International Seed Federation
Seed is Life

Detection of *Clavibacter michiganensis* in Tomato Seed

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Developed by ISHI

Detection of *Clavibacter michiganensis* in Tomato Seed

Crop: Tomato (*Solanum lycopersicum*)

Pathogen(s): *Clavibacter michiganensis* (formerly *C. michiganensis* subsp. *michiganensis*).

Version: 6 (April 2026)

PRINCIPLE

Bacteria are extracted from the seed by stomaching and plated on semi-selective media. Colonies suspected to be *Clavibacter michiganensis* (Cm, formerly *Clavibacter michiganensis* subsp. *michiganensis*; Li *et al.* (2018)), on the basis of their morphology, are tested for pathogenicity on tomato seedlings. Optionally, suspect colonies can be tested by two Cm specific PCRs. If both are negative a colony is not considered to be Cm. A positive result for one or both PCRs should be followed by a pathogenicity assay.

An optional seed extract qPCR assay (SE-qPCR) may be used as a pre-screen directly on bacteria collected from the seed extract. If negative, the seed lot is considered to be healthy. If positive, this SE-qPCR pre-screen assay should be followed by the dilution plating assay to verify the presence of viable and pathogenic Cm. If desired, a single seed sample can be processed by both the SE-qPCR and dilution plating assays. Samples should however be processed on the same day by both methods, since the effect of storing seed extract on test results is not fully known. If the dilution plating assay is performed after a SE-qPCR pre-screen, a new seed sample is required. The full method process workflow is presented in Figure 1.

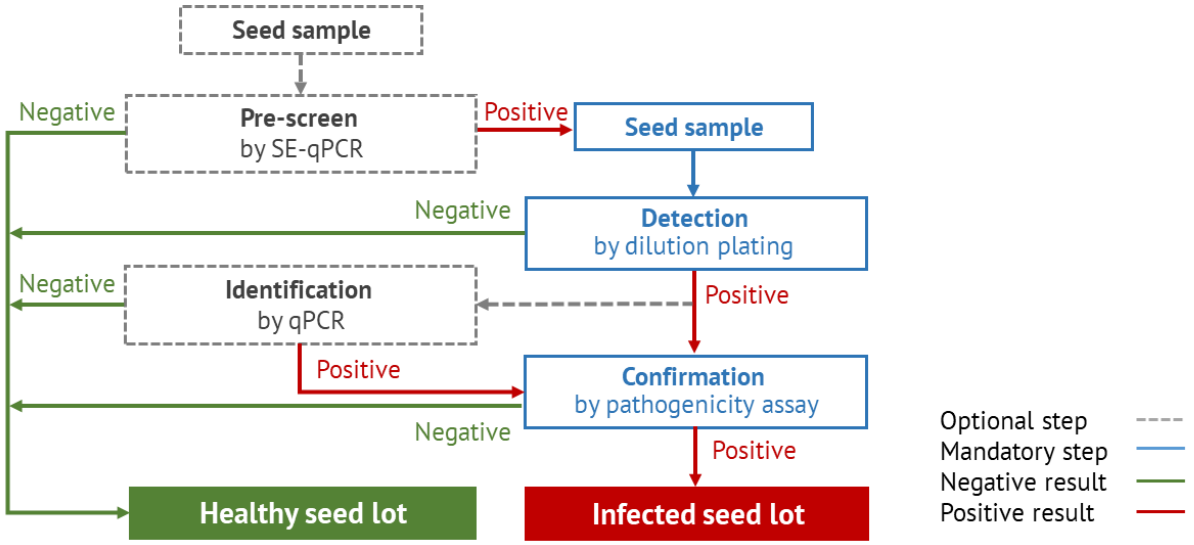

Figure 1. Method process workflow.

METHOD VALIDATION

This method has been peer reviewed by ISHI members and experts outside ISHI. The pre-screen by seed extract qPCR (SE-qPCR) has been validated by ISHI (Lastdrager and Woudt, 2022). Validation included, among others, an interlaboratory comparative test in which seven independent laboratories tested blind samples of naturally infected seed.

The SE-qPCR pre-screening method was initially developed and validated by Naktuinbouw in the EU TESTA project framework (https://cordis.europa.eu/project/rcn/105068/reporting/en). In the current ISHI method, DNA purification using a widely available kit is used as an alternative to the automatic magnetic bead purification used in the Naktuinbouw protocol. The DNA-extraction kit used for validation has wide global availability and is therefore better suited for comparative testing. Other extraction kits and equipment may be equally suitable, but validation of its performance is the responsibility of the user.

A recovery study on 23 *C. michiganensis* strains on CMM1T, SCM and SCMF, including three Cm strains with atypical pinkish (N=2) or whitish (N=1) colony morphology, demonstrated sufficient recovery on these media. Furthermore, the Cm strains with atypical colony morphology were detected by both the PTSSK and MVS21 qPCRs (Koenraadt *et al.*, 2026; Koenraadt *et al.*, in preparation).

RESTRICTIONS ON USE

Before using this protocol routinely, it is necessary to verify its performance, especially when material and consumables from different suppliers are used. Technical details on the reagents/material used in the validation study (e.g., supplier's information) are provided in the protocol and the validation report for SE-qPCR.

The SE-qPCR assay has been developed using pectinase and/or acid extracted seed. The SE-qPCR was not validated for seed treated with protective chemicals, seed disinfectants or biological substances. If a user chooses to test treated seed using this method, it is the responsibility of the user to determine empirically (through analysis, sample spiking, or experimental comparisons) whether the protective chemicals, seed disinfectants or biological substances have an effect on the method results. During development it became apparent that SE-qPCR is not compatible with seeds treated with hypochlorite (ClO^-) because this chemical effectively breaks down bacterial DNA, which is the critical analyte of this assay.

