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Comparison and implementation of detection tests for CLso* in plant tissue samples.

December, 2nd 2015

* '*Candidatus* Liberibacter solanacearum' (CLso) and Zebra chip (ZC)

Content

- Disease facts
- Compare/implementation of test options
- Implementation tested in practice
- Evaluation

Disease facts

From Last two decades of past Millenium:

“Zebra Chip” outbreaks in Central Am./USA potato

Losses up to 60%

EPPO A1 (Solanaceous + specific vector)

Distribution

USA and New Zealand; Haplotype A + B
tamato / potato

Europe, Northern distribution area; Haplotype C
carrot

Europe, Southern distribution area; Hapl. D + E
carrot / celary

Associated, specific vectors (Bc, Ta, Btri, Bn, Btre)

NVWA (= NPPO-NL); Diagnostics preconditions

Reference collection network participation:

Establishment of a in vivo CLso specimen culture in tomato + potato plants; originated from USA (western distribution area)

Infected carrot reference material: provided by IVIA

Participation in EUPHRESCO – IVIA - Test Performance Study detection of "*Candidatus* Liberibacter solanacearum"

CaLsol/100 complete kit of Plant Print Diagnostics S.L. (2012)

Pest status determination survey: tomato (started 2010) carrot (from 2011)

Collection items CLso; grafted tomato plants

Establishment of Collection; Virulent accession From USDA 2012.

Maintained by Grafting (3 Yrs)

Collection items CLso; grafted tomato plants

Potato

Evaluation of Realtime PCR Platforms using the EUPHRESCO - IVIA TPS sample set

Platforms compared

Student Internship Project

Joyce van Assen

Sep-Dec 2013

Bio-Rad
CFX 96

Applied Biosystems
7500

Roche
LC 480

Roche
LC 96

Objects

- Besisdes CLso sample set from the IVIA TPS ; also

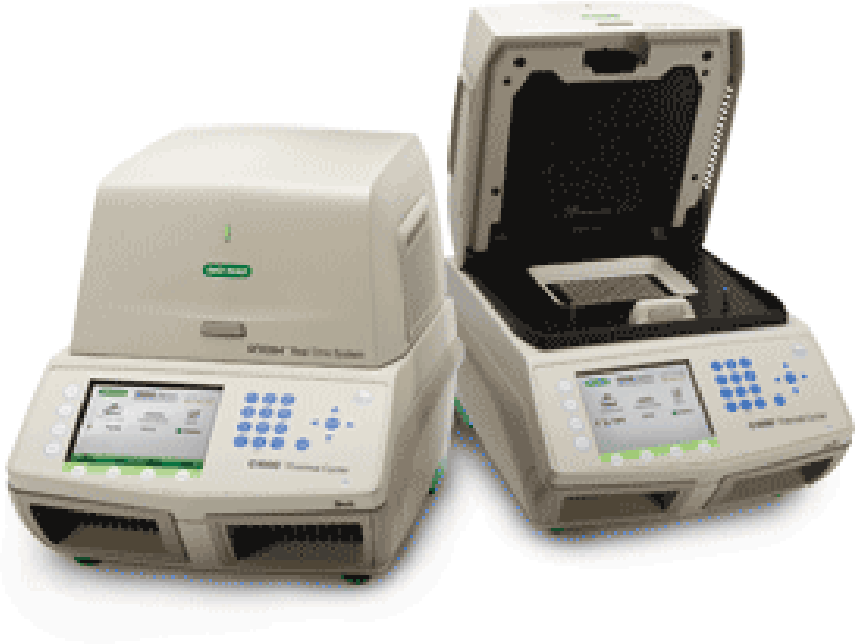
- *Phytophthora ramorum*
- *Clavibacter michiganensis subsp. michiganensis*
- *Spodoptera* spp.
- *Bursaphelenchus xylophilus*
- Pospiviroid

Platform performances in relation to sample ID using CLso/100 complete kit, Pl. Print Diagnost.

