International Seed Federation
Seed is Life

Detection of *Candidatus* Liberibacter solanacearum in Carrot Seed

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Developed by ISHI-Veg

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Crop: *Daucus carota* (carrot)

Pathogen(s): *Candidatus* Liberibacter solanacearum

Revision history: Version 2.1, June 2020
Note: In this version the section Background has been brought up to date

BACKGROUND

In ISHI-Veg'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., several governments imposed emergency measures and import restrictions on carrot and other *Apiaceae* seed. 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., 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 introduction of *Ca*. L. solanacearum. As a trade-enabling measure the World Trade Organisation has been notified of New Zealand's intention to remove 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-Veg has developed a detection method based on real-time PCR, a requirement from some governments to obtain a Phytosanitary Certificate for exporting seed. ISHI-Veg 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 (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/static/media/files/publication/en/2017/05/ISPM_38_2017_En_2017-05-15.pdf.

A qPCR, 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 presumptive positive 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 bio assay, is currently not available for *Ca*. L. solanacearum due to 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.

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.

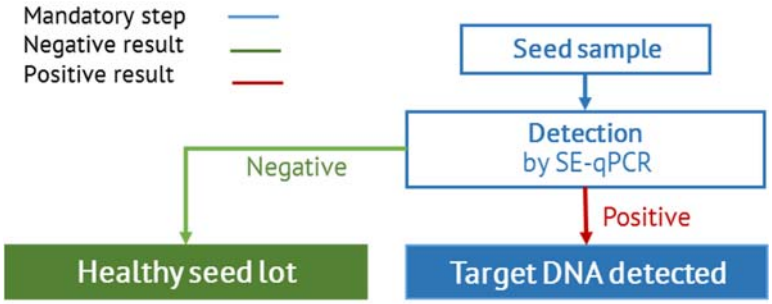

Figure 1: Method process workflow

METHOD VALIDATION

This method has been validated by ISHI-Veg (Chitrampalam et al., 2019). The validation included an inter-laboratory comparative test. The performance of the two main components of the ISHI-Veg assay, namely the Qiagen DNA extraction and the Li et al. (2009) real-time PCR method, has also been validated independently of ISHI-Veg for analytical specificity, sensitivity and repeatability (Loiseau et al., 2015).

RESTRICTIONS ON USE

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 developed by ISHI-Veg for the use of PCR Assays 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 recommended minimum sample size is 20,000 seeds with a maximum sub-sample size of 10,000 seeds.

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

Protocol for detecting *Candidatus* Liberibacter solanacearum in Carrot Seed

SEED-EXTRACT qPCR

Material

Stomacher and stomacher bags	PBS buffer (Table 1)
DNA isolation kit (Qiagen DNeasy Plant Mini kit)	Controls (Table 2)
TaqMan PCR mix, primers (Table 3) and PCR equipment	Centrifuge

Table 1: Phospate 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

Table 2. Controls

Control type	
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 3. Primer sequences and references

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

1. Extraction of bacteria from seeds

1.1. Add the sub-samples, 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 a Negative Extraction Control (NEC) include a sterile stomacher bag with 100 mL sterile PBS buffer only (without seeds)

1.3. As a Positive Extraction control (PEC) and Internal Amplification Control (IAC) add 100 µL of Acat spike (OD600=0.6) to each of the sub-samples, 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 RCF, 1 min) and transfer 1 mL of the supernatant to a new 1.5 mL micro-centrifuge tube.

2.2. Centrifuge at 10,000 RCF 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 dissolved 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 for 5 min at 20,000 RCF.

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

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 for 1 min at 6,000 RCF and discard the flow-through.

2.11. Add 500 µL Buffer AW2 and centrifuge for 1 min at 6,000 RCF 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 for 2 min at 20,000 RCF 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 RCF for 1 min.

Note: The assay has been validated with the Qiagen DNeasy Plant kit. It is the responsibility of the user to check with an in-lab validation study if the sensitivity and selectivity of the assay is not influenced when using a different DNA isolation kit.

3. TaqMan PCR

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

3.1. Test the sub-sample extracts, NTC, PPC, NPC, NEC, PAC1 and PAC2 with the duplex Lso-Acat TaqMan PCR. Table 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 sub-sample 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 5 for the duplex TaqMan.

Table 4. Example PCR mix for the duplex Lso-Acat TaqMan

Component	For 1 reaction (in µL)	Final Concentration
PCR grade H_2O	4.51	
TaqMan Master Mix [1] (2x)	7.50	1x
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**	

[1] Applied TaqMan Universal Mastermix II for example

Table 5. PCR conditions for the duplex Lso-Acat TaqMan

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 need to be determined individually by each laboratory. 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 TaqMan PCR was used for the validation study. Test results are only valid when all included controls presented in Table 2 give the expected result.

Table 6. Interpretation and decision table duplex Lso-Acat TaqMan

Lso TaqMan	Acat TaqMan (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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