International Seed Federation
Seed is Life

Detection of *Xanthomonas campestris* pv. *campestris* and pv. *raphani* on *Brassica* spp. seed

MARCH 2026

Developed by ISHI

Detection of *Xanthomonas campestris* pv. *campestris* and pv. *raphani* on *Brassica* spp. seed

Crop: *Brassica* spp.

Pathogen(s): *Xanthomonas campestris* pv. *campestris* and *Xanthomonas campestris* pv. *raphani*

Version: 7.0 (March 2026)

PRINCIPLE

Detection of viable *Xanthomonas campestris* pv. *campestris* (Xcc) and *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria is typically assessed by dilution plating on two semi-selective media (mFS and mCS20ABN). Extraction of bacteria from the surface and the funiculus of the seed is enhanced by soaking. Suspect bacterial colonies are then confirmed by a pathogenicity assay.

A seed extract qPCR assay (SE-qPCR) may be used as a pre-screen. The test is complete if no Xcc or Xcr are detected, and as such the seed lot is considered healthy. However, as qPCR detects both infectious and non-infectious bacterial particles, as well as viable and non-viable bacterial DNA, a positive pre-screen SE-qPCR is followed sequentially by dilution plating and a pathogenicity assay to determine the presence of viable and pathogenic Xcc or Xcr.

After dilution plating, a qPCR may be used for identification of the suspect bacterial colonies. The test is complete if no Xcc or Xcr bacteria are detected. A positive qPCR must be followed with a pathogenicity assay to determine if the suspect Xcc or Xcr isolate is pathogenic or not. The full method process workflow is presented in Figure 1.

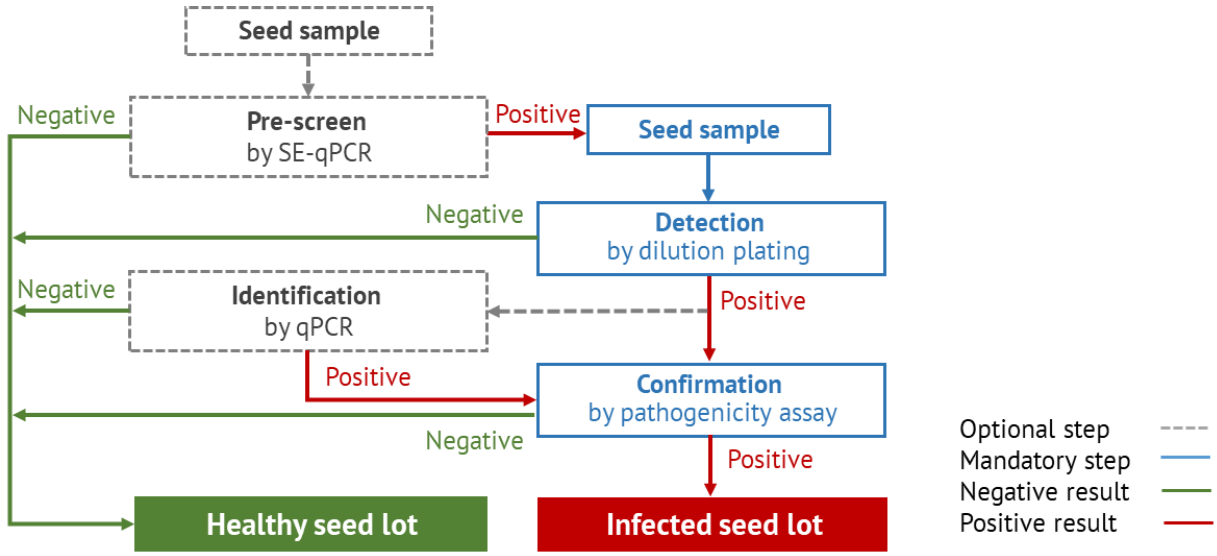

Figure 1. Method process workflow.

METHOD VALIDATION

The dilution plating assay and pathogenicity assay were evaluated for Xcc in cabbage (*Brassica oleracea* var. *capitata*) seed in a comparative test (Koenraadt *et al*., 2005). An additional study on changed mCS20ABN and FS media recipes for Xcc in cabbage seed was performed by Sato *et al.* (2014). The identification qPCR was validated for Xcc and Xcr by Barnhoorn (2018) and the SE-qPCR for Xcc in cabbage seed by Bruinsma *et al.* (2018). Additional validation data to support the inclusion of Xcr in the dilution plating and pathogenicity assay and other *Brassica* species in the protocol can be found in the report of Thomas and Woudenberg (2026) using data generated by Asma and Jonkers in 2025.

An optional pre-screen using a Bio-PCR assay and gel-based PCRs for suspect colony identification were also validated in previous versions of the method (see ISTA Rule 7-019a). In this version, only the pre-screen seed extract qPCR and the identification qPCR assays are included in the method.

RESTRICTIONS ON USE

Before using this protocol routinely, it is necessary to verify its performance, especially when material and consumables from different suppliers are used. Technical details on the reagents/material used in the validation study (e.g., supplier's information) are provided in the protocol and the validation reports.

This test method is suitable for untreated seed. 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.

