Check for updates

DOI: 10.1111/epp.12806

EPPO STANDARD - DIAGNOSTICS

PM 7/99 (2) *Clavibacter insidiosus*

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

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

Specific approval and amendment: Approved as an EPPO Standard in 2010–09. Revision approved in 2021–08.

Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Clavibacter insidiosus is a seed-transmitted pathogen of *Medicago sativa* (lucerne, alfalfa) causing reduction of vigour and growth of the crop and considerably decreasing yield. The pathogen has been reported in most important lucerne production areas in the USA and Canada in the 20th century. It is now no longer present in Canada (EPPO, 2020). Although previously reported in the EPPO region (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 EPPO Global Database (EPPO, 2020).

Medicago sativa is the most important host but *Lotus corniculatus* (common bird's foot trefoil), *Medicago falcata* (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). In the absence of plant material in soil, bacterial cells are quickly inactivated under warm and moist conditions (Nelson & Neal, 1974).

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 an endophyte inside seeds following a systemic infection. Seed transmission appears to be low. Samac et al. (1998) report that *C. insidiosus*-infected seed was recovered from 6.3–7.7% of diseased plants and diseased plants most often produce low quantities of seeds (Samac et al., 1998). The bacterium can spread from plant to plant, particularly via wind-blown soil and debris, irrigation water or harvesting equipment. Infection occurs through wounds such as those made by mowing, freezing and thawing, and feeding by insects and nematodes (Samac et al., 1998). The stem and bulb 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. insidiosus* (EPPO/CABI, 1997) and the EPPO Global Database (EPPO, 2021a). The disease is more prevalent in older lucerne stands, usually stands that are at least 3–4 years old. Experience from the USA suggests *C. insidiosus* can reduce stand life by 3–4 years (Ophel Keller, 2005).

Flow diagrams describing the diagnostic procedure for *C. insidiosus* are presented in Figures 1 and 2.

2 | IDENTITY

Name: *Clavibacter insidiosus* (McCulloch 1925) Li et al., 2018.

Other scientific names: *Clavibacter michiganensis* subsp. *insidiosus* corrig. (McCulloch 1925) Davis et al., 1984, *Corynebacterium insidiosum* (McCulloch 1925) Jensen 1934 (Approved Lists 1980), *Corynebacterium michiganense* pv. *insidiosum* (McCulloch 1925) Dye & Kemp, 1977, *Aplanobacter insidiosum* McCulloch 1925, *Corynebacterium michiganense* subsp. *insidiosum* (McCulloch 1925) Carlson & Vidaver 1982.

Taxonomic position: Bacteria, Actinobacteria, Micrococcales, 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 n°49, EU RNQP (Annex IV).

[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.

© 2021 OEPP/EPPO, *EPPO Bulletin*

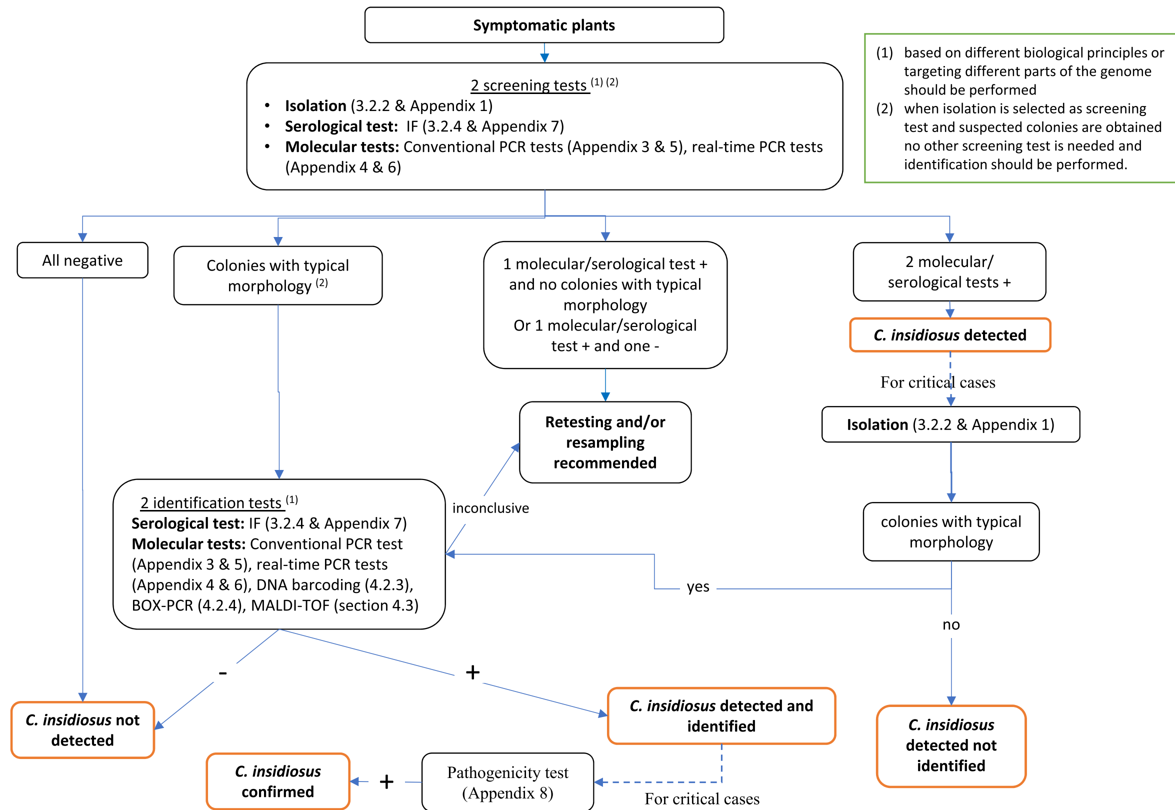

FIGURE 1 Flow diagram describing the diagnostic procedure for *Clavibacter insidiosus* in plant material

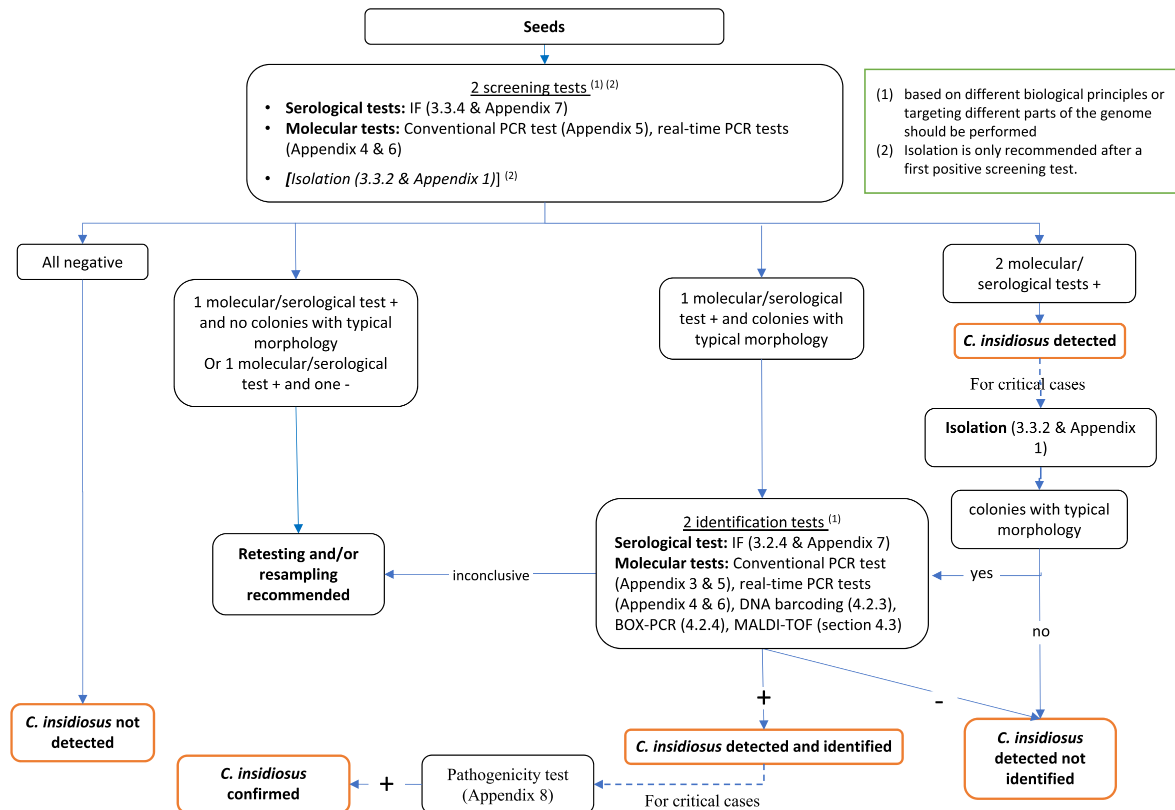

FIGURE 2 Flow diagram describing the diagnostic procedure for *Clavibacter insidiosus* in seeds

3 | DETECTION

3.1 | Disease symptoms

Generally, *C. insidiosus* causes systemic infection of alfalfa plants. In the field, infected plants occur as scattered plants or in patches (Samac et al., 2017). The disease may induce wilting under dry and hot conditions but most often symptoms consist only of stunting and proliferation of stems as described thereafter. Mild symptoms consist of leaf mottling together with slight cupping or curling upwards of the leaflet margins (Figure 3) and some reduction in plant height (Figure 4). Moderately affected plants show mottling and cupping of leaflets, height reduction and often a proliferation of stems giving a 'witches' broom' effect. Severely affected plants are only a few centimetres high, with thin spindly stems and small leaflets that are often distorted and showing bleaching either marginal or entire. When the main taproot of infected plants is cut obliquely a ring of yellow-brown discoloration can be seen at the junction of the cortex and vascular cylinder. The cortical layer when peeled away reveals a yellow-brown discoloration of the inner surface which contrasts markedly with the white colour in healthy plants. Many plants with no above-ground symptoms show either a yellow-brown streaking of the vascular cylinder (Figure 5) or complete vascular discoloration.