The dilution plating assay is suitable for seed that has been extracted using pectinase and/or acid or that has been treated using physical (such as hot water) or chemical (calcium or sodium hypochlorite, tri-sodium phosphate, etc.) processes with the aim of disinfestation/disinfection, provided that any residue, if present, does not influence the assay. It is the responsibility of the user to check for inhibition by analysis, sample spiking, or experimental comparisons.

The ability to recover Cm on plates can be influenced by the presence of other microorganisms. A check for such antagonism must be performed by spiking the most concentrated seed extracts with a known number of Cm-colony forming units (CFUs). See the test method description for details on spiking (Section 2.4 of Dilution Plating).

Guidance for molecular testing methods is provided such that it accommodates modular assay components. DNA isolation, positive extraction control selection, qPCR reaction mixture, cycling parameters, multiplex reactions and evaluation specifications (threshold/cut-offs) are variables that may differ between testing laboratories. It is the responsibility of the testing laboratory to verify that the selected combination of assay components meets the following minimum recovery and detection parameters, *viz.* 100% detection of Cm across triplicate 10,000 seed subsamples in a concentration of 10 CFU/mL[1] non-concentrated seed extract by both Cm qPCR assays.

METHOD EXECUTION

Sample preparation for SE-qPCR and dilution plating are the same. Hence, if desired, a single seed sample can be processed simultaneously by both the SE-qPCR and dilution plating assays.

To ensure process standardization and valid results, it is strongly recommended to follow the best practices developed by ISHI for PCR and Dilution Plating Assays in Seed Health Tests.

In line with the guidance provided in ISF's view on indirect seed health tests, if a sample is determined SE-qPCR positive, the seed lot is considered to be *suspect*. A final decision on the status of the seed lot can be taken only after a follow-up test by dilution plating determining the possible presence of viable Cm in the sample and when its pathogenicity is confirmed.

SAMPLE AND SUBSAMPLE SIZE

The recommended minimum sample size is 10,000 seeds with a maximum subsample size of 10,000 seeds. If the SE-qPCR is positive and dilution plating was not conducted on the same sample, a new sample must be drawn for the confirmatory dilution plating test.

[1] As determined by spiking of a dilution series of a log-phase Cm culture into a Cm-free seed extract. Appropriate dilutions are plated to quantify CFU/mL.

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1	May 2006	First version of the protocol.
2	January 2007	Section *Restriction on use* modified
3	January 2008	Sections *Sample and subsample size, Principles, Sensitivity reference* and *Isolation on semi-selective media* modified.
4	March 2011	Sections *Validation* and *Restriction on use* modified
4	August 2011	Section *Identification by PCR* added (PTSSK and PSA). Sections *Restrictions on use, Extraction of bacteria from the seed, Isolation on semi-selective media* (Spiking added), *Identification by morphology on YDC medium, Identification by pathogenicity assay,* and *Buffers and media* modified. Sections *Sensitivity reference* and *Validation* removed
4.1	January 2014	Sections *Isolation on semi-selective media*, *Identification by PCR*, *Identification by pathogenicity assay*, and *Buffers and media* modified. Critical points identified and indicated.
4.2	August 2015	Section *Method execution* added. Sections *Isolation on semi-selective media*, and *Identification by PCR* modified (MVS 21 qPCR to replace PSA PCR). Critical points removed.
4.3	September 2015	Section *Isolation on semi-selective media* modified according to ISHI Best Practices. Error in the SCM and SCMF media corrected.
4.3.1	August 2016	Section *Identification by PCR* modified according to ISHI Best Practices.
4.3.1	July 2017	Section *Validation* modified.
5	July 2022	SE-qPCR added as optional pre-screen Protocol presented in accordance with ISHI protocol guidelines. Crop name *Lycopersicon esculentum* removed.
6	April 2026	Pictures and description of Cm strains with atypical colony morphology added based on recovery study (Koenraadt *et al.*, in preparation). Pathogen name updated to *Clavibacter michiganensis*. Control names updated to adhere to the new ISHI Best Practices.

Protocol for detecting *Clavibacter michiganensis* in Tomato Seed

I. PRE-SCREEN BY SEED EXTRACT QPCR (OPTIONAL STEP)

For PCR methods, in-house method optimization is often necessary, see Best Practices for PCR Assays in Seed Health Tests for information.

Materials

- Paddle blender and bags (e.g., Bagfilter® or Bagpage® (Interscience, St Nom la Bretèche, France) or an extraction bag with synthetic intermediate layer (e.g., Bioreba, Reinach, Switzerland; Neogen Europe, Ayr, Scotland))
- Seed extraction buffer (Table I.1)
- Phosphate Buffer (Table I.2)
- Refrigerator at 4–7 °C
- Centrifuges capable of spinning 15/50 mL conical tubes and 1.5/2 mL microtubes (Optional: Microtiter plates)
- High speed homogenizer (e.g., Tissuelyser II (Qiagen, Hilden, Germany) or Geno/Grinder (Cole-Parmer, Metuchen, NJ)
- Dry Ø 0.1 mm zirconium/glass beads (e.g., Cole-Parmer; Biospec Products, Bartlesvilles, OK; OPS Diagnostics, Lebanon, NJ)
- DNeasy® Blood & Tissue Mini Spin Column kit (Qiagen, See Section 2 for recommendations on kit use and alternative DNA isolation kits)
- Heating block for 1.5/2 mL microtubes (shaking, otherwise additional vortex is required)
- Controls (Table I.3)
- qPCR mix (e.g., PerfeCTa qPCR Toughmix (Quantabio, Beverly, MA)), primers (Table I.4) and PCR-equipment
- Lab disposables

Table I.1. Seed extraction buffer[a] (pH 7.4).

