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Detection of *Xanthomonas hortorum* pv. *carotae* on Carrot Seed

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Developed by ISHI-Veg

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Crop: Carrot (*Daucus carota*)

Pathogen(s): *Xanthomonas hortorum* pv. *carotae*

Revision history: Version 2, November 2019
Note: In this version the gel-based PCR assay is replaced by a TaqMan assay

PRINCIPLE

Detection of viable *Xanthomonas hortorum* pv. *carotae* (Xhc) is assessed by dilution plating on two semi-selective media (MKM/MD5A or MKM/mTBM). Extraction of bacteria from the seed is enhanced by soaking.

After dilution plating, a TaqMan PCR is used for identification of the suspect bacterial colonies. As qPCR might detect both non-pathogenic and pathogenic bacteria, a positive TaqMan PCR should be followed with a pathogenicity assay to determine if the suspect isolate is pathogenic or not. The complete method process workflow is presented in Figure 1.

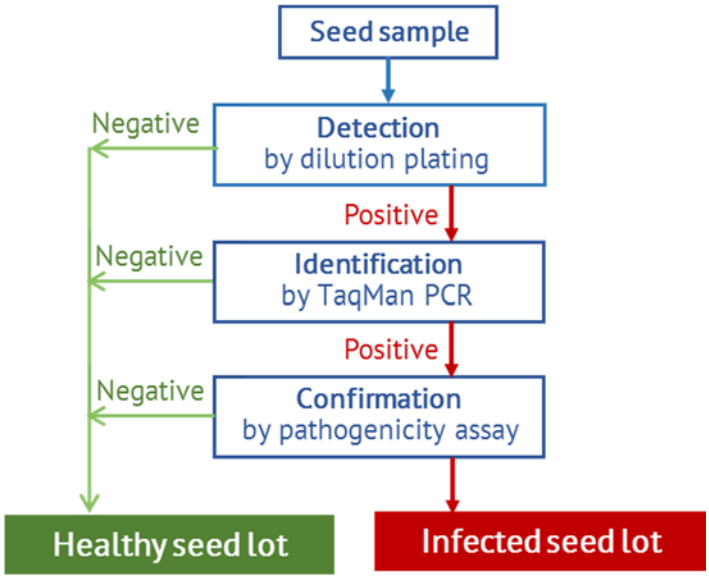

Figure 1. Method process workflow

METHOD VALIDATION

Version 1 of the method was adopted as an ISTA Rule (7-020) in January 2006 (see https://seedhealth.org/seed-health-testing-methods/), and was approved by the US National Seed Health System (NSHS) as a Standard A.

In this version a new Xhc specific TaqMan assay with an internal amplification control (validated by ISHI-Veg; Oosterhof 2017, 2019) replaces the gel-based PCR test from version 1.

RESTRICTIONS ON USE

This test method is suitable for untreated seed.

This test method is suitable for seed that has been treated using physical (hot water) or chemical (chlorine) processes with the aim of disinfestation and disinfection, provided that any residue, if present, does not influence the assay. 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.

METHOD EXECUTION

To ensure process standardization and valid results, it is strongly recommended to follow the best practices described by ISHI-Veg for Dilution Plating Assays and PCR in Seed Health Tests (see https://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg-method-development/).

SAMPLE AND SUB-SAMPLE SIZE

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

Protocol for detecting *Xanthomonas hortorum* pv. *carotae* on Carrot Seed

An older version of this protocol is available at ISTA's website:
ISTA 7-20: Detection of *Xanthomonas hortorum* pv. *carotae* in *Daucus carota* (carrot) seed
(https://www.seedtest.org/en/seed-health-methods-_content---1--1452.html).

SAMPLE PREPARATION

Material

Conical flasks or equivalent	Orbital shaker	Seed Extraction Buffer

Seed Extraction Buffer (SEB)

I 0.85% NaCl with 0.02% Tween™

Compound	Amount/L
NaCl	8.5 g
Tween™ 20 [1]	200 µl

[1]Added after autoclaving

1. Extraction

1.1. Suspend each subsample of seeds in pre-chilled (2–4°C) sterile SEB in a conical flask or equivalent. The volume of SEB should be adjusted according to the number of seeds used (use 10 mL of SEB per 1,000 seeds).

