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

DECEMBER 2021

Developed by ISHI-Veg

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

Crop: Carrot (*Daucus carota*)

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

Revision history: Version 2.1, December 2021

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 qPCR is used for identification of the suspect bacterial colonies. As qPCR might detect both non-pathogenic and pathogenic bacteria, a positive qPCR 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.

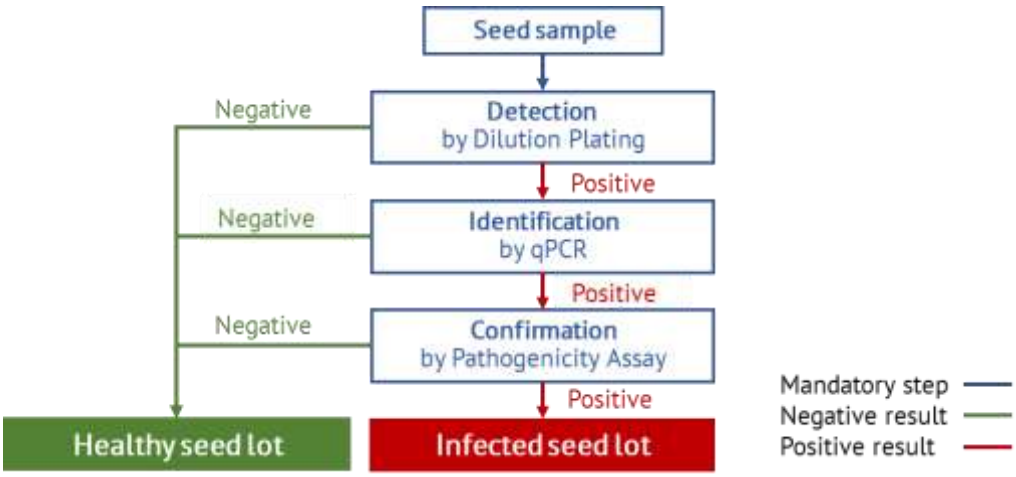

Figure 1. Method process workflow

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 has been approved by the US National Seed Health System (NSHS) as a Standard A.

In version 2 a Xhc specific qPCR assay with an internal amplification control (validated by ISHI-Veg; Oosterhof 2017, 2019) replaces the gel-based PCR test from version 1.

The repeatability, reproducibility and diagnostic performance of the dilution plating assay in combination with the qPCR assay, were validated based on data generated during an inter-laboratory comparative test performed in 2018 by ISHI-Veg (Lê Van et al. 2021).

RESTRICTIONS ON USE

Technical details on the reagents / material used in the validation study (e.g. supplier information) are provided in the protocol and the validation report. If material and consumables from different suppliers are used, it is necessary to verify their performance. For PCR methods, in-house method optimization is often necessary by optimizing parameters as described in Best Practices for PCR Assays in Seed Health Tests.

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, PCR Assays and Biological Assays in Seed Health Tests (see https://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg-method-development/).

SAMPLE AND SUBSAMPLE SIZE

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

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1.0	2002	First version of the protocol.
	2005	Published as ISTA Rule (7-020).
1.0	November 2009	Protocol replaced by reference to ISTA Rule (7-020).
1.0	March 2011	Section *Restrictions on Use* modified.
1.0	August 2011	Section *Restrictions on Use* modified.
1.0.1	July 2017	Section *Validation* updated to include NSHS approval as standard A. Section *Method Execution* added.
2.0	November 2019	Gel-based PCR assay replaced by qPCR assay, this change has not been implemented in ISTA Rule (7-020). Protocol updated to new ISF template.
2.1	December 2021	Reference to comparative test report (Lê Van et al. 2021) added. Protocol presented in accordance with ISHI-Veg's protocol guidelines.

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).

