International Seed Federation
Seed is Life

Detection of *Candidatus* Liberibacter solanacearum in Carrot Seed

DECEMBER 2023

Developed by ISHI

Detection of *Candidatus* Liberibacter solanacearum in Carrot Seed

Crop: *Daucus carota* (carrot)

Pathogen(s): *Candidatus* Liberibacter solanacearum

Version: 2.2 (December 2023)

BACKGROUND

In ISHI's view, there is a lack of evidence for seed transmission of *Candidatus* Liberibacter solanacearum (*Ca.* L. solanacearum) via carrot seed. Seed transmission has been reported in a single publication (Bertolini *et al.*, 2015). Several other studies have challenged this finding (Haapalainen, 2014; Loiseau *et al.*, 2017a and 2017b; Oishi *et al.*, 2017; Mawassi *et al.*, 2018; Fujikawa *et al.*, 2020).

As a consequence of the report by Bertolini *et al.* (2015), several governments started to impose emergency measures and import restrictions on seed of carrot and other *Apiaceae* species. Lately, however, New Zealand's national plant protection organisation reported that its full technical assessment found no new scientific evidence to support the finding by Bertolini *et al.* (2015), that *Ca.* L. solanacearum is seed transmitted on the *Apiaceae* family. As a result, it no longer considers *Apiaceae* (i.e., carrot, celery, chervil, fennel, parsley and parsnip) seed to be a risk pathway for the introduction of *Ca.* L. solanacearum. As a trade-enabling measure New Zealand removed the *Apiaceae* specific requirements from its Import Health Standard (see https://www.mpi.govt.nz/dmsdocument/1151/direct).

To facilitate trade of carrot seed, ISHI has developed a detection method based on real-time PCR, a requirement from some governments to obtain a Phytosanitary Certificate for exporting seed. ISHI calls on researchers seeking to develop detection methods for *Ca.* L. solanacearum to use the primer sets used in this method to harmonise methods used for phytosanitary certification. These primers and probes have been developed by Li *et al.* (2009) and are also recommended in EPPO's Diagnostic Standard PM 7/143 (1) for the detection of *Ca.* L. solanacearum in both asymptomatic and symptomatic plant material or in vectors (see https://onlinelibrary.wiley.com/doi/epdf/10.1111/epp.12611).

According to ISPM 38, *molecular and serological diagnostic methods are considered indirect protocols to detect pests in seeds. These methods may give a positive result even when no viable pests are present. Consequently, when testing seeds with these methods, results should be interpreted carefully. Confirmatory tests or additional tests based on a different biological principle may be required to confirm the presence of a viable pest in a sample* (see https://www.ippc.int/en/publications/84340/.

A qPCR assay, an indirect test, detects both infectious and non-infectious bacterial particles as well as DNA from viable and non-viable bacteria (see http://www.worldseed.org/wp-content/uploads/2015/10/Indirect_Seed_Health_Tests_2013.pdf). A positive SE-qPCR is, therefore, only indicative of a suspected lot. A confirmatory test that determines the presence of

viable and pathogenic *Ca.* L. solanacearum is needed to confirm the health status of the lot. Such a confirmation test, usually a pathogenicity or bioassay, is currently not available for *Ca.* L. solanacearum due to the uncultivable nature of this pathogen. Furthermore, as efforts to show seed transmission have not been successful, it is not possible to develop a direct test that confirms viability of the pathogen and its pathogenicity.

The qPCR has been designed to identify seed lots that are *not* infected with the target pathogen and validated to show there are no false negative results obtained.

PRINCIPLE

Detection of *Ca.* L. solanacearum on carrot seed is assessed by a seed extract qPCR assay (SE-qPCR). Extraction of bacteria located in the phloem sieve tubes of the carrot seed coat is enhanced by stomaching. The test is complete if no *Ca.* L. solanacearum is detected and the seed lot is considered healthy. A positive SE-qPCR is only indicative of the presence of DNA sequence of *Ca.* L. solanacearum and gives no information on viability and pathogenicity.

The method process workflow is presented in Figure 1.

