Method for the Detection of *Xanthomonas* spp. on Pepper Seed

Crop: Pepper (*Capsicum annuum*)

Pathogen: *Xanthomonas euvesicatoria, Xanthomonas vesicatoria and Xanthomonas gardneri*

See (1) and (2) for an account of the re-classification of the *Xanthomonas* species. *X. perforans*, a related species causing bacterial spot on tomato, has not been isolated from pepper nor has it been found to be pathogenic to pepper. *X. gardneri* has been reported a tomato pathogen, but strains were found that were pathogenic to pepper as well (3, 4).

Revision history: Version 5, November 2013

Sample and sub-sample size

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

Principle

- Detection of viable *Xanthomonas* bacteria by dilution plating of the seed extract on semi-selective media.
- Confirmation of suspected bacterial colonies 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 (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 *Xanthomonas* on plates can be influenced by the presence of other microorganisms. It is the responsibility of the user to check for such antagonism by analysis, sample spiking, or experimental comparisons.
- This test method has not been validated for seed treated with protective chemicals 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 or biological substances have an effect on the method results.

Validation

This method has been peer reviewed by ISHI members and experts outside of ISHI. Aspects of this method have been validated by an ISHI laboratory and/or ISHI validation testing.

Note: The sections Restrictions on Use and Method Description have been modified to reflect the inclusion of stomaching treated seed to extract internally located bacteria. The incubation time and buffer volume for extracting bacteria from untreated seed have also been modified.

Method description

1. Extraction of bacteria from the seed

Untreated seed

Incubate each sub-sample for a minimum of 14 hours (max. 24 hours) at 4-10 °C in seed extraction buffer (PBS-Tween) at a ratio of 4 ml of buffer to 1 g of seed (v:w).

Treated seed

Note: the term "treated" refers to seed that has been treated using physical or chemical (calcium or sodium hypochlorite, tri-sodium phosphate, etc.) processes with the aim of disinfestation/disinfection.

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, max. 24 hours) at 4 - 10 °C, and macerate for at least 4 min in a stomacher machine.

- To extract internally located bacteria, the seed should be continuously shaken during stomaching. The force exerted during agitation should be sufficient to release bacteria; it may result in visibly damaging the seed.

2. Isolation on semi-selective media

2.1. Prepare a 10-fold dilution series (to 10-2) of the seed extract in seed extraction buffer. Prepare a 10-fold dilution series of a suspension of a pure culture of a known Xanthomonas reference strain in seed extraction buffer.

2.2. Spread-plate 0.1 ml of the extract onto two plates of each of the two semi-selective media mTMB and CKTM. Spread-plate 0.1 ml of each dilution onto one plate of each of the two semi-selective media. Spread-plate 0.1 ml of dilutions of the reference strain giving between 30 and 300 colonies per plate on each of the semi-selective media.

2.3. Incubate the plates at 28 °C for 4-7 days.

2.4. Check recovery and morphology of the Xanthomonas reference strain on both media.

- Examine the sample plates for the presence of colonies with typical *Xanthomonas* morphology by comparison with the reference strain. Record the number of these suspected colonies as well as of the other colonies and indicate when overgrowth of *Xanthomonas* by the other colonies could have occurred.
- After 4-7 days of incubation on mTMB, *Xanthomonas* colonies are yellow, slightly mucoid, mounded and circular in shape (Fig. 1). *Xanthomonas* utilizes Tween and in 3-7 days a white crystalline halo usually forms around the yellow colony. Some strains of *Xanthomonas* form only a weak halo or only clear the medium under the colony (not visible).
- After 4-7 days of incubation on CKTM, *Xanthomonas* colonies are similar to the colonies on mTMB; yellow, mucoid, mounded and circular in shape (Fig. 2). *Xanthomonas* utilizes Tween and in 3-7 days a white crystalline halo usually forms around the yellow colony. Some strains of *Xanthomonas* form only a weak halo or only clear the medium under the colony (not visible).
- The colony size and color can differ within a sample.

2.5. If present, select at least 2 suspect colonies per medium per sub-sample for further identification on a Yeast extract-dextrose-CaCO3 (YDC) medium.

3. Identification by morphology on YDC medium

3.1. Transfer suspected colonies as well as the reference strain onto YDC medium.

3.2. Incubate YDC plates at 28 °C for 2-3 days.

3.3. Determine whether the transferred colonies have a morphology typical

Xanthomonas by comparing them with the reference strain, and record which of the isolates are still suspected to be *Xanthomonas*.

- On YDC *Xanthomonas* is pale yellow and mucoid (Fig. 3).

3.4. If present, select suspected isolates for further identification with the pathogenicity assay. Select, if present, at least 6 YDC-suspected isolates from all the sub-samples combined. The selection should reflect the distribution of suspected colonies over sub-samples and/or semi-selective media.

4. Identification by pathogenicity assay

4.1. Grow seedlings of a known susceptible pepper cultivar (e.g. Early Cal Wonder) under suitable conditions until the 2-3 true leaf stage (approx. 3-4 weeks after sowing).

4.2. Transfer a small quantity of the selected colonies (in Section 3.4.) to a culture tube with 5-10 ml sterile distilled water. The colonies must not be more than three days old.

4.3. Adjust the inoculum to 10^8 cfu per ml by visual means (to cloudiness) or optical density methods (absorbance of 0.1 to 0.2 at 600 nm).

4.4. Infiltrate a leaf (1–4 sq cm of the surface) of an assay plant with the suspension by gently forcing the liquid into the lower surface of the leaf using a sterile syringe without a needle.

