Detection of *Candidatus* Liberibacter solanacearum on carrot seeds

Crop: Carrot (*Daucus carota*)

Pathogen: *Candidatus* Liberibacter solanacearum (*Ca* L. solanacearum)

Revision history: Version 1.1, March 2017

Note

In ISHI-Veg's view there is a lack of evidence for seed transmission of *Candidatus* Liberibacter solanacearum. The only evidence for seed transmission was observed in one trial which was reported in a single publication (Bertolini *et al*., 2014). Furthermore, a total absence of transmission by carrot seed was observed in experiments designed to reproduce these results (Loiseau *et al*., 2015a).

However, as some governments have imposed emergency measures and import restrictions on carrot seed a protocol is presented below.

Sample and sub-sample size

Sample size: 20,000 seeds

Sub-sample size: 10,000 seeds

NOTE: Bertolini *et al*. (2014) reported detection of *Ca.* L. solanacearum in a sample of 500 seeds and this sample size was used for the survey of commercial seed lots in their publication. Current emergency quarantine measures in Australia for carrot seed importation require a heat treatment of seeds or a PCR test on 20,000 seeds. The draft EPPO protocol recommends a sample size of 20 gr or 10,000 seeds. ISHI-Veg is not aware of any epidemiological information that warrants any sample sizes including 500, 10,000, or 20,000 seeds.

Principle

- Extraction from the seed of bacteria located in the phloem sieve tubes of the carrot seed coat
- DNA extraction of bacteria located in the filtered extract
- Detection of *Ca* L. solanacearum by real-time PCR with *Candidatus* Liberibacter solanacearum specific hydrolysis probe

Restrictions on use

- 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

Note: In this version, additional information based on new publications has been added to the sections Note and Validation. The two new publications have been added to the references.

The use of PMA in the protocol has been deleted, as the efficacy of PMA treatment is less than 100% and some positive PCR results can be obtained in the absence of viable cells due to incomplete removal of DNA from dead bacteria.

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.

Interpretation of the results

Several PCR-protocols use a Ct cut-off value of 45 for the Teresani primers (Teresani *et al*., 2014) and of 40 for the Li primers (Li *et al*., 2009). Teresani *et al*. (2014) showed the detection limits of both assays to be equivalent and to be below a CT-value of 35.

As far as ISHI-Veg is aware there is no data supporting the detection of "biologically relevant" *Ca*. L. solanacearum when using a Ct cut-off value between 35 and 45.

Validation

This method has not yet been validated in an inter-laboratory comparative test. However, the performance of the two main components of the ISHI-Veg assay, namely the Qiagen DNA extraction and the Li *et al*. real-time PCR method, has been validated independently of ISHI-Veg for analytical specificity, sensitivity and repeatability (Loiseau *et al*., 2015b).

Method Description

1. Extraction of bacteria from seeds

1.1. Put subsamples in sterile stomacher bags. Add sterile PBS buffer to the seeds in a ratio of 100ml / 10,000 seeds. Macerate in a stomacher machine until all of the seeds are crushed.

1.2. Pipette 2ml of the extract from the filtered side of the stomacher bag into one tube per sub-sample.

1.3. Include positive (seeds infected with *Ca.* L. solanacearum) and negative control samples.

2. DNA Extraction

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

2.1. Centrifuge the 2 ml filtered extract to pellet large seed debris (500 RCF, 1 minute). Transfer 1 ml of the supernatant to a new 1.5 ml microtube.

2.2. Centrifuge at 10,000 RCF for 5 minutes 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. Add 400 µl Buffer AP1 and 4 µl RNase A stock solution (100 mg/ml). Re-suspend the pellet, vortex and incubate at 65°C for 10 minutes.

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

2.5. Centrifuge the lysate for 5 min at 20,000 RCF.

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

2.7. Transfer the flow-through into a new tube without disturbing the cell-debris pellet.

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

2.9. Pipet 650 µl of the mixture, including any precipitate that may have formed, into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 minute at 6,000 RCF, and discard the flow-through.

2.10. Add 500 µl Buffer AW2, and centrifuge for 1 minute at 6,000 RCF. Discard the flow-through.

2.11. Repeat the wash step by adding 500 µl Buffer AW2 to the DNeasy Mini spin column, and centrifuge for 2 minutes at 20 000 RCF to dry the membrane.

2.12. Elute the extracted and purified DNA by adding 100µl AE Buffer, incubate at room temperature for 5 minutes and centrifuge into a clean 1.5 ml tube at 6,000 RCF for 1 minute.

