Diagnostics
Diagnostic

Clavibacter michiganensis subsp. *insidiosus*

Specific scope

This standard describes a diagnostic protocol for *Clavibacter michiganensis* subsp. *insidiosus*.[1]

Specific approval and amendment

Approved as an EPPO Standard in 2010–09.

Introduction

Clavibacter michiganensis subsp. *insidiosus* is a widespread seed-transmitted disease of *Medicago sativa* (lucerne, alfalfa). It has been reported as an important bacterial disease of *Medicago sativa*, causing reduction of vigour and growth of the crop, and considerably decreasing yield. The pathogen has been reported in most important lucerne-producing areas in the USA and Canada in the 20th Century. Although previously reported in the EPPO region (in Italy, Lithuania, Romania, Russia and the United Kingdom), findings were sporadic and there have been no reports since the 1980s. Further details on the geographic distribution are presented in the EPPO distribution map (see http://www.eppo.org).

Medicago sativa is the most important host but *Lotus corniculatus*, *Medicago falcate* (yellow flowered lucerne), *Medicago* spp., *Melilotus alba* (sweet clover), *Onobrychis viciifolia* (sainfoin) and *Trifolium* sp. are also reported as natural hosts (Bradbury, 1986). Many other *Medicago* spp. were also found to be potential hosts following inoculation (Bradbury, 1986).

The pathogen can survive for up to 10 years in dried plant debris and seeds (Cormack, 1961). There is no documented evidence on length of survival of the bacterium in soil.

The pathogen can be present in seed lots both as a contaminant (on the surface of seeds, in dust or in plant debris) or as systemic infections inside seeds. Seed transmission appears to be low and contaminated plants most often produce low quantities of seeds. The pest can spread from plant to plant in particular via wind-blown soil and debris, irrigation water or harvesting equipment. The nematode *Ditylenchus dipsaci* and root knot nematode *Meloidogyne hapla* are known to favour infections in plots (Hunt *et al.*, 1971). Further information on the host range, geographic distribution and biology can be found in the EPPO data sheet on *C. michiganensis* subsp. *insidiosus* (EPPO/CABI, 1997).

This diagnostic procedure for *C. michiganensis* subsp. *insidiosus* (Figs 1 and 2) describes extraction from plant or seeds, presumptive diagnosis with a rapid test, isolation of presumptive bacterial colonies, identification of presumptive isolates and, where relevant, determination of pathogenicity.

Identity

Name: *Clavibacter michiganensis* subsp. *insidiosus* (McCulloch) Davis *et al.*
Synonyms: *Corynebacterium insidiosum* (McCulloch) Jensen., *Corynebacterium michiganense* pv. *insidiosum* (McCulloch) Dye & Kemp, *Aplanobacter insidiosum, Corynebacterium michiganense* subsp. *insidiosum* (McCulloch) Carlson & Vidaver.
Taxonomic position: Procaryotae, Actinobacteria, Actinomycetales, Microbacteriaceae.

The genus *Clavibacter* was designed to accommodate the plant pathogenic coryneform bacteria of which the cell wall peptidoglycan contains 2,4-diaminobutyric acid as dibasic amino acid (Davis *et al.*, 1984). These strictly aerobic, Gram-positive rods do not produce endospores. V, Y and palisade arrangements of cells are usually observed.
EPPO code: CORBIN.
Phytosanitary categorization: EPPO A2 list no. 49/EU Annex designation II/A2.

Detection

Disease symptoms

Generally, *C. michiganensis* subsp. *insidiosus* causes systemic infection of alfalfa plants. The disease may induce wilting under

[1]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.

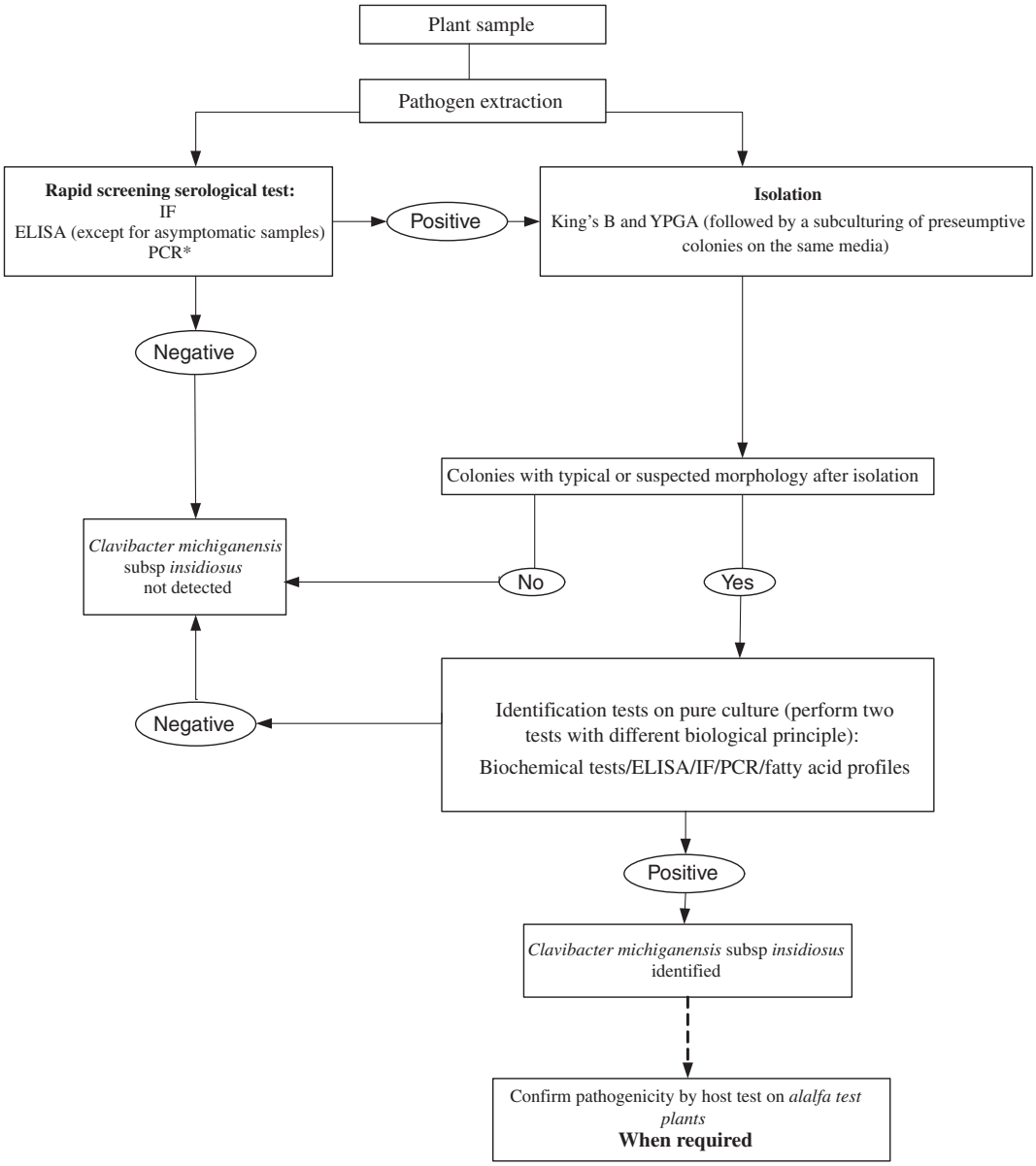

Fig. 1 Flow diagram for the detection and identification of *C. michiganensis* subsp. *insidiosus* on plants.

dry and hot conditions, but most often symptoms consist only of stunting and proliferation of stems. Chlorosis, reduction of size and cupping of leaflets are quite common as well as marginal, papery, white grey necrosis of leaflets (ADAS, 1979).

