I-13-688

Annex 14.2 GSPP Diagnostic protocol for *Clavibacter michiganensis* subsp. *michiganensis* in symptomatic tomato plants

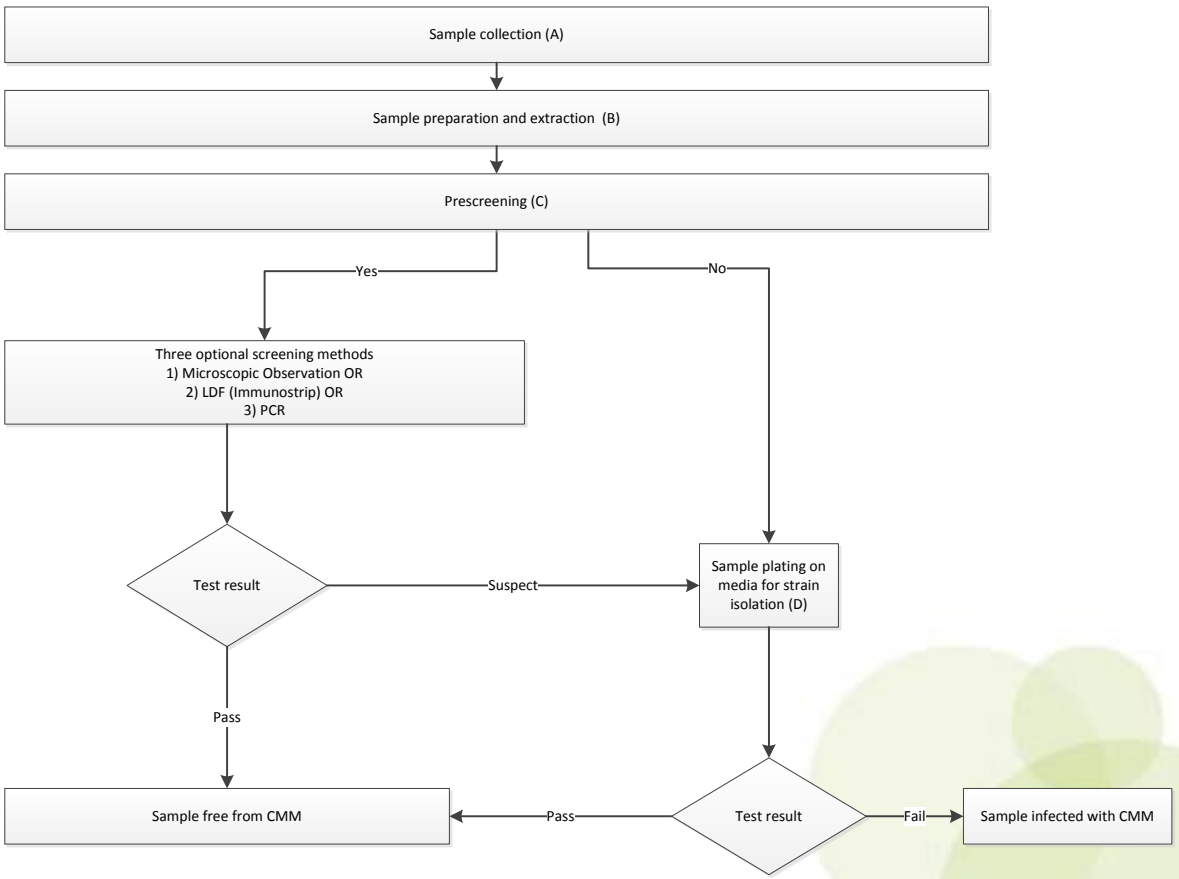
The goal of this document is to describe in detail how symptomatic Cmm suspected plant material can be tested for the presence of Cmm as being the causal agent of the observed disease.

This document describes the procedures sample collection, pre-screening, isolation and confirmation of *Clavibacter michiganensis* subsp. *michiganensis* of symptomatic plant material. For GSPP-participants this protocol is obligatory to apply to confirm Cmm in suspected plant material. In case of filing complaints by non-GSPP participants, this protocol is strongly recommended to be applied for diagnosis. At all times, it should be registered what method has been used for diagnosis of Cmm to determine the reliability of the diagnosis. Conclusions on suspected presence of Cmm can be drawn at many process steps described in this protocol. However, only after meeting the final requirements of a positive pathogenicity assay, the confirmation of Cmm detected is accomplished, as is intended in Koch's postulates. For all other confirmation or detection steps, this final conclusion cannot be made with (sufficient) certainty. This is of particular importance when Quick sticks are used, which application is known to result in false-positive test results occasionally, due to cross reactivity with other micro-organisms.