This test method is not suitable for seed treated using physical techniques (such as hot water) with the aim of disinfestation/disinfection. A test for detecting Xcc and Xcr in disinfested/disinfected *Brassica* spp. seed is available as ISTA Rule (7-019b).

METHOD EXECUTION

To ensure process standardization and valid results, it is strongly recommended to follow the Best Practices for Seed Health Tests developed by ISHI.

SAMPLE AND SUBSAMPLE SIZE

The recommended minimum sample size is 30,000 seeds with a maximum subsample size of 10,000 seeds.

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1.0-6.0		See revision history in ISTA Rule 07-19a.
6.1	September 2019	*Xanthomonas campestris* pv. *raphani* (Xcr) added to the protocol. Optional Bio-PCR pre-screen assay removed. Optional gel-based identification PCR assay removed. Layout updated, no longer ISTA Rule 07-019a.
6.2	October 2019	Editorial corrections.
7.0	March 2026	Control names updated to adhere to the new ISHI Best Practices. Protocol updated to the latest ISHI protocol guidelines. Reference to Thomas and Woudenberg (2026) addendum validation report added. Note on Köhl positive Xcr isolates added.

Protocol for detecting *Xanthomonas campestris* pv. *campestris* and pv. *raphani* on *Brassica* spp. seed

An extended version of this protocol is available on ISTA's website:
ISTA 7-019a: Detection of *Xanthomonas campestris* pv. *campestris* in *Brassica* spp. seed.

I. PRE-SCREEN BY SEED-EXTRACT qPCR (OPTIONAL STEP)

For PCR methods, in-house method optimization is often necessary, see Best Practices for PCR Assays in Seed Health Tests for information.

Materials

- qPCR mix, primers (Table I.1) and PCR equipment
- Controls (Table I.2)
- Conical flasks or equivalent
- Orbital shaker
- NaCl (0.15 M) with 0.02% (v/v) Tween™ 20 added after autoclaving (Table I.3)
- DNA isolation kit e.g., DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany)
- Centrifuge

Table I.1. Primer-sequences and references.

Name	Target	Sequence (5'– 3')	Source
XCC-F	Xcc	GTG CAT AGG CCA CGA TGT TG	Köhl *et al.*, 2011
XCC-R		CGG ATG CAG AGC GTC TTA CA	
XCC-Pr		FAM – CAA GCG ATG TAC TGC GGC CGT G – NFQ-MGB	
DLH153-F	Xcc/Xcr	GTA ATT GAT ACC GCA CTG CAA	Berg *et al.*, 2006
DLH154-R		CAC CGC TCC AGC CAT ATT	
P7		VICrepl – ATG CCG GCG AGT TTC CAC G – BHQ1	
Acat2-F	*P. cattleyae*	TGT AGC GAT CCT TCA CAA G	Koenraadt *et al.*, 2014
Acat2-R		TGT CGA TAG ATG CTC ACA AT	
Acat1-Pr		Texas Red – CTT GCT CTG CTT CTC TAT CAC G – BHQ2	

Table I.2. Types of controls used.

Control type	Description
Inhibition control (IC)[a]	*Paracidovorax cattleyae* spike
Negative buffer control (NBC)[b]	Extraction buffer
Negative process control (NPC)[b]	Brassica seed free of Xcc and Xcr
Non template control (NTC)	PCR reaction mix free of any pathogen or seed
Positive amplification control (PAC)	Xcc DNA *and*
	Xcr DNA
Positive process control (PPC)	Brassica seed infected with Xcc and/or Xcr

[a] The IC also serves as internal amplification control (IAC). Other organisms such as *P. citrulli* may also be used, but the compatibility with the Xcc/Xcr primers in a multiplex qPCR should be verified.
[b] Include at least either the NBC or NPC.

Table I.3. NaCl (0.15 M)[a].

Compound	Amount/L
Sodium chloride (NaCl)	8.5 g

[a] For extraction of seeds, add 20 µL of sterile Tween™ 20 per 100 mL solution after autoclaving.

1. Seed extraction

1.1. Suspend each subsample of seeds, and the positive process control (PPC), separately, in pre-chilled (2–4 °C) sterile NaCl with 0.02% (v/v) Tween™ 20 (Table I.3) in a conical flask or equivalent. The volume of NaCl should be adjusted according to the number of seeds used (use 10 mL of buffer for 1,000 seeds).

1.2. Shake on an orbital shaker at 100–125 rpm for 2.5 hours at room temperature (20–25 °C).

1.3. Shake flasks to mix prior to further processing.

2. Spiking samples

2.1. Transfer 10 mL of seed extract from each subsample into a 15 mL centrifuge tube. Include a seed extract from healthy seeds as a negative process control (NPC) and/or buffer without seeds as a negative buffer control (NBC) (Table I.2).

2.2. Add an inhibition control (IC) spike to all subsamples, the PPC and NPC and/or NBC for seed extract qPCR (e.g., 100 µL of *P. cattleyae* stock of OD_{600} = 0.6) (Table I.2).

3. Concentration of bacteria

3.1. Centrifuge sample tubes at 1,200 × *g* for 5 min at room temperature. Carefully transfer the supernatant to a fresh 15 mL tube and discard the pellet.