Compound	Amount/L
Na_2HPO_4	7.75 g
KH_2PO_4	1.65 g
Tween 20	0.20 mL
$Na_2S_2O_3$[b]	0.50 g

[a] Use deionised water, and autoclave at 121 °C, 15 psi for 15 min.
[b] Recommended when seed extract is in parallel tested by dilution plating. Note however that hypochlorite treatment is not compatible with SE-qPCR.

Table I.2. Phosphate buffer[a] (PB) (pH 7.4).

Compound	Amount/L
Na_2HPO_4	7.75 g
KH_2PO_4	1.65 g

[a] Use deionised water, and autoclave at 121 °C, 15 psi for 15 min.

Table I.3. Types of controls used.

Control type	Description
Inhibition control (IC)[a]	*Clavibacter tessellarius* (ATCC 33566; recommended)
Positive amplification control (PAC)	Known quantity of Cm DNA *and*
	Known quantity of IC DNA
Negative process control (NPC)	Tomato seed free from Cm and the IC
Non template control (NTC)	PCR grade water

[a] The IC also serves as internal amplification control (IAC). Other related gram-positive bacteria may also be used, but compatibility with the primers in a multiplex qPCR should be verified. Since gram-positive bacteria like *Clavibacter* are relatively hard to lyse, the IC must be a related gram-positive bacterium.

Table I.4. Primer-sequences and references.

Name	Target	Sequence (5' – 3')	Source
MVS21-F	Cm	CTA GTT GCT GAA TCC ACC CAG	Sudarshana *et al.*, 2012, modified
MVS21^{+}-R[a]		GTA CCG CTT GAC TCT CGT TTC	
MVS21^{+}-Pr[b]		VIC – CTG CCA CCC GAT GTT GTT GTT CCG – BHQ1	
PTSSK-F		CGT CGC CCG CCC GCT G	Berendsen *et al.*, 2011
PTSSK-R		GGG GCC GAA GGT GCT GGT G	
PTSSK-Pr		FAM – TGG TCG TCC TCG GCG – MGB – NFQ	
Cmt-F	Ct[c]	AAC CCC AGG TCG TCT TGT CGA A	Naktuinbouw, 2016
Cmt-R		GCG CGT CTA CAC GGG CAT CA	
Cmt-Pr		TexRed – TGT CGT CGA TCC AGG CCT CGC CC – BHQ2	

[a] Original MVS21 reverse primer (Sudarshana *et al.* 2012) extended with a single G-nucleotide at the 5'-end to elevate the Tm to align with Tm of PTSSK primers and probe.

[b] Original MVS21 probe (Sudarshana *et al.* 2012) extended with a single G-nucleotide at the 3'-end to elevate the Tm to align with Tm of PTSSK primers and probe.

[c] The use of *C. tesselarius* (Ct) as an IC is recommended but not mandatory. The qPCR assay for detecting Ct strain ATCC® 33566 is offered here. Note that not all Ct strains may react with this assay.

1. Seed extraction

1.1. Put every subsample individually into a sterile stomacher bag with filter and add sterile seed extraction buffer (Table I.1) to each bag at a ratio of 4 mL of seed extract buffer per gram of seed.

Note: A negative process control (NPC) subsample is required to be included (Table I.3).

1.2. Incubate overnight (minimum 14 hours) at 4–7 °C.

1.3. Macerate for at least 4 min in a stomacher machine until the seed extraction buffer becomes milky and white particles become visible as a result of endosperm release (see Figure I.1).

Figure I.1. The seed extraction buffer and seeds after stomaching.

1.4. From behind the filter, transfer 2.5 mL of extract per 1,000 seeds in the subsample to a suitable centrifuge tube.

Note: If desired, dilution plating can be performed on the same extract (starting with Step 1.5 in Section II of this method), but extracts have to be plated immediately before availability of the SE-qPCR result to guarantee survival of Cm in the seed extract. Note that for seeds with a low thousand seed weight (TSW), the amount of extract produced may not be sufficient to perform both assays.

1.5. Spike each subsample extract with approximately 1×10^4 cells of the inhibition control (IC). The bacterial suspension for spiking may be prepared fresh or in advance (e.g., stored in uniform aliquots at -80 °C in a 15% (v/v) glycerol solution).

1.6. Further clear the extract from debris by low-speed centrifugation at 1,000 × *g* for 1 min. This facilitates re-suspension of the pellets in Step 1.9.

1.7. Decant the supernatant into a new centrifugation tube and discard pellet.

1.8. Centrifuge the supernatant with a time and speed sufficient to pellet bacteria (e.g., at 5,000 × *g* for 15 min).

1.9. Remove the supernatant carefully and resuspend the pellet in 1–1.5 mL phosphate buffer (PB, Table I.2).

2. Cell lysis and DNA isolation

The assay has been validated with the DNeasy® Blood & Tissue Mini Spin Column kit (Qiagen), with modifications. Modifications are the consequence of bacterial lysis being achieved by bead beating, which is specific for this Cm SE-qPCR protocol and is done in a detergent-free buffer to limit foam generation. The 96-well plate version of the DNeasy® Blood & Tissue kit was not validated. General instructions can be found in the DNeasy® Blood & Tissue Handbook (Qiagen). Cm SE-qPCR-protocol-specific instructions when using the DNeasy® Blood & Tissue kit are described below.

If a different DNA isolation kit is used, it is necessary to verify its performance. For example, the Nucleospin Tissue 96 wells core kit (Macherey Nagel, Duren, Germany) or a KingFisher protocol

in combination with the Sbeadex plant maxi kit (LGC, Teddington, UK) are good alternatives, for which the steps below might not apply. The Precellys® homogenisator (Bertin instruments, Montigny-Le-Bretonneux, France) is a good alternative for the Tissuelyser (Qiagen) and Geno/Grinder (Cole-Parmer) equipment to perform mechanical cell lysis.

