s at 63°C and 45 s at 72°C, followed by a final extension of 5 min at 72°C.

The test was validated with Platinum DNA polymerase from Invitrogen.

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 potential contamination during nucleic acid extraction: IF buffer (described in EPPO, 2010a).
- Positive isolation control (PIC): IF buffer (described in EPPO, 2010a) or molecular-grade water spiked with *C. michiganensis* subsp. *michiganensis* (reference strains listed in Appendix 3) (10^4–10^5 cfu mL^{-1}).
- Negative amplification control (NAC). Molecular-grade water used in the PCR mix.
- Positive amplification control (PAC). DNA extract of *C. michiganensis* subsp. *michiganensis* cells in molecular-grade water (10^5 cfu mL^{-1})

When it is not known if seeds have been treated, an inhibition control (IC, spiked sample) should be included.

3.2 Interpretation of results of the conventional PCR

Verification of the controls:

- NIC and NAC should produce no amplicons.
- PIC, PAC (and if relevant the IC) should produce amplicons of 268 bp.

When these conditions are met:

- For conventional PCR a test will be considered positive if amplicons of 268 bp are produced.
- A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

For the SYBR Green approach, controls should give expected results and the test will be considered positive if a melting peak comparable to the positive control used is generated. Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria

4.1 Analytical sensitivity

10^3 cfu mL^{-1} to 10^6 cfu mL^{-1}, depending on the seed sample.

4.2 Analytical specificity

Number of *C. michiganensis* subsp. *michiganensis* strains tested: 141.

Number of non-target organisms tested: 72 (2 other tomato pathogens; 12 other *C. michiganensis* subsp.; 1 *Rathayibacter iranicus*; 57 tomato seed saprophytes).

Cross-reacts with 3 tomato seed saprophytes including one '*C. michiganensis* subsp. *michiganensis* look-alike'.

4.3 Diagnostic sensitivity

100% (compared with IF).

94.1% (by comparison with the true status of samples).

4.4 Diagnostic specificity

83.3% (compared with IF).

100% (by comparison with the true status of samples).

4.5 Reproducibility

93% (with 10 samples at the limit of detection in two laboratories).

4.6 Repeatability

95% (with 10 samples at the limit of detection in two laboratories).

Appendix 6 – Bioassay (enrichment) in tomato plantlets

For each IF+/PCR+ seed extract, inoculate 15–25 tomato plantlets of a susceptible cultivar (e.g. cv. Moneymaker or Marmande) at the 2–4 expanded leaf stage. As a positive control, inoculate 5 plantlets with a cell suspension of a reference strain of *C. michiganensis* subsp. *michiganensis* at around 5×10^3 cfu mL^{-1} (this concentration can be verified by IF or by dilution plating). As a negative control, inoculate 2 plantlets with PBS.

It is recommended to inoculate plantlet stems just above the cotyledons using a syringe fitted with a hypodermic needle. Do not water the tomato plantlets for 24–48 h prior to inoculation. Incubate plantlets under a quarantine regime in the greenhouse at 20–30°C, 80% relative humidity. Monitor symptom development in the positive controls. Symptoms are usually distinct after 2 weeks of incubation. Observe symptom development in the plantlets inoculated with the seed extracts. Incubate for up to 30 days.

Virulence of *C. michiganensis* subsp. *michiganensis* strains is variable. Observe plants for the presence of typical symptoms, i.e. systemic wilting, but also localized formation of bacterial cankers without wilting. Perform isolation on a suitable medium (YPGA or YDC) and identify presumptive *C. michiganensis* subsp. *michiganensis* isolates with one rapid test. Isolation should be performed as soon as symptoms are evident.

Note that under certain conditions, in particular when few cells of *C. michiganensis* subsp. *michiganensis* are inoculated into the tomato plantlet or incubation conditions are not optimal, latent infections may develop. If no symptoms have developed after 30 days of incubation, remove a 3 cm section of stem from just above the inoculation site and test as a composite sample by IF, as described previously for diagnosis on symptomatic plants. Isolation can also be performed on a suitable medium (YPGA or YDC) and isolates should be identified. The result of the bioassay test is valid if all controls have given the correct result.

