Method for the Detection of *Clavibacter michiganensis* subsp. *michiganensis* on Tomato seed

Crop: Tomato (*Solanum lycopersicum* ex. *Lycopersicon esculentum* L.)
Pathogen: *Clavibacter michiganensis* subsp. *michiganensis*
Revision history: Version 4.3.1, July 2017

Sample and sub-sample size

The recommended minimum sample size is 10,000 seeds with a maximum sub-sample size of 10,000 seeds.

Principle

- Extraction from the seed of externally and internally located bacteria
- Isolation of viable *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) bacteria by dilution plating seed extract on two different semi-selective media
- Confirmation of suspect bacterial colonies by morphology on a non-selective medium, followed by a PCR and by a pathogenicity assay

Restrictions on use

- This test method is suitable for untreated seed
- This test method is suitable for seed that has been treated using physical or chemical (acid extraction, 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 such inhibition by analysis, sample spiking, or experimental comparisons
- The ability to recover Cmm 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 Cmm-colony forming units (CFUs). See test method description for details on spiking (see sections 2.6 to 2.11).
- This test method has not been validated for seed treated with protective chemicals or biological substances. If a lab chooses to test treated seed using this method, it is the lab's responsibility to determine empirically (through analysis, sample spiking, or experimental comparisons) whether the protective chemicals or biological substances have an effect on the method results.

Method execution

Following the best practices described by ISHI-Veg for the reliable use of molecular techniques and dilution-plating assays in seed health testing (see http://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg/) is strongly recommended to ensure process standardization and valid results.

Note: The section **Validation** has been updated.

Validation

This method has been peer reviewed by ISHI-Veg members and experts outside ISHI-Veg. It is in use by GSPP (www.gspp.eu).

This method has also been approved by the US National Seed Health System (NSHS) as a Standard A (see http://seedhealth.org/seed-health-testing-methods/).

Method description

1. Extraction of bacteria from the seed

1.1 Put sub-samples in sterile stomacher bags. Add sterile seed extraction buffer to each bag at a ratio of 4 ml of seed extract buffer to 1 g of seed (v:w). Incubate overnight (minimum 14 hours) at 4°C, and macerate for at least 4 min in a stomacher machine until the extraction buffer becomes milky and white particles become visible as a result of endosperm release (see Figure 1).

Figure 1. The seed extraction buffer and seeds after stomaching

2. Isolation on semi-selective media

2.1. Filter coarse particles from the required volume of extract using a filter bag (e.g. Bagfilter® or Bagpage® from Interscience (France) or an extraction bag with synthetic intermediate layer from Bioreba (Switzerland) or Neogen Europe (Scotland)). Alternately, use a stomacher bag with filter.

2.2. Filter the extract by a low speed centrifugation for 1 min at 1,000 g. This removes fungal spores and facilitates re-suspension of the pellets. Remove the supernatant carefully and use it for dilution plating.

2.3. Prepare a 10-fold dilution of the seed extract in sterile seed extraction buffer. For sub-samples larger than 2,000 seeds prepare a 10-fold concentrated extract also. Centrifuge the filtered extract with a time and speed sufficient to pellet bacteria (e.g. for 5 minutes at 5,000 g). Remove the supernatant carefully and re-suspend the pellet in 1/10 of the original centrifuged volume of sterile seed extraction buffer.

2.4. Plate 0.1 ml of the concentrated (if applicable), the undiluted and diluted extracts on two semi-selective media CMM1T and, either SCM or SCMF.

2.5. Prepare a 10-fold dilution series of a suspension of a pure culture of a known Cmm reference strain in sterile seed extraction buffer. Plate 0.1 ml 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 2.9).

2.6. As a check on the ability to recover Cmm, spike Cmm into (a separate aliquot of) the most concentrated extract of each subsample at a level of 20 – 100 CFU per 0.1 ml. Plate 0.1 ml of these spiked extracts on the two semi-selective media. Use similarly spiked seed extraction buffer as a control.

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

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

2.8. Check recovery and morphology of the Cmm reference strain on both media.

2.9. Examine the sample plates for the presence of colonies with typical Cmm morphology by comparing them with the reference Cmm strain. Record the number of suspected colonies as well as other colonies.

