Method for the Detection of *Xanthomonas* spp. in Tomato seed

Crop: Tomato *(Lycopersicon esculentum* L. now *Solanum lycopersicum)*

Pathogen: *Xanthomonas euvesicatoria, Xanthomonas vesicatoria, Xanthomonas perforans and Xanthomonas gardneri*
(See references 1 and 2 for an account of the reclassification of *Xanthomonas* species)

Revision history: Version 4, August 2011

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 of externally and internally located bacteria from the seed
- Isolation of viable *Xanthomonas* bacteria by dilution plating of seed extract on two different semi-selective media
- Confirmation of suspected bacterial colonies by a pathogenicity assay

Restrictions on Use

- This test method is suitable for untreated seed.
- The ability to recover *Xanthomonas* spp. on plates can be influenced by the presence of other microorganisms and/or inhibitory chemicals used for seed disinfestation/ disinfection (acid extraction, calcium or sodium hypochlorite, tri-sodium phosphate, etc.). It is the responsibility of the user to check for such antagonism and/or inhibition 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.

Method description

1. Extraction of bacteria from the seed

1.1. Put every sub-sample into a sterile stomacher bag. Add sterile seed extraction buffer to each bag at a ratio of 3 ml of buffer to 1 g of seed. (v:w). Incubate overnight (minimum 14 hours) at 4°C, and macerate for at least 7 minutes in a stomacher machine.

2. Isolation on semi-selective media

2.1. Taking further steps into account, filter coarse particles from the required volume of extract using a filter bag (Bagfilter® or Bagpage® from Interscience, France or an extraction bag with synthetic intermediate layer from Bioreba, Switzerland or Neogen Europe, Scotland). If a stomacher bag with filter is used, this is automatically achieved. Prepare a 10-fold dilution series (to 10^{-2}) of the seed extract in sterile seed extraction buffer.

2.1.1 For sub-samples larger than 5,000 seeds also prepare a 10-fold concentrated extract. Centrifuge at least 2 ml of the filtered extract for 5 minutes at 5,000 g. Carefully remove the supernatant and re-suspend the pellet in 1/10 of the original centrifuged volume of sterile seed extraction buffer.

Prepare a 10-fold dilution series of a suspension of a pure culture of a known *Xanthomonas* reference strain in sterile seed extraction buffer.

2.2. Spread plate 0.1 ml of the concentrated, the undiluted and diluted extract 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 medium.

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. *Xanthomonas* utilizes Tween and in 3-7 days a white crystalline halo usually forms around the yellow colony (Fig. 1). 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 suspected colonies per medium per sub-sample for further identification on 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 transferred colonies have typical *Xanthomonas* morphology by comparison 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 tomato cultivar (e.g. Cal Ace) under suitable conditions until the 2-3 true leaf stage (appr. 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 (absorbence of 0.1 to 0.2 at 600 nm).

4.4. Infiltrate a leaflet of an assay plant with the suspension by gently forcing the liquid into the underside of the leaflet using a sterile syringe without a needle.

4.5. Infiltrate a leaflet 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.

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 per batch. Compare the purity of the old and new batch and the recovery of the target pathogen as well.
- Antibiotics are not stable in time. Therefore, add antibiotics at relative 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 (3)

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 (4, 5)

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[12]	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 (6, 7)

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

References

1. Jones, J., Lacy, G., Bouzar, H., Stall, R. and N Schaad (2004) Reclassification of the Xanthomonads associated with bacterial spot disease of tomato and pepper. *Systematic and Applied Microbiology* **27**: 755-762.
2. Anon. (2006). List of new names and new combinations previously effectively, but not validly, published. *International Journal of Systematic and Evolutionary Microbiology* **56**: 925-927.
3. McGuire, R.G., Jones, J.B. and M. Sasser (1986) Tween medium for semiselective isolation of *Xanthomonas campestris* pv. *vesicatoria* from soil and plant material. *Plant Disease* **70**: 887.
4. Sijam, K., Chang, C. J. and R.D. Gitaitis (1991) An agar medium for the isolation and identification of *Xanthomonas campestris* pv. *vesicatoria* from seed. *Phytopathology* **81**: 831-834.
5. Sijam, K., Chang, C. J. and R.D. Gitaitis (1992) A medium for differentiating tomato and pepper strains of *Xanthomonas campestris* pv. *vesicatoria*. *Canadian Journal of Plant Pathology* **14**: 182-184.
6. Schaad, N.W., Jones, J. B. and W. Chun (eds) (2001) Laboratory Guide for Identification of Plant Pathogenic Bacteria, Third Edition. St Paul, USA: American Phytopathological Society Press.
7. Wilson, E.E., Zeitoun, F.M. and D.L. Fredrickson (1967) Bacterial phloem canker, a new disease of Persian walnut trees. *Phytopathology* **57**: 618-621.

Fig. 1. Colonies of *Xanthomonas* on mTMB medium

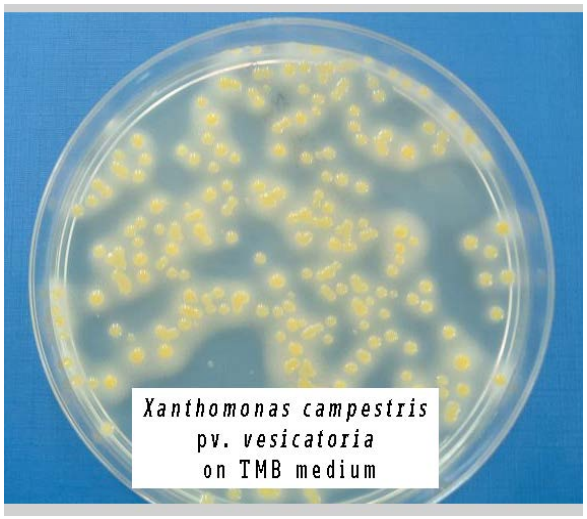

Fig. 2. Colonies of *Xanthomonas* on CKTM medium

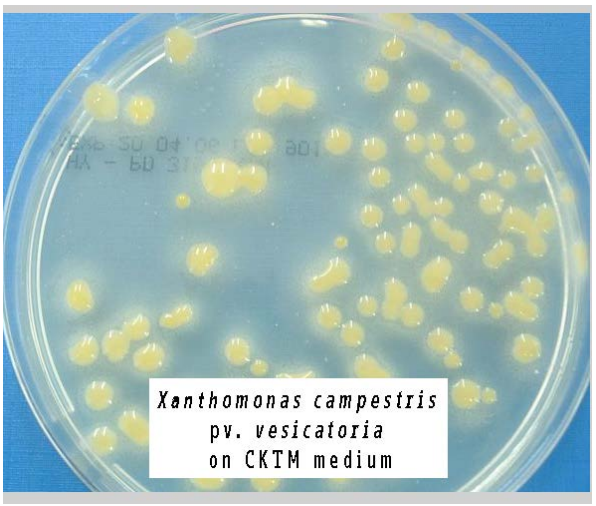

Fig. 3. Colonies of *Xanthomonas* on YDC medium