FIGURE 3 Leaf cupping caused by *Clavibacter insidiosus*. Courtesy of K. Ophel-Keller

FIGURE 4 Healthy (left) and infected (right) plants. Courtesy of K. Ophel-Keller

FIGURE 5 Cross-sections of a healthy root (right) and diseased roots (left and centre) of lucerne infected by *Clavibacter insidiosus*. Courtesy of APS, St Paul (US)

Wilt symptoms caused by *C. insidiosus* may be confused with other systemic diseases caused by *Verticillium albo-atrum* lucerne strains.

3.2 | Detection in plant material other than seeds

Recommendations for testing are only provided for symptomatic plants. For detection at least two tests with different biological principles or targeting different parts of the genome should be performed. However, when isolation is selected as a screening test and suspected colonies are obtained no other screening test is needed and identification should be performed (see Section 4).

3.2.1 | Test sample requirement for symptomatic plants

Wilted 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). Prior to analysis, field samples should be stored in a cool environment, with temperature not exceeding 14–16°C.

Roots should be carefully washed using tap water to remove soil and contamination by saprophytes avoided 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. Small sections of symptomatic vascular tissue should be excised and transferred into a small volume of sterile distilled water or phosphate buffer (PB, 50 mM; 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 Section 3.2.2), PCR (see Appendix 2 for DNA extraction and Section 3.2.3) or immunofluorescence (see Section 3.2.4). 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.

3.2.2 | Isolation

3.2.2.1 | Media

For isolation both a supplemented generic and a semiselective media should be used. Media are described in Appendix 1.

Recommended generic media supplemented with cycloheximide[2] are:

- King's B supplemented with 200 mg L^{-1} of cycloheximide
- YPGA supplemented with 200 mg L^{-1} of cycloheximide.

Recommended semi-selective media are:

- Glucose-yeast carbonate agar (GYCA) supplemented with kanamycin and cycloheximide. This medium has been evaluated by Coertze et al. (2015). It proved to be more suitable for distinguishing the yellow pigmented *C. insidiosus* colonies, which frequently have a blue centre (Coertze et al., 2015), from other bacterial species present. The use of kanamycin and cycloheximide together allows a better inhibition of saprophytes without affecting the growth of *C. insidiosus*. In addition, colonies are easier to detect on this nontransparent medium than on other media (Coertze et al., 2015).
- TBY Agar medium is recommended by Samac et al. (2017).
- NCP-88 (De la Cruz et al., 1992) can be used for isolating *C. insidiosus* (Kolodziejska, pers. comm.) although some isolates are sensitive to Polymyxin B, which is a component of this medium (Coertze et al., 2015).
- MTNA (Jansing & Rudolph, 1998).
- BCT (Ftayeh et al., 2011).

As culture plates may be overgrown by quicker growing saprophytes, dilutions are likely to be required for isolation (i.e. streaking in sectors or spreading of serially diluted macerate).

Spread or streak 100 µL of the macerate and dilutions of macerate (1/10, 1/100) on the selected media. As a reference, plates with a diluted cell suspension of a *C. insidiosus* reference strain should also be prepared. Plates should be incubated at 21–25°C for up to 8 days. Colonies appearing up to 72 h should be discarded as the isolation of this bacterium takes longer. Generally, 2–3 mm colonies of *C. insidiosus* develop within 4–5 days.

Presumptive colonies should be purified by subculturing on King's B, ND, GYCA, YDC or YPGA media (Appendix 1).

3.2.2.2 | Colony description (Figures 6–11)

Colonies are usually light yellow, convex and semifluidal, round or irregular. They become deeper yellow, opaque and glistening with longer incubation. However, some exceptions and variations are reported. For example, Ftayeh et al. (2011) report that on BCT medium the strain NCPPB 1634 produces pink pigmented colonies with violet internal flecks.

On NCP-88 the yellow colour is darker than on other media and nonmucoid colonies may be produced. On this medium, the pigment appears more rapidly than on YPGA and, depending on the strain, brown centres can develop (Figure 8). In addition, some nonpigmented strains on YPGA produce pigment on NCP-88. This pigmentation is very useful for separating presumptive *C. insidiosus* colonies in the presence of saprophytes.

Characteristic dark bluish granules of indigoidine (Lelliott & Stead, 1987) may be produced on YPGA agar medium (Figure 8), but granules may not be produced on media with low sugar content.

The Gram-negative bacterium *Pseudomonas corrugate*, which may be isolated from alfalfa plants, produces a transient blue pigment on TBY, but this pigmentation fades after 2–4 days whereas the pigment produced by *C. insidiosus* intensifies over time (Lukezic, 1979; Samac et al., 2017).

Figures 6–8 show that differences in colour, shape and growth of colonies can be observed between strains and for a same strain on different media.

3.2.3 | Molecular tests

Several molecular tests have been described on plant material. DNA extraction is described in Appendix 2.

Ward et al. (2008) describe a conventional and a real-time PCR on plant material. The conventional PCR is described in Appendix 3 and the real-time PCR in Appendix 4.

Samac et al. (2017) recommend a conventional and a real-time PCR based on primers of Marefat et al.

[2]Nystatin may be used as an alternative to cycloheximide but the amount of nystatin equivalent to 200 mg L^{-1} for cycloheximide has not been evaluated for *C. insidiosus*. 200 mg L^{-1} is recommended in PM 7/127 *Acidovorax citrulli* (EPPO, 2016a) and 100 mg L^{-1} in PM 7/042 *Clavibacter michiganensis* subsp. *michiganensis* (EPPO, 2016b).

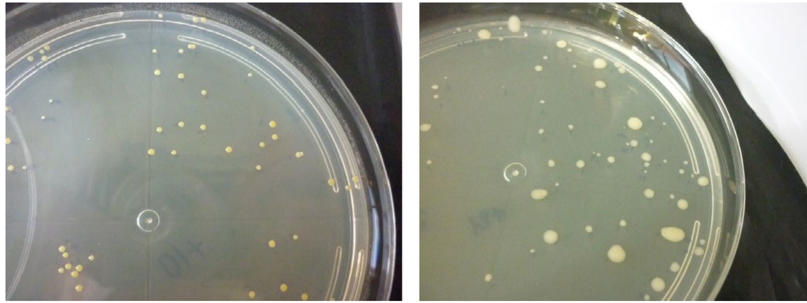

FIGURE 6 Colonies of *Clavibacter insidiosus* on non-selective medium (YPGA) after 6 days of incubation at 25°C. Left, strain NCPPB 1109; right, strain CFBP 6491 with variable speed growth

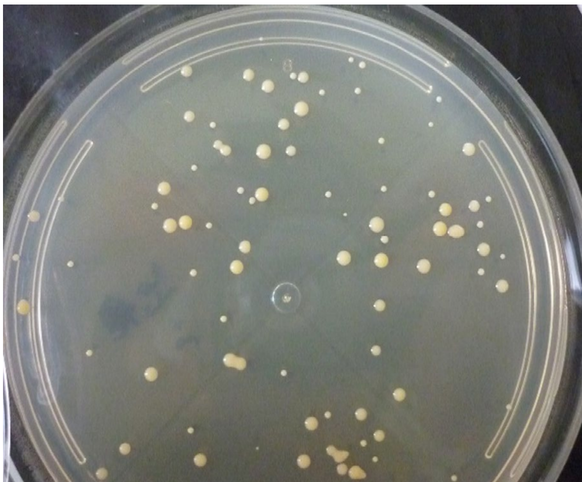

FIGURE 7 Strain CFBP 6491 on NCP-88 after 6 days of incubation

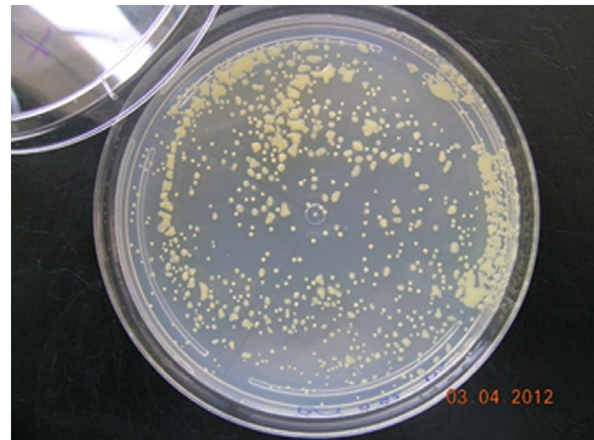

FIGURE 9 Strain LSV 40.74 on BCT medium (incubation time not known)