Then:

- the result of the bioassay test is negative if the plantlets do not develop infection by *C. michiganensis* subsp. *michiganensis* after 30 days and the bacterium was not detected in the bioassay verification test
- the result of the bioassay test is positive if the plantlets have developed infection by *C. michiganensis* subsp. *michiganensis* within 30 days and the bacterium could be re-isolated or if the bacterium was re-isolated from symptomless plants. The bacterium should be re-isolated and identified.

Performance criteria

A study was conducted in France in 2010 (ANSES, Plant Health Laboratory and GEVES-SNES) based on the procedures as described. Seed macerates, artificially contaminated at a final concentration of 6×10^2 to 6×10^6 cfu mL^{-1} of the CFBP 1940 strain, were inoculated into tomato plants as described in Commission Directive 2006/63/CE amending Annexes II to VII to Council Directive 98/57/EC on the control of *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* (EU, 2006). Symptoms were observed to develop on the plants following inoculations at concentrations as low as 600 cfu mL^{-1} and the bacterium could be re-isolated. Four seed lots naturally infested with *C. michiganensis* subsp. *michiganensis*, confirmed by the isolation test (Procedure A), were tested in the same conditions (infestation level evaluated at approximately 10^6 cells per mL evaluated by IF). The bioassay was negative for these 4 contaminated seed lots: no symptoms could be observed in the bioassay and no *C. michiganensis* subsp. *michiganensis* colonies were isolated. A negative bioassay result should therefore be interpreted with caution.

In the framework of the Clavitom project, the minimum detection threshold of the bioassay in IF-positive extracts was determined as 6×10^2 cfu mL^{-1} (result obtained on seed extract artificially contaminated with serial dilutions of *C. michiganensis* subsp. *michiganensis*).

Appendix 7 – Conventional PCR test for identification of *C. michiganensis* subsp. *michiganensis* isolates (adapted from Pastrik & Rainey, 1999)

1. General information

1.1 Pastrik & Rainey (1999) designed the primers PSA-R and PSA-4 for specific identification of *Clavibacter michiganensis* subsp. *michiganensis*. PSA-R, located in the 23S rRNA gene, is a universal primer, at least for Gram-positive bacteria.

1.2 Specificity of the primer pair is provided by the forward primer PSA-4, located in the internal transcribed spacer (ITS) of the 16S and 23S rRNA genes. A single nucleotide substitution at the 3′-end of the primer provides specificity of PSA-4 compared with other *C. michiganensis* subspecies. Bacteria other than *C. michiganensis* subsp. *michiganensis*, originating from samples, regularly tested positive in the PCR with PSA-4 and PSA-R. In order to increase the specificity, the forward primer PSA-4 was therefore modified by extension by 3 bases at the 3′-end and removing 3 bases at the 5′-end (Woudt, unpublished information). This modified primer was proposed as PSA-8, as Pastrik and Rainey used numbers up to PSA-7 in their paper. PSA-8 combined with PSA-R did not react with DNA of non-target bacteria that were positive with PSA-4/PSA-R. It was

concluded that PSA-8/PSA-R provides higher specificity than PSA-4/PSA-R. All the other characteristics related to this modified PCR are similar to the descriptions in Pastrik & Rainey (1999).

1.3 Primers:

PSA-8: 5′-TTG GTC AAT TCT GTC TCC CTT C-3′

PSA-R: 5′-TAC TGA GAT GTT TCA CTT CCC C-3′

1.4 Internal control primers:

Bac-1492R: 5′-TAC GGC TAC CTT GTT ACG ACT T-3′

Bac-8F:5′-GAA GAG TTT GAT CCT GGC TCA G-3′

1.5 An amplicon of 268 bp is generated when PSA-8/PSA-R primers are used and an amplicon of approximately 1500 bp is generated when primers Bac-1492R/Bac-8F are used.

1.6 This PCR may be done in real-time PCR by using SYBR Green master mix. Verification (or validation) according to PM 7/98 should be conducted.