Abbreviations:

CCP - Critical Control Point, is a described process step which cannot be deviated from.
Cmm - *Clavibacter michiganensis* subsp. *michiganensis*
IF - Immuno fluorescence
PCR - Polymerase Chain Reaction
ISHI - International Seed Health Initiative
EtOH - Ethanol
NaCl - Sodium chloride

Figure 1: Flow chart for diagnosis and isolation of Cmm out of suspected plant material.



A. Sample collection procedure

1. The person who takes the samples must take care of the right hygienic conditions during the sampling process.
   1.1. He or she must use the required protective clothes, gloves, footwear and disinfectants (e.g. 70 % EtOH, 1% household bleach) to prevent accidental spread of the pathogen(s).
2. Record the relevant information on the GSPP-submission form for Cmm-suspected plants (see appendix 1).
   2.1. Take a picture of the suspected plant(s) if possible.
   2.2. Mark the sampling location (spot) to be able to find the sampled plant or its former location back, when additional observations are necessary.
3. Because Cmm is often not traceable in all plant parts, a complete plant must be collected and sent to a laboratory that is approved for Cmm diagnosis by the GSPP-foundation for Cmm diagnosis.
   3.1. If the plant is very big/long, then the leaves and fruits can be removed from the stem.
   3.2. The stem can be folded or cut into sections for packing and shipment. Then include a few symptomatic leaves and/or fruits for diagnosis separately.
   3.3. Avoid contamination of plant material with potting soil or substrate as this may make the diagnosis more difficult.
   3.4. When necessary, pack the samples in enough absorbing paper to avoid dehydration or rotting, as dehydration makes diagnosis more difficult.
4. Pack the sampled plant immediately on the spot of collection in a closed plastic bag to avoid contamination of other plants with the pathogen.
5. Mark the sample bag (and put a label inside the sample bag too) to have a unique relation with the submission/registration form.
6. Disinfect all the used materials and the outside of the sample bag with an appropriate disinfection (e.g. 70% EtOH or 1% household bleach) at the place of sampling.
7. Dispose the hand gloves and the protective clothing in a closed plastic bag in a waste bin before leaving the entity.
8. Send the sample to an approved laboratory as soon as possible to avoid deterioration of the plant.
   8.1. When the sample cannot be transported immediately, then keep it refrigerated or at least out of direct sunlight.
   8.2. Store the samples as short as possible in the closed plastic bag. Longer storage decreases the likelihood of Cmm extraction. (note: acceptable duration differs between samples, it is up to the Phytopathologist to decide if the sample is still in acceptable condition for Cmm isolation).
9. At all circumstances the procedures for packing, transport and shipment of Cmm suspect plant material of the local authorities and the authorities of the country where the sample is sent (if different) should be followed (**CCP**).

B. Sample preparation and extraction

1. A list of procedures for the isolation from Cmm suspected tomato plants in the laboratory is described below.
2. It is important to note that Cmm might not be present in all parts of the stem or plant at all times.
3. Selection of vascular discoloured stem tissue for extraction of Cmm increase the probability of recovery.
4. Extractions from the lower portion of the stem, just above the soil line, or at the graft, will have the highest probability of recovery of Cmm.
5. When diagnosing multiple plants, sterilize cutting tools or use a new cutting tool for each plant. This will assure that cross contamination between plants will not occur and isolate integrity can be maintained for each plant **(CCP).**
6. From each individual plant, test at least 8 plant pieces, as indicated above, or until Cmm was detected.
   6.1. Extraction from stem tissue:
      6.1.1. Select a piece of stem tissue closely to a canker or the soil base.
      6.1.2. Cut a small section from the stem
      6.1.3. Cut stem tissue diagonally using a sterile knife, razor blade, scalpel or equivalent.
   6.2. Extraction from wilted leaf tissue:
      6.2.1. Take the entire leaf from the stem.