1.2. Soak subsamples overnight (16–18 h) at 4–7°C.

1.3. Shake on an orbital shaker set at 200 rpm for 5 minutes at room temperature (20–25°C).

1.4. Proceed with dilution plating.

DILUTION PLATING

Material

Dilution bottles or tubes	Incubator: operating at 28–30°C
NaCl 0,85%	pH meter
Sterile spreader	Controls (Table 1)
Pipettes and tips	Plates of MKM/MD5A or MKM/mTBM and YDC media (recipes below)

Table 1: Controls

Control type	
Positive Control (PC)	A known strain of Xhc
Negative Process Control (NPC)	SEB (NaCl with Tween™ 20)

Media recipes

I MKM semi-selective medium (pH 6.6) (modified KM-1 medium, Kim et al., 1982)

Compound	Amount/L
NH_4Cl	1.0 g
K_2HPO_4	1.2 g
KH_2PO_4	1.2 g
Lactose monohydrate	10.0 g
D(+) Trehalose dihydrate	4.0 g
Yeast extract	0.5 g
2-Thiobarbituric acid	0.2 g
Agar (BD Bacto™ Agar)	17.0 g
Tobramycin sulphate[a] (2 mg/mL in 70% (v/v) ethanol)	2 mg (1.0 mL)
Cephalexin monohydrate[a] (20 mg/mL in 70% (v/v) ethanol)	10 mg (0.5 mL)
Bacitracin[a] (50 mg/mL in 70% (v/v) ethanol)	50 mg (1.0 mL)
Nystatin[a,b] (10 mg/mL in 70% (v/v) ethanol)	35 mg (3.5 mL)

Notes apply to all media recipes tables:

[a] add after autoclaving (15 psi for 15 min at 121°C).

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

[c] The quality of skimmed milk powder greatly affects the efficacy of mTBM. Milk sources that work well are BBL, Oxoid or Sigma. Autoclave Skimmed milk separately.

II MD5A semi-selective medium (pH 6.4) (Cubeta & Kuan, 1986)

Compound	Amount/L
$MgSO_4 \cdot 7H2O$	0.3 g
NaH_2PO_4	1.0 g
NH_4Cl	1.0 g
K_2HPO_4	3.0 g
Agar (BD Bacto™ Agar)	17.0 g
Cellobiose[a] (100 mg/mL in distilled/deionised water)	10.0 g (100 mL)
L-glutamic acid[a] (5 mg/mL in distilled/deionized water)	5 mg (1 mL)
L-methionine[a] (1 mg/mL in distilled/deionized water)	1 mg (1 mL)
Cephalexin monohydrate[a] (20 mg/mL in 70% (v/v) ethanol)	10 mg (0.5 mL)
Bacitracin[a] (50 mg/mL in 70% (v/v) ethanol)	10 mg (0.2 mL)
Nystatin[a,b] (10 mg/mL in 70% (v/v) ethanol)	35 mg (3.5 mL)

III **mTBM semi-selective medium (pH 7.4)** (modified Tween™ Medium B, McGuire et al., 1986)

Compound	Amount/L
H_3BO_3	0.3 g
KBr	10.0 g
Peptone	10.0 g
Agar (BD Bacto™ Agar)	17.0 g
Skimmed milk solution[a,c] 10.0 g/100ml water	100 ml
Tween™ 80	10.0 ml
Cephalexin monohydrate[a] (20 mg/mL in 70% (v/v) ethanol)	65 mg (3.25 mL)
5-Fluorouracil[a] (10 mg/mL in 70% (v/v) ethanol)	12 mg (1.2 mL)
Nystatin[a,b] (10 mg/mL in 70% (v/v) ethanol)	35 mg (3.5 mL)

IV **Yeast dextrose chalk (YDC) agar (Wilson et al. 1967)**

Compound	Amount/L
Agar (BD Bacto™ Agar)	15.0 g
Yeast extract	10.0 g
$CaCO_3$ (light powder)	20.0 g
D-Glucose (dextrose)	20.0 g
Deionized water to a final volume of	1000 mL

Preparation

1. Weigh all ingredients, except the antibiotics.
2. Dissolve them in a suitable container in distilled/ultra-pure water.
3. Adjust pH to 6.6 for MKM, 6.4 for MD5A, and 7.4 for mTBM, if necessary.
4. Autoclave at 15 psi for 15 min at 121°C.
5. Prepare antibiotics and sterilize as appropriate.

Note: Antibiotics stock solutions and other supplements prepared in distilled/de-ionized water must be sterilized using a 0.2 µm bacterial filter. Solutions prepared in ethanol don't need sterilization.