3.2. Centrifuge the supernatant at a minimum of 3,400 × *g* for 20 min at room temperature.

3.3. Carefully decant as much supernatant as possible without disturbing the pellet.

3.4. Use the pellet for the isolation of DNA.

Note: The pellet is not always stable. Take tubes carefully out of the centrifuge.

4. DNA isolation

4.1. Re-suspend the bacterial pellet in lysis buffer and extract the bacterial DNA, as described in the manual of the DNA extraction kit.

Note: The assay has been validated with the DNeasy Blood and Tissue kit (Qiagen). If a different DNA isolation kit is used, it is necessary to verify its performance.

5. qPCR

Notes: Work on ice whenever possible and minimize probe's exposure to light.

If different qPCR mixtures and amplification programs are used, it is necessary to verify their performance.

5.1. Prepare the qPCR mix (Table I.4). Use the Xcc primer sets from Köhl *et al.* (2011) and from Berg *et al.* (2006) and the *P. cattleyae* primer set from Koenraadt *et al.* (2014) (Table I.1).

5.2. Test each DNA extraction from each subsample in duplicate (technical replicates). Include both positive amplification controls (PAC) and a non-template control (NTC) (Table I.2).

5.3. Cover the plate after adding DNA and perform the qPCR according to the PCR conditions described in Table I.5.

Table I.4. qPCR mix for the SE-qPCR[a].

Component	Per reaction (in µL)	Final concentration
PCR grade water	9.95	
PerfeCTa Multiplex qPCR ToughMix (5×)	5.00	1×
XCC-F (10 µM)	0.75	0.30 µM
XCC-R (10 µM)	0.75	0.30 µM
XCC-Pr (10 µM)	0.50	0.20 µM
DLH153-F (10 µM)	0.75	0.30 µM
DLH154-R (10 µM)	0.75	0.30 µM
P7 (10 µM)	0.50	0.20 µM
Acat2-F (10 µM)	0.40	0.16 µM
Acat2-R (10 µM)	0.40	0.16 µM
Acat1-Pr (10 µM)	0.25	0.10 µM
Sample	5.00	
Total	**25.00**	

[a] Use of a triplex qPCR, as described in this method, is preferred. If, however, the PCR machine used is not appropriate for a triplex, two duplex PCR reactions (target and spike) can be performed, which however will need to be internally validated.

Table I.5. PCR conditions SE-qPCR.

Step	Temperature	Duration
hold	95 °C	2 min
40 cycles	95 °C	15 sec
	60 °C	48 sec

6. Interpretation and decisions

Cut-off values must be established by each laboratory for their positive and inhibition controls (IC) prior to the assay being used on routine samples. For recommendations on setting cut-off values, see Real-time PCR, an 'indirect' test used for pre-screening in seed health methods. The cut-off for *P. cattleyae* is set preferably between Cq 28 and 32.

A cut-off value of Cq 35 for the SE-qPCR of samples was used for the validation study.

For interpretation and decision making, results from both primer sets need to be considered, as described in Table I.6. Test results are only valid when all included controls presented in Table I.2 give the expected results.

Table I.6. Interpretation and decision table SE-qPCR.

Köhl qPCR	Berg qPCR	qPCR result	Follow-up
Positive	Positive	Target DNA for Xcc detected[a]	Dilution plating
Negative[a]	Positive	Target DNA for Xcr detected	Dilution plating
Positive	Negative	Inconclusive	Dilution plating
Negative	Negative	No target DNA for Xcc/Xcr detected	No follow up required

[a] Some Köhl qPCR positive Xcr isolates are known (ISHI, internal communication).

II. DETECTION BY DILUTION PLATING

Materials

- Dilution bottles
- Incubator: operating at 28–30 °C
- NaCl (0.15 M) (Table I.3)
- pH meter
- Sterile spreader
- Controls (Table II.1)
- Plates of mFS, mCS20ABN and YDC media (Tables II.2-II.4)

Table II.1. Types of controls used.

Control type	Description
Positive control (PC)	A known strain of Xcc *and*
	A known strain of Xcr
Negative buffer control (NBC)	Extraction buffer (NaCl with Tween™ 20) *and*
	Dilution buffer (NaCl)

Table II.2 mFS semi-selective medium (Schaad *et al.,* 1989).

Compound	Amount/L
K_2HPO_4	0.8 g
KH_2PO_4	0.8 g
KNO_3	0.5 g
$MgSO_4.7H_2O$	0.1 g
Yeast extract	0.1 g
Methyl green (1% (w/v) aqueous solution)	1.5 mL
Soluble starch (Merck 1252)	25.0 g
Agar (BD Bacto™ Agar)	15.0 g
Nystatin[a] (10 mg/mL in 50% (v/v) ethanol)	35 mg (3.5 mL)
D-Methionine[a] (1 mg/mL in 50% (v/v) ethanol)	3 mg (3.0 mL)
Pyridoxine HCl[a] (1 mg/mL in 50% (v/v) ethanol)	1 mg (1 mL)
Cephalexin[a] (20 mg/mL in 50% (v/v) ethanol)	50 mg (2.5 mL)
Trimethoprim[a] (10 mg/mL in 70% (v/v) ethanol)	30 mg (3 mL)

[a] Added after autoclaving.