In some varieties, the vascular root system may be discoloured yellowish-brown. Moist or full-ring discolourations may appear in the outer cortex of cut roots. Wilt symptoms caused by *C. michiganensis* subsp. *insidiosus* may be confused with other systemic diseases caused, for instance, by *Verticillium albo-atrum* lucerne strains.

Detection in symptomatic plant material other than seeds

Extraction from symptomatic plants

Wilting or stunted plants should be uprooted and the upper part of the stem and small roots removed. Laboratory analysis should be performed as soon as possible (preferably within 72 h). The sample should be kept in appropriate conditions prior to analysis (e.g. avoid excessive temperatures). Roots should be carefully washed using tap water to remove

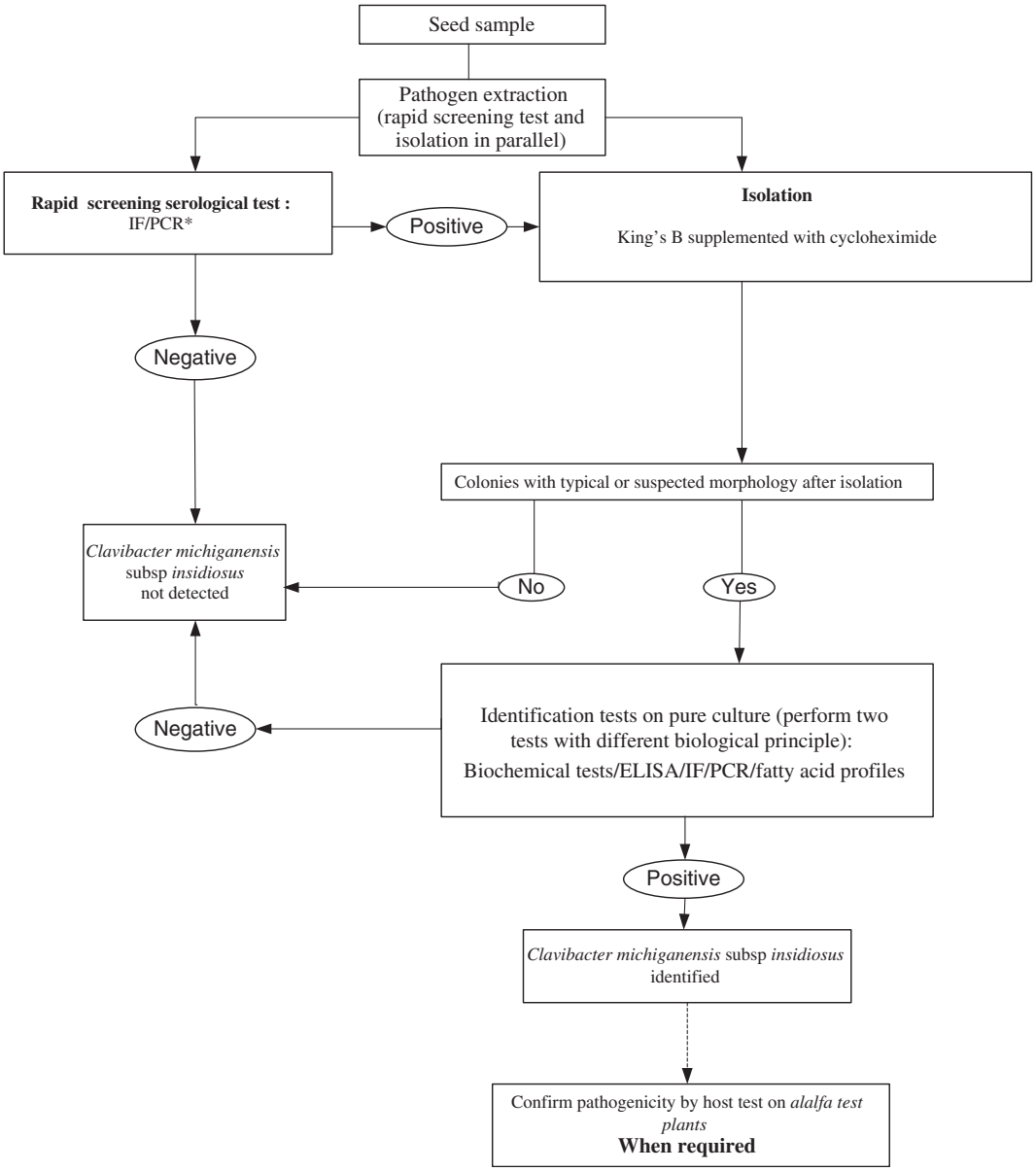

Fig. 2 Flow diagram for the detection and identification of *C. michiganensis* subsp. *insidiosus* on seeds.

soil and avoid contamination by saprophytes as far as possible. Main roots and stem bases should be cut transversely with a clean disinfected blade and examined for the presence of vascular discolouration.

Where symptoms are seen on cut sections, the epidermis should be carefully removed and small sections of symptomatic vascular tissue extracted and transferred into a small volume of sterile distilled water or phosphate buffer (50 mM PB; see Appendix 1). The tissue is then comminuted with a clean and disinfected scalpel to allow bacterial diffusion for 5–10 min. This suspension should be used, preferably immediately, for isolation (see below), immunofluorescence (see PM 7/97), ELISA (see Appendix 2) or PCR (see Appendix 3). Commercial antisera are available (e.g. Florilab, Neogen-Agden, Plant Research International). If necessary, the suspension can be refrigerated for up to 24 h. For longer preservation, the suspension should be stored below −18°C with 10–30% glycerol.

Dilution plating

Both King's B and YPGA media supplemented with 200 mg L^{-1} of cycloheximide are appropriate for isolation of *C. michiganensis* subsp. *insidiosus* from diseased vascular tissues (see Appendix 1). As culture plates may be overgrown by quicker growing saprophytes, dilution techniques are likely to be required for isolation (i.e. streaking in sectors or spreading of serially-diluted macerate).