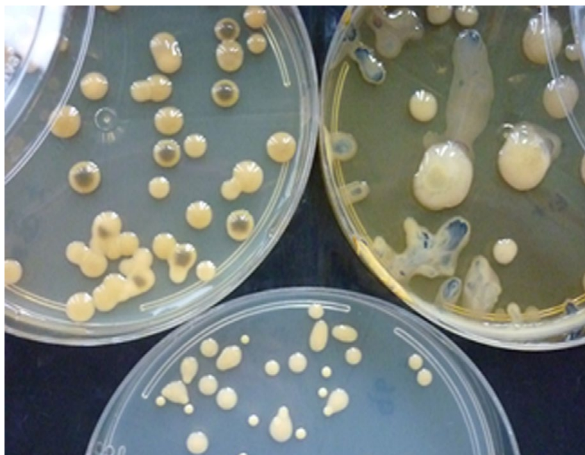

FIGURE 8 Strain CFBP 6491 on NCP-88 (top left), on YPGA (top right) and on King's B (bottom) after 15 days of incubation

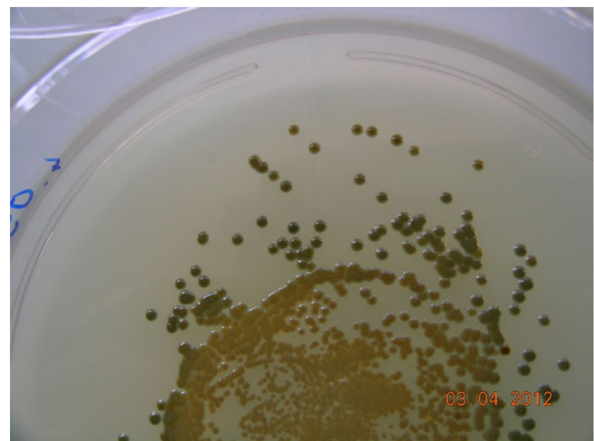

FIGURE 10 Strain NCPPB 83 on NCP88 medium (incubation time not known)

(2007) which can be used for plant material as well as for seeds. The conventional PCR is described in Appendix 5. The primers of this conventional PCR are considered easier to use than the primers published in Samac et al. (1998) which amplify a smaller DNA fragment (Samac, personal communication, 2020). The real-time PCR test from Marefat et al. (2007) is described in Appendix 6.

None of these tests have been evaluated by laboratories in the EPPO region on plant material, but some validation data is available in the publications.

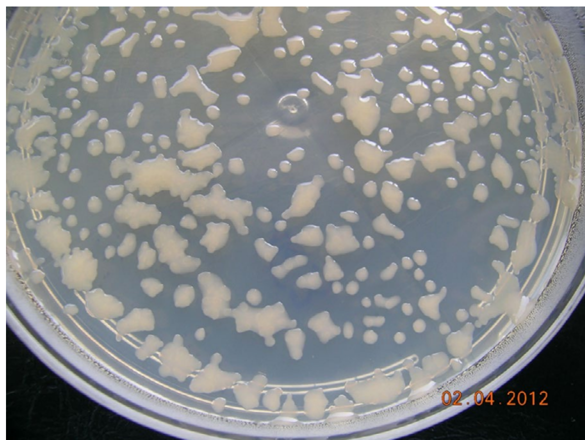

FIGURE 11 Strain CFBP 6490 on MTNA medium (incubation time not known)

3.2.4 | Serological tests

Instructions to perform an Immunofluorescence (IF) test are provided in the EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009). A commercial antiserum for IF is available at Prime Diagnostics (see Appendix 7 for performance characteristics).

A commercial antiserum for ELISA is available at Nano Diagnostics, but there is little experience in the EPPO region with using ELISA for the detection of *C. insidiosus*, thus it is not recommended in this protocol.

3.3 | Detection from seeds

For detection from seeds at least two tests with different biological principles or targeting different parts of the genome should be performed. Isolation is only recommended after a first positive screening test (see Section 3.3.2).

3.3.1 | Test sample requirement

ISPM 31 (IPPC, 2008) reports that for lots larger than 200 000 units, a standard sample of 5000 units allows a 99% confidence level of detecting an infection at 0.1% with a 95% of efficacy of detection. Therefore, the recommended sample size for detecting *C. insidiosus* in seeds is 5000. This corresponds to approximately 10 g for lucerne or clover seeds.

3.3.1.1 | Sample preparation for isolation and/or IF

The sample of 5000 seeds should be divided into subsamples of a maximum of 1000 seeds each. Each subsample 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 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 approximately 5°C. Experience at Anses (FR) shows that maceration at room temperature increases the number of bacteria present, thus facilitating reading of IF slides. However, as the number of saprophytes is increased, recovery of *C. insidiosus* isolates is more difficult. Consequently, the preparation of separate macerates per sample for isolation and IF is required. The extraction period allows for the retrieval of bacteria, but saprophyte contamination may occur. Approximately 1–1.5 mL of each extract should be kept in an Eppendorf or equivalent sterile microtube for reference at approximately 5°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 Section 3.3.2) or immunofluorescence (see Section 3.3.3).

Samac et al. (2017) recommend grinding of seed subsamples to a fine powder. Ground seeds are then soaked in 30 mL of Luria Bertani (LB) broth (see Appendix 1) with gentle shaking at room temperature (22–25°C) for 30 min. When analysing seed extracts by IF, the presence of starch and other seed components may make slide observation difficult. Consequently, the sample should be diluted further or seed grinding should be replaced by seed soaking (see above).

3.3.1.2 | Sample preparation for molecular tests

Two subsamples of 5 g of seed (i.e. approximately 2500 seeds) are incubated in 20 mL of YGM broth (Appendix 1) with agitation for 4 h, refrigerated overnight at 4°C without agitation and then warmed to room temperature with agitation for 3 h (Ward et al., 2008)[3]. From each subsample, an aliquot of 4 mL of broth is centrifuged to concentrate microbial cells, and the pellet is resuspended in 500 µL of sterile water and used to prepare the DNA template.

DNA extraction is described in Appendix 2.

3.3.2 | Isolation

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. insidiosus* based on phenotypical characteristics. Isolation is consequently only recommended after a first positive screening test. At least two semiselective media should be used containing different antibiotics. The recommended semiselective media are described in Section 3.2.2.

[3]Samac et al. (2017) describe testing based on a sample of 50 g. The drafting team considered that there was not sufficient experience with such sample size to recommend it in this protocol.

3.3.3 | Serological test

Instructions to perform an IF test are provided in the EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009). A commercial antiserum for IF is available from Prime Diagnostics.

3.3.4 | Molecular tests

There is little experience on direct seed testing in the EPPO region. DNA extraction is described in Appendix 2.

Ward et al. (2008) describe a conventional and real-time PCR but for regulatory seed testing they recommend using the real-time PCR. The real-time PCR test of Ward et al. (2008) is described in Appendix 4.

A conventional and a real-time PCR are described in Samac et al. (2017) and are based on primers from Marefat et al. (2007). The conventional PCR test is described in Appendix 5 and the real-time PCR in Appendix 6.

None of these tests have been evaluated by laboratories in the EPPO region on seeds, but some validation data is available in the publications.

4 | IDENTIFICATION

For identification at least two tests with different biological principles or targeting different parts of the genome should be performed.

4.1 | Serological tests

A commercial antiserum for IF is available at Prime Diagnostics. Instructions for performing an immunofluorescence test are provided in EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009). Validation data is given in Appendix 7. ELISA is not recommended for identification.

4.2 | Molecular tests

PCR tests can be used to identify pure cultures of *C. insidious*.

4.2.1 | Conventional PCR

Two different conventional PCR tests are described in Appendix 3 (Ward et al., 2008) and Appendix 5 (Samac et al., 2017).

Although described in the previous version of this Standard, the conventional PCR developed by Borowicz (2001) is not included in this revised version as it is not used in the region and validation data is not available. Samac et al. (1998) and Pastrik and Rainey (1999) are also no longer described in this Standard as during an evaluation conducted at Anses (FR) it was noted that one *C. insidiosus* strain was not detected with these tests.

4.2.2 | Real-time PCR

Three real-time PCR tests have been published (Bach et al., 2003; Marefat et al., 2007; Ward et al., 2008). In comparative studies, Marefat et al. (2007) compared their test with the test of Bach et al. (2003), which was shown to be less sensitive. The test from Marefat et al. (2007) is described in Appendix 6 and that from Ward et al. (2008) in Appendix 4.