Table II.3. mCS20ABN semi-selective medium.

Compound	Amount/L
Soya peptone	2.0 g
Tryptone (BD Bacto™ Tryptone)	2.0 g
KH_2PO_4	2.8 g
$(NH_4)_2HPO_4$	0.8 g
$MgSO_4.7H_2O$	0.4 g
L-Glutamine	6.0 g
L-Histidine	1.0 g
D-Glucose (dextrose)	1.0 g
Soluble starch (Merck 1252)	25.0 g
Agar (BD Bacto™ Agar)	18.0 g
Nystatin[a] (10 mg/mL in 50% (v/v) ethanol)	35 mg (3.5 mL)
Neomycin sulphate[a] (20 mg/mL in distilled/deionised water)	40 mg (2.0 mL)
Bacitracin[a] (50 mg/mL in 50% (v/v) ethanol)	100 mg (2.0 mL)

[a] Added after autoclaving.

Media preparation

1. Weigh all ingredients, except the antibiotics, pyridoxine-HCl and D-methionine.

Note: For starch hydrolysis, the source of starch used in the selective media is critical. Verify that each new batch of starch gives clear zones of hydrolysis with reference cultures of Xcc.

2. Dissolve them in a suitable container in distilled/deionised water.
3. Adjust pH to 6.5, if necessary.
4. Autoclave at 15 psi for 15 min at 121 °C.
5. Prepare antibiotics, pyridoxine-HCl and D-methionine solutions, and filter sterilize as appropriate.

Note: Antibiotics stock solutions and other supplements prepared in distilled/deionised water must be sterilized using a 0.2 µm bacterial filter. Alternatively, add the antibiotic powder to autoclaved distilled/deionised water. Solutions prepared in 70% (v/v) ethanol do not need sterilization.

6. Allow medium to cool to approximately 50 °C prior to adding antibiotics, pyridoxine-HCl and D-methionine solutions.
7. Mix the molten medium thoroughly but gently to avoid air bubbles and pour plates (18 mL per 90 mm plate).
8. Leave plates to cool down and dry under sterile conditions.

Note: Storage conditions and duration may affect antibiotic activity, which can influence the performance of the test. Depending on the source of starch, pre-storage of plates in the refrigerator (4 °C) for at least four days before use may result in more easily visible zones of starch hydrolysis.

Table II.4. 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

Preparation

1. Weigh all ingredients and put them into a suitable oversized container (e.g., 250 mL of medium in a 500 mL bottle/flask) to allow swirling of the medium just before pouring.
2. Dissolve in 1 L of distilled/deionised water by steaming the mix.
3. Autoclave at 15 psi for 15 min at 121 °C and allow the medium to cool to approximately 50 °C.
4. Swirl the bottle/flask to ensure an even distribution of $CaCO_3$ and avoid air bubbles. Pour 22 mL on to each 90 mm plate.
5. Leave plates to cool down and dry under sterile conditions.

1. Dilution and plating

1.1. Perform the seed extraction as described in Section I.1.

1.2. Prepare two 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 give a 10^{-1} dilution and ii.) Pipetting 0.5 mL of the 10^{-1} dilution into 4.5 mL of sterile NaCl to give a 10^{-2} dilution. Vortex all dilutions well.

1.3. Pipette 100 µL of each dilution and the undiluted seed extract onto plates of the mFS and mCS20ABN selective media and spread over the surface.

1.4. Incubate plates at 28–30 °C and examine after 3–4 days.

2. Positive control (PC) - culture or reference material

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

Note: Xcc and Xcr cannot be distinguished by their colony morphology.

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

2.3. Pipette 100 µL of appropriate dilutions onto plates of both semi-selective media (mFS, mCS20ABN) and spread over the surface.

2.4. Incubate plates with the sample plates (as in Section 1.4).

3. Negative buffer control (NBC) – sterility check

3.1. Pipette 100 µL of the extraction buffer (NaCl plus Tween™ 20) and dilution buffer (NaCl) onto plates of both semi-selective media (mFS and mCS20ABN) and spread over the surface.

3.2. Incubate plates with the sample (as in Section 1.4).

4. Examination of the plates

4.1. Examine the NBC and PC plates. There should be no growth on the NBC plates . For the PC plates, the number of bacteria on the dilution plates should be consistent with the dilution, i.e., it should decrease approximately ten-fold with each dilution.

4.2. Examine the sample plates for the presence of typical Xcc/Xcr colonies by comparing them with the PC plates. Dilution plates, prepared from the PC isolates or reference material, should give single colonies with typical morphology. The numbers of colonies on dilution plates prepared from the PC isolates or reference material should be similar on both media.

4.3. After 3–4 days Xcc/Xcr colonies on mFS are small, pale green, mucoid and surrounded by a zone of starch hydrolysis. This zone appears as a halo that may be easier to evaluate with a black background (Figure II.1a). Colonies may show marked variation in size and may be visible on mFS after three days; if not, incubate for an additional day.