2.1. Transfer the cell suspension obtained in Step 1.9 to 2 mL Safelock tubes with round bottom. Spin down (e.g., at 5,000 × *g* for 5 min) the cells and resuspend in 230 µL phosphate buffer (PB). Add 360 µL of dry Ø 0.1 mm zirconium/glass beads and beat in a Tissuelyser II (Qiagen) at 30 Hz for 3 min or in a Geno/Grinder (Cole-Parmer) at 1500 rpm for 3 min (settings of alternative tissue homogenizers need to be determined by in-lab validation. It is the responsibility of the user to secure adequate lysis). Short spin the samples to collect beads and liquid at the bottom of the tubes.

Note: Zirconium/glass beads bind DNA. Low binding Ø 0.1 mm zirconium beads are recommended to minimize DNA loss to the beads.

2.2. From the DNeasy® Blood & Tissue Mini Spin Column kit (Qiagen), add 205 µL buffer ATL and 25 µL proteinase K to each tube and incubate at 56 °C (minimum 3 hours to maximum overnight (~16 hours)). Vortex at least every hour or use a shaking incubator. Do not premix buffer ATL and proteinase K more than 10–15 min before addition to the sample. Proteinase K tends to self-digest in buffers without substrate. Short spin the samples to collect beads and liquid at the bottom of the tubes.

2.3. Add 460 µL buffer AL and mix by vortexing. Short spin the samples to collect beads and liquid at the bottom of the tubes. Pipet 560 µL lysate without taking any beads and mix with 280 µL EtOH (96–100% (v/v)) in a clean reaction tube and mix by vortexing.

2.4. Continue with Step 4 of the protocol "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)", page 30 in the DNeasy® Blood & Tissue Handbook (Qiagen). Load and spin the 840 µL volume in two portions, not exceeding 600 µL for either portion. Empty the collection tube after the first portion to accommodate the flow-through of the second. Do this with care to minimize risk of cross-contamination.

2.5. Finally elute DNA in 100 µL elution buffer.

3. qPCR assay

The qPCR assays have been validated with the PerfeCTa qPCR Toughmix (Quantabio). If different qPCR mixtures and amplification programs are used, it is necessary to verify their performance. The annealing temperature is critical and should be optimised for the PCR mix used. Since the MVS21$^+$ qPCR primers have a lower Tm than those of the PTSSK qPCR an annealing temperature gradient should be performed to select the temperature 1 °C lower than the point where MVS21$^+$ qPCR Cq values start to increase.

3.1. Perform, in duplicate, a multiplex qPCR reaction on DNA of each subsample, combining the two Cm qPCR assays with the qPCR assay detecting the IC. See Table I.5. for the PCR mixture. A positive amplification control (PAC) for Cm and the IC as well as a non-template control (NTC) are required (Table I.3).

Table I.5. Example of a PCR mix for MVS21⁺/PTSSK/Ct multiplex qPCR.

Component	Per reaction (in µL)	Final concentration
MVS21-F (100 µM)	0.10	0.40 µM
MVS21⁺-R (100 µM)	0.10	0.40 µM
MVS21⁺-Pr (100 µM)	0.10	0.40 µM
PTSSK-F (100 µM)	0.10	0.40 µM
PTSSK-R (100 µM)	0.10	0.40 µM
PTSSK-Pr (100 µM)	0.10	0.40 µM
Cmt-F (100 µM)	0.10	0.40 µM
Cmt-R (100 µM)	0.10	0.40 µM
Cmt-Pr (100 µM)	0.05	0.20 µM
qPCR master mix (2×)	12.50	1×
PCR grade water	6.65	
DNA template	5.00	
Total	**25.00**	

3.2. Perform the PCR reaction in a real-time PCR instrument. An example of the PCR conditions is shown in Table I.6.

Table I.6. qPCR cycling parameters.

Step	Temperature	Duration
hold	95 °C	10 min
40 cycles	95 °C	15 sec
	67 °C[a]	48 sec

[a] PCR reagent mix dependent, see note on annealing temperature optimization above.

4. qPCR assay evaluation

Cut-off values must be established by each laboratory for the positive control and the inhibition control prior to the assay being used on routine samples such that the minimal analytical sensitivity of detecting 10 CFU of Cm per mL non-concentrated seed extract is achieved (see Section "Restrictions on use"). For recommendations on setting cut-off values, see Real-time PCR, an 'indirect' test used for pre-screening in seed health methods.

Test results are only valid when all included controls presented in Table I.3 give the expected results. The NTC must be negative for all qPCR assays (no detection of Cm or the IC). The NPC must be negative for Cm. The IC/PAC results must fall within an expected range. Control ranges are determined per laboratory. For the IC, the Cq cut-off should be below Cq 35, with a preferable expected Cq range of 29–32.

4.1. A sample is considered suspect for Cm if one or more subsamples show amplification of Cm DNA by one or both Cm primer sets.

4.2. If a sample is determined qPCR Cm suspect, dilution plating, either on the same extract or on a new sample is required for a final conclusion (see Method principles).

II. DETECTION BY DILUTION PLATING

Materials

- Paddle blender and bags (e.g., Bagfilter® or Bagpage® (Interscience, St Nom la Bretèche, France) or an extraction bag with synthetic intermediate layer (Bioreba, Reinach, Switzerland; Neogen Europe, Ayr, Scotland))
- Refrigerator at 4–7 °C
- Incubator: operating at 26–28 °C
- Filter bags
- Centrifuge
- pH meter
- Seed extraction buffer (Table II.1)
- Controls (Table II.2)
- Plates of CMM1T, SCM or SCMF and YDC media (Tables II.3-II.6)
- Lab disposables

Table II.1. Seed extraction buffer[a] (pH 7.4).

Compound	Amount/L
Na_2HPO_4	7.75 g
KH_2PO_4	1.65 g
Tween 20	0.20 mL
$Na_2S_2O_3$[b]	0.50 g

[a] Use deionised water, and autoclave at 121 °C, 15 psi for 15 min.
[b] Recommended when seeds have been treated with hypochlorite.