4.2.3 | DNA barcoding

Comparisons of sequenced PCR products amplified from a combination of two housekeeping gene loci [*16S* ribosomal DNA (rDNA) and *gyrB*], can support the identification of *C. insidiosus* (Zaluga et al., 2011). General procedures for sequencing are described in the EPPO Standard PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* (Appendix 2) (EPPO, 2021b). Sequences are available in the EPPO-Q-bank (https://qbank.eppo.int/bacteria/).

4.2.4 | BOX-PCR

Presumptive isolates identified as *C. 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 *Rep-PCR tests for identification of pure cultures of bacteria*. (EPPO, 2010)

4.3 | Matrix-assisted laser desorption/ionization-time of flight mass spectrometry MALDI-TOF

Proteomic analysis using MALDI-TOF mass spectrometry allowed an accurate identification of *C. insidiosus*. Both tests are described in Zaluga et al. (2011).

4.4 | Other tests

4.4.1 | Biochemical characteristics

C. insidiosus is Gram-positive, oxidase negative and catalase positive. Differential biochemical characteristics that can support the identification of *C. insidiosus* are described in Yasuhara-Bell and Alvarez (2015).

4.4.2 | Pathogenicity test

For critical cases, a pathogenicity test may be performed and is described in Appendix 8.

5 | REFERENCE MATERIAL

The Type strain is available from the following collections:

- NCPPB 1109 - NCPPB, National Collection of Plant Pathogenic Bacteria, Fera, Sand Hutton, York YO411LZ, UK; https://www.fera.co.uk/ncppb
- CFBP 2404 – CIRM-CFBP, International Center for Microbial Ressources – French Collection for Plant-associated Bacteria, IRHS UMR 1345 INRAE-ACO-UA, 42 Rue Georges Morel, 49070 Beaucouze Cedex, France; https://www6.inrae.fr/cirm
- LMG 3663 - LMG, Belgian Co-ordinated Collections of Microorganisms (BCCM)/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium; http://bccm.belspo.be/
- ICMP 2621 – ICMP, International Collection of Microorganisms from Plants, PO Box 69040, Lincoln, New Zealand https://scd.landcareresearch.co.nz/.

Additional strains are listed in Appendices 3 and 7.

6 | REPORTING AND DOCUMENTATION

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

7 | PERFORMANCE CRITERIA

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

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

V Olivier, ANSES, Plant Health Laboratory Angers, FR (valerie.olivier@anses.fr), A Aspin, Fera, York, GB (andrew.aspin@fera.co.uk) and E Stefani, UNIMORE, IT (emilio.stefani@unimore.it).

9 | FEEDBACK ON THIS DIAGNOSTIC PROTOCOL

If you have any feedback concerning this Diagnostic Standard, or any of the tests included, or if you can provide additional validation data for tests included in this Standard 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 Standards. Standards 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.

ACKNOWLEDGEMENTS

This Protocol was originally drafted by D Caffier, National Laboratory for Plant Health, Angers, France. It was revised by a drafting group composed of A Aspin (Fera, York, GB), ML Cruz (INIAV, PT), V Olivier (lead author) and F Poliakoff (Anses, Plant Health Laboratory, Angers, FR), M Rosello Perez (Plant Health Laboratory, Valencia, ES) and E Stefani (UNIMORE, Reggio Emilia, IT). We would like to thank D Samac (USDA-ARS, Plant Science Research Unit, US) and K Ophel-Keller (Sustainable Systems Division at South Australian Research and Development Institute, AU) for the information provided during the preparation of this revision.

REFERENCES

Bach HJ, Jessen I, Schloter M & Munch JC (2003) A TaqMan-PCR protocol for quantification and differentiation of the pathogenic *Clavibacter michiganensis* subspecies. *Journal of Microbiological Methods* 52, 85–91.

Bradbury JF (1986) *Guide to plant pathogenic bacteria*. CAB International Mycological Institute 332 p.

Borowicz BP (2001) Use of the DNA sequence of the intergenic spacer region between the 16S and 23S rRNA genes for the identification of *Clavibacter michiganensis* subsp. *insidiosus* at the molecular level. *EPPO Bulletin* 31, 489–491.

Coertze S, Jensen T, Kotzé TN & McLeod A (2015) Establishing the status of *Clavibacter michiganensis* subsp. insidiosus in lucerne in South Africa. *Journal of Plant Pathology* 97, 283–290.

Cormack MW (1961) Longevity of the bacterial wilt organism in alfalfa hay, pod debris, and seed. *Phytopathology* 5, 260–261.

Cormack MW, Peake RW & Downey RK (1957) Studies on methods and materials for testing alfalfa for resistance to bacterial wilt. *Canadian Journal of Plant Science* 37, 1–11.

Davis MJ, Gillaspie AG, Vidaver AK & Harris RW (1984) Clavibacter: a new genus containing some phytopathogenic coryne from bacteria, including *Clavibacter xyli* subsp. *xyli*. sp. nov., subsp. nov. and *Clavibacter xyli* subsp. *cynodontis* subsp. nov. *International Journal of Systematic Bacteriology* 34, 107–117.

de la Cruz AR, Wiese MV & Schaad NW (1992) A semi-selective medium for the isolation of *Clavibacter michiganensis* subsp. *sepedonicus* from potato tissue. *Plant Disease* 75, 830–834.

Dye DW (1962) The inadequacy of the usual determinative tests for the identification of *Xanthomonas* spp. *New Zealand Journal of Agricultural Research* 5, 393–416.

Dye DW & Kemp WJ (1977) A taxonomic study of plant pathogenic *Corynebacterium* species. *New Zealand Journal of Agricultural Research* 20, 563–582.

EPPO/CABI (1997) *Clavibacter michiganensis* subsp. *insidiosus*. In: *Quarantine Pests for Europe* (second edition). Eds. IM Smith, DG McNamara, PR Scott & M Holderness, pp 977–980. CAB International, Wallingford (GB).

EPPO (2009) PM 7/97 (1): Indirect immunofluorescence test for plant pathogenic bacteria. *EPPO Bulletin* 39, 413–416. https://doi.org/10.1111/j.1365-2338.2009.02344.x

EPPO (2010) PM 7/100(1): Rep-PCR tests for identification of bacteria. *EPPO Bulletin* 40, 365–368. https://doi.org/10.1111/j.1365-2338.2010.02409.x

EPPO (2016a) PM 7/127 (1) *Acidovorax citrulli*. *EPPO Bulletin* 46, 444–462. https://doi.org/10.1111/epp.12330

EPPO (2016b) PM 7/42 (3) *Clavibacter michiganensis* subsp. *michiganensis*. *EPPO Bulletin* 46, 202–225. https://doi.org/10.1111/epp.12302

EPPO (2020) EPPO Global Database https://gd.eppo.int/taxon/CORBIN/distribution (last accessed 19/11/2021).

EPPO (2021a) EPPO Global Database (available online). https://gd.eppo.int

EPPO (2021b) PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests. *EPPO Bulletin* 51, 100–143. https://doi.org/10.1111/epp.12724.

Ftayeh RM, von Tiedemann A & Rudolph KWE (2011) A new selective medium for isolation of *Clavibacter michiganensis* subsp. *michiganensis* from tomato plants and seed. *Phytopathology* 101, 1355–1364.

Hunt OJ, Griffin GD, Murray JJ, Pederson MW & Peaden RN (1971) The effects of root knot nematodes on bacterial wilt in alfalfa. *Phytopathology* 61, 256–259.

IPPC (2008) *ISPM no. 31 Methodologies for sampling of consignments*. IPPC, FAO, Rome (IT). https://www.ippc.int/static/media/files/publication/en/2016/01/ISPM_31_2008_En_2015-12-22_PostCPM10_InkAmReformatted.pdf [last accessed on 2 December 2020].

Jansing H & Rudolph K (1998) Physiological capabilities of *Clavibacter michiganensis* subsp. *sepedonicus* and development of a semi-selective medium. *Journal of Plant Pathology* 105, 590–601.

King ED, Ward MK & Raney DE (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *The Journal of Laboratory and Clinical Medicine* 44, 301–307.

Lelliott RA & Stead DE (1987) *Methods for the diagnosis of bacterial diseases of plants*. Blackwell, London, UK, 216 p.

Li W & De Boer S (1995) Selection of polymerase chain reaction primers from an RNA intergenic spacer region for specific detection of *Clavibacter michiganensis* subsp, *sepedonicus*. *Phytopathology* 85, 837–842.

Li X, Tambong J, Yuan KX, Chen W & Xu H (2018) Re-classification of *Clavibacter michiganensis* subspecies on the basis of whole-genome and multi-locus sequence analyses. *International Journal of Systematic and Evolutionary Microbiology* 68, 234–240.