4.4. After 3–4 days, Xcc/Xcr colonies on mCS20ABN are pale yellow, mucoid and surrounded by a zone of starch hydrolysis (Figure II.1b). Colonies may show marked variation in size. Depending on the number of colonies present, it may be easier to evaluate plates after three days, before coalescence of starch hydrolysis zones that can make identification of suspect colonies more difficult.

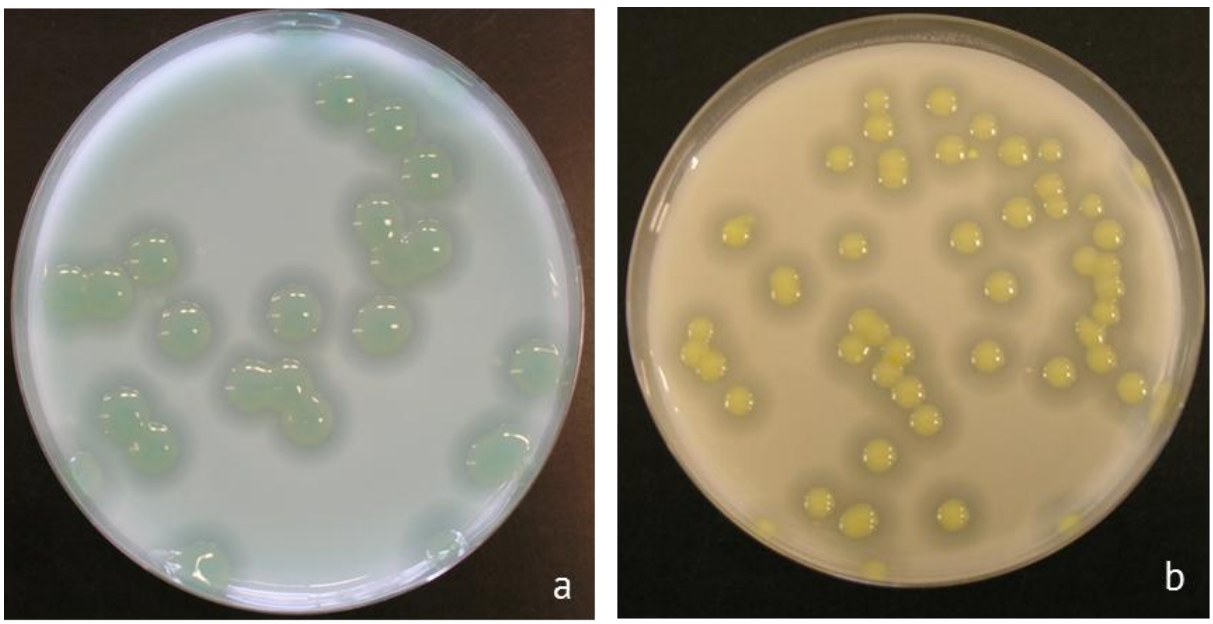

Figure II.1. Plates of mFS (a) and mCS20ABN (b) after five days of incubation at 28 °C showing typical colonies of Xcc surrounded by zones of starch hydrolysis.

4.5. Incubation of the plates at 4 °C for several hours before recording may result in sharper zones of starch hydrolysis with some starch sources.

4.6. Verify that the plates are readable, according to the Best Practices for Dilution Plating Assays in Seed Health Tests, and record the presence of suspect colonies.

Note: Test results are only valid when all included controls presented in Table II.1 give the expected results.

5. Confirmation identification of suspect colonies

5.1. Subculture suspect colonies to sectored plates of YDC. To avoid the potential for cross-contamination of isolates, use a new sectored plate for each subsample. The precise numbers of colonies subcultured will depend on the number and variability of suspect colonies on the plate: if present, at least six colonies should be subcultured per subsample.

5.2. Subculture the PC isolate to a sectored plate for comparison.

5.3. Incubate sectored plates for 24–48 hours at 28–30 °C.

5.4. Compare the appearance of growth with the PC. On YDC, Xcc/Xcr colonies are pale yellow and mucoid/fluidal (Figure II.2). The PC isolate(s) or reference material should give colonies with typical morphology on YDC.

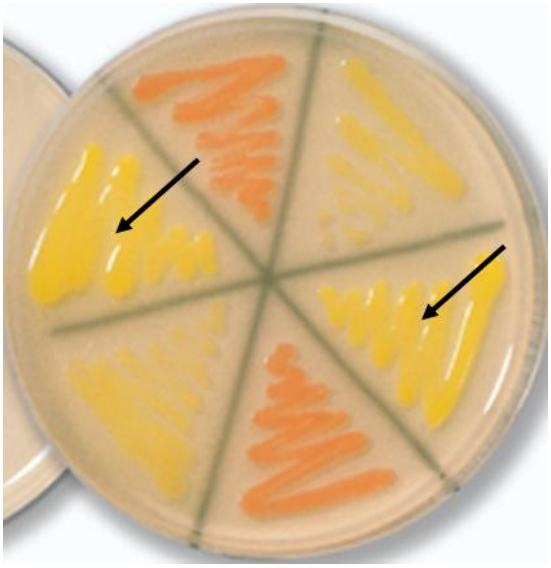

Figure II.2. Typical yellow mucoid growth of isolates of Xcc on a sectored plate of YDC after three days at 28 °C. Suspect cultures are indicated by arrows.