Table II.2. Types of controls used.

Control type	Description
Positive control (PC)	A known Cm reference strain
Negative buffer control (NBC)	Seed extraction buffer
Inhibition (spike) control (IC)[a]	A known Cm reference strain *or*
	An antibiotic resistant Cm-strain *or*
	A *Clavibacter* strain of a species other than Cm

[a] The use of an antibiotic resistant Cm-strain as IC is an option to rapidly verify possible false-positives due to cross-contamination. Use of a different *Clavibacter* species such as *C. tessellarius*, with a morphology that is distinct from Cm and from common saprophytes is another option. Such a species can be spiked directly into the most concentrated seed extracts. The selected strain must be suppressed or overgrown similarly to Cm on the semi-selective media in the presence of antagonists.

Table II.3. CMM1T pH 7.7[a] (Alvarez *et al.,* 1999; Koenraadt *et al.,* 2009).

Compound	Amount/L
Sucrose	10.0 g
Trizma base (Tris base)[b]	3.32 g
Tris-HCl[b]	11.44 g
$MgSO_4.7H_2O$	0.25 g
LiCl	5.0 g
Yeast extract	2.0 g
NH_4Cl	1.0 g
Casein hydrolysate	4.0 g
Agar	15 g
Polymyxin B sulphate[c] (10 mg/mL in deionised water)	10 mg (1.0 mL)
Nalidixic acid (salt)[c] (10 mg/mL in 0.1 M NaOH)	28 mg (2.8 mL)
Nystatin[c] (100 mg/mL in 50% (v/v) DMSO / 50% (v/v) ethanol)	100 mg (1 mL)

Notes apply to Tables II.3-II.5:

[a] Recommended pH before autoclaving.

[b] The pH should be set by using both Tris-base and Tris-HCl rather than taking either one of the two compounds and setting with NaOH or HCl as that would increase salt concentration. Most importantly, this approach secures correct pH without any need for adjusting it when all ingredients have been added. The pH is very critical, and most pH meters are less suitable for Tris buffers at high molarity.

[c] Added after autoclaving (Temperature < 50 °C).
Antibiotics stock solutions and other supplements prepared in distilled/deionised water must be sterilized using a 0.2 µm bacterial filter. Alternatively, add the antibiotic powder to autoclaved distilled/deionised water. Solutions prepared in 70% (v/v) ethanol do not need sterilization.
Storage conditions and duration may affect antibiotic activity, which can influence the performance of the test.

[d] This is a guideline. The concentration of potassium tellurite varies depending on the manufacturer and adjustment of the volume may be needed. The pH of the medium may influence the recovery of Cm in the presence of potassium tellurite.

Table II.4. SCM pH 7.3[a] (Fatmi and Schaad, 1988).

Compound	Amount/L
Sucrose	10.0 g
K_2HPO_4	2.0 g
KH_2PO_4	0.5 g
$MgSO_4.7H_2O$	0.25 g
H_3BO_3 (boric acid)	1.5 g
Yeast extract	0.1 g
Agar	18.0 g
Potassium tellurite[c, d] (1% (w/v) solution)	10 mg (1 mL)
Nicotinic acid[c] (20 mg/mL in deionised water)	100 mg (5 mL)
Nalidixic acid (salt)[c] (10 mg/mL in 0.1 M NaOH)	30 mg (3 mL)
Nystatin[c] (100 mg/mL in 50% (v/v) DMSO and 50% (v/v) ethanol)	100 mg (1 mL)

Table II.5. SCMF pH 7.3[a] (Fatmi and Schaad, 1988; Koenraadt *et al.,* 2009).

Compound	Amount/L
Sucrose	10.0 g
K_2HPO_4	2.0 g
KH_2PO_4	0.5 g
$MgSO_4.7H_2O$	0.25 g
H_3BO_3 (boric acid)	1.5 g
Yeast extract	2.0 g
Agar	18.0 g
Potassium tellurite[c,d] (1% (w/v) solution)	10 mg (1 mL)
Trimethoprim[c] (10 mg/mL in 100% methanol)	80 mg (8 mL)
Nicotinic acid[c] (20 mg/mL in deionised water)	100 mg (5 mL)
Nalidixic acid (salt)[c] (10 mg/mL in 0.1 M NaOH)	20 mg (2 mL)
Nystatin[c] (100 mg/mL in 50% (v/v) DMSO and 50% (v/v) ethanol)	100 mg (1 mL)

See notes Table II.3.

Table II.6. Yeast dextrose chalk (YDC) agar (Wilson *et al.,* 1967; Schaad *et al.,* 2001).

Compound	Amount/L
Agar	15.0 g
Yeast extract	10.0 g
$CaCO_3$ (light powder)	20.0 g
D-Glucose (dextrose)	20.0 g

1. Seed extraction

1.1. Put every subsample individually into a sterile stomacher bag with filter and add sterile seed extraction buffer (Table II.1) to each bag at a ratio of 4 mL of seed extract buffer per gram of seed.

1.2. Incubate overnight (minimum 14 hours) at 4-7 °C.

1.3. Macerate for at least 4 min in a stomacher machine until the seed extraction buffer becomes milky and white particles become visible as a result of endosperm release (Figure I.1).

1.4. From behind the filter, transfer the required volume of extract to a suitable centrifuge tube.

1.5. Further clear the extract from debris by low-speed centrifugation at 1,000 × *g* for 1 min. This removes fungal spores and facilitates re-suspension of the pellets. Remove the supernatant carefully and use it for dilution plating.