- After 10 days of incubation on CMM1T, Cmm colonies are yellow, mucoid and convex (see Figure 2).
- After 10 days of incubation on SCM, Cmm colonies are translucent grey, mucoid, eventually often irregularly shaped, with internal black flecks (see Figure 3).
- After 10 days of incubation on SCMF, Cmm colonies are mucoid and translucent grey, often irregularly shaped. The center of the colony can be grey to grey-black depending on the strain (see Figure 4).
- The colony size and color can differ within and between samples. It is particularly influenced by other colonies growing nearby. Isolates may vary in the speed at which they grow.

2.10. From sample plates, select, if present, at least 6 suspect colonies per medium per sub-sample for further identification on YDC medium.

2.11. Examine the plates of the spiked extracts of each subsample for the presence of colonies with typical Cmm morphology. Spiking is valid when at least 5 colonies are present on any one medium. A negative sub-sample test result can only be concluded if the spiking of the same sub-sample is valid. In the case that no Cmm is identified and the spiking is invalid, test a new subsample after (further) treatment/disinfestations of the seed lot.

3. Identification by morphology on YDC medium

3.1. Transfer selected suspect colonies as well as the reference strains on the YDC medium. Incubate YDC plates at 26 - 28°C for 2-3 days.

3.2. Determine whether transferred colonies have typical Cmm morphology by comparing them with the reference strains and record which of the isolates are still suspected to be Cmm.

- On YDC Cmm is yellow, domed (convex) and mucoid in appearance.

3.3. Identify all suspect isolates directly by the pathogenicity assay (section 5) or by a pre-screening using PCR (section 4) followed by the pathogenicity assay.

4. Identification by PCR

4.1. Prepare a slightly turbid cell suspension (with an OD_{600nm} of approximately 0.05) in sterile distilled water from each suspect colony on YDC medium and the positive control. Cultures should not be older than 5 days after plating. In addition, use as controls: a non-suspect isolate as a negative control, a positive amplification control and a positive process control. Heat the suspensions for 10 min at 95–100°C. The suspensions can be stored at -20°C until the PCR analysis. Two PCRs (4.2 and 4.3) are performed for each suspect isolate. The two Taqman assays may be combined

in a single multiplex PCR-reaction; to distinguish between the results of the two PCR-reactions, different probe fluorophores should be used in the reaction.

4.2. **MVS21 Taqman® PCR**

4.2.1. Use the following specific primers (6). The probe should be used with the nucleic acid sequence exactly as indicated below; the probe fluorophore and quencher, however, may be modified.

MVS21 Forward: 5′-CTAgTTgCTgAATCCACCCAg-3′

MVS21 Reverse: 5′-TACCgCTTgACTCTCgTTTC-3′

MVS21 Probe: 5′-FAM-CTgCCACCCgATgTTgTTgTTCC-TAMRA-3′

4.2.2. Use Internal Amplification Control (IAC) primers, such as the following universal bacterial primers (adapted from 8), to validate the PCR reaction

Wu Forward: 5′- CAACgCgAAgAACCTTACC-3′

Wu Reverse: 5′- ACgTCATCCCCACCTTCC-3′

Wu Probe 1: 5′-VIC- ACgACAACCATgCACCACCTg-QSY-3′

Wu Probe 2: 5′-VIC- ACgACAgCCATgCAgCACCT-QSY-3′

4.2.3. Carry out PCR reactions in a real-time PCR instrument. An example of reagent compositions and reaction conditions is described in Appendix 1A.

4.2.4. Determine Ct values; Ct values of positive controls should consistently be lower than 30. The cut-off Ct value of the internal amplification control 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 Ct values from reactions on suspect isolates should indicate at least a 10-fold higher concentration of bacterial DNA than the IAC Ct values from the NTC reactions; the difference between Ct values should be more than 3.3.

4.3. **PTSSK Taqman® PCR**

4.3.1. Use the following specific primers (2). The probe should be used with the nucleic acid sequence and the MGB non-fluorescent quencher exactly as indicated below. The probe fluorophore, however, may be modified.

Primer RZ_ptssk 10: 5′ ggg gCC gAA ggT gCT ggT g 3′

Primer RZ_ptssk 11: 5′ CgT CgC CCg CCC gCT g 3′

Probe RZ_ptssk 12: 5′ FAM-Tgg TCg TCC TCg gCg-MGB-NFQ 3′

4.3.2. Use Internal Amplification Control (IAC) primers, such as the following universal bacterial primers (adapted from 8), to validate the PCR reaction

Wu Forward: 5′- CAACgCgAAgAACCTTACC-3′

Wu Reverse: 5′- ACgTCATCCCCACCTTCC-3′

Wu Probe 1: 5′-VIC- ACgACAACCATgCACCACCTg-QSY-3′

Wu Probe 2: 5′-VIC- ACgACAgCCATgCAgCACCT-QSY-3′

4.3.3. Carry out PCR reactions in a real-time PCR instrument. An example of reagent compositions and reaction conditions is described in Appendix 1B.