TPS sample	7900HT (AB)		7500 (AB)		CFX96 (Bio-Rad)		LC480 (Roche)		LC96 (Roche)		Identity
	set 1	set 2	set 1	set 2	set 1	set 2	set 1	set 2	set 1	set 2	
sample 01	-	35,8	36,0	36,7	35,0	35,1	36,0	-	33,8	34,7	+
sample 02	-	-	-	-	-	-	-	-	-	-	-
sample 03	-	-	-	-	-	-	-	-	-	-	-
sample 04	34,5	33,2	35,1	35,6	31,0	31,0	-	33,0	34,1	-	+
sample 05	33,5	31,9	34,6	36,3	31,1	31,0	33,5	33,0	33,5	-	+
sample 06	-	-	-	-	-	-	-	-	-	-	-
sample 07	35,0	35,6	37,8	35,3	33,4	33,3	-	33,8	35,8	-	+
sample 08	-	-	-	-	-	-	-	-	-	-	-
sample 09	34,1	34,2	35,2	36,0	40,0	33,5	-	35,9	34,7	36,1	+
sample 10	-	-	-	-	-	-	-	-	-	-	-

Platform selection

Since January 2013
Bio-Rad CFX 96



C1000 thermocycler from Bio-Rad (for cPCR; not evaluated)

Literature comparison of tests

Seven detection test versions (4 references) nominated from literature to be compared (to select NVWA standard for Clso).

Besides analytical performance criteria (sensitiviety, specificity, selectivity) comparison from literature data, also focused on:

- Homogenization for crude extract preparation,
- DNA extraction,
- Target locus,
- Reaction mixes,
- and Thermocycler conditions.

Some common qPCR and cPCR from literature

Table 2 Sensitivity of two qPCR and three cPCR assays for detection of Lso in 101 DNA samples extracted from potato tubers from regions in NZ affected by TPP/Liberibacter

Number of samples positive for Lso					
Number of samples	cPCR			qPCR	
	Standard[a]	Nested[b]	Semi-nested[c]	TaqMan[d]	SYBR Green[e]
101	42	45	59	80	78

[a] Standard cPCR with OA2-OI2c (Liefting et al. 2009a)

[b] Two-round nested PCR as described by Liefting, et al. (Liefting et al. 2009b)

[c] Single-tube semi-nested cPCR with OA2-Lib16S01F-Lib16S01R

[d] TaqMan qPCR as described by Li, et al. (Li et al. 2009)

[e] Single-tube semi-nested SYBR Green qPCR with LsoF-Lso16SF-Lso16SRI

Literature info matrix; reference LOD set point

Liefting etal, 2009. Theoretical reference

Author	Method	Target	internal control (plant)	Sensitivity	Theoretical LOD	Specificity
Liefting *et al*. 2009	PCR	16S rDNA	N.A.	unknow n	100	Negative for 'Ca. L. asiaticus', 'Ca. L. africanus', or 'Ca. L. americanus'

Theoretical "Limit of Detection" (LOD) relative to Liefting et al

Literature info matrix; first selection basis

Two Real-time PCR detection assays and two conventional PCR selected for experimental comparison.

Author	Method	Target	internal control (plant)	Sensitivity	Theoretical LOD	Specificity
Liefting *et al.* 2009	PCR	16S rDNA	N.A.	unknown	100	Negative for 'Ca. L. asiaticus', 'Ca. L. africanus', or 'Ca. L. americanus'
Li *et al.* 2009	PCR	16S rDNA	N.A.	10X PCR Liefting	10	18 Ca L. solanacearum (positive Ct 20.6-35.2) 3 Ca. L asiaticus, 3 Ca L. africanus, 3 Ca L. americanus (negative) Potato leaf roll virus, Clover proliferation phytoplasma, Candidatus Phytoplasma americanum, Xylella fastidiosa PD strain, Xylella fastidiosa CVC strain (negative)
	real-time PCR (TaqMan)	16S rDNA	COX	10X PCR Li	1	
Ravindran *et al.* 2011	PCR	16S–23S rDNA ITR	β-TUB	10X PCR Li	1	1 Ca. L. asiaticus (negative)
	PCR	adenylate kinase	β-TUB	10X PCR Li	1	
Beard *et al.* 2012	semi-nested real-time PCR (SYBR)	16S rDNA	EF-1α	50X cPCR Liefting	2	3 Ca. L solanacearum (positive) 1 Ca. L. americanus, 1 Ca. L. africanus, 1 Ca. L. asiaticus, 1 Ca. L. europaeus (negative) WX phytoplasma, PoiBI phytoplasma, WWB phytoplasma, Ca. Phytoplasma australiense, Pectobacterium atrosepticum, Pseudomonas fluorescens (1 each, negative)
	semi nested PCR	16S rDNA	28S	1/10X semi-nested qPCR Beard	20	

Selection for Experimental comparison

Choise of methods from literature comparison is mainly based on:

- Analytical sensitivity (most sensitive; qPCRs 100% specific), and
- results from routine testing presented in different papers.