Spread or streak the macerate and dilutions of macerate (1/10, 1/100) over the surface of the medium. As a reference, also inoculate plates with a diluted cell suspension of the *C. michiganensis* subsp. *insidiosus* reference strain. Incubate the plates at 24°C for up to 14 days. Disregard colonies appearing up to 72 h as the isolation of this bacterium takes longer. Generally, 2–3 mm colonies of *C. michiganensis* subsp. *insidiosus* develop within 4–5 days. These are light yellow, flat and semi-fluidal, round or irregular. Colonies become deeper yellow, opaque and glistening with longer incubation. Many strains may produce a distinctive indigo diffusible pigment (indigoidine) on certain media (including YPGA and YDC). Presumptive colonies should be purified by sub culturing on King's B or YPGA media.

Molecular tests
Several molecular tests exist to identify pure cultures of *C. michaganensis* subsp. *insidiosus*. However, none of them have their DNA extraction method validated for naturally infected plant material. Therefore, they should not be used without prior validation.

Detection from asymptomatic plant material other than seeds

Extraction from asymptomatic plants
In asymptomatic material, the same process as described above should be followed by excising and macerating in sterile water or phosphate buffer with small sections of symptomless vascular tissue from the main root. The macerate should be used immediately for isolation (see below) or immunofluorescence (see PM 7/97). Optimum size sample for latent testing and sampling period has not been determined.

Dilution plating
See process described for symptomatic plant material. On asymptomatic plants, the probability of success of isolation is much lower than for symptomatic plants.

Molecular tests
Several molecular tests exist to identify pure cultures of *C. michaganensis* subsp. *insidiosus*. However, none of them have their DNA extraction method validated for naturally infected plant material. Therefore, they should not be used without prior validation.

Detection from seeds

Extraction
The standard sample size is 5000 seeds. The sample should be divided into sub samples of a maximum of 1000 seeds each. Each sub sample of 1000 seeds is transferred into a sterilized screw cap flask or into a sterile-adapted plastic bag (e.g. Stomacher® bags). Approximately 10–20 mL of sterile sample phosphate buffer (see Appendix 1) is added to the seeds. Bags or flasks are placed onto a rotary shaker for 72 h at 100–150 rpm at 5°C (±4°C). The extraction period allows for the retrieval of bacteria but saprophyte contamination may occur. Care should be made to store bags securely during soaked incubation, allowing air exchange without spilling macerate or cross-contaminating (an appropriate bag holder should be used).

Approximately 1–1.5 mL of each extract in an Eppendorf or equivalent sterile microtube, should be kept for reference at 5°C (±4°C) for up to 48 h. Alternatively for longer storage, the extract can be kept frozen below −18°C after addition of 10–30% glycerol. The remaining extract should be used immediately for isolation (see below) or immunofluorescence (see PM 7/97).

Dilution plating
King's B agar supplemented with cycloheximide has been used routinely with good results from seed extracts (Nemeth *et al.*, 1991). Nevertheless it should be noted that isolation from seeds as a screening test is difficult because seeds are often overloaded with many saprophytic bacteria including a number of Gram-positive ones. These are often not easily distinguishable from *C. michiganensis* subsp. *insidiosus* based on phenotypic characteristics. No selective or semi selective media are available to date for isolation of *C. m*. subsp. *insidiosus* from *Medicago sativa* seeds. See the process described for symptomatic plant material.

Other tests
A double antibody sandwich ELISA test has been designed (Gooden & Ophel Keller, 2005) for the detection of *C. michiganensis* subsp. *insidiosus,* using a monoclonal antibody for coating and a polyclonal antibody for detection on artificially contaminated seeds and pure cultures. It has not yet been validated on naturally infected seeds and should not be used without prior validation for detection in seeds. The availability of the monoclonal antibody is not known and consequently the test is not described in full.

Regarding PCR tests, trials carried out by Gooden & Ophel Keller (2005) and Caffier (unpubl. results) on artificially contaminated seeds indicate that the threshold is at the best about 10^5–10^6 cfu mL^{-1} of seed extract. Independent from all available primer sets the sensitivity varies according to PCR conditions. Therefore, existing PCR tests should not be used without prior validation.

Identification

From the tests given below, two tests with different biological principles should be chosen for identification of presumptive colonies.

Biochemical characteristics

The following phenotypic properties which are universally present or absent in *C. michiganensis* subsp. *insidiosus* should be determined using the methods of Dye & Kemp (1977).

Some strains of *C. michiganensis* subsp. *insidiosus* may produce a highly characteristic blue, diffusible pigment on King's B agar medium, but this is not always the case. Biochemical characteristics that should be checked are given in Table 1.

Table 1 Biochemical characteristics for *C. michiganensis* subsp. *insidiosus*

Gram stain	+
O/F metabolism of glucose	O
Catalase	+
Cytochrome C oxidase	–
Aesculin hydrolysis	+
Acid from mannose	+
Acid from mannitol	–
Acid from rhamnose	–
Acid from galactose	+
Acid from xylose	+
Acid from sorbitol	–
Utilization of sodium acetate	–
Utilization of sodium succinate	–
Utilization of lactate	–
Growth in 6% NaCl	–
Hydrolysis of potato starch	+
H_2S from peptone	–

– = negative result, + = positive result (from Vidaver & Starr, 1981; Lelliott & Stead, 1987; Bradbury, 1986).

Serological tests

IF test

Commercial antisera are available (e.g. Florilab, Neogen-Agden, Plant Research International). The IF test is described in PM 7/97.

ELISA test

Commercial antisera are available (e.g. Florilab, Neogen-Agden, Plant Research International). The test is described in Appendix 2.

PCR tests

PCR tests can be used to identify pure cultures of *C. michiganensis* subsp. *insidious*. Three different conventional PCR tests are described in Appendix 3 (Samac *et al.*, 1998; Pastrik & Rainey, 1999; Borowicz, 2001). Two real-time PCR tests have been published (Bach *et al.*, 2003; Marefat *et al.*, 2007). They are described in Appendix 4.

An international ring test has been performed on reference strains and strains isolated from seeds with the tests Samac *et al.* (1998) and Pastrik & Rainey (1999). This ring test has shown that those PCR tests may lead to false positive identifications (Caffier, unpubl. results), depending on genotypic variation and amplification conditions. Therefore, it is recommended that each test is first adapted and validated to the PCR conditions in force in the laboratory (reagents and cyclers) and that at least two sets of primers are used for identification. A PCR test should only be declared positive for *C. michiganenisis* subsp. *insidiosus* if two positive results with different primer sets are obtained.