4.3.4. Determine Ct values; Ct values of positive controls should consistently be lower than 30. The cut-off Ct value of the internal amplification control 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 Ct values from reactions on suspect isolates should indicate at least a 10-fold higher concentration of bacterial DNA than the IAC Ct values from the NTC reactions; the difference between Ct values should be more than 3.3.

4.4. Combine the results of both PCRs (sections 4.2 and 4.3). If both PCRs result in a negative reaction, and the internal amplification controls are positive in both reactions, the suspect colony is considered not to be Cmm. If one or both PCRs result in a positive reaction, the pathogenicity assay should be performed to reach a final conclusion about the identity of the suspect colony. Possible PCR outcomes and the conclusions are summarized in the table below.

Table 1: Interpretation and decision table

MVS21 Taqman	RZ-PTSSK Taqman	qPCR Result	Follow-up
Positive	Positive	Expected result for Cmm	Pathogenicity test needed for confirmation
Negative	Positive	Inconclusive	Pathogenicity test needed for confirmation
Positive	Negative	Inconclusive	Pathogenicity test needed for confirmation
Negative	Negative	Expected result for non-Cmm	Negative PCR-result, isolate is not Cmm

5. Identification by pathogenicity assay

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

5.2. Dip a sterile toothpick directly in a fresh suspect colony on YDC medium and inoculate 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. Include a reference Cmm strain as a positive control and do a mock inoculation with a clean toothpick as a negative control.

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

5.4. Observe the plants for symptoms starting 1 week after inoculation. Final observation is 3 weeks after inoculation. Compare with the positive and negative control.

- Typical symptoms caused by Cmm are canker formation at the site of inoculation, yellowing and marginal necrosis, and/or wilting of true leaves.

Buffers and media

- Use de-ionized water.
- Autoclave buffers and media at 121°C, 15 psi for 15 minutes.
- Antibiotics are not stable in time. Therefore, add antibiotics to the media at a relatively low temperature (< 50°C). Use plates within a month to maintain the selectivity of the media.

Seed extraction buffer (pH 7.4) per liter

Compound	Amount per liter
Na_2HPO_4	7.75 g
KH_2PO_4	1.65 g
Tween 20	0.2 ml
$Na_2S_2O_3$ [1]	0.5 g

[1] Recommended when seeds have been treated with hypochlorite

CMM1T (pH 7.7) per liter (1, 4)

Compound	Amount per liter	Stock solution
Sucrose	10.0 g	
Trizma base (Tris base)*	3.32 g	
TrisHCl*	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[1]	10 mg	10 mg/ml in distilled water
Nalidixic acid (salt)[1]	28 mg	sodium salt, 10 mg/ml in 0.1 M NaOH
Nystatin[1]	100 mg	100 mg/ml in 50% DMSO/50% ethanol

[1] Add after autoclaving

* The pH should be set by using both Tris base and TrisHCl 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.

SCM (pH 7.3*) per liter (3)

Compound	Amount per liter	Stock solution
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 g	
Potassium tellurite[1]	10 mg[2]	1 ml of 1% solution
Nicotinic acid[1]	100 mg	20 mg/ml in distilled water
Nalidixic acid (salt)[1]	30 mg	sodium salt, 10 mg/ml in 0.1 M NaOH
Nystatin[1]	100 mg	100 mg/ml in 50% DMSO and 50% ethanol

* Recommended pH before autoclaving

[1] Add after autoclaving

[2] 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 Cmm in the presence of potassium tellurite.

SCMF (pH 7.3*) per liter (3, 4)

Compound	Amount per liter	Stock solution
Sucrose	10.0 g	
K_2HPO_4	2.0 g	
KH_2PO_4	0.5 g	
H_3BO_3 (boric acid)	1.5 g	
Yeast extract	2.0 g	
$MgSO_4.7H_2O$	0.25 g	
Agar	18 g	
Potassium tellurite[1]	10 mg[2]	1 ml of 1% solution
Trimethoprim[1]	80 mg	10 mg/ml in 100% methanol
Nicotinic acid[1]	100 mg	20 mg/ml in distilled water
Nalidixic acid (salt)[1]	20 mg	sodium salt, 10 mg/ml in 0.1 M NaOH
Nystatin[1]	100 mg	100 mg/ml in 50% DMSO and 50% ethanol

* Recommended pH before autoclaving

[1] Add after autoclaving

[2] 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 Cmm in the presence of potassium tellurite.