Chosen methods for further testing:

- 2x cPCR, both Ravindran et al, 2011 (adenylate kinase and ITS)
- Semi-nested (SYBR) Real-time from Beard et al 2011 (16S rDNA)
- Real-time from Li et al 2012 (16S rDNA)

Mixer Mill (Retsch): Crude extract preparation

Effect of carrot tissue maceration using a Mixer Mill MM301 (Retsch); [NAC, 2x NiC, leaves, petioles, root, NiC]

Left side: effet of one large bead (4 mm). Right: 6-8 small (2 mm) beads.

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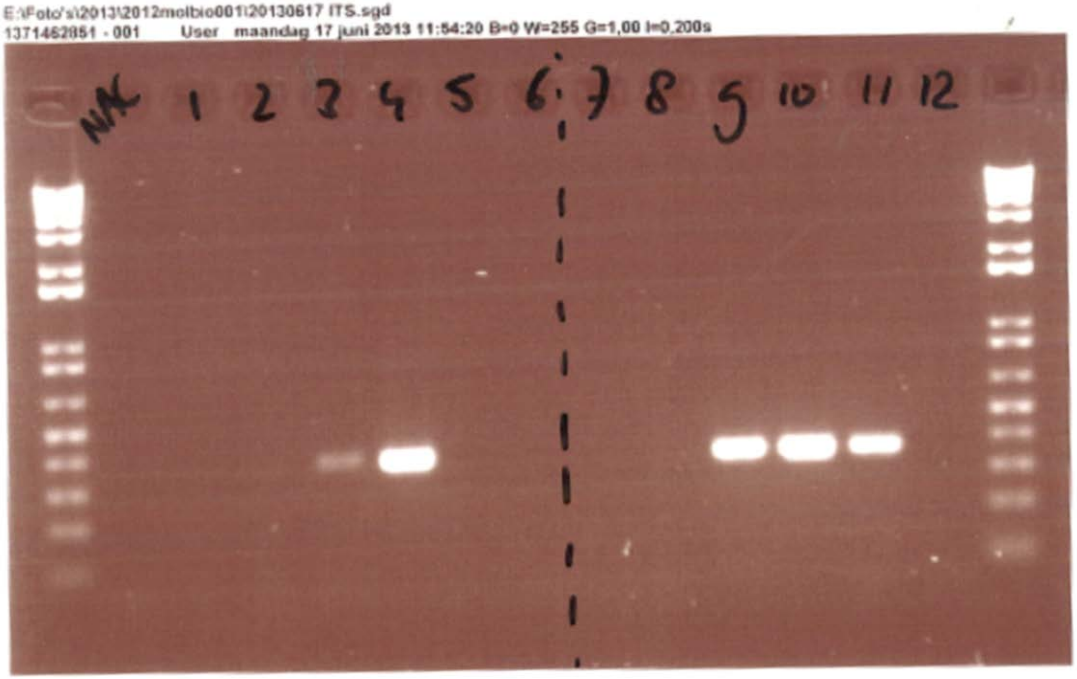
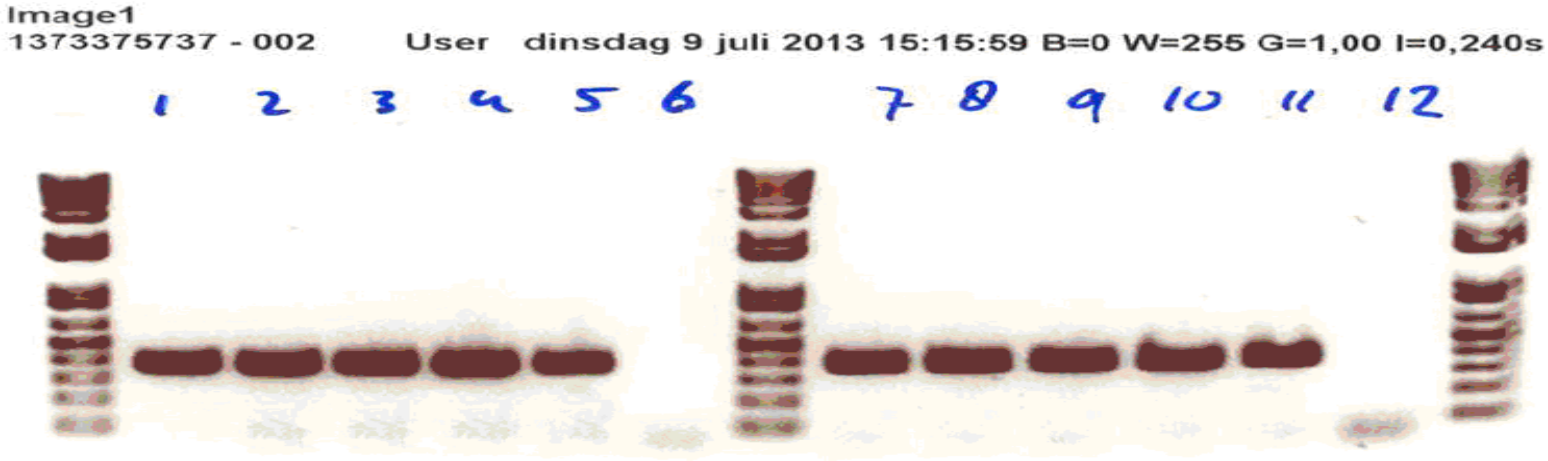