2. Methods

2.1 Nucleid acid extraction

Prepare a cell suspension from each putative *C. michiganensis* subsp. *michiganensis* isolate. Lyse bacterial cells by heating, i.e. 15 min at 95°C, and subsequently cool on ice for 5 min. Centrifuge for 1 min at 10 000 *g* and use 5 µL of supernatant as target DNA in the PCR.

2.2 Polymerase chain reaction

2.2.1 Master mix

Reagent	Working concentration	Volume (µL) in 25 µL	Final concentration
10× buffer* (Invitrogen)	1×	2.50	1×
$MgCl_2$ (Invitrogen)	50 mM	0.75	1.5 mM
dNTPs	2.5 mM	1.00	100 µM
Primer PSA-8	20 µM	0.50	0.40 µM
Primer PSA-R	20 µM	0.50	0.40 µM
Primer Bac-8F	20 µM	0.50	0.40 µM
Primer Bac-1492R	20 µM	0.50	0.40 µM
Taq polymerase	5U μL^{-1}	0.125	625 U
Bacterial suspension	–	5.00	–
Molecular-grade water	–	To make up to 25.00	–
Total	–	25.00	–

*10× buffer: Tris-HCl (pH 9.0) 750 mM; $(NH_4)_2$ SO_4 200 mM; Tween 20, 0.1% (v/v).

2.2.2 PCR conditions

Initial 5 min incubation at 95°C followed by 35 cycles of 15 s at 95°C, 15 s at 63°C and 45 s at 72°C followed by a final extension of 5 min at 72°C.

The PCR amplicon can be detected on agarose gel, or detected in a real-time PCR machine with the use of SYBR Green.

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 potential contamination during nucleic acid extraction and subsequent amplification in sterile extraction buffer.
- Positive isolation control (PIC): to ensure that nucleic acid of sufficient quantity and quality is isolated. Perform DNA extraction from a *C. michiganensis* subsp. *michiganensis* reference strain, for example PD233 (Appendix 3).
- Negative amplification control (NAC): to rule out false positives due to contamination during the preparation of the reaction mix. The molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC): to monitor the efficiency of the amplification. Add nucleic acid of a *C. michiganensis* subsp. *michiganensis* reference strain, for example PD233, to a PCR reaction (Appendix 3).

Internal positive controls (IPCs) are used with each sample to ensure successful DNA extraction and PCR amplification. Co-amplification of endogenous nucleic acid can occur when conserved primers amplify non-target nucleic acid that is also present in the sample (e.g. bacterial 16S rDNA). Universal bacterial primers are therefore used to give a product of approximately 1500 bp. (Adapted from Eden *et al.*, 1991.)

3.2 Interpretation of results of the conventional PCR

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of 268 bp.
- IPC should produce an amplicon of approximately 1500 bp.

When these conditions are met:

- For conventional PCR a test will be considered positive if amplicons of 268 bp are produced.
- A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained (if both the IPC and specific target

do not give a signal no conclusion can be made and the PCR should be repeated).

For the SYBR Green approach, a melting peak comparable to the positive control used should be generated. If both the internal control and specific target do not give a signal no conclusion can be drawn and the PCR should be repeated.

4. Performance criteria

The PCR test for the identification of *C. michiganensis* subsp. *michiganensis* has been validated according to EPPO Standard PM 7/98, for both the conventional approach and the SYBR Green approach.

4.1 Analytical sensitivity data

The limit of detection (LOD) is 5×10^3 cfu per reaction, calculated based on the mean LOD of 5 isolates of *C. michiganensis* subsp. *michiganensis* + 3 standard deviations.

This data was generated from pure cultures.

