Detection of '*Candidatus* Liberibacter solanacearum' using real-time PCR test

1. Scope

The test is used for detection of '*Candidatus* Liberibacter solanacearum' from plant sample. The test is validated for potato tubers but it is also tested to be suitable for carrot seeds and petioles, and potato stolons.

2. Principle and process description

The method is developed at Plant Analytics Unit (Plant Pest Section, KAJA) based on the publications of Li *et al*. 2009, Teresani *et al.* 2014 and Bertolini *et al*. 2015. Deviations from the original papers are described in 14., References and deviations. Sample DNA is extracted using KingFisher Flex robot with a commercial kit and DNA is tested with a real-time PCR test. Principle of the real-time PCR test is described in Evira 7547.

3. Possible sources of error

Sources of error related to the PCR tests and the KAJA mode of operation for eliminating them are explained in detail in working instructions LAB 4015 ('Performing PCR-tests').

In the PCR tests, potential sources of error are nucleic acid contaminations that cause false positive results. In real-time PCR, contamination can occur during the DNA isolation or the preparation of PCR reaction mixtures and the addition of template DNA.

In this assay the total DNA is isolated from samples using a commercial DNA isolation kit. In KAJA there is a lot of experience of using these kits and established procedures to avoid contamination. Good hygiene and care must always be exercised when working. A healthy plant sample that goes through the same steps as the samples from DNA isolation to PCR run should always be included in the test.

Generally, the largest contamination risk is caused by PCR reaction products in which DNA is very concentrated. Since real-time PCR products are on a sealed plate and the seal is not opened after the PCR run, the risk of contamination is lower than in the conventional PCR. In real-time PCR, however, the risk of contamination may be greater than usual during plate pipetting since the wells are sealed only after the pipetting is completed. To control contaminations, each PCR run must include a negative amplification control (water used for premix).

False negative results can be caused by a failure of DNA isolation and purification or by the DNA degrading, although DNA is generally very durable. To control the success of DNA isolation, each sample is amplified with COX primers and probe that recognize the plant cytochrome oxidase gene (Weller *et al*. 2000).

The consistency of a faulty PCR reaction mixture or PCR run program and disturbances in the equipment cause false - mainly negative - results. Such errors can easily be detected because positive controls are included in the test. If the whole sample series gives a negative result, the error is likely to be in the reaction mixtures or PCR run. Such errors are corrected by making a new test.

4. Work safety

KingFisher Flex robot contains very strong permanent magnets. People wearing a pacemaker or metallic prostheses should not use this apparatus. A pacemaker or prostheses may be affected or damaged if it comes in close contact with a strong magnetic field. Chemicals, reagents, tools and equipment are handled with caution and care. Protective gloves and clothing should be used.

5. Environmental conditions

DNA isolation and PCR testing require very good hygiene and sterile working to avoid DNA and DNase contamination. Pipetting is done in laminar flow cabinets. The samples are ground in the plant pathology laboratory (D 106). The DNA is then isolated in the laminar flow cabinet and KingFisher Flex robot of the analytical laboratory (D 113). PCR reaction mixtures are prepared in the laminar flow cabinets of the PCR laboratory (D112) in accordance with working instructions LAB 4015. Sample tubes and PCR plates are always transported sealed from one place to another. Otherwise, tubes and plates will not be kept open unless it is necessary.

6. Instruments and equipment

DNA isolation

- Precision scale
- 50 ml tubes
- Platform rocker (e.g. Stuart See-saw rocker SSL4, Bibby Scientific)
- Small filter bags "universal" (Bioreba, Art. No. 430100)
- Homex 6 homogenizer (Bioreba)
- 12 ml tubes Sarstedt 60.9922.936 white cap
- 12 ml tubes Sarstedt 60.9922.937 yellow cap
- Centrifuge esim. Jouan MR22
- Basic equipment for DNA isolation (pipettes, tubes, vortex, laminar flow cabinet, centrifuge for Eppendorf tubes, etc.)
- Shaking heat bath (esim. Eppendorf Thermomixer)
- Minicentrifuge (e.g. Eppendorf Centrifuge 5417 R)
- KingFisher Deep Well 96 plates Cat. No. 95040450 (Thermo Fischer Scientific)
- KingFisher 96 tip comb for DW Magnets Cat. No. 97002534 (Thermo)
- KingFisher Flex robot (Thermo)
- Film for sealing the KF plates e.g. Microtiter Plate Sealers Cat. #3501 (Thermo)
- Refrigerator 6 ± 2℃, freezer –19 ± 3 °C (if desired, deep freezer –78 ± 2℃)

PCR (these instruments are only used for the preparation of PCR reactions)

- Basic equipment for PCR work (pipettes, tubes, cold blocks, vortex, racks, minisentrifuges, laminar flow cabinets, refrigerator 5 ± 2℃, freezer –19 ± 3 ° C (from –16 to –25 °C) etc.)
- PCR plates (Multiplate PCR Plates, 96 well, clear, BioRad Cat. # MLL9601) and film for sealing them (Microseal 'B' Seals BioRad Cat. # MSB1001)
- Mini Plate Spinner MPS 1000 (Labnet) for spinning PCR platesq
- Real-time PCR equipment (CFX96TM Real-Time System and C1000TM Thermal Cycler and CFX96TM Real-Time System and C1000 TouchTM Thermal Cycler, Bio-Rad)

7. Calibration

Pipettes, incubators and readers are calibrated according to the calibrating plan.