5.5. The identity of the isolates can be confirmed by the qPCR. The pathogenicity of the isolates should be confirmed using known susceptible *Brassica* seedlings by 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) and to test the pathogenicity of all *Xanthomonas*-like subcultured isolates by a pathogenicity or qPCR assay.

5.6. Record results for each colony subcultured.

III. SUSPECT COLONY IDENTIFICATION BY qPCR (OPTIONAL STEP)

For PCR methods, in-house method optimization is often necessary, see Best Practices for PCR Assays in Seed Health Tests for information.

Materials

- Controls (Table III.1)
- qPCR mix, primers (Table I.1 and III.2) and equipment
- NaCl (0.15M) (Table I.3)
- NaOH (0.5M) (Table III.3)
- Tris-HCl (1 M), pH 8.0
- Centrifuge
- Optical Density (OD) meter

Table III.1. Types of controls used.

Control type	Description
Positive process control (PPC)	Freshly prepared suspension of Xcc *and*
	Freshly prepared suspension of Xcr
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 III.2. Primer-sequences and references.

Name	Target	Sequence (5'– 3')	Source
Wu-F	Bacterial 16S rRNA	CAA CGC GAA GAA CCT TAC C	Wu *et al.*, 2008
Wu-R		ACG TCA TCC CCA CCT TCC	
Wu-Pr1		TEXAS RED – ACG ACA ACC ATG CAC CAC CTG – BHQ2	
Wu-Pr2		TEXAS RED – ACG ACA GCC ATG CAG CAC CT – BHQ2	

Table III.3. NaOH (0.5 M).

Compound	Amount/L
NaOH	20 g

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 (OD_{600} ~ 0.05) in 1.0 mL of sterile NaCl from the suspected cultures on YDC medium and the positive controls (Table III.1). In addition, a non-suspect isolate should be used as a negative process control (NPC).

1.2. Centrifuge bacterial suspensions at 8,000 rpm for 5 min at room temperature. Discard the supernatant and resuspend the pellet with 500 µL of 0.5 M NaOH.

1.3. Incubate for 10 min at 100 °C.

1.4. Suspensions can be stored at −20 °C until qPCR.

2. qPCR

2.1. Prepare the reaction mixture (Table III.4 and III.5 for duplex reactions). Use the Xcc specific primer sets from Köhl *et al.* (2011) and from Berg *et al.* (2006) (Table I.1). and the universal primer set from Wu *et al.* (2008) (Table III.2) to validate the PCR reactions.

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

Table III.4. PCR mix qPCR Berg/Wu duplex.

Component	Per reaction (in µL)	Final concentration
DLH153 (10 µM)	1.25	0.5 µM
DLH154 (10 µM)	1.25	0.5 µM
P7 (10 µM, FAM)	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 (TexRed, 10 µM)	0.50	0.2 µM
Wu-Pr2 (TexRed, 10 µM)	0.50	0.2 µM
PerfeCTa Multiplex qPCR ToughMix (5×)	5.00	1×
PCR grade water	10.00	
Template DNA	5.00	
Total	**25.00**	

Table III.5. PCR mix qPCR Köhl/Wu duplex.

Component	Per reaction (in µL)	Final concentration
XCC-F (10 µM)	0.5	0.2 µM
XCC-R (10 µM)	0.5	0.2 µM
XCC-Pr (10 µM, Vic)	0.5	0.2 µM
Wu-F (10 µM)	0.5	0.2 µM
Wu-R (10 µM)	0.5	0.2 µM
Wu-Pr1 (TexRed, 10 µM)	0.5	0.2 µM
Wu-Pr2 (TexRed, 10 µM)	0.5	0.2 µM
PerfeCTa Multiplex qPCR ToughMix (5×)	5.0	1×
PCR grade water	11.5	
Template DNA	5.0	
Total	**25.0**	

2.2. Perform the PCR reactions in a real-time PCR instrument according to the prescribed PCR conditions (Table III.6).

Note: If different PCR mixtures and amplification programs are used, it is necessary to verify their performance. Validation studies showed that PCR results were more dependent on laboratory conditions than on the PCR protocol itself when different PCR mixes, and amplification products were used in laboratories.

Table III.6. PCR conditions qPCR.

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

2.5. Cut-off values must be established by each laboratory for their positive and internal amplification controls prior to the assay being used on routine samples. For recommendations on setting cut-off values, see Real-time PCR, an 'indirect' test used for pre-screening in seed health methods. The Cq values of positive controls should consistently be lower than 30. 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 be at least 3.3 Cq value lower than the IAC Cq values from the NTC reactions.

5. Interpretation and decisions

For interpretation and decision making, the results from both primer sets need to be considered, as described in Table III.7. Test results are only valid when all included controls presented in Table III.1 give the expected results.

Table III.7. Interpretation and decision table for the qPCR.