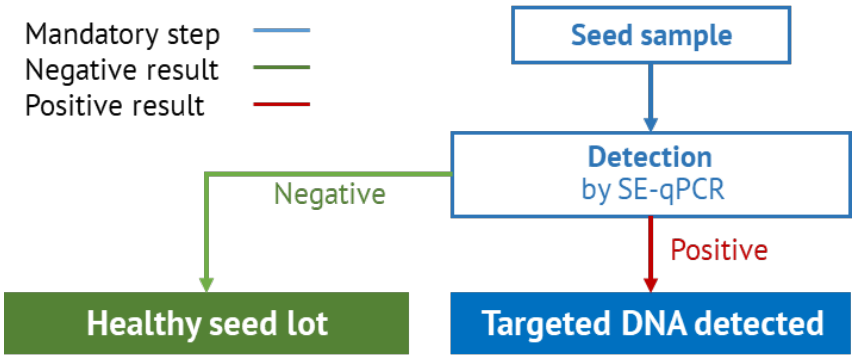

Figure 1. Method process workflow.

METHOD VALIDATION

This method has been validated by ISHI (Chitrampalam *et al.*, 2020). The validation included an inter-laboratory comparative test. The performance of the two main components of the ISHI assay, namely the Qiagen DNA extraction and the Li *et al.* (2009) real-time PCR method, has also been validated independently of ISHI for analytical specificity, sensitivity and repeatability (Loiseau *et al.*, 2015).

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 report.

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.

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 20,000 seeds with a maximum subsample size of 10,000 seeds.

NOTE: ISHI is not aware of any epidemiological information that warrants seed samples of any particular size.

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1.0	January 2015	First version of the protocol.
1.1	July 2016	Additional information from new publications added, including references to the new publications. The use of PMA has been deleted as its efficacy is less than 100%. Positive PCR results can be obtained due to incomplete removal of DNA from dead bacteria.
2.0	November 2019	An internal amplification control is added to the SE-qPCR.
2.1	June 2020	Background section updated to latest information.
2.2	December 2023	Protocol updated in accordance with ISHI protocol guidelines.

Protocol for detecting *Candidatus* Liberibacter solanacearum in Carrot Seed

I. DETECTION OF TARGET DNA BY SEED-EXTRACT qPCR

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

Materials

- Stomacher and stomacher bags
- DNA isolation kit e.g., Qiagen DNeasy Plant Mini kit
- qPCR mix, primers (Table I.1) and PCR equipment
- Controls (Table I.2)
- PBS buffer (Table I.3)
- Centrifuge

Table I.1. Primer sequences and references.

Name	Sequence (5' – 3')	Source
Lso-F	GTC GAG CGC TTA TTT TTA ATA GGA'	Li *et al.*, 2006, 2009
HLBr	GCG TTA TCC CGT AGA AAA AGG TAG	
HLBp	FAM – AGA CGG GTG AGT AAC GCG – BHQ1	
Acat2-F	TGT AGC GAT CCT TCA CAA G	Koenraadt *et al.*, 2014
Acat2-R	TGT CGA TAG ATG CTC ACA AT	
Acat1-Pr	HEX – CTT GCT CTG CTT CTC TAT CAC G – BHQ2	

Table I.2. Types of controls used.

Control type	Description
Positive extraction control (PEC)	*Acidovorax cattleyae* spike
Negative extraction control (NEC)	PBS buffer
Internal amplification control (IAC)	*Acidovorax cattleyae* spike
Positive process control (PPC)	Seeds infected with *Ca.* L. solanacearum, or healthy seed lots spiked with *Ca.* L. solanacearum extracts
Negative process control (NPC)	Healthy seeds (*Ca.* L. solanacearum negative seeds)
Positive amplification control 1 (PAC1)	*Ca.* L. solanacearum DNA
Positive amplification control 2 (PAC2)	*Acidovorax cattleyae* DNA
Non template control (NTC)	Nucleic acid free water

Table I.3. Phosphate buffered saline (PBS) buffer.

Compound	Amount/L
NaCl	8.00 g
KH_2PO_4	0.24 g
Na_2HPO_4	1.44 g
KCl	0.20 g

1. Extraction of bacteria from seeds

1.1. Add the subsamples, the positive process control (PPC) and the negative process control (NPC) to sterile stomacher bags with 100 mL sterile PBS buffer (ratio of 100 mL buffer/10,000 seeds).

1.2. As negative extraction control (NEC), include a sterile stomacher bag with 100 mL sterile PBS buffer only (without seeds).

1.3. As positive extraction control (PEC) and internal amplification control (IAC), add 100 µL of Acat spike (OD600=0.6) to each of the subsamples, the PPC, the NPC and the NEC.

1.4. Macerate all samples and controls (including NEC without seeds) in a stomacher machine until all the seeds are crushed.