Louws FJ, Bell J, Medina-Mora CM, Smart CD, Opgenorth D, Ishimaru CA, Hausbeck MK, de Bruijn FJ & Fulbright DW (1998) Rep-PCR-mediated genomic fingerprinting: a rapid and effective method to identify *Clavibacter michiganensis*. *Phytopathology* 88, 862–868.

Lukezic FL (1979) *Pseudomonas corrugata*, a pathogen of tomato, isolated from symptomless alfalfa roots. *Phytopathology* 69, 27–31.

Marefat A, Ophel Keller K & McKay A (2007) A real time PCR assay for detection of *Clavibacter michiganensis* subsp. *insidiosus* in lucern. *Australasian Plant Pathology* 36, 1–8.

Nelson GA & Neal JL (1974) Persistence of a streptomycin-resistant variant of *Corynebacterium insidiosum* in soil and alfalfa roots in soil. *Plant and Soil*, 40, 581–588.

Ophel Keller K (2005) *Bacterial wilt of lucerne A market access issue for lucerne seed growers*. Report for the Rural Industries Research and Development Corporation. RIRDC Publication No 05/101 https://www.agrifutures.com.au/wp-content/uploads/publications/05-101.pdf

Pastrik KH & Rainey FA (1999) Identification and differentiation of *Clavibacter michiganensis* subspecies by Polymerase Chain Reaction-based techniques. *Journal of Phytopathology* 147, 687–693.

Pastrik KH (2000) Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by multiplex PCR with coamplification of host DNA. *European Journal of Plant Pathology* 106, 155–165.

Pearse PG, Howard RJ, Hwang SF & Northover PR (2005) Survey of bacterial wilt pathogen in alfalfa seed producers in Alberta, Saskatchewan and Manitoba in 2003 and 2004. *Canadian Plant Disease Survey* 85: 60–61.

Samac D, Nix RJ & Oleson AE (1998) Transmission frequency of *Clavibacter michiganensis* subsp. *insidiosus* to alfalfa seeds and identification of the bacterium by PCR. *Plant Disease* 82, 1362–1367.

Samac DA, Ophel-Keller K & Caffier D (2017) *CHAPTER 16: Detection of* Clavibacter michiganensis *subsp.* insidiosus *in Alfalfa Seeds in Detection of Plant-Pathogenic Bacteria in Seed and Other Planting Material*, Second Edition. January 2017, 103–107.

Stolp H & Starr MP (1964) Bacteriophage reaction and speciation on phytopathogenic xanthomonads. *Phytopathologische Zeitschrift* 51, 442–478.

Víchová J & Kozová Z (2004) The virulence of *Clavibacter michiganensis* subsp. *insidiosus* strains and test of alfalfa varieties for resistance to the wilt pathogen. *Journal of Plant Protection Research* 44, 147–154.

Ward LJ, Gourley J & De Boer S (2008) Molecular detection of *Clavibacter michiganenis* ssp. *insidiosus* in alfalfa seed. *Canadian Journal of Plant Pathology* 30, 492–497.

Yasuhara-Bell J & Alvarez AM (2015) Seed-associated subspecies of the genus *Clavibacter* are clearly distinguishable from *Clavibacter michiganensis* subsp. *michiganensis*. *International Journal of Systematic and Evolutionary Microbiology* 65, 811–826. https://doi.org/10.1099/ijs.0.000022

Zaluga J, Heylen K, Van Hoorde K, Hoste B, Van Vaerenbergh J, Maes M & De Vos P (2011) *GyrB* sequence analysis and MALDI-TOF MS as identification tools for plant pathogenic *Clavibacter*. *Systematic and Applied Microbiology* 34, 400–407.

APPENDIX 1 - MEDIA AND BUFFERS

1. Media

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

Store media and stock solutions of antibiotics at 4°C in the dark and use within 1 month.

1.1. Generic media

King's B medium (King et al., 1954):

Proteose peptone N°3	20.0 g
Glycerol	10.0 mL
K_2HPO_4	1.5 g
$MgSO_4{\cdot}7H_2O$	1.5 g
Microbiological grade agar	15.0 g
Distilled water	1 L

Adjust pH to 7.0–7.2.

*Nutrient dextrose agar (ND) (*Lelliott & Stead, 1987*)*

D-glucose	10.0 g
Oxoid nutrient agar	28.0 g
Distilled water	1 L

*Yeast extract-dextrose-calcium carbonate agar medium (YDC) (*Stolp & Starr, 1964*):*

Yeast extract	10.0 g
Dextrose (glucose)	20.0 g
$CaCO_3$ (fine powder)	20.0 g
Microbiological grade agar	15.0 g
Distilled water to	1 L
Adjust to pH 7.0.	
The autoclaved medium should be cooled to 50°C in a water bath, and $CaCO_3$ suspended by swirling before pouring the plates.	

Luria-Bertani (LB) broth

Yeast extract	5.00 g
Tryptone	10.00 g
NaCl	0.02 g
Distilled water	to 1 L

Adjust pH to 7.2.

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

Yeast extract	5.0 g
Peptone	5.0 g
D(+) glucose	10.0 g
Microbiological grade agar	15.0 g
Distilled water	1 L

Adjust to pH 6.5–7.0.

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

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

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

For the broth same as above but with no microbiological grade agar.

1.2. Semi selective media

BCT (Ftayeh et al., 2011):

Yeast extract	2.0 g
Mannitol	2.5 g
K_2HPO_4	1.0 g
KH_2PO_4	0.1 g
NaCl	0.05 g
$MgSO_4{\cdot}7H_2O$	0.1 g
$MnSO_4{\cdot}H_2O$	0.1 g
$FeSO_4{\cdot}7H_2O$	0.015 g
H_3BO_3	0.6 g
Microbiological grade agar	15.0 g
Distilled water	to 1 L

Dissolve ingredients, adjust pH to 7.0–7.1. After autoclaving and cooling down to 50°C, add the supplements.

Trimethoprim	0.1 g
Nalidixic acid	0.02 g
Polymyxin B sulphate (8 120 international units IU/mg)	0.02 g
Opus Top[a] (fungicide-BASF)	50 µL

[a]It should be noted that fenpropimorph (active substance of Opus Top) is no longer authorized in EU countries. The drafting team considered that cycloheximide or nystatin may be good alternatives but no data is currently available. This should be verified by the laboratories.

Glucose-yeast carbonate agar (GYCA) (Dye, 1962; Pearse et al., 2005*)*

Glucose	10 g
Yeast extract	5 g
Precipitated chalk ($CaCO_3$)	30 g
Microbiological grade agar	15 g
Distilled water	1 L

After autoclaving the medium is cooled to 50°C, and 10 mg L^{-1} kanamycin and 200 mg L^{-1} cycloheximide[4] are added.

Plates should preferably be used immediately after preparation or kept refrigerated for a period of up to 1 month.

MTNA (Jansing & Rudolph, 1998).

Yeast extract	2.00 g
Mannitol	2.50 g
K_2HPO_4	0.25 g
KH_2PO_4	0.25 g
NaCl	0.05 g
$MgSO_4{\cdot}7H_2O$	0.10 g
$MnSO_4{\cdot}H_2O$	0.015 g
$FeSO_4{\cdot}7H_2O$	0.005 g
Microbiological grade agar	16.00 g
distilled water	to 1.0 L

Dissolve ingredients, adjust pH to 7.2. After autoclaving and cooling down to 50°C, add the antibiotics.

Trimethoprim	0.06 g
Nalidixic acid	0.002 g
Amphotericin B	0.01 g

Durability of basal medium is 3 months. After antibiotics are added durability is 1 month when stored refrigerated.

NCP-88 (De la Cruz et al., 1992).

Yeast extract	2.0 g
K_2HPO_4	2.0 g
KH_2PO_4	0.5 g
$MgSO_4{\cdot}7H_2O$	0.25 g
D-Mannitol	5.0 g
Difco nutrient agar	23.0 g
Distilled water	1000 mL

After autoclaving and cooling down to 50°C, add the antibiotics.

Polymyxin B sulphate (7900 IU/mg)	0.003 g
Nalidixic acid sodium salt	0.008 g
Cycloheximide	0.2 g

TBY agar medium (Samac et al., 2017)

Tryptone	10.0 g
Yeast extract	5.0 g
Microbiological grade agar	15.0 g
NaCl	5.0 g
Distilled water	1 L

After autoclaving the medium is cooled to 50°C. Then 25 mL of 20% glucose is added, and 7 mg L^{-1} kanamycin and 200 mg L^{-1} cycloheximide[5] are added.

1.3. Performance characteristics available for media

1.3.1. Data from Anses, FR (generated in 2012 and 2013 unless otherwise stated)

1.3.1.1. Analytical sensitivity data

On three seed macerates (varieties Franken Neu, Equipe, Fee). 100% recovery was obtained at 3×10^3 cfu mL^{-1} (NCP- 88), 10^3 cfu mL^{-1} (MTNA), 3×10^5 cfu mL^{-1} (BCT) and 10^5 cfu mL^{-1} (King's B).