6.2.2. Cut the petiole lengthways from the base of the petiole of the leaf tip, including the vascular tissue.

6.3. Extraction from fruits:

6.3.1. Select a fruit with 'birds eye' symptoms.

6.3.2. Cut out the birds eye or take a piece of the center core of the fruit

C. Pre-screening (optional)

The pre-screening sections 1, 2 and 3 are optional and exchangeable, it is not necessary to carry out all sections as a pre-screen.

Pre-screening steps 2 and 3 can be carried out in the field, and are not necessarily performed in a laboratory.

1. Pre-screening with Microscopically observation (Optional):

1.1. Observe vascular stem or petiole tissue for discoloration.

1.2. When discoloration is present, process the sample as described below, to observe bacterial streaming out of the vascular tissue.

1.3. Cut a very thin section horizontally from (discoloured) vascular tissue, using a sterile razor blade or scalpel to prepare a "wet mount" microscope slide.

1.3.1. Thin slices are easier to use for the detection of bacterial streaming, due to a reduced focus field of thicker pieces.

1.3.2. There is no need to use a larger surface area of the sample.

1.3.3. A 100x magnification is sufficient for the detection of bacterial streaming in a dark field.

1.4. Observe the tissue under a compound microscope for bacterial streaming.

1.5. When no bacterial streaming is observed, proceed with extraction of Cmm from vascular tissue or repeat this step with another tissue sample.

1.6. When bacterial streaming is observed, proceed with extraction of Cmm from vascular tissue.

Picture 1; Bacterial streaming of Cmm from plant tissue visible in microscope

2. Pre-screening with LDF (Optional, e.g. Pocket Diagnostics, Agdia)

2.1. It is recommended to surface-disinfect selected plant tissue for isolation (e.g. 1% household bleach or 70% EtOH) for maximally 60s

2.2. After a bleach surface sterilisation, thoroughly rinse tissue with sterile water (3 times) to remove residual disinfectant (**CCP**).

2.2.1. Note: Residual household bleach or EtOH will reduce the recovery of Cmm.

2.2.2. Note: Bacteria can be isolated from tissue without surface sterilization. However this may lead to higher levels of saprophytic bacteria, which can make isolation of Cmm more difficult.

2.3. Dry tissue on paper towel.

2.4. Cut a cross-section out of the disinfected vascular tissue.

2.5. Perform sample preparation according manufactures instructions

2.6. In case of a PASS, the sample is regarded Cmm-free

2.7. In case of a FAIL, the sample is regarded Cmm-suspected, continue with the isolation procedure for confirmation (D).

2.8. Note that the buffers from the pre-screen kits contain preservatives that instantly kill microorganisms and therefor these extracts cannot be used for media-isolation.(**CCP**)

3. Pre-screening with PCR (Optional);

3.1. It is recommended to surface-disinfect selected plant tissue for isolation (e.g. 1% household bleach or 70% EtOH) for maximally 60s

3.2. After bleach soak, thoroughly rinse tissue with sterile water (3 times) to remove residual disinfectant. (**CCP**).

3.2.1. Note: Residual household bleach or EtOH will reduce the recovery of Cmm.

3.2.2. Note: Bacteria can be isolated from tissue without surface sterilization. However this may lead to higher levels of saprophytic bacteria, which can make isolation of Cmm more difficult.

3.3. Dry tissue on paper towel.

3.4. Cut a cross-section out of the disinfected vascular tissue.

3.5. Add approximately 0.5 ml sterile 0.85% NaCl (see ref.), sterile water or DNA- extraction buffer in a sterile Petri dish or 2 ml-tube.

3.6. Macerate (**CCP**) suspected tissue using a sterile scalpel or pestle.

3.7. Cook the macerate for 10 min and put at 4ºC immediately, or, extract DNA according to manufacturer's instructions when a commercial DNA extraction kit is used.

3.8. Centrifuge the extract before the PCR

3.9. Perform PCR as described in the most recent ISHI-protocol for colony confirmation.

3.9.1. In case of a PASS, the sample is regarded Cmm-free

3.9.2. In case of a FAIL, the sample is regarded Cmm-suspected, continue with the isolation procedure for confirmation.

D. Sample plating on media for strain isolation.

1. It is recommended to surface-disinfect selected plant tissue for isolation (e.g. 1% household bleach or 70% EtOH) for maximally 60s After bleach soak, thoroughly rinse tissue with sterile water (3 times) to remove residual disinfectant. (**CCP**).