Genomic fingerprinting

Presumptive isolates identified as *C. michiganensis* subsp. *insidiosus* can be further characterized with fingerprint patterns generated by BOX-PCR. Four distinct groups were identified by polymorphism in the 1 Kb region (Louws *et al.*, 1998). The test is described in PM 7/100 on *Rep-PCR tests for identification of pure cultures of bacteria.*

Fatty acid profiling (FAP)

This test is described in Appendix 5.

Pathogenicity test

When required for a diagnosis, this test is meant to confirm a positive identification of *C. michiganensis* subsp. *insidiosus* that is based on two of above-mentioned tests with different biological principles. It is described in Appendix 6.

Strain characterization methods

Genomic fingerprint and fatty acid profiling can be used for strain characterization.

Reference strain

NCPPB 1109 (equivalent strain designations: LMG 3663, CFBP 2404, ICMP 2621).

Reporting and documentation

Guidance on reporting and documentation is given in EPPO Standard PM7/77 (1) *Documentation and reporting on a diagnosis.*

Further information

Further information on this organism can be obtained from:

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This protocol was originally drafted by:

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Appendix 1 – Preparation of media and buffers

Media

Nutrient glucose agar		Yeast peptone glucose agar	
Difco nutrient agar	11.5 g	Difco yeast extract	2.5 g
D(+) glucose	5.0 g	Difco bacto peptone	2.5 g
Distilled water	500 mL	D(+) glucose	5.0 g
		Difco bacto agar	7.5 g
		Distilled water	500 mL

King's B medium (King *et al.*, 1954):		Yeast Dextrose Chalk agar (YDC)	
Proteose peptone N°3	10 g	Difco yeast extract	5.0 g
Glycerol	5 mL	D-glucose	10.0 g
K_2HPO_4	0.75 g	Precipitated chalk ($CaCO_3$)	10.0 g
$MgSO_4 \cdot 7H_2O$ 1.5 g	0.75 g	Oxoid agar no.3	6.0 g
Agar	7.5 g	Distilled water	500 mL
Distilled water	500 mL		
Adjust pH to 7.0–7.2.			

Prepare 0.5 L of medium in 1 L Erlenmeyers or flasks.
Dissolve ingredients and sterilize by autoclaving at 121°C for 15 min.

Buffers

50 mM phosphate buffer (PB), pH 7.0	
Na_2HPO_4	4.26 g
KH_2PO4	2.72 g
Distilled water	1 L

Dissolve ingredients, check pH and adjust to pH 7.0 if necessary and sterilize by autoclaving at 121°C for 15 min.
50 mM phosphate buffer Tween® (PB-T), pH 7.0
Add Tween® 20 to phosphate buffer 50 mM pH 7.0 (see before) to a final concentration of 0.2%.

Carbonate buffer pH = 9.6	
Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
Distilled water	1.0 L

Adjust pH to 9.6 with HCl (HCl solution volume is deduced from distilled water volume). Sterilize by autoclaving.

Conjugate buffer	
Bovine serum albumin	0.02 g
PB-T	10 mL

Adjust pH to 7.4. The buffer should not autoclaved. Prepare just before use.

Substrate buffer (for alkaline phosphatase)	
Diethanolamine	97.0 mL
distilled water	to make up to 1.0 L

Adjust pH to 9.8 with concentrate HCl solution (HCl solution volume is deduced from distilled water volume). Sterilize by autoclaving.

Just before use add para nitrophenyl phosphate (pNPP) to reach a final concentration of 1 mg mL^{-1}.

Appendix 2 – ELISA

For extraction of the bacteria from plant samples or colonies, use a buffer recommended by the kit supplier. According to the kit, DAS ELISA or Antigen Coated Plate ELISA (ACP- ELISA) may be used.

For ACP ELISA and when no protocol is given by the provider, use the following procedure.

Add 200 µL of each sample in carbonate buffer pH = 9.6 (see Appendix 1) for coating the plates. Incubate at 37°C for 4 h (±15 min) preferably in a closed box and wash 3 times for 5 min (±1 min) with PBS-T (see Appendix 1).

Add 200 µL of an appropriate dilution of specific rabbit antiserum to each empty well, incubate at 37°C for 4 h (±15 min) as described above and wash as above.

Add 200 µL of an appropriate dilution of goat anti-rabbit antibodies conjugated with alkaline phosphatase in conjugate buffer (see Appendix 1) to each empty well, incubate at 37°C for 4 h as above (±15 min).

Prepare a 1 mg mL^{-1} alkaline phosphatase substrate (para nitrophenyl-phosphate) in substrate buffer (see Appendix 1). Wash ELISA plates as above and add 200 µL of the phosphatase substrate to each well, incubate at room temperature, preferably not in direct light and read at 405 nm preferably when positive controls or positive samples give an OD value between 1.2 and 1.6.

Note that the test can also be done with volumes of only 100 µL at each step, but with lower detection sensitivity.

Samples are positive when their OD is at least twice that obtained with the plant negative control sample.

Appendix – 3 Conventional PCR tests

These tests have only been validated for pure cultures and if they are used for plant material extraction and controls have to be adapted

After extraction/purification of DNA from samples, transfer the requested volume of samples to PCR vials. Add PCR mix to the samples according to descriptions below and mix by gentle aspiration in the pipette tip.

Yield of amplification may vary according to the DNA polymerases, PCR master mix, $MgCl_2$ concentrations etc. used, and therefore PCR should be validated in each laboratory prior to routine use.

Modification of the duration of the amplification cycles may be required for the use of other thermal cyclers than those described by the authors.

Reveal DNA amplicons after electrophoresis with appropriate weight markers using ethidium bromide staining and UV transillumination.

In all cases, nucleic acid source is a bacterial colony, Molecular Grade Ultra Pure Water (MGUPW) is used for PCR mix.

(1) Preparation of DNA samples.

Suspend a single colony from each presumptive isolate, in sterile microvials containing 100 µL of sterile distilled water. Alkaline treatment assists cell lysis (Zhang & Goodwin, 1997). Add 50 µL of 0.25N NaOH to 100 µL of suspended bacteria. Vortex. Close vials and heat for 6 min at 95°C. After cooling, add 50 µL of 0.25N HCl. Vortex. Add 25 µL of 0.5 M Tris-HCl (pH 8.0) – Tween20 (1% v/v). Close vials and heat for another 6 min at 95°C. Pulse centrifuge after cooling on ice. Heat closed vials at 95°C for 12 min to disrupt cells. Transfer immediately heated suspensions to melting ice (or equivalent) for at least 1 min. Pulse centrifuge after cooling.

(2) PCR runs.

(A) PCR test (Samac *et al.*, 1998).