1.3.1.2. Analytical specificity data

Inclusivity

- NCP-88

  100% recovery with at least one colony per plate in a first evaluation performed on 15 *C. insidiosus* strains in 2012. However, in a second evaluation in 2013, recovery was only 50%. After some experiments, the hypothesis made regarding the lack of reproducibility was the high sensitivity of *C. insidiosus* to antibiotic/component concentrations or brand, but this could not be demonstrated.

  In the first evaluation in 2012 the percentage of colony retrieved compared to YPGA with 15 strains was 74% (ranging from 10% to 137% depending on the strain).
- BCT

  62.5% recovery with at least one colony per plate (16 strains of *C. insidiosus*).
- MTNA

  100% recovery with at least one colony per plate (16 strains of *C. insidiosus*).

  Additional data

  Percentage of colony retrieved for five strains of *C. insidiosus* compared to King's B medium:
  - MTNA: 70% (from 56% to 100% with growth of the five strains).
  - BCT: 46.5% (from 19% to 80% growth of the four strains out of five).
  - NCP-88: 50% (with the growth of one strain out of five).

Exclusivity:

- NCP-88

  95.2%, performed in 2012 with 16 nontarget strains including phylogenetically close bacteria, other plant pathogenic bacteria and alfalfa seed saprophytes. Cross-reaction was noted with one strain of *Curtobacterium flaccumfaciens* subsp. *flaccumfaciens* (CFBP3411). The recovery rate of that strain compared to YPGA was 4%.

[4]Nystatin may be used as an alternative to cycloheximide but the amount of nystatin equivalent to 200 mg L^{-1} for cycloheximide has not been evaluated for *C. insidiosus*. 200 mg L^{-1} is recommended in PM 7/127 *Acidovorax citrulli* (EPPO, 2016a) and 100 mg L^{-1} in PM 7/042 *Clavibacter michiganensis* subsp. *michiganensis* (EPPO, 2016b).

[5]Nystatin may be used as an alternative to cycloheximide but the amount of nystatin equivalent to 200 mg L^{-1} for cycloheximide has not been evaluated for *C. insidiosus*. 200 mg L^{-1} is recommended in PM 7/127 *Acidovorax citrulli* (EPPO, 2016a) and 100 mg L^{-1} in PM 7/042 *Clavibacter michiganensis* subsp. *michiganensis* (EPPO, 2016b).

1.3.2. Data from Coertze et al. (2015) on seed extracts

The data was obtained with spiked seed lots. Seeds were soaked in a bacterial suspension (approximately 10^9 cfu mL^{-1}). The seed were subsequently mixed with pathogen-free seed to provide six infection rates (0.1%, 0.5%, 1.5%, 2%, 2.5% and 3%), where a 0.1% infection rate consisted of one infected seed in 1000 seeds. These concentrations were selected based on the report that natural infection of seed occurs at frequencies of 0.21–0.55% (Samac et al., 1998).

The analytical sensitivity of the serial dilution plating method was dependent on the selective medium that was used. On King's B *C. insidiosus* could not be detected due to overgrowth by saprophytic bacteria present in the seed extracts. On TBY the detection limit was 0.5%. On GYCA the detection level was 0.1%. The identity of the *C. insidiosus* colonies detected at the lowest concentrations were confirmed to be *C. insidiosus* through KOH testing and PCR. The identity of a subset of these colonies was further confirmed through sequence analyses of the ITS region.

2. Buffers

Referred to in Section 3.2.1.

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

Na_2HPO_4 (anhydrous)	4.26 g
KH_2PO_4	2.72 g
Distilled water	to 1 L
Adjust pH to 7.0 before autoclaving.	

Tris-HCl buffer

Referred to in Appendixes 2–4

1 M Tris-HCl is commercially available and can be diluted to 10 mM and adjusted to pH 8 to prepare this lysozyme stock solution.

APPENDIX 2 - DNA EXTRACTION

DNA extraction from plant material or seed extracts
Controls

Extraction methods should be able to extract amplifiable target DNA from a known negative plant extract to which between 10^3 and 10^4 cfu mL^{-1} of a reference strain of *C. insidiosus* has been added.

Options for DNA extraction are presented below:

DNA extraction from plant material or seed macerates can be performed using the Invitrogen Easy DNA kit, according to the manufacturer's instructions, and with the addition of 5 mg 100 μL^{-1} of lysozyme in the lysis buffer and heating to 37°C for 30 min, as validated by Pastrik (2000).

Alternative extraction kits are, for example, DNeasy Plant Mini Kit (Qiagen), MoBio Laboratories Power plant DNA isolation kit (Qiagen).

When another specific extraction method is used this is indicated in the corresponding appendix.

DNA extracts should preferably be used immediately or stored at approximately −20°C.

DNA extraction from colonies

There are many extraction methods possible. One possibility is described below.

From a bacterial suspension (around 10^5–10^6 cfu mL^{-1}), thermal lysis for Gram-positive bacteria can be performed at 95°C for 15 min then cooled in ice. Then 80 µL of lysozyme stock solution (50 mg mL^{-1} lysozyme in 10 mM Tris HCl, pH 8.) is added and the sample is incubated for 30 min at 37°C.

The DNA is then purified using a commercial kit (e.g. the Easy DNA Extraction kit by Invitrogen or the DNeasy Blood and Tissue kit by Qiagen), following the manufacturers' instructions. Nucleic acid concentration may be estimated using the NanoDrop 2000 microvolume spectrophotometer (Thermo Fisher Scientific, Germany) or similar devices.

APPENDIX 3 - CONVENTIONAL PCR (WARD ET AL., 2008)

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

1. General information

1.1. The test was developed by Ward et al. (2008) for the detection of *C. insidiosus* in plant material and suspected isolates. PCR conditions have been adapted by Anses, FR, to optimize analytical specificity (35 cycles (94°C 30 s/53°C 30 s), instead of 35 (94°C 30 s/71°C 30 s). Both PCR conditions are presented.

1.2. The target gene is the internal transcribed spacer (ITS) region in the *rrn* operon (Li & De Boer, 1995). The expected amplicon size is 135 bp.

1.3. Oligonucleotides:

Primer name	Sequence	Amplicon size
CMI-F	5′-GAC CGC ATC TTT CGG GGT GTG-3′	135
CMI-R	5′-CGG AAA CCC GGT GAA TCT AAG-3′	

2. Methods

2.1. Nucleic acid extraction and purification

DNA is extracted from 500 µL of plant extract by first adding 1 mL of Tris-HCl 10 mM extraction buffer (pH 8.0) to 0.035 mg L^{-1} proteinase K, 3.5% SDS and 0.088 mol/L EDTA and incubating the mixture for >3 h (maximum, overnight) at 55–60°C to disrupt bacterial cells. Extraction is completed using the KingFisher processor (ThermoFisher Scientific, Walthan, MA, USA) with Magnesil KFGenomic System reagents following the supplier's instructions.

Other extraction methods are also included in Appendix 2**.**

2.2. Conventional PCR

2.2.1. Master Mix

Ward et al. (2008) Mastermix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular grade water	N.A.	16.35	N.A.
PCR buffer (*BD Advantage 2 PCR buffer*, *Clontech, CA. USA*)	10×	2.5	1×
dNTPs *(BD Advantage 2 PCR master mix*, *Clontech, CA, USA)*	mix 50×	0.50	1× (10 mM each)
Forward primer CMI-F	20 μM	1.25	1 μM
Reverse primer CMI-R	20 μM	1.25	1 μM
Polymerase (*BD Advantage 2 polymerase mix*, *Clontech, CA, USA*)	50×	0.5	1×
BSA	50 μg μL^{-1}	0.15	0.3 μg μL^{-1}
Subtotal		22.5	
Genomic DNA extract		2.5	
Total		25	

Anses, FR, mastermix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular grade water	N.A.	16.00	N.A.
PCR buffer (*Platinum Invitrogen*)	10×	2.5	1×
$MgCl_2$ (or alternatives) (*Platinum Invitrogen*)	50 mM	0.75	1.5 mM
dNTPs	20 mM	1.00	0.8 mM
Forward primer CMI-F	20 μM	1.25	1 μM
Reverse primer CMI-R	20 μM	1.25	1 μM
Polymerase (*Platinum Invitrogen*)	5 U μL^{-1}	0.1	0.5 U
BSA	50 μg μL^{-1}	0.15	0.3 μg μL^{-1}
Subtotal		23	
Genomic DNA extract		2	
Total		25	

N.A. : Not applicable.

2.2.2. PCR conditions

- Publication (Ward et al., 2008): PCR: 95°C 120 s, 35 cycles (94°C 30 s/71°C 30 s), 69°C for 4 min.
- Adapted by Anses, FR: PCR: 95°C 120 s, 35 cycles (94°C 30 s/53°C 30 s); 69°C for 4 min.

3. Essential procedural information

3.1. Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: *C. insidiosus* equivalent to a concentration of approximately 10^4 cfu mL^{-1}.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

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

a duplicate sample spiked with the target nucleic acid.