1.1. Note: Residual household bleach or EtOH will reduce the recovery of Cmm.

1.2. Note: Bacteria can be isolated from tissue without surface sterilization. However this may lead to higher levels of saprophytic bacteria, which can make isolation of Cmm more difficult.

2. Dry tissue on paper towel.
3. Cut a cross-section out of the disinfected vascular tissue.
4. Add approximately 0.5 ml sterile 0.85% NaCl (see ref.) or sterile water in a sterile Petri dish or 2 ml-tube.
5. Macerate (**CCP**) suspected tissue using a sterile scalpel, pestle or hand homogenizer.
6. Dilute the extract 10x and 1000x in sterile 0.85% NaCl (see ref.) or sterile water.
7. Use these dilutions for plating on media, For isolation of Cmm from diseased vascular tissue, symptomatic leaves or fruits, it is recommended to use semi-selective media although general media can be used. Appropriate and validated semi-selective media are described in detail in the ISHI method description (see reference).
8. The ISHI method is developed and validated for seed testing and you will find the description of the preparation of extracts from seeds. The seed extract preparation is different from preparation of extracts from plant material and cannot be used here. However the dilution plating and identification of Cmm extracted from plant and seed extracts is identical.
9. Proceed from here using the same protocol as in use for isolation of Cmm from tomato seeds as published by ISHI in the "Manual for seed health testing methods" on the ISF website.
10. There you find the description of remaining steps for the identification of Cmm.
11. These remaining steps define recommended semi selective media, identification on YDC medium, PCR and the pathogenicity assay.
12. Perform PCR as described in the most recent ISHI-protocol for colony confirmation.
    12.1. In case of a PASS, the sample is regarded Cmm-free
    12.2. In case of a FAIL, the sample is regarded Cmm-contaminated.
13. **A suspected plant is defined as contaminated with *Clavibacter michiganensis* subsp. *michiganensis* if the final part(s) of the test, PCR and/or the pathogenicity assay are positive**.

References

Norms OEPP. EPPO standards. Diagnostics PM 7/42. *Clavibacter michiganensis* subsp.*michiganensis* (EPPO website)

ISF (International Seed Federation), Method for the Detection of *Clavibacter michiganensis* subsp. *michiganensis* on Tomato seed. **(latest version of the ISHI-protocol at the time of testing)** Website: http://www.worldseed.org/isf/ishi_vegetable.html

Appendix 1. Example of an identification form; GSSP submission form for suspected plants.

Crop: Species .....

Cultivar .....

Location .....

Sample: ID number: ..... Sample description: .....

Infection: Cultivated area / number of plants .....

Loss (% / number) .....

Symptoms	**Affected parts**	**Soil / Subst.**	**Distribution disease**	start disease? _____
wilting ☐	stems ☐	soil ☐	entire cultivation ☐	increase of disease? _____
yellowing ☐	roots ☐	loam ☐	edge of cultivation ☐	country of origin _____
galls ☐	leafs ☐	clay ☐	random ☐	age of plants? _____
dieback ☐	flowers ☐	peat ☐	high patches ☐	preceding crop? 1 _____
rot ☐	Seeds / fruits ☐	sand ☐	low patches ☐	2 _____
marginal burns ☐		_____ ☐	wet patches ☐	3 _____
leaf / needle drop ☐	**Irrigation**		dry patches ☐	
leaf spots ☐	sprinkler ☐	substrate	sunny patches ☐	
streak ☐	flooding ☐	rock wool ☐	shadow patches ☐	outdoor / field ☐
mosaic ☐	drip ☐	potting soil ☐	specific cultivar ☐	greenhouse ☐
blight ☐	recirculation yes/no*	_____ ☐	greenhouse bench ☐	pot cultivation ☐
_____ ☐	disinfection yes/no*		_____ ☐	_____ ☐

Chemicals / Fertilizers:

Describe nature problem:

Sample(s) taken by:

Sent by:

Sent to:

Date of sample collection:

Digital photographs made? **yes / no**