4.2 Analytical specificity data

In total 67 strains of *C. michiganensis* subsp. *michiganensis* and other closely related bacteria (other *C. michiganensis* subspecies and look-alikes) were used to test the specificity of this real-time PCR. More specifically:

- 41 different *C. michiganensis* subsp. *michiganensis* isolates, covering different geographical origins, which are all positive in pathogenicity tests on tomato.
- 22 different *C. michiganensis* look-alikes which are all negative in pathogenicity tests on tomato.
- 4 different *C. michiganensis* isolates from different host plants: *C. michiganensis* subsp. *insidiosus*, *C. michiganensis* subsp. *tessellarius*, *C. michiganensis* subsp. *nebraskensis* and *C. michiganensis* subsp. *sepedonicus*.

100% of the *C. michiganensis* subsp. *michiganensis* were found positive, so no false negative results were obtained. For the non-*C. michiganensis* subsp. *michiganensis* strains tested, 1 strain isolated and provided by Syngenta gave a false positive result.

4.3 Data on repeatability and reproducibility

Repeatability and reproducibility of the conventional PCR were found to be 100%. All 4 isolates of *C. michiganensis* subsp. *michiganensis* and 4 *Clavibacter michiganensis* look-alikes tested gave the expected results.

Appendix 8 – Real-time PCR test (Oosterhof & Berendsen, 2011)

1. General information

1.1 This real-time PCR test has been targeted on the putative two-component system sensor kinase (PTSSK) using sequence data acquired from *C. michiganensis* subsp. *michiganensis* strain NCPPB 382.

1.2 The specific amplicon location (first base pair, including primer sequences) is defined from 22792142 to 2792274.

1.3 The PCR test should be performed with the primers RZ_ptssk 10/RZ_ptssk 11 and the probe RZ_ptssk 12 (Applied Biosystems).

Forward RZ_ptssk 10: 5′-GGGGCCGAAGGTGCTG GTG-3′

Reverse RZ_ptssk 11: 5′-CGTCGCCCGCCCGCTG-3′

Probe RZ_ptssk 12: 6FAM-TGGTCGTCCTCGGC G-MGBNFQ

1.4 Internal control primers:

CMT-F 5′-GGG CCG CAC CTT CG-3′

CMT-R5′-CGT TTC GCC TCC CCT AGA-3′

CMT probe Texas red-TCG TCC CTG AGT GGA TGG TGG TG-BHQ2

1.5 The amplicon size in base pairs (including primer sequences) is 132 bp.

1.6 The nucleic acid source is pure cultures.

1.7 Real-time PCR system used for validation: CFX96 Real Time System (Bio-Rad).

2. Method

2.1 Nucleid acid extraction

Prepare a cell suspension from each putative *C. michiganensis* subsp. *michiganensis* isolate. Lyse bacterial cells by heating for approximately 15 min at 95°C and cool on ice. Alkaline or sodium hydroxide treatment may improve cell lysis. Centrifuge for 1 min at 10 000 *g* and use 2 µL of the supernatant for real-time PCR.

2.2 Polymerase chain reaction

2.2.1 Master mix

Reagent	Working concentration	Volume (µL) in 25 µL	Final concentration
TaqMan master mix (Applied Biosystems)	2×	12.5	1×
RZ_ptssk 10	20 µM	0.3	0.24 µM
RZ_ptssk 11	20 µM	0.3	0.24 µM
RZ_ptssk 12	20 µM	0.3	0.24 µM
Bacterial suspension	–	2.0	–
Molecular-grade water	–	To make up to 25.0	–
Total	–	25.0	–

2.2.2 PCR conditions

Incubation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. The ramp speed should be 5°C s^{-1}.

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 potential contamination during nucleic acid extraction. Nucleic acid extraction and subsequent amplification of sterile extraction buffer.
- Positive isolation control (PIC): to ensure that nucleic acid of sufficient quantity and quality is isolated. Perform DNA extraction from a *C. michiganensis* subsp. *michiganensis* reference strain, for example PD233 (Appendix 3).
- Negative amplification control (NAC): to rule out false positives due to contamination during the preparation of the reaction mix. The molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC): to monitor the efficiency of the amplification. Add nucleic acid of *C. michiganensis* subsp. *michiganensis* reference strain, for example PD233, to a PCR reaction (Appendix 3).