2. Dilution and plating

2.1. Prepare a ten-fold dilution of the seed extract in sterile seed extraction buffer (Table II.1). For subsamples larger than 2,000 seeds, prepare also a ten-fold concentrated extract. Centrifuge the filtered extract with a time and speed sufficient to pellet bacteria (e.g., at 5,000 × *g* for 5 min). Remove the supernatant carefully and resuspend the pellet in 1/10 of the original centrifuged volume of sterile seed extraction buffer.

2.2. Pipette 100 µL of the concentrated (if applicable), the undiluted and diluted extracts on two plates of both the CMMT1 and either SCM or SCMF semi-selective media and spread over the surface.

2.3. Prepare a ten-fold dilution series of a pure culture of a known Cm reference strain in sterile seed extraction buffer (positive control (PC), Table II.2). Plate 100 µL of the dilutions on each medium to give at least one plate of 30–300 colonies on each medium for use as reference plates (see Section 3.1).

2.4. As a check on the ability to recover Cm, spike an inhibition control (IC) into (a separate aliquot of) the most concentrated extract of each subsample at a level of 20–100 CFU per 100 µL (Table II.2). Plate 100 µL of these spiked extracts on the two semi-selective media. Use similarly spiked seed extraction buffer as a control.

2.5. Plate 100 µL of seed extraction buffer on the two semi-selective media as negative buffer control (NBC).

2.6. Incubate plates in the dark at 26–28 °C for 10 days. Check plates at 7–10 days.

3. Examination of the plates

3.1. Check recovery and morphology of the Cm reference strain on both media.

After 10 days of incubation on CMM1T, Cm colonies are typically yellow, mucoid, and convex (see Figure II.1A and B). However, atypical strains can also be detected with mucoid and convex colonies, with colour ranging from white to pinkish (see Figure II.1C and D).

After 10 days of incubation on SCM or SCMF, Cm colonies are translucent grey, mucoid, eventually often irregularly shaped. The centre of the colony can be grey to grey-black depending on the strain (see Figure II.1E–L).

There should be no growth of target pathogens on the NBC plates.

Test results are only valid when all included controls (Table II.2) give the expected results.

3.2. Examine the plates of each subsample spiked with the IC for the presence of colonies with typical morphology. Spiking is valid when at least five colonies are present on any one of the used media. A negative subsample test result can only be concluded if the spiking of the same subsample is valid. In the case that no IC colonies are identified, and the spiking is invalid, test a new subsample, optionally after (further) treatment/disinfestations of the seed lot.

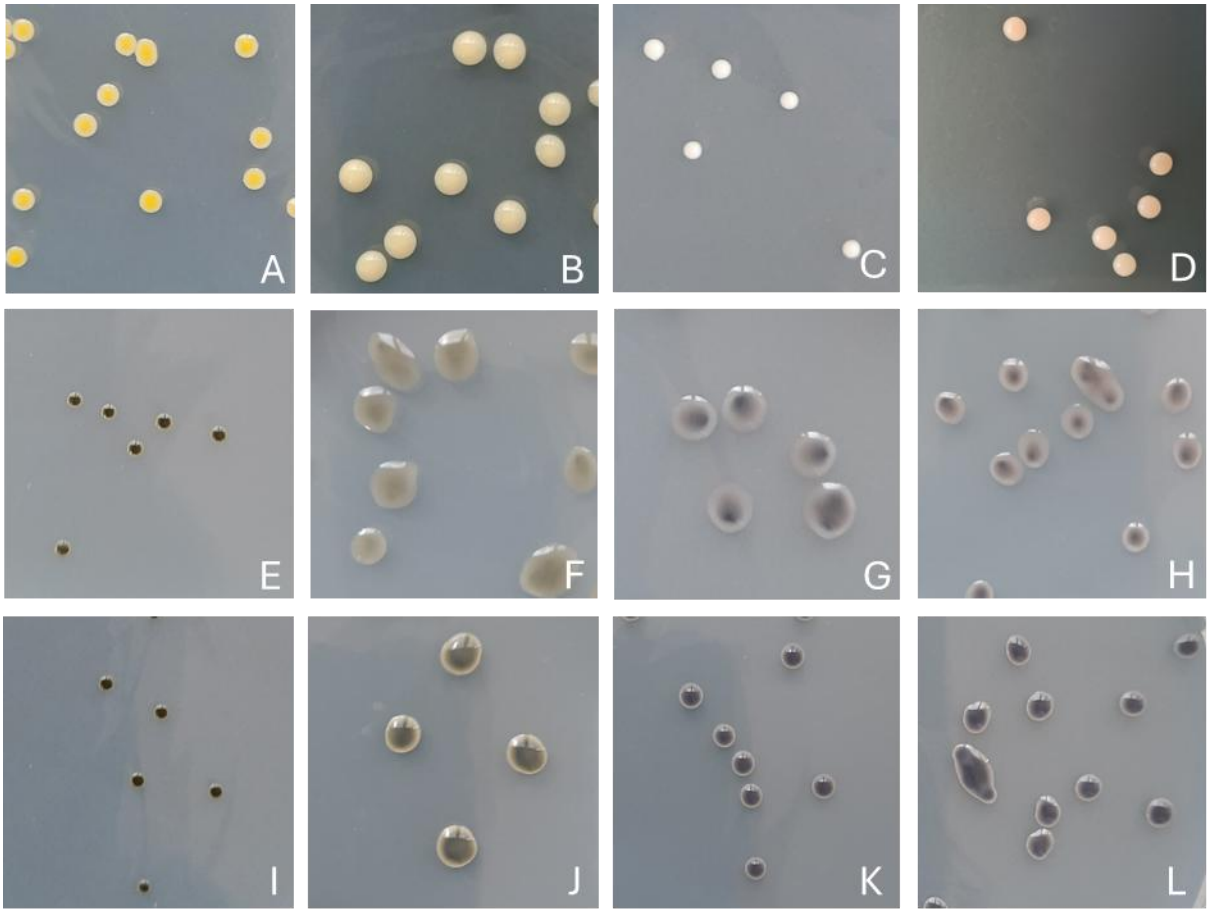

Figure II.1. Colony morphology of four Cm strains (IPO 500: A, E, I; IPO 542: B, F, J; ZTO 735: C, G, K; CFBP 9078: D, H, L) after 10 days of incubation at 28 °C on CMM1T (A–D), SCM (E–H) and SCMF (I–L).