3. Detection by PCR

The DNA extracts are tested in real-time PCR with the *Ca* L. solanacearum specific primers-probe described by Li *et al.* (2009). The reaction conditions described below were those used for the development and validation of the method in the Vilmorin SA laboratory.

3.1. Test the extracts with LsoF-HLBr/HLBp Taqman® based real-time PCR. A positive control DNA extract should be included as a positive PCR control. Use the following specific primers and probe (Li *et al.*, 2009)

Oligonucleotides	Sequence 5'-3'
LsoF	GTC GAG CGC TTA TTT TTA ATA GGA
HLBr	GCG TTA TCC CGT AGA AAA AGG TAG
HLBp	FAM-AGA CGG GTG AGT AAC GCG-BHQ1

3.2. Prepare the reaction mixture according to the table below.

	Volume (µL)	Concentration finale
TaqMan® Universal Master Mix II	7.5	1x
LsoF 20µM	0.3	400nM
HLBr 20µM	0.3	400nM
HLBp 5µM	0.24	80nM
Water	4.66	
DNA (extraction)	2	
Total	15	

3.3. PCR conditions

Denaturation	95°C/10min

Denaturation	95°C/15s	40 cycles
Hybridisation / Elongation	60°C/60s	

3.4. Interpretation of PCR results

The amplification curves are analyzed with a threshold fixed above the background fluorescence within the exponential amplification phase of the amplification curves. The cycle threshold (Ct) value is used to identify positive reactions. True positive reactions show a typical exponential increase in fluorescence.

PCR result	Interpretation	Conclusion
Ct ≤ 40 Positive	Positive result for *Ca.* L. solanacearum	Presence of *Ca*. L. solanacearum[1]
Ct > 40 Negative	Negative result for *Ca.* L. solanacearum	Negative

[1] The Ct cut-off value of <40 is set to identify true (PCR) positive samples. It still needs to be determined what the epidemiological relevant Ct cut-off value should be. As a cut-off value is dependent on equipment, material and chemistry it needs to be verified in each laboratory when implementing the test.

Material and buffer

Phosphate Buffered Saline

Oligonuleotides: LsoF, HLBr, HLBp

Applied Biosystems TaqMan® Universal Master Mix II

Stomacher bags, Stomacher

Pippettes, 2 ml and 1.5 ml microtubes

Real-time PCR machine (for example Qiagen Rotorgene Q)

Qiagen DNeasy Plant Mini DNA extraction kit

Phosphate buffered saline (PBS buffer) (1L)

Ingredient	Quantity for 1 l
Sodium chloride (NaCl)	8.0 g
Potassium phosphate monobasic (KH_2PO_4)	0.24 g
Sodium phosphate dibasic (Na_2HPO_4)	1.44 g
Potassium chloride (KCl)	0.2 g
Demineralised water	1 000 ml

References

Bertolini, E., Teresani, G. R., Loiseau, M., Tanaka, F. A. O., Barbé, S., Martínez, C., Gentit, P., López, M. M. and Cambra, M. 2014. Transmission of '*Candidatus* Liberibacter solanacearum' in carrot seeds. *Plant Pathology*. doi: 10.1111/ppa.12245.

Li, W., Abad, J. A., French-Monar, R. D., Rascoe, J., Wen, A., Gudmestad, N. C., Secor, G. A., Lee, I. M., Duan, Y., and Levy, L. 2009. Multiplex real-time PCR for detection, identification and quantification of '*Candidatus* Liberibacter solanacearum' in potato plants with zebra chip. *Journal of Microbiological Methods* 78:59-65.

Loiseau, M., Renaudin, I., Cousseau-Suhard, P., Lucas, P.M., and Gentit, P. 2015a. Essai de transmission de *Candidatus* Liberibacter solanacearum par la semence de carotte. Internal Report, Laboratoire de la Santé des Végétaux.

Loiseau, M., Renaudin, I., Cousseau-Suhard, P., Lucas, P.M., and Gentit, P. 2015b. Assessment of performance criteria of *Candidatus* Liberibacter solanacearum detection methods. In: Testa-EPPO Conference on diagnostics for plant pests. (http://archives.eppo.int/MEETINGS/2015_conferences/testa.htm).

Teresani, G. R., Bertolini, E., Alfaro-Fernández, A., Martínez, C., Tanaka, F. A. O., Kitajima, E. W., Roselló, M., Sanjuán, S., Ferrándiz, J. C., López, M. M., Cambra, M., and Font, M. I. 2014. Association of '*Candidatus* Liberibacter solanacearum' with a vegetative disorder of celery in Spain and development of a real-time PCR method for its detection. *Phytopathology* 104:804-811.