1. General information
   - 1.1 The oligonucleotide primers are derived from IS 1122, a 1.1 kbp-long sequence largely repeated in the genotype of *C. michiganensis* subsp. *insidiosus*.
   - 1.2 The amplicon size from *C. michiganensis* subsp. *insidiosus* DNA is 132 bp.
   - 1.3 Oligonucleotides:
     Forward primer CIRS 1: 5′- TTC AAC CGC ACC CTC GCG AC - 3′
     Reverse primer CIRS 2: 5′- CGT CAG CCC GTG GCT CGA GT - 3′
   - 1.4 Eurogentec Goldstar Red DNA polymerase (5 U μL^{-1}) for instance, but Perkin Elmer AmpliTaq and 10x PCR buffer can also be used at the same concentrations.
   - 1.5 Thermal cycler: Applied Biosystems, Foster City, CA, US Perkin Elmer model 480 or 9700 cyclers.

1.6 The test was developed with 3 strains of *C. michiganensis* subsp. *insidiosus* (strains 33114, 10253, Ci4). It should be noted that the test produced a barely detectable amount of amplification product with *C. michiganensis* subsp. *sepidonicus* but a larger quantity of product was formed when the template was increased 2000-fold. However, these IS*11*22 derived products from *C. michiganensis* subsp. *sepidonicus* did not hybridize with ^{32}P-labelled CIRS-3. No amplification product was observed for the other *C. michiganensis* subspecies tested: *michiganensis, nebraskensis* and *tessellarius.* Further tests with DNA from uninfected alfalfa and 39 other bacteria including *Agrobacterium tumefaciens, Agromyces ramosus, Arthrobacter ilicis, A. globiformis, Auroebacterium testaceum, Bacillus cereus, B. subtilis, Brevibacterium casei, Clavibacter xyli cynodontis, Corynebacterium glutamicum, Curtobacterium flaccumfaciens* subsp. *betae, C. flaccumfaciens* subsp. *laccumfaciens, C. flaccumfaciens* subsp. *oortii, C. flaccumfaciens* subsp. *poinsettiae, Erwinia carotovora* subsp. *amylovora, E. carotovora* subsp. *carotovora, E. herbicola, E. stewartii, Escherichia coli, Microbacterium lacticum, Micrococcus luteus, Mycobacterium phlei, Pseudomonas aurantiaca, Ralstonia. solanacearum, Rathayibacter iranicus, R. rathayi, R. toxicus, R. tritici, Rhizobium phasioli, Rhodococcus fascians, m, Serratia marcescens, and S. scabies* and showed that the test was selective for *C. michiganensis* subsp. *insidiosus.*

2. Methods

Amplification conditions are the following:

2.1 Total standard reaction volume of a single PCR reaction 50.0 µL.

2.2 Adequate volume of PCR-grade water so that final volume is equal to 50 µL per tube.

2.3 5 µL of 10x PCR buffer

2.4 1.5 µM $MgCl_2$

2.5 0.2 µM dNTPs

2.6 0.75 units of Taq polymerase

2.7 1 µM Forward Primer

2.8 1 µM Reverse Primer

2.9 10 µL DNA sample (the solution should be fresh).

2.10 PCR cycling parameters for Applied Biosystems Perkin Elmer model 480 cycler: 30 cycles (4 cycles 2 min, 26 cycles of 60 s) at 94°C (denaturation of DNA), 60 s 58°C (annealing of primers), 60 s (first 29 cycles) then 5 min (last cycle) 75°C (elongation of DNA) hold at 4°C.

On a thermal cycler Applied Biosystems PE 9700, the following cycle can be used (Caffier, unpubl. results): 1 cycle 3 min 94°C, 35 cycles 40 s 94°C, 40 s 65°C, 40 s 72°C, 1 cycle 5 min 72°C, hold at 4°C. With this cycler, it is therefore possible to run this amplification and the one described in part B of this Appendix at the same time on the same thermal cycler. Nevertheless, this protocol is not suitable for multiplex PCR.

3. Essential procedural information

3.1 Controls: every time a PCR-based test is performed, the following controls should be included:

Essential:

- A contamination ('negative') control
- A test ('positive') control from a reference culture of *C. michiganensis* subsp. *insidiosus*.

Recommended:

- An extraction contamination control for every batch of samples tested. This consists of performing a nucleic acid (NA) extraction using a known 'blank' sample that does not include target NA (e.g. or clean extraction buffer)
- An extraction inhibition control used to monitor for the co-extraction of assay inhibitors. This can include testing extracted NA with a PCR-based assay known to amplify a non-target specific sequence (e.g. a conserved host gene or a 'universal' ITS gene). Alternatively, where available, a synthetic Internal Amplification Control can be used from a culture of *C. michiganensis* subsp. *insidiosus* strain.

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

- A PCR test will be considered positive if it produces amplicons of 132 bp and provided that the contamination controls are negative
- A PCR test will be considered negative, if it produces no band or no band of a similar size and provided that the assay and extraction inhibition controls are positive
- Tests should be repeated if any contradictory or unclear results are obtained.

A faint 132 bp band may be produced by cultures of *C. michiganensis* subsp. *sepidonicus.* To verify that the fragment is not from *C. michiganensis* subsp *insidiosus* hybridization can be carried out according to Samac *et al.* (1998)

(B) PCR protocol (Pastrik & Rainey, 1999).

1. General information

1.1 The oligonucleotide primers are derived from the 16S-23S rRNA intergenic spacer region.

1.2 The amplicon size from *C. michiganensis* subsp. *insidiosus* DNA is 393 bp

1.3 Oligonucleotides:

Forward primer PSA-5: 5′- CCC TTT CCG TCG TCC CGG A - 3′

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

1.4 Taq DNA polymerase (Life Technology, DE)

1.5 Thermal cycler: Applied MJ Research PTC 200.

1.6 This test was developed with three strains of *C. michiganensis* subsp *insidiosus*. Other *C. michiganensis* subsp were tested: 7 strains of *C. michiganensis* subsp *sepedonicus,* 6 strains of *C. michiganensis* subsp *michiganensis,* 1 strain of *C. michiganensis* subsp *tessellarius* and 1 strain of *C. michiganensis* subsp *nebraskensis.* These subsp. gave bands that were different sizes to that of *C. michiganensis* subsp *insidiosus* other than for

C. michiganensis subsp *nebraskensis* which gave a band that was the same size but, as a pest of maize, *C. michiganensis* subsp *nebraskensis* should not be found on alfalfa. The test was also carried out on 5 strains of *Ralstonia solanacearum,* 4 strains of *Pectobacterium atrosepticum,* 4 strains of *Pectobacterium carotovorum* subsp. *carotovorum*, 2 strains of *Erwinia* sp., 2 strains of *Erwinia rhapontici*, 2 strains of *Pseudomonas syringae* pv. *atrofaciens*, 1 strain of *Pseudomonas syringae* pv. *morsprunorum* and 1 strain of *Pseudomonas syringae* pv. *phaseolicola* and showed that the test was selective for *C. michiganensis* subsp. *insidiosus* on alfalfa.