6. Allow medium to cool to approx. 50°C prior to adding antibiotics.
7. Mix the molten medium thoroughly but gently to avoid air bubbles and pour plates. For the YDC: swirl the bottle/flask repeatedly to ensure an even distribution of $CaCO_3$.
8. Pour approximately 22 mL on to each 90 mm plate.
9. Leave plates to cool down and dry under sterile conditions.
10. Store inverted plates in polythene bags at 4°C. Antibiotic activity will drop over time during storage, and should not exceed 8 weeks.

1. Dilution and plating

1.1. Prepare a serial ten-fold dilutions from the seed extract by:

i.) Pipetting 0.5 mL of the extract into 4.5 mL of sterile NaCl to a 10^1 dilution

ii) Pipetting 0.5 mL of the 10^1 dilution into 4.5 mL of sterile NaCl to give a 10^2 dilution.

iii) Vortex well all dilutions.

1.2. Pipette 100 µL per plate of each dilution and undiluted seed extract onto plates of the MKM/MD5A or MKM/mTBM semi-selective media and spread over the surface.

1.3. Incubate plates at 28–30°C and examine after 4–8 days.

2. Positive control (culture or reference material)

2.1. Prepare a suspension of a known Xhc strain in sterile NaCl or reconstitute standardized reference material according to the supplier's instructions.

2.2. Dilute sufficiently to obtain dilutions containing approx. 10^2 to 10^4 CFU/mL.

2.3. Pipette 100 µL per plate of appropriate dilutions onto plates of both semi-selective media (MKM/MD5A or MKM/mTBM) and spread over the surface.

2.4. Incubate plates with the sample plates.

3. Sterility check (Negative Process Control)

3.1. Prepare a dilution series from a sample of the SEB without seeds. Plate 100 ul of each dilution on the two semi-selective media, spread over the surface and incubate (as in 1.2. and 1.3.).

4. Examination of the plates

4.1. Examine the sterility check and positive control plates. There should be no growth on dilution plates being used as a sterility check. The number of bacteria on the dilution plates should be consistent with the dilution, i.e. it should decrease approx. ten-fold with each dilution.

4.2. Examine the sample plates for the presence of typical Xhc colonies by comparing them with the positive control plates. Dilution plates prepared from the positive control isolate or reference material, should give single colonies with typical morphology.

4.3. After 4-6 days Xhc colonies on MKM appear light yellow-cream, light brown to peach yellow, glistening, round and 2–4 mm in diameter (Figure 2a).

4.4. After 7-8 days, Xhc colonies on MD5A appear straw yellow, glistening, round smooth, convex with entire margins, and 2–3 mm in diameter (Figure 2b).

4.5. After 7-8 days, Xhc colonies on mTBM appear white or yellow or white-yellow, glistening, round, smooth, convex with entire margins, 1–2 mm in diameter and surrounded by a large clear zone of casein hydrolysis (Figure 2c). Casein hydrolysis on mTBM is not always present.

4.6. The colony size and color can differ within a sample.

4.7. Verify that the plates are readable according to the dilution plating best practices and record the presence of suspect colonies.

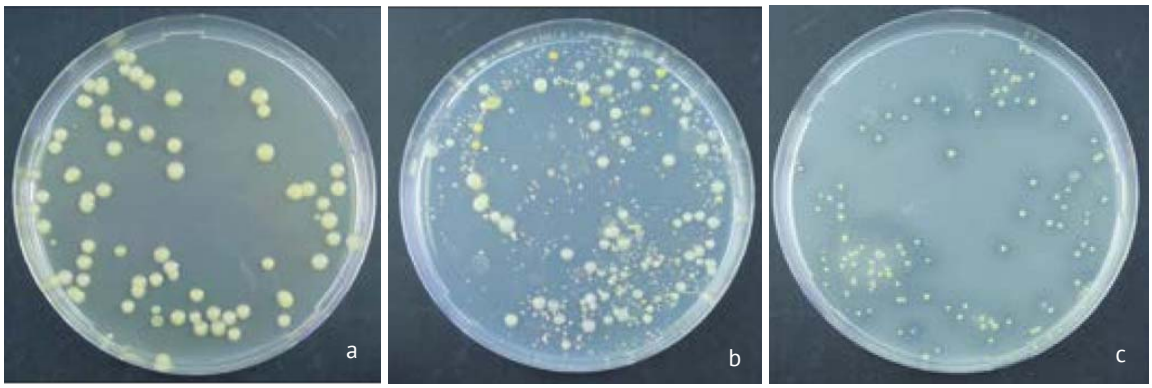

Figure 2. Plates of MKM (a), MD5A (b) and mTBM (c) after incubation of resp. 6, 7 and 7 days at 28°C showing typical colonies of Xhc.