NVWA – modified; Crude extract preparation

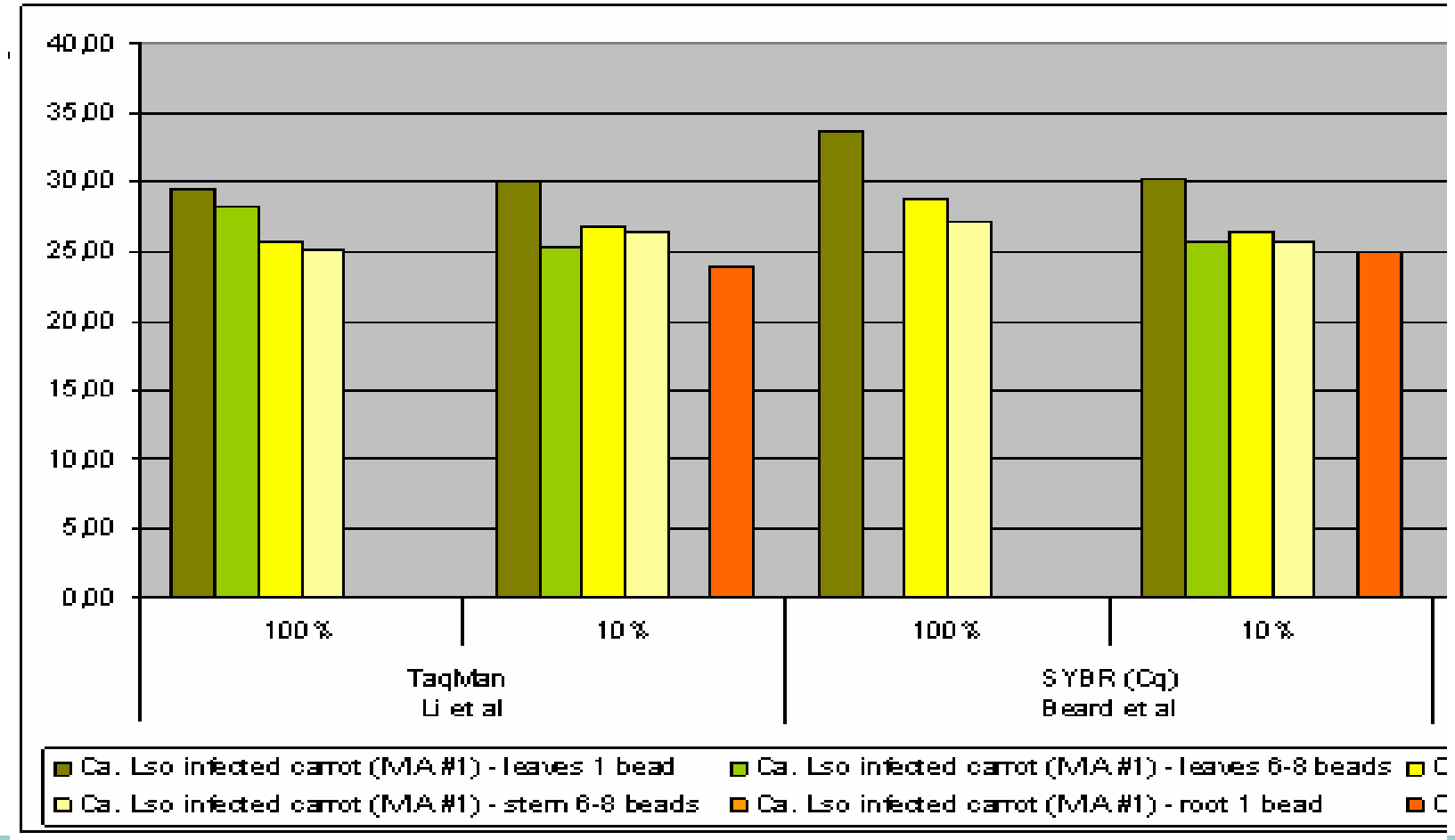
homogenization for crude plant extract preparation according to NVWA-NRC (left) and Munyaneza, (right);