Note: With InterScience Bag mixer good results were obtained by stomaching for 4 min at speed 3 and gap 5.

1.5. Pipet 2 mL of the extract from the filtered side of the stomacher bag into a 2 mL tube.

2. DNA extraction

2.1. Centrifuge the 2 mL filtered extract to pellet large seed debris (500 × *g*, 1 min) and transfer 1 mL of the supernatant to a new 1.5 mL micro-centrifuge tube.

2.2. Centrifuge at 10,000 × *g* for 5 min to pellet the bacteria. Remove the supernatant and continue the DNA extraction with the Qiagen DNeasy kit on the pellet in each tube.

2.3. Check that the salts in the Qiagen DNeasy Plant Mini Kit are fully suspended in the AP1 buffer, if necessary, place at 65 °C, until the salts are dissolved.

2.4. Add 400 µL Buffer AP1 and 4 µL RNase-A stock solution (100 mg/mL) to the pellet. Re-suspend the pellet, vortex and incubate at 65 °C for 10 min.

2.5. Add 130 µL Buffer P3 to the lysate, mix, and incubate for 5 min on ice or at +4 °C.

2.6. Centrifuge the lysate at 20,000 × *g* for 5 min.

2.7. Pipet the lysate into the QIA shredder Mini spin column (lilac) placed in a 2 mL collection tube, and centrifuge at 20,000 × *g* for 2 min.

2.8. Transfer the flow-through into a new 1.5 mL tube without disturbing the cell-debris pellet.

2.9. Add 1.5 volumes of Buffer AW1 to the cleared lysate and mix by pipetting.

2.10. Pipet 650 µL of the mixture, including any precipitate that may have formed, into the DNeasy Mini spin column (white) placed in a 2 mL collection tube and centrifuge at 6,000 × *g* for 1 min and discard the flow-through.

2.11. Add 500 µL Buffer AW2 and centrifuge at 6,000 × *g* for 1 min and discard the flow-through.

2.12. Repeat the wash step by adding 500 µL Buffer AW2 to the DNeasy Mini spin column, and centrifuge at 20,000 × *g* for 2 min to dry the membrane.

2.13. Elute the extracted and purified DNA by adding 100 µL AE Buffer, incubate at room temperature for 5 min and centrifuge into a clean 1.5 mL tube at 6,000 × *g* for 1 min.

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

3. qPCR

Note: Work on ice whenever possible and minimize probes exposure to light.

3.1. Test the subsample extracts, NTC, PPC, NPC, NEC, PAC1 and PAC2 with the duplex Lso-Acat qPCR. Table I.4 provides an example for the reaction mixtures. However, reaction mixture and conditions need to be checked and/or optimized within each laboratory.

3.2. Add *in duplicate* the subsample extracts, NTC, PPC, NPC, NEC, PAC1 and PAC2 to the PCR reaction mixture in a suitable PCR reaction tube or plate.

3.3. Run the assay based on the conditions described in Table I.5 for the duplex qPCR.

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

Table I.4. Example qPCR mix for the duplex Lso-Acat qPCR.

Component	Per reaction (in µL)	Final concentration
PCR grade H_2O	4.51	
qPCR Master Mix (2×)[a]	7.50	1×
Lso-F (25 µM)	0.18	0.3 µM
HLBr (25 µM)	0.18	0.3 µM
HLBp (25 µM)	0.12	0.2 µM
Acat2-F (25 µM)	0.18	0.3 µM
Acat2-R (25 µM)	0.18	0.3 µM
Acat1-Pr (10 µM)	0.15	0.1 µM
Sample	2.00	
Total	**15.00**	

[a] TaqMan™ Universal Master Mix II (Applied Biosystems) for example.

Table I.5. PCR conditions for the duplex Lso-Acat qPCR.

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

4. Interpretation and decisions

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 cut-off for the IAC *Acidovorax cattleyae* (Acat*)* is set preferably below Cq 35. A cut-off value of Cq 40 for the Lso qPCR was used for the validation study. See Table I.6 for the interpretation of the qPCR results.

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 duplex Lso-Acat qPCR.

Lso qPCR	Acat qPCR (IAC)	qPCR result
Cq < 40	Not relevant	Target DNA for *Ca.* L. solanacearum detected
Cq ⩾ 40	Cq ⩾ 35	Inconclusive, repeat PCR
Cq ⩾ 40	Cq < 35	No target DNA for *Ca.* L. solanacearum detected

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