Köhl qPCR	Berg qPCR	qPCR Result	Follow-up
Positive	Positive	Target DNA for Xcc detected[a]	Pathogenicity test for confirmation
Negative[a]	Positive	Target DNA for Xcr detected	Pathogenicity test for confirmation
Positive	Negative	Inconclusive	Pathogenicity test for confirmation
Negative	Negative	No target DNA for Xcc/Xcr detected	Negative, no follow up

[a] Some Köhl qPCR positive Xcr isolates are known (ISHI, internal communication).

IV. CONFIRMATION BY PATHOGENICITY ASSAY

Materials

- Brassica seedlings: susceptible to all races of the pathogen (e.g., *B. oleracea* 'Wirosa')
- Sterile cocktail sticks (toothpicks) or insect pins
- Controls (Table IV.1)

Table IV.1. Types of controls used.

Control type	Description
Positive control (PC)	A known strain of Xcc *and*
	A known strain of Xcr
Negative buffer control (NBC)	Sterile cocktail stick or insect pin

1. Pathogenicity assay

1.1. Grow seedlings of a *Brassica* cultivar known to be susceptible to all races of Xcc (e.g., cabbage 'Wirosa', see Vicente *et al.,* 2001) in small pots or modules until at least 3–4 true leaf stage.

1.2. Scrape a small amount of bacterial growth directly from a 24–48 hours YDC culture (e.g., sectored plate) with a sterile cocktail stick or insect pin.

1.3. Inoculate six of the major veins at a point near the leaf edges on the two youngest leaves by stabbing with the cocktail stick or insect pin.

Note: The number of plants which should be inoculated will depend on the variability of the cultivar and experience of the operator, but one to three plants per isolate should usually be sufficient. It is better to inoculate more isolates with one plant per isolate than fewer isolates with three plants per isolate.

1.4. Inoculate with the positive control (PC) isolate and stab with a sterile cocktail stick or insect pin as a negative buffer control (NBC). PC isolates as well as a NBC should be included in every pathogenicity test (Table IV.1).

1.5. Grow on plants at 20–25 °C.

1.6. Examine plants after 10–14 days for the appearance of typical progressive V-shaped, yellow/necrotic lesions with blackened veins caused by the vascular pathogen Xcc (Figure IV.1a) and for the appearance of circular dark spots that become lighter and are occasionally surrounded by a chlorotic halo for the detection of Xcr (Figure IV.1b). Symptoms may be visible earlier depending on temperature and the aggressiveness of the isolate. Compare against positive controls.

Note: Test results are only valid when all included controls give the expected results. The positive control isolate should give typical symptoms in the pathogenicity test. It is important to discriminate between the progressive lesions caused by Xcc and the limited dark necrotic lesions at the inoculation site caused by leaf spot Xcr (See Figure IV.1b; Kamoun *et al.,* 1992; Alvarez *et al.,* 1994; Tamura *et al.,* 1994; Vicente *et al.,* 2001; Roberts *et al.,* 2004).

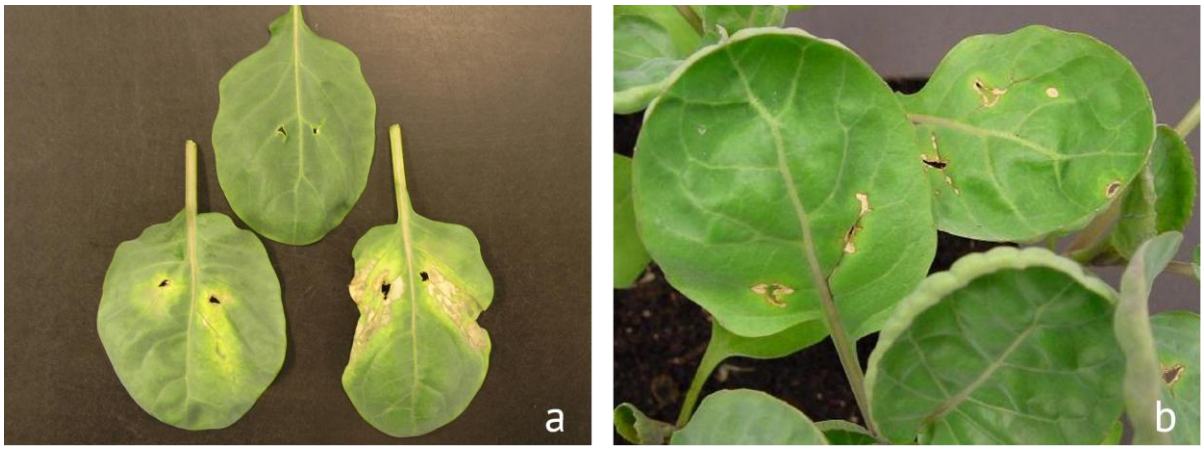

Figure IV.1. Cabbage leaves seven days post-inoculation with Xcc (a) and Xcr (b). a. The top leaf was used as a negative buffer control, typical symptoms of Xcc are black veins, wilting and chlorosis and typical symptoms of Xcr are circular dark spots that become lighter and are occasionally surrounded by a chlorotic halo.