YDC (Yeast extract - dextrose - $CaCO_3$ Agar) per liter (5, 7)

Compound	Amount per liter
Yeast extract	10.0 g
D-glucose (dextrose)	20.0 g
$CaCO_3$	20.0 g
Agar	15.0 g

References

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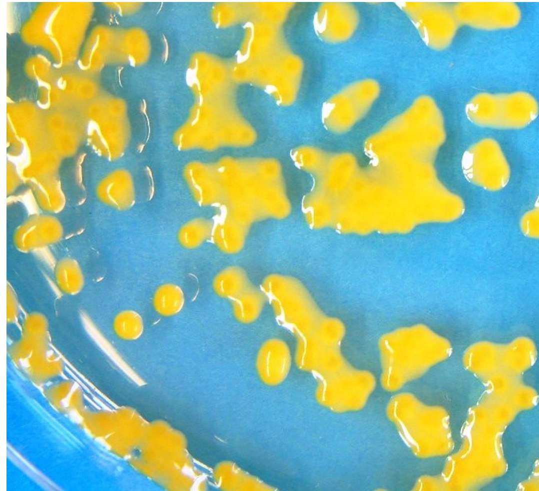

Figure 2. Cmm morphology after 10 days of incubation on CMM1T

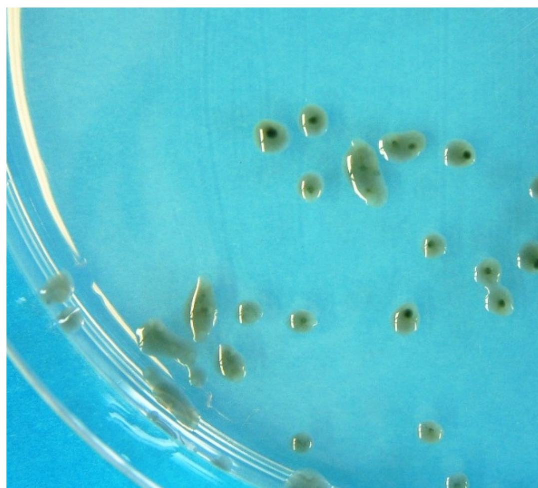

Figure 3. Cmm morphology after 10 days of incubation on SCM

Figure 4. Cmm morphology after 10 days of incubation on SCMF

Appendix 1

A. Example of a Reaction Mixture for MVS21 TaqMan® PCR (4.2.3)

Compound	Final concentration	Volume (µl) in 25 µl
Sterile Milli Q water		6.9875
MVS21 Forward (100 pmol/µl)	0.50 µM	0.125
MVS21 Reverse (100 pmol/µl)	0.50 µM	0.125
MVS21 Probe (100 pmol/µl)	0.25 µM	0.0625
TaqMan® master mix (2x)		12.50
Wu-Forward (100 pmol/µl)	0.20 µM	0.05
Wu-Reverse(100 pmol/µl)	0.20 µM	0.05
Wu-Probe 1 (100 pmol/µl)	0.20 µM	0.05
Wu-Probe 2 (100 pmol/µl)	0.20 µM	0.05
Bacterial suspension		5.00

PCR conditions used during method validation: 5 min incubation at 95°C followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. Ramp speed at 5°C/s.

B. Preparation of Reaction Mixture for PTSSK TaqMan® PCR (4.3.3)

Compound	Final concentration	Volume (µl) in 25 µl
Sterile Milli Q water		6.9875
PTSSK Forward (100 pmol/µl)	0.50 µM	0.125
PTSSK Reverse (100 pmol/µl)	0.50 µM	0.125
PTSSK Probe (100 pmol/µl)	0.25 µM	0.0625
TaqMan® master mix (2x)		12.50
Wu-Forward (100 pmol/µl)	0.20 µM	0.05
Wu-Reverse(100 pmol/µl)	0.20 µM	0.05
Wu-Probe 1(100 pmol/µl)	0.20 µM	0.05
Wu-Probe 2(100 pmol/µl)	0.20 µM	0.05
Bacterial suspension		5.00

PCR conditions used during method validation: 5 min incubation at 95°C followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. Ramp speed at 5°C/s.

The PTSSK PCR has been validated on a strain collection using an annealing temperature of 60°C, a temperature that is 8-10 C below the maximum annealing temp (2).