5. Confirmation identification of suspect colonies

5.1. Subculture suspect colonies to sectored plates of YDC. To avoid the possibility for cross-contamination of isolates, use a new sectored plate for each subsample. The exact numbers of colonies sub-cultured will depend on the number and variability of suspect colonies on the plate: if present, at least six colonies (including each type) should be sub-cultured per subsample.

5.2. Subculture the positive control isolate on a sectored plate for comparison.

5.3. Incubate sectored plates for 24–72 h at 28–30°C.

Figure 3. Typical yellow mucoid growth of isolates of Xhc on a sectored plate of YDC after 72 h at 28°C.

5.4. Compare the morphology and growth to the positive control. On YDC, Xhc colonies are pale yellow and mucoid / fluidal (Figure 3). The positive control isolate(s) or reference material should give colonies with typical morphology on YDC.

5.5. The identity of isolates must be confirmed by the TaqMan PCR. The pathogenicity of the isolates should be confirmed using known susceptible carrot seedlings in a pathogenicity assay.

Note: As non-pathogenic isolates may also be present in seed lots it is essential to subculture at least the minimum number of suspect colonies specified (six per subsample per type), and to test the identity and pathogenicity of all Xanthomonas-like sub-cultured isolates.

5.6. Record results for each colony sub-cultured.

TAQMAN PCR FOR IDENTIFICATION OF SUSPECT XHC COLONIES

Material

TaqMan PCR mix, primers (Table 3) and equipment	Optical Density (OD) meter
Controls (Table 4)	NaCl 0.85%

Table 3. Primer and probe-sequences

Name	Sequence	Source
MVSXhc3F (10 µM)	5' – CCA AAG CAG TCG CAA ACT TGA – 3'	Barnhoorn 2014
MVSXhc3R (10 µM)	5' – AAT TGC GGA TTC CCA ACA AA – 3'	
MVSXhc3P (10 µM)	5' – VIC – TGG CCC TAA GCT TCA A – NFQ-MGB – 3'	
Xhc-q2F (10 µM)	5' – GCA TGA AGG CAA TAC AGC G – 3'	Temple et al. 2013
Xhc-q2R (10 µM)	5' – CGA TCC AGC TGA TGT TCT CCG AA – 3'	
Xhc-q2P (10 µM)	5' – FAM – TCA AGC TCA GAC GAA ACC GGC GTC – BHQ1 – 3'	
Wu-F (10 µM)	5' – CAA CGC GAA GAA CCT TAC C – 3′	Wu et al. 2008
Wu-R (10 µM)	5' – ACG TCA TCC CCA CCT TCC – 3′	
Wu-Pr1 (10 µM)	5' – TEXAS RED – ACG ACA ACC ATG CAC CAC CTG – BHQ2 – 3′	
Wu-Pr2 (20 µM)	5′ – TEXAS RED – ACG ACA GCC ATG CAG CAC CT – BHQ2 – 3′	

Table 4. Controls

Control type	
Positive Process Control (PPC)	Freshly prepared suspension of Xhc
Negative Process Control (NPC)	Freshly prepared suspension of non-target colony
Internal Amplification Control (IAC)	Universal bacterial primers (Wu et al. 2008)
Non Template Control (NTC)	Nucleic acid-free water

1. DNA isolation

The template DNA from single colonies for qPCR can be obtained by several means.

1.1. Make a slightly turbid cell suspension (e.g. OD600 nm approximately 0.05) in 1.0 mL sterile NaCl from the suspected cultures on YDC medium and the positive controls (Table 4). In addition, a non-suspect isolate should be used as a negative process control (NPC).

1.2. Incubate for 10 min at 100°C.

1.3. Suspensions can be stored at –20°C until identification.

2. TaqMan PCR

2.1. Use the Xhc specific primers and probes from Barnhoorn 2014 (MVSXhc3 set) and from Temple et al. 2013 (Xhc-q2 set) (Table 3).