REFERENCES

Alvarez, A. M., Benedict, A. A., Mizumoto, C. Y., Hunter, J. E. and Gabriel, D. W. (1994). Serological, pathological, and genetic diversity among strains of *Xanthomonas campestris* infecting crucifers. *Phytopathology*, **84**, 1449–1457.

Barnhoorn, R. (2018). Validation study to add TaqMan PCR as an option to the conventional PCR test for the identification of *Xanthomonas campestris* pv. *campestris* and pv. *raphani* in *Brassica* seed supporting the proposal C.7.1 to Modify Seed Health Method 7-019a. Method Validation Reports on Rules Proposals for the International Rules for Seed Testing 2019 Edition. International Seed Testing Association (ISTA), Bassersdorf, Switzerland.

Berg, T., Tesoriero, L. and Hailstones, D. L. (2006). A multiplex real-time PCR assay for detection of *Xanthomonas campestris* from brassicas. *Letters in Applied Microbiology*, **42**, 624–630.

Bruinsma, M., Baldwin, T. and Ponzio, C. (2018). Validation study to use bio-PCR and seed extract PCR for pre-screening in the detection of *Xanthomonas campestris* pv. *campestris* and pv. *raphani* in *Brassica* seed; supporting the proposal C.7.1 to modify Seed Health Method 7-019a. Method Validation Reports on Rules Proposals for the International Rules for Seed Testing 2019 Edition. International Seed Testing Association (ISTA), Bassersdorf, Switzerland.

Kamoun, S., Kamdar, H. V., Tola, E. and Kado, C. I. (1992). Incompatible interactions between crucifers and *Xanthomonas campestris* involve a vascular hypersensitive response: role of the *hrpX* locus. *Molecular Plant- Microbe Interactions,* **5**, 22–33.

Koenraadt, H., van Bilsen, J. G. P. M. and Roberts, S. J. (2005). Comparative test of four semi-selective agar media for the detection of *Xanthomonas campestris* pv. *campestris* in brassica seeds. *Seed Science and Technology*, **33**, 115–125.

Koenraadt, H., van Vliet, A., Jodlowska, A., Woudt, B., Ebskamp, M. and Bruinsma, M. (2014). Detection of *Acidovorax citrulli* in seed extracts of melon and watermelon with TaqMan PCR. 7th ISTA Seed Health Symposium. Edinburgh, United Kingdom, 12–14 June 2014.

Köhl, J., Vlaswinkel, M., Groenenboom-de Haas, B. H., Kastelein, P., van Hoof, R. A., van der Wolf, J. M. and Krijger, M. (2011). Survival of pathogens of Brussels sprouts (*Brassica oleracea* Gemmifera Group) in crop residues. *Plant Pathology*, **60**, 661–670.

Roberts, S. J., Brough, J., Everett, B. and Redstone, S. (2004). Extraction methods for *Xanthomonas campestris* pv. *campestris* from *Brassica* seed. *Seed Science and Technology*, **32**, 439–453.

Sato, M., Asma, M. and Politikou, L. (2014). Proposal for replacement of mCS20ABN and FS media recipes in ISTA Tule 7-019a (*Xanthomonas campestris* pv. *campestris* detection in *Brassica* spp. seed lots) by adapted versions. Method Validation Reports on Rules Proposals for the International Rules for Seed Testing 2015 Edition. International Seed Testing Association (ISTA), Bassersdorf, Switzerland.

Schaad, N. W. (1989). Detection of *Xanthomonas campestris* pv. *campestris* in Crucifers. In *Detection of bacteria in seeds and other planting material* (eds. A. W. Saettler, N. W. Schaad & D. A. Roth), pp. 68– 75. American Phytopathological Society, St. Paul, Minnesota, USA.

Sudarshana, P. (2010). ISHI *Aac* Primers Evaluation. ISHI-Veg meeting, July 2010.

Tamura, K., Takikawa, Y., Tsuyumu, S. and Goto, M. (1994). Bacterial spot of crucifers caused by *Xanthomonas campestris* pv. *raphani*. *Annals of the Phytopathological Society of Japan,* **60**, 281–287.

Thomas, L. A. and Woudenberg, J. H. C. (2026). Detection of *Xanthomonas campestris* pv. *campestris* and *Xanthomonas campestris* pv. *raphani* in *Brassica* spp. seed. Addendum Validation report, International Seed Federation (ISF), Nyon, Switzerland. https://www.worldseed.org/our-work/seed-health/ishi-method-development-and-validation/

Vicente, J. G., Conway, J., Roberts, S. J. and Taylor, J. D. (2001). Identification and origin of *Xanthomonas campestris* pv. *campestris* races and related pathovars. *Phytopathology,* **91**, 492–499.

Wilson, E. E., Zeitoun, F. M. and Fredrickson, D. L. (1967). Bacterial phloem canker, a new disease of Persian walnut trees. *Phytopathology*, **57**, 618–621.

Wu, Y-D., Chen, L-H., Wu, X-L., Shang, S-Q., Lou, J-T., Du, L-Z. and Zhao, Z-Y. (2008). Gram stain-specific probe-based real-time PCR for diagnosis and discrimination of bacterial neonatal sepsis. *Journal of Clinical Microbiology*, **46**, 2613–2619.