amounts of homogenate (µL): 50, 100, 200, 400, 100 c.



No qualitative effects on PCR efficiëncy observed; not by method nor by plant tissue volume.

DNeasy plant Mini Kit (Qiagen); Inhibit. Beard; root



DNA extraction from Crude plant extract

Using DNeasy Plant Mini Kit (Qiagen) for both (Li and Beard) failed detection of undiluted carrot root extract.

10x dilutions of those difficult extracts gives the same qualitative results as the conventional PCRs. This suggists that inhibition plays a role in the DNA extracts obtained with the DNeasy Plant Mini Kit.

Both, in experiments and in practice

the automated QuickPick Plant DNA kit (Bionobile) (with additionally PVPP purification) proved fit for purpose.

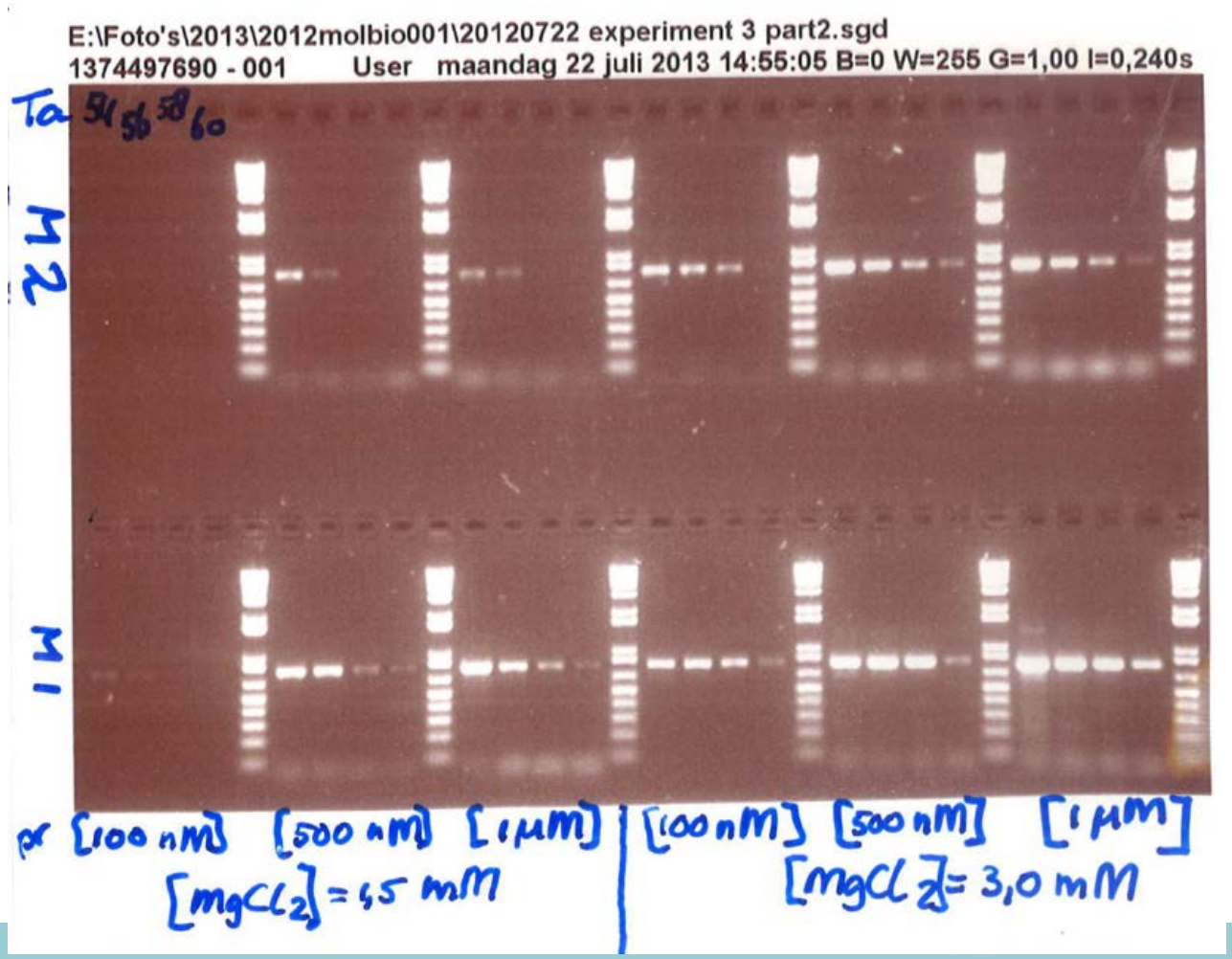
Optimization cPCR (Ravindran et al 2011)