3.3. Examine the sample plates for the presence of colonies with Cm morphology by comparing them with the PC. The colony size and colour can differ greatly within and between samples. It is particularly influenced by other colonies growing nearby. Isolates may also vary in the speed at which they grow.

3.4. From sample plates, select, if present, at least six suspect colonies per medium per subsample for further identification on YDC medium.

4. Morphology identification of suspect colonies

4.1. Subculture suspect colonies as well as the reference strain on the YDC medium.

4.2. Incubate plates 2–3 days at 26–28 °C.

4.3. Compare the appearance of growth with the PC. On YDC, Cm colonies are typically yellow, domed (convex) and mucoid in appearance. However, atypical strains with domed (convex), mucoid, whitish and pink coloured colonies have also been reported (Haghverdi *et al.*, 2025; Kaneshiro *et al.*, 2006) and should be tested further (See Figure II.2). The PC(s) or reference material should give colonies with typical morphology on YDC.

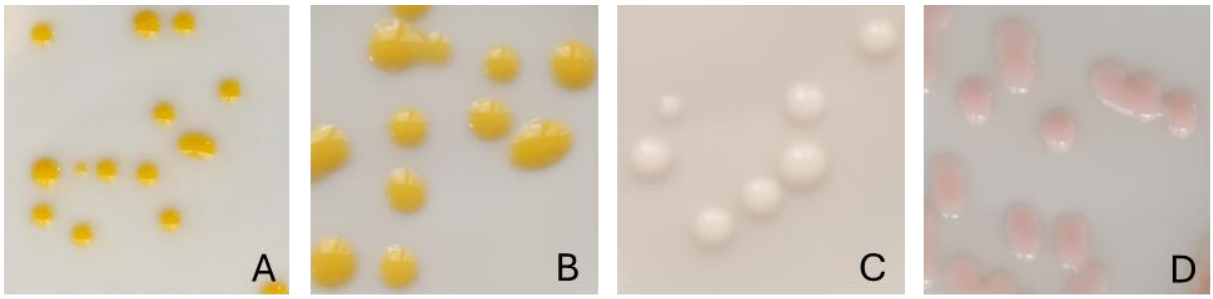

Figure II.2. Colony morphology of four Cm strains (IPO 500: A; IPO 542: B; ZTO 735: C; CFBP 9078: D) after 3 days of incubation at 28 °C on YDC.

4.4. If no suspects remain, check if all the suspect colonies on semi-selective media have been tested on YDC (see Section 3.4). If not, start testing all suspects. If all suspects have been tested negative on YDC, the subsample is tested negative for Cm.

4.5. Identify all isolates with Cm morphology on YDC directly in the pathogenicity assay (Section IV), or by a colony identification pre-screening using qPCR (Section III) followed by the pathogenicity assay (Section IV).

III. SUSPECT COLONY IDENTIFICATION BY qPCR (OPTIONAL STEP)

For PCR methods, in-house method optimization is often necessary, see Best Practices for PCR Assays in Seed Health Tests for information.

Materials

- Sterile distilled/deionised water
- Optical Density (OD) meter
- Controls (Table III.1)
- Centrifuge
- qPCR mix, primers (Table III.2) and equipment
- Thermo block (95–100 °C)
- Lab disposables

Table III.1. Types of controls used.

Control type	Description
Positive process control (PPC)	Freshly prepared suspension of Cm
Negative process control (NPC)	Freshly prepared suspension of non-target colony
Non template control (NTC)	Nucleic acid-free water
Internal amplification control (IAC)	Universal bacterial primers (e.g., Wu *et al.*, 2008)

Table III.2. Primer-sequences and references.

Name	Target	Sequence (5' – 3')	Source
MVS21-F[a]	Cm	CTA GTT GCT GAA TCC ACC CAG	Sudarshana *et al.*, 2012
MVS21-R[a]		TAC CGC TTG ACT CTC GTT TC	
MVS21-Pr[a]		FAM – CTG CCA CCC GAT GTT GTT GTT CC – TAMRA	
PTSSK-F		CGT CGC CCG CCC GCT G	Berendsen *et al.*, 2011
PTSSK-R		GGG GCC GAA GGT GCT GGT G	
PTSSK-Pr		FAM – TGG TCG TCC TCG GCG – MGB – NFQ	
Wu-F	Bacterial 16S rRNA	CAA CGC GAA GAA CCT TAC C	Wu *et al.*, 2008
Wu-R		ACG TCA TCC CCA CCT TCC	
Wu-Pr1		VIC – ACG ACA ACC ATG CAC CAC CTG – QSY	
Wu-Pr2		VIC – ACG ACA GCC ATG CAG CAC CT – QSY	

[a] The original MVS21 qPCR assay by Sudarshana *et al.* (2012) is used for colony confirmation, while the modified MVS21^{+} assay is used in the SE-qPCR assay (Table I.4). SE-qPCR assays are run with a different PCR program compared to the program used for colony confirmation (Tables I.5 and III.5, respectively). The MVS21^{+} assay was not validated for colony confirmation, hence the assays used in SE-qPCR and colony confirmation are different.

1. Sample preparation

The template DNA from single colonies for qPCR can be obtained by several means. The method presented here was used for the validation of this protocol.

1.1. Make a slightly turbid cell suspension (OD_{600}~ 0.05) in sterile distilled/deionised water from the suspected cultures on YDC medium and the positive process control (PPC, Table III.1). In addition, a non-suspect isolate should be used as a negative process control (NPC).