I. DETECTION BY DILUTION PLATING

Material

- Conical flasks or equivalent
- Orbital shaker
- Plates of MKM/MD5A or MKM/mTBM and YDC media (recipes Table I.1 – I.4)
- Seed extraction buffer (Table I.5)
- Bottles or tubes to prepare dilutions in
- Incubator: operating at 28–30 °C
- NaCl 0.85% solution
- pH meter
- Sterile spreader (e.g. Drigalski spatula or (glass) beads)
- Controls (Table I.6)
- Pipettes and tips

Table I.1 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 (e.g. 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)

[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.

Table I.2. 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 (e.g. 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)

[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.

Table I.3. 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 (e.g. 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)

[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.

Table I.4. Yeast dextrose chalk (YDC) agar (Wilson et al. 1967)

Compound	Amount/L
Agar (e.g. 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 media Table I.1 to I.4.

1. Weigh all ingredients, except the antibiotics.
2. Dissolve them in a suitable container in distilled/deionised 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. Alternatively, add the antibiotic powder to the autoclaved distilled/de-ionized water. Solutions prepared in 70% ethanol need no 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.

Note: Storage conditions and duration may affect antibiotic activity, which can influence the performance of the test.

Table I.5. Seed extraction buffer (0.85% NaCl with 0.02% Tween™)

Compound	Amount/L
Sodium chloride (NaCl)	8.5 g
Tween™ 20[a]	200 µL
Deionized water to a final volume of	1000 mL

[a] Added after autoclaving

Table I.6. Types of controls used

Control type	Description
Positive Control (PC)	A known strain of Xhc
Negative Process Control (NPC)	Seed extraction buffer (NaCl with Tween™ 20)

1. Extraction

1.1. Suspend each subsample of seeds in pre-chilled (2–4 °C) sterile seed extraction buffer (Table I.5) in a conical flask or equivalent. The volume of seed extraction buffer should be adjusted according to the number of seeds used (use 10 mL of seed extraction buffer per 1,000 seeds).

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

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

1.4. Proceed with dilution plating.

2. Dilution and plating

2.1. Prepare a ten-fold serial dilution series from the seed extract by e.g.:

i) Pipetting 0.5 mL of the extract into 4.5 mL of sterile NaCl to give 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.

2.2. 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.

2.3. Incubate plates at 28–30 °C.

3. Positive control (culture or reference material)

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

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

3.3. 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.

3.4. Incubate plates with the sample plates.

4. Sterility check (Negative Process Control)

4.1. Prepare a dilution series from a sample of the seed extraction buffer (Table I.5) without seeds. Plate 100 µL of each dilution on the two semi-selective media, spread over the surface and incubate (as in 2.2. and 2.3.).

5. Examination of the plates

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

5.2. 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.

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 I.1a).

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 I.1b).

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 I.1c). However, casein hydrolysis on mTBM is not always present for all strains.

The colony size and color can differ within a sample.

5.3. Verify that the plates are readable according to the dilution plating best practices at the final reading date and record the presence of suspect colonies.

Note: Test results are only valid when all included controls, presented in Table I.6, give the expected result.

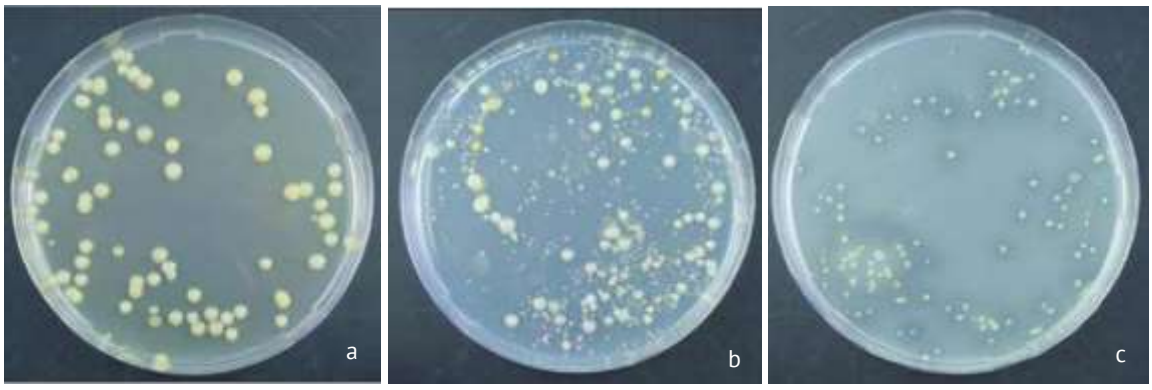

Figure I.1. Plates of MKM (a), MD5A (b) and mTBM (c) after incubation of resp. 6, 7 and 7 days at 28 °C showing typical colonies of Xhc.