2. Methods
   - 2.1 Amplification conditions are the following: total reaction volume of a single PCR reaction is 25.0 µL and test can be carried out with a total volume of 50 µL; in that case volumes of all reagents may be doubled and volume of PCR grade water adjusted to a final total volume of 50 µL.
   - 2.2 Adequate volume of PCR grade water so that final volume is equal to 25 µL per tube.
   - 2.3 2.5 µL of PCR buffer
   - 2.4 1.5 µM $MgCl_2$
   - 2.5 100 µM dNTPs
   - 2.6 1 unit of Taq polymerase
   - 2.7 0.2 µM Forward Primer
   - 2.8 0.2 µM Reverse Primer
   - 2.9 10 µL of DNA sample (the solution should be fresh).
   - 2.10 PCR cycling parameters for Applied MJ Research PTC 200: 1 cycle of 90 s 94°C, 30 cycles 30 s 94°C, 20 s 64°C, 45 s 72°C, 1 cycle of 5 min at 72°C. Hold at 4°C. On a thermal cycler Applied Biosystems PE 9700, the following cycle can be used (Caffier, unpubl. results): 1 cycle 3 min 94°C, 35 cycles 40 s 94°C, 40 s 65°C, 40 s 72°C, 1 cycle 5 min 72°C, hold at 4°C. With this cycler, it is therefore possible to run this amplification and the one described in part A of this annex at the same time on the same thermal cycler. Nevertheless, this protocol is not suitable for multiplex PCR.
3. Essential procedural information
   - 3.1 Controls: every time a PCR-based test is performed, the following controls should be included:

Essential:

- A contamination ('negative') control
- A test ('positive') control from a reference culture of *C. michiganensis* subsp. *insidiosus*.

Recommended:

- An extraction contamination control for every batch of samples tested. This consists of performing a NA extraction using a known 'blank' sample that does not include target NA (e.g. clean extraction buffer)
- An extraction inhibition control used to monitor for the co-extraction of assay inhibitors. This can include testing extracted NA with a PCR-based assay known to amplify a non-target specific sequence (e.g. a conserved host gene or a 'universal' ITS gene). Alternatively, where available, a synthetic Internal Amplification Control can be used from a culture of *C. michiganensis* subsp. *insidiosus* strain

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

- A PCR test will be considered positive if it produces amplicons of 393 bp and provided that the contamination controls are negative
- A PCR test will be considered negative, if it produces no band or no band of a similar size and provided that the assay and extraction inhibition controls are positive
- Tests should be repeated if any contradictory or unclear results are obtained.

(C) PCR test (Borowicz, 2001).

1. General information
   - 1.1 The oligonucleotide primers are derived from the 16S-23S rRNA intergenic spacer region.
   - 1.2 The amplicon size from *C. michiganensis* subsp. *insidiosus* DNA is 218 bp
   - 1.3 Oligonucleotides:
     Forward primer CMI-III/BB1: 5′- GAG GGA CCG GAC CGC ATC TTT CGG GG - 3′
     Reverse primer CMI-III/BB2: 5′- GAT TGA TTC GTT TCG CCT CCC CTA GA - 3′
   - 1.4 Recombinant DNA polymerase (TaKaRa Taq from Takara Shuzo Co, Kyoto, Japan.)
   - 1.5 Thermal cycler from Biometra, Göttingen, DE.
   - 1.6 The following bacteria were used: *C. michiganensis insidiosus*, *C. michiganensis ichiganensis* subsp. *michiganensis*, *C. michiganensis* subsp. *sepedonicus*, *Pseudomonas* sp. and *Pseudomonas syringae* pv. *pisi*. These bacteria were obtained from the Culture Collection of the Institute of Plant Protection in Poznan (PL).
2. Methods
   - 2.1 Amplification conditions are the following: total reaction volume of a single PCR reaction is 20.0 µL and the test can be carried out with a total volume of 50 µL; in that case volumes of all reagents may be adapted and volume of PCR grade water adjusted to a final total volume of 50 µL.
   - 2.2 Adequate volume of PCR grade water so that final volume is equal to 20 µL per tube.
   - 2.3 2 µL of PCR buffer (Mg^{++} free, from Takara Shuzo Co.)
   - 2.4 1.2 µL of 25 mM $MgCl_2$
   - 2.5 1.6 µL of 2.5 mM dNTPs each
   - 2.6 0.17 µL of Takara Taq DNA polymerase (5 units per µL)
   - 2.7 2 µL of 5 pM μL^{-1} forward primer
   - 2.8 2 µL of 5 pM μL^{-1} reverse primer
   - 2.9 2 µL of DNA (the solution should be fresh).
   - 2.10 PCR cycling parameters for the given cycler is: 34 cycles 60 s at 95°C, 60 s at 65°C, 1 cycle of 2 min at 72°C, the last elongation at 72°C during 10 min. Hold at 4°C.

3. Essential procedural information
   3.1 Controls: every time a PCR-based test is performed, the following controls should be included:

Essential:

- A contamination ('negative') control
- A test ('positive') control from a reference culture of *C. michiganensis* subsp. *insidiosus*.

Recommended:

- An extraction contamination control for every batch of samples tested. This consists of performing a NA extraction using a known 'blank' sample that does not include target NA (e.g. an extraction buffer)
- An extraction inhibition control used to monitor for the co-extraction of assay inhibitors. This can include testing extracted NA with a PCR-based assay known to amplify a non-target specific sequence (e.g. a conserved host gene or a 'universal' ITS gene). Alternatively, where available, a synthetic Internal Amplification Control can be used from a culture of *C. michiganensis* subsp. *insidiosus* strain.

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

- A PCR test will be considered positive if it produces amplicons of 218 bp and provided that the contamination controls are negative
- A PCR test will be considered negative, if it produces no band or no band of a similar size and provided that the assay and extraction inhibition controls are positive
- Tests should be repeated if any contradictory or unclear results are obtained.

Appendix – 4 Real-time PCR

These tests have only been validated for pure cultures and if they are used for plant material extraction and controls have to be adapted

(A) Real-time PCR (Marefat *et al.*, 2007).