4.5. Infiltrate a leaf with the pathogenic isolate (positive control) and blank water (negative control).

4.6. Incubate the inoculated plants at 90–100 % humidity and at 27-32 °C with 8-12 h light per day.

4.7. Observe the plants daily and record the day that water-soaked lesions appear. *Xanthomonas* will develop a water-soaked lesion in 48–96 hours. Non-pathogenic bacteria will produce a hypersensitive reaction in 24 hours or will not develop a lesion at all (Fig. 4a and 4b).

Buffers and media

- Use de-ionized water.
- Autoclave buffers and media at 121 °C, 115 psi for 15 min.
- Especially the activity of antibiotics (units/mg) is crucial for the recovery of *Xanthomonas*. The purity of antibiotics and therefore, its activity can vary from batch to batch. Compare the purity of the old and new batches and the recovery of the target pathogen as well.
- Antibiotics are not stable in time. Therefore, add antibiotics at a relatively low temperature (<50 °C) and store plates before use in polythene bags at 4 °C in the dark. Use plates within a month to maintain the selectivity of the media.

Seed extraction buffer (PBS-Tween) per liter

PO_4 buffer pH 7.2	0.05 M
NaCl	8.5 g
Tween 20	0.2 ml

mTMB (modified Tween Medium B) per liter (5)

Bacto Peptone	10.0 g
H_3BO_3 (boric acid)	0.1 g
KBr	10.0 g
$CaCl_2$ anhydrous	0.25 g
Bacto agar	15.0 g
Tween 80[1,2]	10.0 ml

Cephalexin[2]	65 mg
5-Fluorouracil[2]	12 mg
Tobramycin sulphate	0.2 mg
Nystatin[2,3]	35 mg

[1] Autoclave separately

[2] Added after autoclaving.

[3] Use 100 mg/L cycloheximide, instead of nystatin, when fungal growth on the selective media is not completely inhibited by 35 mg/L nystatin.

CKTM medium per liter (6, 7)

Soya Peptone	2.0 g
Tryptone	2.0 g
Glucose	1.0 g
L-Glutamine	6.0 g
L-Histidine	1.0 g
$(NH4)_2HPO_4$	0.8 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.4 g
$CaCl_2$ anhydrous	0.25 g
Bacto agar	15.0 g
Tween 80[1,2]	10.0 ml
Cephalexin[2]	65 mg
5-Fluorouracil[2]	12 mg
Tobramycin sulphate[2]	0.4 mg
Bacitracin[2]	100 mg
Neomycin sulphate[2]	10 mg
Nystatin[2,3]	35 mg

[1] Autoclave separately

[2] Added after autoclaving

[3] Use 100 mg/L cycloheximide, instead of nystatin, when fungal growth on the selective media is not completely inhibited by 35 mg/L nystatin

YDC (Yeast extract Dextrose $CaCO_3$) medium per liter (8, 9)

Yeast extract	10.0 g
$CaCO_3$	20.0 g
D-glucose (Dextrose)	20.0 g
Bacto agar	17.0 g

References

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4. Schornack, S., Minsavage, G. V., Robert E., Stall, R. E., Jones J. B. and Lahaye, T. (2008) Characterization of AvrHah1, a novel AvrBs3-like effector from *Xanthomonas gardneri* with virulence and avirulence activity. *New Phytologist* **179**: 546–556
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Fig. 1. Colonies of *Xanthomonas* on mTMB medium

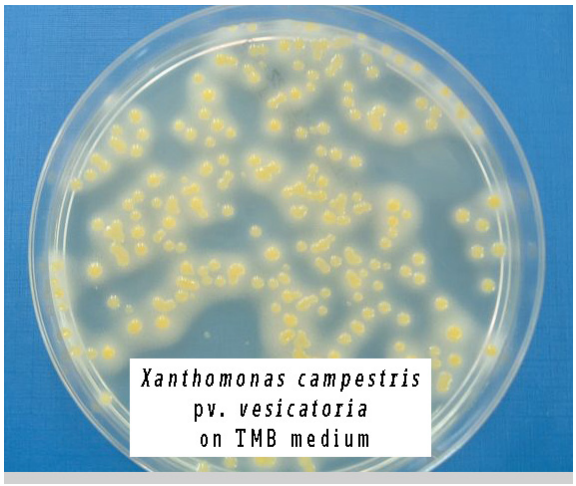

Fig. 2. Colonies of *Xanthomonas* on CKTM medium

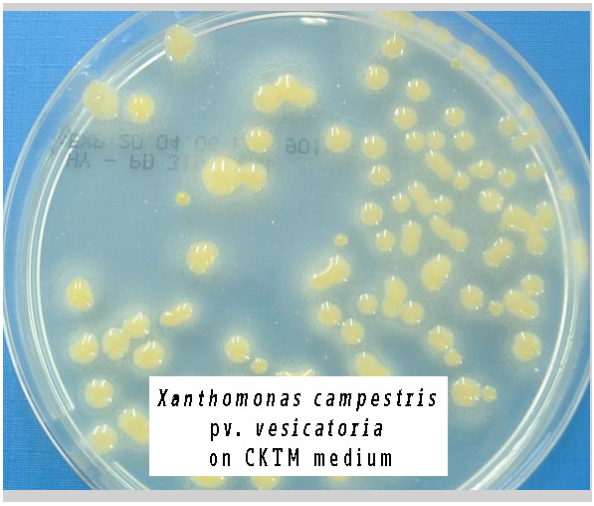

Fig. 3. Colonies of *Xanthomonas* on YDC medium

Fig. 4a. Pathogenic reaction on the upper surface of a pepper leaf

Fig. 4b. Pathogenic reaction on the lower surface of the same pepper leaf