8. Reagents

DNA extraction:

- PBST (LAB 4207)
- QuickPick Plant DNA-kit 53022 or QuickPick XL Plant DNA Reagents 53100–53600 (BioNobile)

For carrot seeds also:

- 0.5 % Triton X-100 (prepared fresh, 500 µl Sigma T8787 + 100 ml UP-water)
- UP-water

qPCR:

- Sterile nuclease-free water
- Maxima Probe qPCR Master Mix (K0261–K0263,Thermo Fischer Scientific)
- Sample-DNA

CLso primers (Li *et al*. 2006, Li *et al*. 2009)

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

or CLso primers (Teresani *et al*. 2014)

- Primer CaLsppF 5'-GCA GGC CTA ACA CAT GCA AGT-3'
- Primer CaLsppR 5'-GCA CAC GTT TCC ATG CGT TAT-3'
- Probe CaLsolP 5'FAM-AGC GCT TAT TTT TAA TAG GAG CGG CAG ACG-3'BHQ1

Plant primers (Weller *et al*. 2000, Tomlinson *et al*. 2005)

- Primer COX-F 5'-CGT CGC ATT CCA GAT TAT CCA-3'
- Primer COX-RW 5'-CAA CTA CGG ATA TAT AAG RRC CRR AAC TG-3'
- Probe COX-P 5'Cy5-AGG GCA TTC CAT CCA GCG TAA GCA-3'BHQ2

Negative controls:

- Fresh or frozen negative plant sample ground in PBST buffer for DNA extraction
- Water used in premix preparation instead of sample for PCR control

Positive controls:

- Fresh or frozen positive plant sample ground in PBST buffer for DNA extraction
- Previously extracted positive DNA for PCR (optional)

DNA extraction and PCR controls are performed on every sample using COX primers and probe.

9. Procedure

Sample preparation:

Potato tuber sample can be taken from a single tuber, or from the heel end core sample for ring rots (LAB4001) directly or after the ring rots have been extracted. Heel end cores are homogenized in Bioreba extraction bag without the buffer, 25 ml PBST is added and the bacteria are extracted by rubbing the buffer into the tuber pulp inside the bag. The bacteria are concentrated from the extract by 2-step centrifugation. In the first step (clarification) the coarse plant material is removed from the sample by 100×g 5 min centrifugation at 10 °C, the bacteria are then pelleted from the supernatant (~8.5 ml) by 7000×g 5 min centrifugation at 10 °C. Bacterial pellet is suspended in 500 µl PBST buffer, 200 µl of the suspension is used for DNA extraction and the rest can be frozen in case the analysis needs to be repeated.

A single tuber sample is taken from the heel end or bud site, homogenized with Homex 6 and extracted 1:2 to PBST buffer. It is important to get as much vascular tissue as possible into the sample. Symptomatic sample does not need concentration.

Carrot petioles and potato stolons are homogenized in in Bioreba extraction bag with Homex 6 and extracted 1:2 in PBST. 1 cm pieces from the petioles are cut circa 3 cm above the root. 200 µl of the unconcentrated extract is used for DNA extraction.

Carrot seeds are washed before grinding to remove the seed dressing, in addition the washing softens the seeds and thus aids the grinding.

One gram of seeds is weighed into 50 ml centrifuge tube and 30–40 ml 0.5 % Triton X-100 is added. Larger volume reduces the flow of liquid in the mixing and results in poor washing result. The tube is shaken 30 minutes on a platform rocker. The liquid is carefully poured out, the tube is filled with UP-water, shaken vigorously and the water is carefully decanted. The rinsing is performed three times total, on the third time the seeds are poured with the water into Bioreba extraction bag and the water is subsequently decanted from the bag. The seeds are ground to fine powder with Homex 6, 10 ml PBST is added and the bacteria are extracted by rubbing the buffer into the seed powder inside the bag. 200 µl of the extract is used for DNA extraction. Color from seed dressing may remain in the sample, it does not interfere with the extraction.

Samples ground in PBST buffer can be stored in freezer for months or in the deep freezer for years. Although deep freezing is generally recommended for plant samples, symptomatic tuber sample stored for five years in –20 °C freezer worked well with this method.

ATTENTION! Concentration of the samples is tested only with tuber material. The yield is 10–92 %, average 42 %, median 35 %.