1.2. Incubate for 10 min at 95-100 °C. Alternatively, cell suspensions can be subjected to a validated DNA extraction method. It is the responsibility of the user to validate any alternative method relative to the reference method given.

1.3. Suspensions can be stored at -20 °C until further use.

2. qPCR

2.1. Two qPCRs are performed for each suspect isolate, the MVS21 qPCR (Sudarshana *et al.* 2012) and PTSSK qPCR (Berendsen *et al.* 2011). The two qPCR assays may be combined in a single multiplex qPCR-reaction; to distinguish between the results of the two qPCR-reactions, different probe fluorophores should be used in the reaction.

2.2. Use internal amplification control (IAC) primers, such as the universal bacterial primers from Wu *et al.* (2008) (Table III.2), to validate the qPCR reaction.

2.3. Include a non template control (NTC) (see Table III.1).

2.4. Prepare the reaction mixture (Table III.3 and III.4 for duplex reaction).

Note: Ensure that the template DNA is at room temperature when added to the mix to prevent temperature linked chemical reactions prior to PCR.

2.5. Perform the PCR reaction in a real-time PCR instrument. An example of the PCR conditions is indicated in Table III.5.

Note: If different PCR mixtures and amplification programs are used, it is necessary to verify their performance.

Table III.3. Example of a PCR mix for MVS21/Wu duplex qPCR.

Component	Per reaction (in µL)	Final concentration
MVS21-F (100 µM)	0.125	0.50 µM
MVS21-R (100 µM)	0.125	0.50 µM
MVS21-Pr (100 µM)	0.0625	0.25 µM
Wu-F (100 µM)	0.05	0.20 µM
Wu-R (100 µM)	0.05	0.20 µM
Wu-P1 (100 µM)	0.05	0.20 µM
Wu-P2 (100 µM)	0.05	0.20 µM
qPCR master mix (2×)	12.50	1×
PCR grade water	6.9875	
Bacterial suspension	5.00	
Total	**25.00**	

Table III.4. Example of a PCR mix for PTSSK/Wu duplex qPCR.

Component	Per reaction (in µL)	Final concentration
PTSSK-F (100 µM)	0.125	0.50 µM
PTSSK-R (100 µM)	0.125	0.50 µM
PTSSK-Pr (100 µM)	0.0625	0.25 µM
Wu-F (100 µM)	0.05	0.20 µM
Wu-R (100 µM)	0.05	0.20 µM
Wu-P1 (100 µM)	0.05	0.20 µM
Wu-P2 (100 µM)	0.05	0.20 µM
qPCR master mix (2×)	12.50	1×
PCR grade water	6.9875	
Bacterial suspension	5.0	
Total	**25.0**	

Table III.5. qPCR conditions.

Step	Temperature	Duration
hold	95 °C	5 min
40 cycles	95 °C	15 sec
	60 °C	30 sec

3. Interpretation and decisions

Cut-off values must be established by each laboratory for their positive control and internal amplification control prior to the assay being used on routine samples. For recommendations on setting cut-off values, see Real-time PCR pre-screening in seed health methods.

The Cq values of positive controls should consistently be lower than 30. The cut-off Cq value of the internal amplification control (IAC) should be below 35, and the expected range is to be determined by the user based on experimental data.

In the case of universal bacterial primers, positive reactions may occur in non template controls (NTC) due to the presence of residual DNA in Taq enzyme reagents. The IAC Cq values from reactions on suspect isolates should at least be 3.3 Cq value lower than the IAC Cq values from the NTC reactions.

For interpretation and decision making, the results from both primer sets need to be taken into account (see Table III.6). Test results are only valid when all included controls presented in Table III.1 give the expected results.

Table III.6. Interpretation and decision table for the qPCR.

MVS21 qPCR	PTSSK qPCR	qPCR Result	Follow-up
Positive	Positive	Target DNA for Cm detected	Pathogenicity test for confirmation
Negative	Positive	Inconclusive	Pathogenicity test for confirmation
Positive	Negative	Inconclusive	Pathogenicity test for confirmation
Negative	Negative	No target DNA for Cm detected	Negative, no follow up

IV. CONFIRMATION BY PATHOGENICITY ASSAY

Materials

- Tomato cultivar: susceptible to the pathogen (e.g., Moneymaker)
- Sterile toothpicks
- Controls (Table IV.1)
- Lab disposables

Table IV.1 Types of controls used.

Control type	Description
Positive control (PC)	A reference Cm strain
Negative process control (NPC)	A non-target strain
Negative buffer control (NBC)	Sterile toothpick

1. Pathogenicity assay

1.1. Grow seedlings of a known susceptible cultivar (e.g., Moneymaker) under suitable conditions until 2–3 true leaves have developed (about 3–4 weeks after sowing).

1.2. Dip a sterile toothpick directly in a fresh suspect colony on YDC medium and inoculate at least two tomato seedlings by stabbing the toothpick into the stem between the cotyledons and the first true leaf. Use the sharp (rather than blunt) end of the toothpick to avoid excessive damage while introducing inoculum.

1.3. Include at least one reference Cm strain as a positive control (PC), a non-target strain as negative process control (NPC) and stab with a sterile toothpick as a negative buffer control (NBC).

1.4. Incubate the inoculated plants at 25–32 °C with a minimum of 8 hours daylight.

1.5. Observe the plants for symptoms starting one week after inoculation. Final observation is three weeks after inoculation. Compare with the PC and NPC.

Note: Test results are only valid when all included controls presented in Table IV.1 give the expected results. The PC should give typical symptoms in the pathogenicity test. Typical symptoms caused by Cm are canker formation at the site of inoculation, yellowing and marginal necrosis, and/or wilting of true leaves. The NPC and NBC should give no symptoms.

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