Other possible controls

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

3.2. Interpretation of results

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC and PAC (and if relevant IC): a band of 135 bp is visualized.

When these conditions are met

- A test will be considered positive if a band of 135 bp is visualized.
- A test will be considered negative if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

A. Data generated by Anses, FR (2013)

4.1. Analytical sensitivity data

Pure cultures.

10^5 cells mL^{-1} (presence of weak bands at 10^4 cells mL^{-1}).

Range evaluated was 10^3 to 10^5 cells mL^{-1} with several bacterial suspensions.

4.2. Analytical specificity data

- Inclusivity

100% evaluated with 17 target strains in duplicate (10^5 cells mL^{-1} DNA extracted by thermal lysis):

12 reference strains from collections: *Clavibacter insidiosus* LMG 3676, NCPPB 1109, LMG7324T1, NCPPB 83, NCPPB 1660, LMG 7325T2, CFBP 6489, CFBP 2404, CFBP 6490, CFBP 6492, CFBP6488, CFBP 6491.

Five *Clavibacter insidiosus*: strains from Poland (one isolated in 1964; four in 2008).

- Exclusivity

88.2% evaluated with 19 non-target strains:

Genetically related bacteria: *C. tessellarius* CFBP 3499, *C. nebraskensis* CFBP 3493, *C. sepedonicus* CFBP 1154, *C. m.* subsp. *michiganensis* CFBP 2498, *Clavibacter tritici* = *Rathayibacter tritici* CFBP 1385.

Other plant pathogenic bacteria from reference or laboratory collections: *Curtobacterium flaccumfaciens* subsp. *flaccumfaciens* CFBP 3411, *Curtobacterium flaccumfaciens* subsp. *betae* CFBP 2402, *Pseudomonas syringae* pv. *pisi* LNPV 05.42, *Ralstonia solanacearum* CFBP 3857, *Rhodococcus fascians* CFBP 2100, *Xanthomonas arboricola* pv. *pruni* CFBP 3892, *Xanthomonas axonopodis* pv. *phaseoli* CFBP 2534.

Four alfalfa saprophytes isolated from seeds

Cross-reactions observed with *C. tessellarius* CFBP 3499 and *C. nebraskensis* CFBP 3493.

No cross-reaction with alfalfa seed saprophytes.

4.3. Data on repeatability: no data.

4.4. Data on reproducibility: no data.

B. Data from Ward et al. (2008)

In their evaluation of *Clavibacter insidiosus* detections, Ward et al. inoculated several cultivars of alfalfa seedlings, noted development of foliage symptoms that were useful diagnostic markers and tested tissue from inoculated plants with both the conventional and real-time PCR tests. In the four cultivars tested ('Algonquin', 'Apica', 'Dupuit' and 'Rhizoma'), symptom development was non-uniform and varied from plant to plant.

Symptom development in four alfalfa cultivars 5 weeks after root inoculation with *Clavibacter insidiosus* and detection of the bacterium by conventional and real-time PCR.

	Alfalfa cultivar			
Test criterion	Algonquin	Apica	Dupuit	Rhizoma
Symptoms observed	10	15	16	11
Symptom type				
Stunting	3[a]	0	12	0
Leaf scorch	5	3	8	4
Yellowing or mottling[b]	2	9	3	1
Wilt	2	4	6	7
Molecular detection				
Conventional PCR	2	8	9	8
Real-time PCR	2	8	9	9

Values are numbers of plants from a total of 16 with each symptom type and positive PCR detection.

[a] Numbers may appear not add up because some plants expressed more than one symptom type.

[b] Mentioned as yellowing or mosaic in Ward et al. (2008).

APPENDIX 4 - REAL-TIME PCR (WARD ET AL., 2008)

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

1. General information

1.1. The test was developed by Ward et al. (2008) for the detection of *C. insidiosus* in plant material, seeds and suspected isolates based on primers published by Samac et al. (1998). The probe of Samac et al. (1998) has been slightly modified by Ward et al. (2008) and labelled with 5′-Cal-Fluor-610 and 3′-Iowa Black RQ. The test description is based on the publication of Ward et al. (2008) and is a real-time Taqman PCR test with subsequent confirmation of amplicon identity by melting peak analysis (addition of EvaGreen to the reaction mix).

1.2. The oligonucleotide primers are derived from IS 1122, a 1.1 kbp long sequence with multiple copies in the genome of *C. insidiosus*.

1.3. Oligonucleotides

Primer/probe name	Sequence	Amplicon size
CMI-F	5′-GAC CGC ATC TTT CGG GGT GTG-3′	135
CMI-R	5′-CGG AAA CCC GGT GAA TCT AAG-3′	
CIRS-1	5′-TTC AAC CGC ACC CTC GCG AC-3′	132
CIRS-2	5′-CGT CAG CCC GTG GCT CGA GT-3′	
CIRS-3A	5′-Cal-Fluor-610-AGA ACC GAC GCC CTT GAT CCG TGG-3′-Iowa Black RQ	

2. Methods

2.1. Nucleic acid extraction and purification

DNA is extracted from 500 µL of either the resuspended pellet from the seed extract or liquid from the plant homogenate by first adding 1 mL of Tris-HCl 10 mM extraction buffer (pH 8.0) to 0.035 mg mL^{-1} proteinase K, 3.5% SDS and 0.088 mol/L EDTA and incubating the mixture for >3 h (maximum, overnight) at 55–60°C to disrupt bacterial cells. Extraction is completed using the KingFisher processor (ThermoFisher Scientific, Walthan, MA, USA) with Magnesil KFGenomic System reagents following supplier's instructions.

Other extraction methods are also included in Appendix 2.

2.2. Real-time PCR

The test can be performed as simplex or duplex.

2.2.1. Master Mix

2.2.1.1. Simplex

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	5.85	N.A.
Ready to use Master Mix (*iQ supermix, Biorad)*	2×	12.5	1×
Forward primer CIRS-1	10 µM	1.25	0.5 µM
Reverse primer CIRS-2	10 µM	1.25	0.5 µM
Taqman probe CIRS-3A	10 µM	0.25	0.1 µM
BSA	50 µg µL^{-1}	0.15	0.3 µg µL^{-1}
EvaGreen (*Biotium, CA, USA*)	20 × (25 µM)	1.25	1 × (1.25 µM)
Subtotal		22.5	
Genomic DNA extract		2.5	
Total		25	

2.2.1.2. Duplex

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	3.40	N.A.
Ready to use Master Mix (*iQ supermix, Biorad)*	2×	12.5	1×
Forward primer CIRS-1	10 µM	1.25	0.5 µM
Reverse primer CIRS-2	10 µM	1.25	0.5 µM
Taqman probe CIRS-3A	10 µM	0.25	0.1 µM
CMI-F	10 µM	1.25	0.5 µM
CMI-R	10 µM	1.25	0.5 µM
BSA	50 µg µL^{-1}	0.15	0.3 µg µL^{-1}
EvaGreen (*Biotium, CA, USA*)	20 × (25 µM)	1.25	1 × (1.25 µM)
Subtotal		22.5	
Genomic DNA extract		2.5	
Total		25	

N.A. : Not applicable.

2.2.2. PCR conditions

95°C for 2 min, 45 cycles (95°C for 5 s and 69°c for 30 s), 69°C for 3 min.

Melting curve analysis of PCR products: 25 s soak at 80°C and 0.2°C/s ramp from 80 to 99°C.

3. Essential procedural information

3.1. Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: *C. insidiosus* equivalent to a concentration of approximately 10^4 cfu mL^{-1}

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

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

Other possible controls

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

3.2. Interpretation of results:

Verification of the controls

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

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- The T_m value should be as expected.
- CMI-F/CMI-R (peak 1): 88.6 (simplex) and 87.6 (duplex)
- CIRS-1/CIRS-2 (peak 2): 90.5 (simplex) and 89.7 (duplex).
- Tests should be repeated if any contradictory or unclear results are obtained

4. Performance characteristics available

From Ward et al. (2008).

4.1. Analytical sensitivity

C. insidiosus was consistently detected in 5 g seed samples, each spiked with a single inoculated seed. These tested positive in repeated tests on seven different batches of alfalfa seed.

Of seven non-spiked seed samples run as negative controls in the same experiment, only three yielded an amplification product that registered above the fluorescence threshold, but the Ct was >35, which is greater than the values obtained for spiked samples. The melting temperatures for two of these were outside the range obtained for spiked samples, but for one sample with a Ct of 36.2, the melting temperature was 89.42°C, suggesting a weak positive result requiring further investigation.

4.2. Analytical specificity

A PCR of pure cultures with primer pair CIRS-1/CIRS-2 in the presence of EvaGreen followed by melting curve analysis only produced amplification and melting peaks with *C. insidiosus* (T_m = 90.5) and *C. sepedonicus* (T_m = 92) templates and not with DNA templates from other *Clavibacter* species evaluated (*C. michiganensis* subsp. *michiganensis*, *C. nebraskensis* and *C. tessellarius*). The amplification of *C. sepedonicus* template with the *C. insidiosus*-directed primers had also been observed by Samac et al. (1998). However, with the use of EvaGreen for the melting peak analysis, the amplicons for *C. insidiosus* and *C. sepedonicus* were readily distinguishable.