2.2. Use the universal primers and probes from Wu et al. (2008) (Table 3) to validate the PCR reaction.

2.3. Prepare the reaction mixture (Table 5).

Table 5. Example PCR mix TaqMan PCR: MVSXhc3 / q2 / Wu triplex

Component	For 1 reaction (in µL)	Final concentration
MVSXhc3F (10 µM)	2.25	0.9 µM
MVSXhc3R (10 µM)	2.25	0.9 µM
MVSXhc3P (10 µM)	0.625	0.25 µM
Xhc-q2F (10 µM)	1.00	0.4 µM
Xhc-q2R (10 µM)	1.00	0.4 µM
Xhc-q2P (10 µM)	0.50	0.2 µM
Wu-F (10 µM)	0.50	0.2 µM
Wu-R (10 µM)	0.50	0.2 µM
Wu-Pr1 (10 µM)	0.50	0.2 µM
Wu-Pr2 (10 µM)	0.50	0.2 µM
ABI Gene Expression Master mix (2x)*	12.50	1x
PCR grade H_2O	0.875	
Template DNA	2.00	
Total	**25.00**	

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

2.4. Perform the PCR reaction in a real-time PCR instrument according to the PCR conditions (Table 6).

Note: The preparation of the PCR mixture and the amplification PCR program should be adapted to available material and equipment of individual laboratories testing for Xhc under the condition that results will be validated by PCR controls.

Table 6. PCR conditions TaqMan PCR

Step	Temperature	Duration
hold	95°C	10 min
40 cycles	95°C	15 sec
	60°C	30 sec

2.5. Determine the cut off values. Cq values of positive controls should consistently be lower than 32. 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.

Note: 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 indicate at least a 10-fold higher concentration of bacterial DNA than the IAC Cq values from the NTC reactions; the difference between Cq values should be more than 3.3.

3. Interpretation and decisions

For interpretation and decision making, the results from both primer sets need to be considered, see Table 7. Test results are only valid when all included controls presented in Table 4 give the expected result.

Table 7. Interpretation and decision table for the TaqMan PCR

MVSXhc3 Taqman	Q2 Taqman	Wu TaqMan[a]	qPCR Result	Follow-up
Positive	Positive	NA	Target DNA for Xhc detected	Pathogenicity test for confirmation
Negative	Positive	NA	Target DNA for Xhc detected	Pathogenicity test for confirmation
Positive	Negative	NA	Target DNA for Xhc detected	Pathogenicity test for confirmation
Negative	Negative	Positive	No target DNA for Xhc detected	Negative, no follow up
Negative	Negative	Negative	Invalid	Repeat PCR or continue with pathogenicity test for confirmation

[a] NA: Not applicable

PATHOGENICITY ASSAY

Material

Carrot seedlings: susceptible of the pathogen (e.g. *carrot* 'Napoli')	Spraying device
Sterile tap water	Controls (Table 8)

Table 8. Controls

Control type	
Positive Process Control (PPC)	A known strain of Xhc
Negative Control (NC)	sterile-tap water

1. Pathogenicity assay

1.1. Grow seedlings of a carrot cultivar known to be susceptible to Xhc (e.g. carrot 'Napoli') in small pots or modules until at least 3–4 true leaf stage (approximately 3-4 weeks after sowing).

1.2. Prepare a suspension in sterile tap water from each suspect bacterial culture on YDC medium and dilute to a concentration containing approximately 2×10^6 CFU/mL.

The same procedure should be used for the Positive Process Control (PPC) isolate.

1.3. Inoculate plants by spraying until runoff. Use one small pot with 3–4 plants per isolate.

A PPC isolate as well as a Negative Control (NC) should be included in every pathogenicity test (Table 8).

It is important not to rub the leaves after spraying, since this will cause false positive results.

1.4. Grow inoculated plants at 27–28°C enclosed in plastic bags/tents (to provide conditions near 100 % RH). After 48 h, remove the bags during daytime and replace at night.

1.5. Record symptoms after 7–10 days incubation. Typical Xhc symptoms first appear as small irregular yellowish water-soaked areas with a tiny light brown spot in the center on inoculated leaves. Later, affected areas enlarge, become brown, and are often surrounded by a yellow halo (Figure 4). Compare to the PPC and NC.

Note: The PPC isolate should give typical symptoms in the pathogenicity test, while the NC should not show any symptoms.

Figure 4. Typical Xhc symptoms in a pathogenicity test indicated by small brown irregular areas surrounded by a yellow halo

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