Poor results firstly obtained from literature protocol

Aspects optimized (Conventional adk PCR):

- Annealing temperature gradient (54, 56, 58, 60° C),
- $MgCl_2$ conc. (1.5 mM and 3.0 mM) and
- Primer conc. (100 µM; 0.5 µM; 1.0 µM)

cPCR Ravindran et al



cPCR Ravindran et al; circomstances

Table: Semi quantitative results. optimisation adk test Ravindran *et al*. 2011.

			Ta			
			54 °C	56 °C	58 °C	60 °C
M1 (*Ca*. Lso infected carrot petioles)						
	1.5 mM	100 nM	z+	z+	-	-
		500 nM	+	+	z+	z+
		1 µM	+	+	+	z+
	3.0 mM	100 nM	+	+	+	z+
		500 nM	++	++	++	+
		1 µM	++	++	++	+
M2 (*Ca*. Lso infected carrot root)						
	1.5 mM	100 nM	-	-	-	-
		500 nM	+	z+	-	-
		1 µM	+	z+	-	-
	3.0 mM	100 nM	+	+	z+	-
		500 nM	+	+	+	z+
		1 µM	+	+	+	z+

selected: 54° C; 0.5 µM Primer; 3.0 mM $MgCl_2$.

Chemistry; Real-time PCR

Choice of chemistry for Real-time PCR by Li et al, 2009.

No results obtained and only background noise when used:

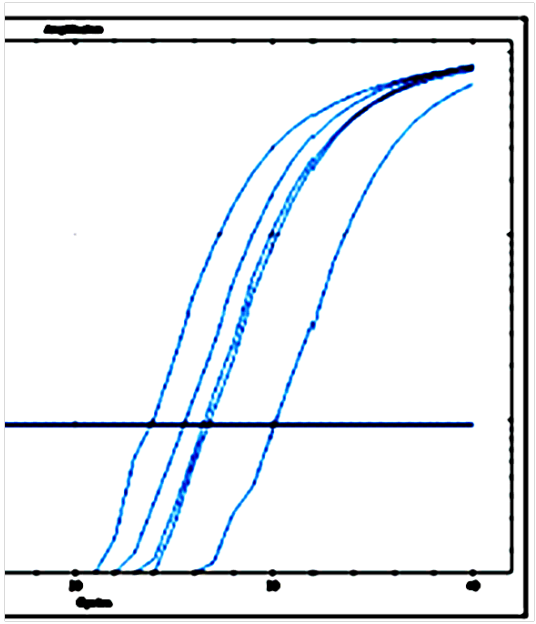
- Ex Taq Premix (Perfect Real-time) from TAKARA.