APPENDIX 5 - CONVENTIONAL PCR (SAMAC ET AL., 2017) BASED ON PRIMERS FROM MAREFAT ET AL. (2007).

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

1. General information

1.1. The test description is based on the publication Samac et al. (2017).

1.2. The forward (CMIF241005) and reverse (CMIR241005) oligonucleotide primers are derived from the 16S-23S rRNA intergenic spacer region.

1.3. The test can be performed on plant material, seeds and suspected colonies.

1.4. Oligonucleotides

Primer name	Sequence	Amplicon size
Forward primer CMIF241005	5′-GTC AGG CGT TTG TCC TGG T-3′	224 bp
Reverse primer CMIR241005	5′-CCA CCA CCA TCC ACT CCG-3′	

1.5. Thermal cycler: ABI Prism 7900HT.

2. Methods

2.1. Nucleic acid extraction and purification

See Appendix 2.

2.2. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	11	N.A.
PCR buffer (iQ Supermix, Bio-Rad)	10×	2.5	1×
dNTPs	10 mM	1.5	0.6 mM
Forward primer CMIF241005	10 µM	1.25	0.5 µM
Reverse primer CMIR241005	10 µM	1.25	0.5 µM
Taq polymerase (iQ Supermix, Bio-Rad)	5 U µL^{-1}	2.5	0.5 U
Subtotal		20	
Genomic DNA extract		5	30 ng
Total		25	

N.A. : Not applicable

2.3. PCR conditions

95°C for 15 min, 30 cycles of 95°C for 45 s, 60°C for 45 s and 72°C for 1 min; 72°C for 10 min.

3. Essential procedural information

3.1. Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: *C. insidiosus* equivalent to a concentration of approximately 10^4 cfu mL^{-1}.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

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

Other possible controls

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

3.2. Interpretation of results

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC, PAC (and if relevant IC): a band of 224 bp is visualized.

When these conditions are met

- A test will be considered positive if a band of 224 bp is visualized.

- A test will be considered negative if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

From Samac et al. (2017).

4.1. *Analytical sensitivity*

30 cells/reaction.

No information available for other performance criteria.

APPENDIX 6 - REAL TIME PCR (SAMAC ET AL., 2017) BASED ON PRIMERS FROM MAREFAT ET AL. (2007)

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

1. General information

1.1. The forward (CMIF241005) and reverse (CMIR 241005) oligonucleotide primers are derived from the 16S-23S rRNA intergenic spacer region.

1.2. The test can be performed on plant material seeds and suspected colonies.

1.3. The amplicon size from *C. insidiosus* DNA is 224 bp.

1.4. Oligonucleotides

Primer/probe name	Sequence	Amplicon size
CMIF241005:	5′-GTC AGG CGT TTG TCC TGG T-3′	224 bp
CMIR241005	5′-CCA CCA CCA TCC ACT CCG-3′	
BW151205	FAM-5′ CTG CTA GTA CGC CTC CTT GTG G-3′ MGB[a]	

[a] FAM, fluorescein, six-isomer; MGB, minor groove binder.

1.5. Thermal cycler: ABI Prism 7900HT.

2. Methods

2.1. Nucleic acid extraction and purification

See Appendix 2.

2.2. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	3	N.A.
QuantiTect Probe PCR Master Mix (Qiagen Pty Ltd)	2×	10	1×
Forward primer CMIF241005	10 µM	0.8	0.4 µM
Reverse primer CMIR241005	10 µM	0.8	0.4 µM
Probe BW151205	10 µM	0.4	0.2 µM
Subtotal		15	
Genomic DNA extract		5	30 ng
Total		20	

N.A. : Not applicable

2.3. PCR conditions

95°C for 15 min, 45 cycles[6] of 95°C for 15 s and 65°C for 1 min.

3. Essential procedural information

3.1. Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample

[6]The number of cycles is as in the publication, but the drafting team considered that inconsistent results may be obtained above 40 cycles.

separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

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

Other possible controls

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

3.2. Interpretation of results

Verification of the controls

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

When these conditions are met

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

4. Performance characteristics available

From Marefat et al. (2007).

4.1. Analytical sensitivity

The average minimum number of cells detected by the test when performed on pure cultures using 40 cycles of amplification was 3.4 (mean values of three replicates) cells per PCR reaction. Similar sensitivity was achieved for plant extracts spiked with *C. insidiosus* and incubated for 2 h.

4.2. Analytical specificity

Inclusivity 100%.

The test was developed with 13 strains of *C. insidiosus* from different geographical origins, 11 from Australia, one from the USA and one from the UK.

Exclusivity 100% (no cross reaction observed).

It also included *C. 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.

APPENDIX 7 - SEROLOGICAL TESTS

Data from prime diagnostics indirect immunofluorescence test serum 9742A (evaluation made on plant material)

Analytical sensitivity 4.5×10^3 cfu mL^{-1}

Analytical specificity

Inclusivity evaluated on five strains of *Clavibacter insidiosus*.

Exclusivity evaluated on four strains of *Clavibacter michiganensis* subsp. *michiganensis*, four strains of *C. sepedonicus* and two strains of *Xylella fastidiosa*.

Cross-reaction observed with one strain of *C. michiganensis* subsp. *michiganensis*.

Reproducibility 100%

Repeatability 100%

Data from Anses, FR Indirect Immunofluorescence test Antiserum Prime Diagnostic reference 2970; Conjugate FITC: SIGMA 079K4804. Evaluation made in seed extracts.

Analytical sensitivity: 100% at 10^4 to 10^5 cells mL^{-1} (2012).

Diagnostic sensitivity: 100%.

Evaluation performed with 10 seed lots.

Five seeds lots (varieties Capri, Concerto, Europe, Fado, Symphonie) spiked with bacterial concentration of strain LMG 3676 from 10^4 to 10^6 cells mL^{-1}.

Five seeds lots (varieties Azzura, Crioula, Delta, Europe, Pondus) spiked with bacterial concentration of strains LMG 3676 and LSV40.75 from 10^3 to 10^5 cells mL^{-1}.

Analytical specificity

Inclusivity: 100% (17 strains).

Twelve reference strains from collections: *Clavibacter insidiosus* LMG 3676, NCPPB 1109, LMG7324T1, NCPPB 83, NCPPB 1660, LMG 7325T2, CFBP 6489, CFBP 2404, CFBP 6490, CFBP 6492, CFBP6488, CFBP 6491 and five *Clavibacter insidiosus* strains from Poland (one isolated in 1964, four in 2008).

Exclusivity: 93.8% cross-reaction noted with *C. nebraskensis* strain LMG 5625 (CFBP 3493).

Genetically related bacteria: *C. tessellarius* LMG 7300 (CFBP 3499), *C. nebraskensis* LMG 5625 (CFBP 3493), *C. sepedonicus* CFBP 1154, *C. m.* subsp. *michiganensis* CFBP 2498, *Clavibacter tritici* = *Rathayibacter tritici* CFBP 1385.

Other plant pathogenic bacteria from reference or laboratory collections: *Curtobacterium flaccumfaciens* subsp. *flaccumfaciens* CFBP 3411, *Curtobacterium flaccumfaciens* subsp. *betae* CFBP 2402, *Pseudomonas syringae* pv. *pisi* LNPV 05.42, *Ralstonia solanacearum* CFBP 3857, *Rhodococcus fascians* CFBP 2100, *Xanthomonas arboricola* pv. *pruni* CFBP 3892, *Xanthomonas axonopodis* pv. *phaseoli* CFBP 2534.

Reproducibility: not evaluated.

Repeatability: 100%.

APPENDIX 8 - PATHOGENICITY TESTS

Grow susceptible alfalfa test plants (varieties Europe and Orca are reported to be highly susceptible, according to 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 ten, five to six weeks old plantlets should be used to test the presumptive isolates 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 phosphate buffer (see Appendix 1) 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 phosphate buffer).

Although different methods can be used for inoculation, Cormack et al. (1957) concluded that root inoculation is more efficient than stem inoculation, thus this method is described below.

Cut root tips of test plants with a sterile scissor and immediately dip them into the bacterial suspension for 17–18 h (Víchová & Kozová, 2004). Inoculated test plants are then potted in peat soil and kept under observation for at least 6–8, weeks in order to check possible development of typical symptoms.

From the fourth week make at least weekly observations for wilting. Attempt isolation from wilting plants by removing a 1-cm stem section from 2 cm above the plant collar and suspending in 2–3 mL of phosphate buffer. Soak the stem sections in buffer for 15–20 min and then, perform dilution plating on King's B or YPGA media. Subculture presumptive isolates and undertake identification tests to confirm they are *C. insidiosus*.