1. General information
   1.1 The forward (CMIF) and reverse (CMIR) oligonucleotide primers are derived from the 16S-23S rRNA intergenic spacer region.
   1.2 The amplicon size from *C. michiganensis* subsp. *insidiosus* DNA is 224 bp
   1.3 Oligonucleotides:
   Forward primer CMIF: 5′- GTC AGG CGT TTG TCC TGG T - 3′
   Reverse primer CMIR: 5′- CCA CCA CCA TCC ACT CCG - 3′
   Probe BW: FAM – 5′ - CTG CTA GTA CGC CTC CTT GTG G - 3′ TAMRA[B].
   1.4 Thermal cycler: ABI Prism 7900HT.
   1.5 The test was developed with 13 strains of *C. michiganensis* subsp. *insidiosus* from different geographical origin 11 from Australia, one from the US one from the United Kingdom. It also included *C. michiganensis* subsp. *nebraskensis*, *C. michiganensis* subsp. *michiganensis, Arthrobacter ilicis, Curtobacterium flaccumfaciens*, *Rathayibacter iranicus*, *R. rathayi*, *R. tritici* and *R. toxicus,* and *Corynebacterium agropyri.* Other bacteria occurring naturally in and around lucerne plants were also included. The average minimum number of cells detected by the test when performed on pure cultures was 3.4 (mean values of three replicates) cells per PCR reaction. Similar sensitivity was achieved for plant extracts spiked with *C. michiganensis* subsp. *insidiosus* and incubated for 2 h. In comparative studies this test was shown to be more sensitive than the test of Bach *et al.* (2003) described in part B.
2. Methods
   The reaction mixture contains:
   2.1 1 µL of bacterial cell suspension (approx 10^8 cells per mL) or plant extract.
   2.2 5 µL of QuantiTect Probe PCR Master Mix (Qiagen Pty Ltd, Victoria, AU).
   2.3 0.2 µM of TaqMan probe and 0.4 µM of each primer in a final volume of 10 µL.
   The PCR cycling conditions are the following: 95°C for 15 min to activate the Taq DNA polymerase, followed by 40 cycles of 95°C for 15 s and 65°C for 1 min.
3. Essential procedural information
   3.1 Controls: every time a PCR-based test is performed, the following controls should be included:

Essential:

- A contamination ('negative') control
- A test ('positive') control from a reference culture of *C. michiganensis* subsp. *insidiosus*.

Recommended:

- An extraction contamination control for every batch of samples tested. This consists of performing a NA extraction using a known 'blank' sample that does not include target NA (e.g. clean extraction buffer)
- An extraction inhibition control used to monitor for the co-extraction of assay inhibitors. This can include testing extracted NA with a PCR-based assay known to amplify a non-target specific sequence (e.g. a conserved host gene or a 'universal' ITS gene). Alternatively, where available, a synthetic Internal Amplification Control can be used from a culture of *C. michiganensis* subsp. *insidiosus* strain.

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

- A test will be considered positive if it produces a Ct below 40 (undiluted samples) and provided that the contamination controls are negative
- A test will be considered negative, if it produces no signal below Ct40 provided that the assay and extraction inhibition controls are positive.
- Tests should be repeated if any contradictory or unclear results are obtained.

(B) Real time PCR (Bach *et al.*, 2003).

1. General information
   1.1. This test was developed for the identification of subspecies of *Clavibacter michiganensis* in a single PCR run.
   1.2 The forward (FP Cm) and reverse (RP Cm) oligonucleotide primers are derived from the 16S-23S rRNA intergenic spacer region.
   1.3 The amplicon size from *C. michiganensis* subsp. *insidiosus* DNA is 223 bp
   1.4 Oligonucleotides:
   Forward primer FP Cm: 5′- TGT CGA GGG CAT GTT GCA CG -3′
   Reverse primer RP Cm: 5′- GGA GAC AGA ATT GAC CAA TGA T- 3′
   Probe BW: FAM –5′ - TTC CGT CGT CCC GGA GTG GAT - 3′ TAMRA.
   FAM is fluorescein, 6 isomer, TAMRA is tetraethylrhodamine 6 isomer.
   1.5 Thermal cycler: ABI 7700 Sequence Detection System.
   1.6 The test was developed with 6 strains of *Clavibacter michiganensis* subsp. *Sepedonicus*, 2 strains of *C. michiganensis* subsp. *Michiganensis*, 3 strains of *C. michiganensis* subsp. *Nebraskensis*, 2 strains of *C. michiganensis* subsp. *insidiosus,* 3 strains of *C. michiganensis* subsp. *Tessellarius, Rathayibacter iranicus, R. rathayi, R. tritici, R. toxicus, Frigoribacter faeni, Cellulomonas biazotea, C. fimi, C. turbata, Arthrobacter globiformis, Nocardioides simplex, N. jensenii, Brevibacterium sp., Curtobacterium citreum, C. luteum, Rhodococcus fascians* and *Streptomyces griseus* subsp. *griseus.* These were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). Analytical sensitivity is 8.0×10^5 starting copy numbers per mL (mean values).
2. Methods
   The reaction mixture contains per 50 µL:
   2.1 5 µL of bacterial cell suspension (approx 10^8 cells per mL, corresponding to 1 ng of DNA) or plant extract.
   2.2 10 nmol of dNTPs.
   2.3 10 pmol of forward primer.
   2.4 20 pmol of reverse primer.
   2.5 5 pmol of TaqMan probe.
   2.6 5 µL of 10x reaction buffer.
   2.7 40 mM of $MgCl_2$.
   2.8 1 U of Ampli Taq Gold DNA Polymerase (Applied Biosystems).
   2.9 Molecular grade water to a total volume of 50 µL.
   The PCR cycling conditions are the following:
   95°C for 10 min for denaturation of DNA and activation of polymerase, followed by 35 cycles of 95°C for 20 s and 66°C for 60 s.
3. Essential procedural information
   3.1 Controls: every time a PCR-based test is performed, the following controls should be included:

Essential:

- A contamination ('negative') control
- A test ('positive') control from a reference culture of *C. michiganensis* subsp. *insidiosus*.

Recommended:

- An extraction contamination control for every batch of samples tested. This consists of performing a NA extraction using a known 'blank' sample that does not include target NA (e.g. clean extraction buffer)
- An extraction inhibition control used to monitor for the co-extraction of assay inhibitors. This can include testing extracted NA with a PCR-based assay known to amplify a non-target specific sequence (e.g. a conserved host gene or a 'universal' ITS gene). Alternatively, where available, a synthetic Internal Amplification Control can be used from a culture of *C. michiganensis* subsp. *insidiosus* strain.

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

- A test will be considered positive if it produces a Ct below 40 (undiluted samples) and provided that the contamination controls are negative
- A test will be considered negative, if it produces no signal below Ct40 provided that the assay and extraction inhibition controls are positive
- Tests should be repeated if any contradictory or unclear results are obtained.

Appendix – 5 Fatty acid profiling protocol for *C. michiganensis* subsp. *insidiosus* and *C. michiganensis* subsp. *michiganensis*

Fatty acid profiling has been widely used for rapid, fairly cost effective identification of plant pathogenic bacteria. Standard methods exist for the preparation of fatty acid profiles (FAP), mostly based on the use of the MIDI Microbial Identification System (MIDI, Newark, USA). The technique can be broken down into a series of steps which involve cell culture and harvesting, saponification of the lipids, methylation of the fatty acids to methyl esters (FAMEs), extraction into an organic solvent, some simple clean-up procedures, and separation and identification of the FAMEs by gas chromatography. MIDI software identifies and quantifies the peaks produced and compares the pattern obtained with profile libraries of known strains. These libraries can be purchased from MIDI, e.g. TSBA 6 Aerobic library, or can be self-generated. The key to successful use of FAPs in identification is standardization of all parts of the method.