6. Confirmation identification of suspect colonies

6.1. 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.

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

6.3. Incubate sectored plates for 24–72 hours at 28–30 °C.

6.4. Compare the morphology and growth to the positive control. On YDC, Xhc colonies are pale yellow and mucoid / fluidal (Figure I.2). The positive control isolate(s) or reference material should give colonies with typical morphology on YDC.

6.5. The identity of isolates must be confirmed by the qPCR. 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.

6.6. Record results for each sub-cultured colony.

Figure I.2. Typical yellow mucoid growth of isolates of Xhc on a sectored plate of YDC after 72 hours at 28 °C.

II. IDENTIFICATION BY qPCR OF SUSPECT XHC COLONIES

For PCR methods, in-house method optimization is often necessary by changing certain parameters as described in Best Practices for PCR Assays in Seed Health Tests

Material

- Optical Density (OD) meter
- Controls (Table II.1)
- qPCR mix, primers (Table II.2) and equipment
- NaCl 0.85% solution
- Lab disposables

Table II.1. Types of controls used

Control type	Description.
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

Table II.2. 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´	

1. DNA isolation

The template DNA from single colonies for qPCR can be obtained by several means. The method presented here was used for the validation of this protocol.

1.1. Make a slightly turbid cell suspension (e.g. OD 600 nm approximately 0.05) in 1.0 mL sterile NaCl-solution from the suspected cultures on YDC medium and the positive controls (Table II.1). 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. qPCR

2.1. Use the Xhc specific primers and probes from Barnhoorn 2014 (MVSXhc3 set) and from Temple et al. 2013 (Xhc-q2 set) (Table II.2).

2.2. Use the universal primers and probes from Wu et al. (2008) (Table II.2) to validate the PCR reaction.

2.3. Prepare the reaction mixture (Table II.3).

2.4. Perform the PCR reaction in a real-time PCR instrument according to the PCR conditions (Table II.4).

Note: If different PCR mixtures and amplification programs are used, it is necessary to verify their performances.

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.

Notes: Cut-off values must be established by each laboratory for their positive and internal amplification controls (IAC) prior to the assay being used on routine samples. For recommendations on setting cut-off values, see: 'Real-time PCR pre-screening in seed health methods'.

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 be 3.3 Cq value lower than the IAC Cq values from the NTC reactions.

Table II.3. Example qPCR mix: MVSXhc3 / Xhc-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.

Table II.4. qPCR conditions

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

3. Interpretation and decisions

For interpretation and decision making, the results from both primer sets need to be considered, see Table II.5. Test results are only valid when all included controls presented in Table II.2 give the expected result.

Table II.5. Interpretation and decision table for the qPCR

MVSXhc3	Xhc-q2	Wu[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

III. CONFIRMATION BY PATHOGENICITY ASSAY

Material

- Carrot seedlings: susceptible to the pathogen (e.g. carrot 'Napoli')
- Spraying device
- Sterile tap water
- Controls (Table III.1)

Table III.1. Types of controls used

Control type	Description
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 III.1).

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 hours, 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 III.1). Compare to the PPC and NC.

Note: Test results are only valid when all included controls presented in Table III.1 give the expected result. The PPC isolate should give typical symptoms in the pathogenicity test, while the NC should not show any symptoms.

Figure III.1. Typical Xhc symptoms in a pathogenicity test indicated by small brown irregular areas surrounded by a yellow halo

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