DNA extraction with KingFisher Flex and QuickPick™ Plant DNA-kit (BioNobile)

1. Add 300 µl Lysis Buffer and 20 µl proteinase K to 200 µl sample
2. Incubate 30 minutes in thermal shaker 700 rpm at 65℃
3. Pipet KingFisher Deepwell plates (Cat. 95040450) during incubation

   Sample: 500 µl Binding Buffer + 20 µl Magnetic Particles
   Wash 1: 900 µl Wash Buffer
   Wash 2_1: 800 µl Wash Buffer
   Wash 2_2: 800 µl Wash Buffer
   Elution: 50 µl Elution Buffer

4. Centrifuge the samples 5 minutes 18000×g at room temperature. Pipet 450 µl of the supernatant carefully to the Sample plate wells avoiding the sediment.
5. Start KingFisher Flex and run PURE_DNAPlant_Flex96 program
6. Pipet the DNA from Elution plate to eppendorf tube and use for real-time PCR analysis. The DNA can be kept short time (1–2 weeks) in the fridge or longer times in the freezer for further use.

Real-time PCR

Prepare PCR reaction in D112 and run the PCR in D103. Follow general instructions in LAB 4015. Keep the reagents cold and the probe protected from light.

Make at least one technical replicate of each sample and control. One reaction for COX gene for each sample is sufficient.

PCR reaction for one well

Reagent	Conc.	Volume (µl)	Final conc.
Nuclease free water		4.0	
Maxima Probe qPCR Master Mix	2×	12.5	1×
CaLsppF OR LsoF OR COX-F	5 µM	1.5	0.3 µM
CaLsppR OR HLBr OR COX-RW	5 µM	1.5	0.3 µM
CaLsolP OR HLBp OR COX-P	5 µM	0.5	0.1 µM
Total		**20.0**	
DNA		5.0	
Total		**25.0**	

PCR program

Step	Time	Temp °C
1	00:10:00	95
2	00:00:15	95
3	00:01:00	60
4	go to 2, 45 times	
5	end	

Plate is read during step 3

10. Results

The cut-off value between positive and negative sample is 38 cycles. The plant control cut-off value is 18 cycles, or 21 cycles for non-concentrated samples.

Requirements for a valid results are:

- Exponential amplification curves from positive DNA extraction (and PCR) controls
- Negative DNA extraction control and PCR control are negative (Cq≥40)
- Positive control is less than cut-off value (Cq<38)
- Plant control is less than cut-off value (Cq<18, non-concentrated <21)
- Amplification curves from technical replicates are uniform

When the results are valid:

- Result is positive if amplification curves are exponential and Cq<38
- Result is negative if amplification curves are not exponential and Cq>38

<u>If the results are invalid, test will be repeated</u>

11. Validation of the method

The method is validated for potato tubers in the Plant Analysis Unit in 2018

12. The status of the method

- The method is in accordance with a Council Directive ☐
- The method is in an international method collection (e.g. EPPO) ☐
- Internal method ☒
- Accredited method ☐

13. Quality assurance procedures

- Commercial international comparative tests (e.g. FAPAS) ☐
- Comparative analyses between laboratories ☒
- Comparisons of methods ☐
- Use of comparative/control strains ☐
- Inoculated samples ☐
- Parallel determination ☐
- Blind tests ☒
- The laboratory's own control samples ☒

14. References and deviations

Bertolini E, Teresani GR, Loiseau M, Tanaka FAO, Barbé S, Martínez C, Gentit P, López MM and Cambra M. (2015) Transmission of ‘*Candidatus* Liberibacter solanacearum’ in carrot seeds. Plant Pathology, 64: 276–285.

Li W, Hartung JS, Levy L. (2006) Quantitative real-time PCR for detection and identification of *Candidatus Liberibacter* species associated with citrus huanglongbing. Journal of Microbiological Methods, 66: 104–115. (***HLBr primer, HLBp probe***)

Li W, Abad JA, French-Monar RD, Rascoe J, Wen A, Gudmestad NC, Secor GA, Lee IM, Duan Y, 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. (***LsoF primer***)

Teresani GR, Bertolini E, Alfaro-Fernández A, Martínez C, Tanaka FA, Kitajima EW, Roselló M, Sanjuán S, Ferrándiz JC, López MM, Cambra M, Font MI. (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. (***CaLsppF and CaLsppR primers, CaLsolP probe***)

Tomlinson JA, Boonham N, Hughes KJD, Griffin RL, Barker I. (2005) On-site DNA extraction and real-time PCR for detection of *Phytophthora ramorum* in the field. Applied and Environmental Microbiology, 71: 6702–6710. (***COX-RW primer and COX-P probe***)

Weller, S. A., Elphinstone, J. G., Smith, N. C., Boonham, N. & Stead, D. E. 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Applied and Environmental Microbiology, 66: 2853–2858. (***COX-F primer***)

Deviations from the references:

- Sample preparation
- DNA extraction
- PCR reagents and reaction volume
- Primer and probe concentrations
- BHQ1-quencher is used instead of TAMRA for CaLsolP probe
- Plant probe COX-P is labeled with Cy5
- PCR program (Teresani *et al*. 2014 is also used for Li *et al*. 2009 primers)