Culture bacteria on Trypticase Soy agar (BBL Trypticase Soy broth plus 1.5% Bacto agar) for a defined period at 28°C. If comparing with the MIDI libraries, then since these are generally prepared using 24 h cultures, culture for precisely 24 h. For slower growing bacteria such as *Clavibacter michiganensis*, 48 or 72 h cultures may be used if not comparing with the MIDI libraries. Full details of the method are given on the MIDI website (http://www.midi-inc.com/pdf/MIS_Technote_101.pdf).

Even if comparing the isolated colonies with libraries, it is best to include reference strains.

Table 2 Fatty acid profiles for *Clavibacter, Rathayibacter* and *Curtobacterium* species. This table was generated with the standard procedure

	Taxon Mean percentage (SD)						
Fatty acid	Cmi	Cmm	Cmn	Cms	Cmt	Cff	Rr
14:0 Iso	0	t	0.4 (0.1)	t	0.5 (0.2)	0.4 (0.1)	1.4 (0.1)
15:1 Anteiso A	1.45 (1.4)	5.1 (1.4)	2.0 (0.9)	6.9 (2.8)	1.1 (0.8)	0	0.7 (0.1)
15:0 Iso	0	0.75 (0.3)	1.0 (0.6)	t	0.6 (0.3)	2.1 (0.6)	2.9 (0.4)
15:0 Anteiso	44.2 (1.8)	42.9 (2.2)	45.0 (1.3)	39.0 (3.2)	46.3 (0.8)	25.2 (1.7)	44.2 (1.2)
15:0	0	0.5 (0.4)	0.8 (0.4)	t	t	0	0
16:0 Iso	3.6 (1.0)	17.1 (3.1)	20.0 (1.0)	14.9 (1.6)	23.0 (2.1)	7.6 (2.4)	21.6 (1.0)
16:1 A	0	0	0	0	0	0.5 (0.1)	0
16:0	1.4 (0.6)	2.3 (0.4)	1.8 (0.3)	2.9 (0.9)	2.7 (1.0)	0.6 (0.1)	2.9 (1.6)
17:0 Iso	0	0.6 (0.2)	0.7 (0.3)	t	0.4 (0.3)	0.6 (0.1)	2.9 (0.4)
17:0 Anteiso	48.3 (2.4)	30.1 (2.1)	27.7 (2.2)	35.0 (2.6)	25.2 (4.0)	19.6 (2.6)	22.8 (1.7)
17:0	0	t	0.7 (0.2)	t	t	0	t
18:1w7c	0	0	0	0	0	43.0 (6.3)	0
No. strains	12	14	5	14	10	6	4

Cmi, *C. michiganensis* subsp. *insidiosus;* Cmm, *C. michiganensis* subsp. *michiganensis;* Cmn, *C. michiganensis* subsp. *nebraskensis;* Cms, *C. michiganensis* subsp. *sepedonicus;* Cmt, *C. michiganensis* subsp. *tesselarius;* Cff, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens;* Rr, *Rathayibacter rathayi;* t, trace quantities

There are several references that discuss fatty acid profiles of *Clavibacter michiganensis* and other plant pathogenic Gram-positive bacteria. These include Gitaitis & Beaver (1990), Henningson & Gudmestad (1991), Stead *et al.*, 1992; Weller *et al.*, 2000 and Dickstein *et al.* (2001). Fatty acid profiles of several *Clavibacter michiganensis* subspecies are presented in Table 2 together with profiles from *Rathayibacter rathayi* and *Curtobacterium flaccumfaciens* pv *flaccumfaciens*. These are taken from the self generated NCPPB 3 library (Stead, DE, unpubl. results).

As can be seen from Table 1, the profiles of the different taxa are similar. All *Clavibacter michiganensis* subspecies and some *Rathayibacter* species have 15: anteiso A, which is not found in *Curtobacterium*. Most *Curtobacterium flaccumfaciens* strains have 18:1w7c which is not found in *Clavibacter michiganensis* subspecies and *Rathayibacter* species. Differences between *Clavibacter michiganesis* subspecies are quantitative rather than qualitative. *C. michiganensis* subsp. *insidiosus* has much lower amounts of 16:0 iso and much higher amounts of 17:0 anteiso than other subspecies. *C. michiganensis* subsp. *michiganensis* and *C. michiganensis* subsp. *sepedonicus* have significantly higher amounts of 15:1 Anteiso A than the other subspecies. In a validation study of a library containing the profiles shown in Table 2, 31 out of 31 strains of *C. michiganensis* subsp. *sepedonicus* and 12 out of 15 *C. michiganensis* subsp. *michiganensis* strains were listed as first choice (Stead *et al.*, 1992).

Results with low similarity indexes should be interpreted with caution.

Appendix – 6 Pathogenicity tests

Grow susceptible alfalfa test plants (varieties Europe and Orca seem to be highly susceptible; Víchová & Kozová, 2004) in pots with enough substrate, at approximately 20–25°C (day) and >70% relative humidity in a glasshouse or growth chamber. At least 10 5–6 week old plantlets should be used for each pathogenicity test. Use a reference strain (known to be pathogenic) as a positive control to inoculate a series of 10 plantlets for each experiment as well as a series of plantlets inoculated with sterile physiological saline as a negative control.

Prepare an appropriate volume of approximately 10^9 cfu mL^{-1} suspension of the presumptive 24–72 h old isolates and of the reference strain in sterile physiological saline (24–48 h old culture).

Three methods can be used for inoculation.

- Method 1: dip a previously disinfected pair of scissors into the bacterial suspension and use them to cut the plantlets. Dip the scissors as many times as required into the suspension to be sure that enough suspension is present.
- Method 2: cut the plantlets with a disinfected pair of scissors and add a drop of bacterial suspension on each wound with a pipette.
- Method 3: dip shortened roots of test plants into the bacterial suspension for 17–18 h (Víchová & Kozová, 2004).

Cormack *et al.* (1957) concluded that root inoculation is more efficient than stem inoculation, at least for breeding tests. But this method is not easy to perform.

Immediately after inoculation, place a plastic bag over the plantlets or use any other appropriate system to keep the plantlets under high moisture conditions for 24 h, the optimum temperature being 17–24°C. After this, plantlets can be kept under normal glasshouse conditions.

Keep the plants under observation for at least 6–8 weeks.

From the fourth week, make at least weekly observations for wilting. Isolate from wilting plants by removing a 1-cm stem section from 2 cm above the inoculation point and suspending in phosphate buffer (see Appendix 1). Perform dilution plating on King's B or YPGA media. Subculture presumptive isolates and undertake identification tests to confirm they are *C. michiganensis* subsp. *insidiosus*.