Internal positive controls (IPCs) are used with each sample to ensure adequate DNA extraction and PCR amplification. Samples spiked with non-*C. michiganensis* subsp. *michiganensis* exogeneous nucleic acid should be included. The bacterial suspension of *C. michiganensis* subsp. *michiganensis* should be spiked with a related bacterium and also tested with primers for this bacterium. The related bacterium should be added in a 100× lower concentration to avoid competition with the specific target. For example, *C. michiganensis* subsp. *tessellarius* can be used, together with Cmt-primers.

3.2 Interpretation of the real-time PCR results

The cycle (Ct) cut-off value is set at 35, and was obtained using the equipment/materials and chemistry as described in this appendix. When necessary the Ct cut-off value should be determined for the required control. The Ct cut-off value needs to be verified in each laboratory when implementing the test for the first time.

Verification of the controls

- The PIC and PAC amplification curves should be exponential.
- NIC and NAC should be negative (Ct > cut-off value).
- PIC, PAC should have a Ct value below 30.
- IPC should have a Ct value below the expected value for the related bacteria chosen.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve and a Ct value below the cut-off value.
- A test will be considered negative if it produces no exponential amplification curve and a Ct value equal to or greater than the cut-off value.
- Tests should be repeated if any contradictory or unclear results are obtained (if both the internal control and specific target do not give a signal no conclusion can be made and the PCR should be repeated).

4. Performance criteria available (the real-time PCR for the identification of *C. michiganensis* subsp. *michiganensis* has been validated according to EPPO Standard PM 7/98)

4.1 Analytical sensitivity data

The limit of detection (LOD) is 2×10^3 cfu mL^{-1}, based on the mean LOD of 5 *C. michiganensis* subsp. *michiganensis* isolates + 3 standard deviations.

This data was generated from pure cultures.

4.2 Analytical specificity data

In total 67 strains of *C. michiganensis* subsp. *michiganensis* and other closely related bacteria (other *C. michiganensis* subspecies and look-alikes) were used to test the specificity of this real-time PCR. More specifically:

- 41 different *C. michiganensis* subsp. *michiganensis* isolates, covering different geographical origins, which were all positive in pathogenicity on tomato.
- 22 different *C. michiganensis* look-alikes which were all negative in pathogenicity on tomato.
- 4 different *C. michiganensis* isolates from different host plants: *C. michiganensis* subsp. *insidiosus*, *C. michiganensis* subsp. *tessellarius*, *C. michiganensis* subsp. *nebraskensis* and *C. michiganensis* subsp. *sepedonicus*.

The primer–probe combination gave positive results for all the *C. michiganensis* subsp. *michiganensis* isolates. No false positives were found for the non-*C. michiganensis* subsp. *michiganensis* isolates tested. The method was found to be very specific.

4.3 Repeatability and reproducibility

Repeatability and reproducibility of the real-time PCR were found to be optimal. All 4 isolates of *C. michiganensis* subsp. *michiganensis* and 4 *C. michiganensis* look-alikes tested gave the expected results. Ct values ranged between 20.18 and 22.78 for *C. michiganensis* subsp. *michiganensis*.

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CORRIGENDUM

Corrigendum - PM 7/42 (3) *Clavibacter michiganensis* subsp. *michiganensis*

An error was noted in the Standard PM 7/42 (3) *Clavibacter michiganensis* subsp. *michiganensis* (EPPO, 2016).

In the description of Procedure B: IF, PCR and Bioassay, section B1 on extraction from seeds, under preparation of samples (page 210) it was written 'Add 7.5 mL of **0.1 M** PBS (Appendix 2) per gram of seeds.'

This should have read 'Add 7.5 mL of **0.01 M** PBS (Appendix 2) per gram of seeds.'

The correct concentration was given in Appendix 2 (which describes the Buffers and Media used in the protocol). The EPPO Secretariat apologizes for this error and thanks the reader who highlighted this inconsistency.

REFERENCE

EPPO (2016) PM 7/42 (3) *Clavibacter michiganensis* subsp. *michiganensis*, *EPPO Bulletin* 46, 202–225.