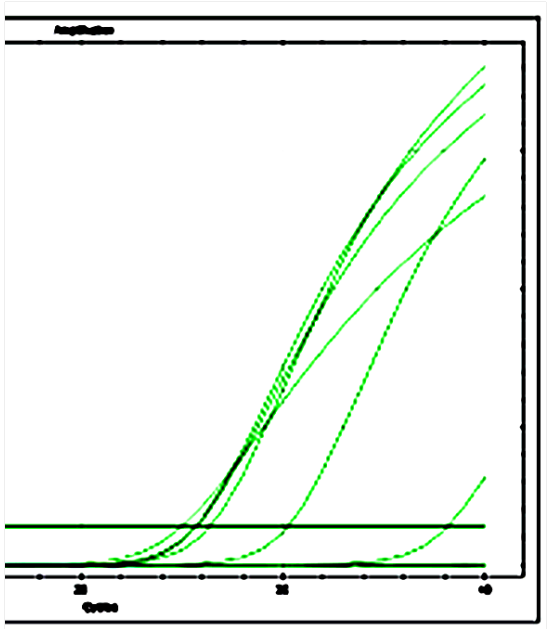
Nice exponential curves and the expected qualitative results obtained with:

- TaqMan Universal Master Mix chemistry.

Curves

Left: Li et al, 2009. Right: Beard et al, 2012.





Ct values for the Li and Beard assays are about the same; no significant difference.

National survey on CaLso and vectors 2011-15

Inspections: tomato and carrots

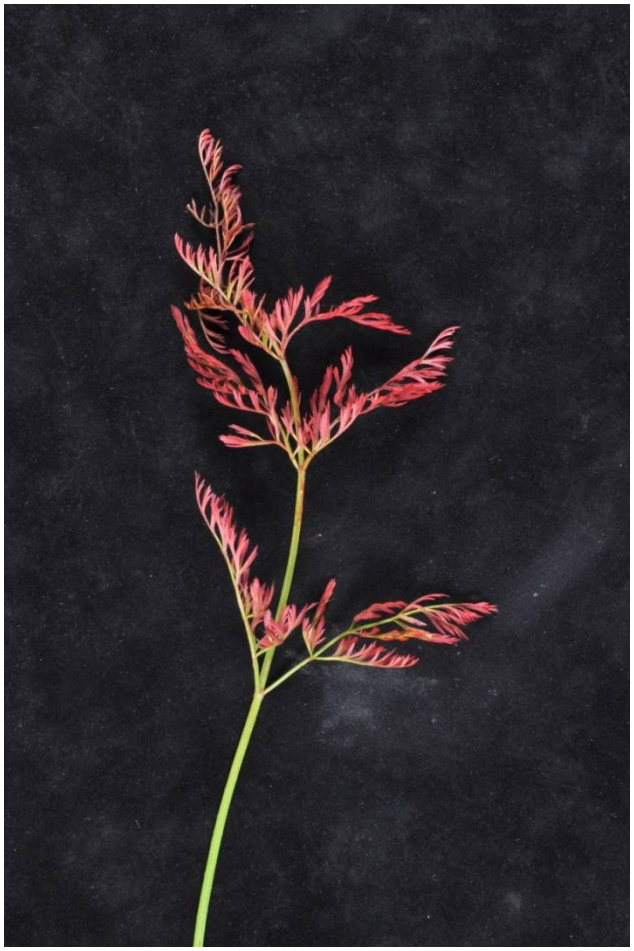
No suspect symptoms observed in solanaceous crops

Suspect and other unusual carrot plants were sent to NVWA-NRC for testing on CLso infections

Suspect insects send to NVWA-NRC for determination (and for bacterial testing if vector found)

Remark: No carrot psyllid nor potato psyllid identified so far

Suspect carrot plants in national survey



Evaluation in practice: Survey, routine diagnosis

Survey crops: Tomato and Carrot

symptomatic samples:

tested: 150 over survey period (only carrot)

3 samples received from company in two other EU counties where

samples positive: Three (the foreign) samples.

Tested Stolbur phytoplasma. (found: Aster Yellows)

Carrot survey samples CLso 2011 - 2015 were simultaneously tested on phytoplasma in general, and specific on stolbur phytoplasma. The same DNA extracts used.

phytoplasma was detected (>50%); No stolbur phytoplasma.

ID by sequence analysis (c. 20% of those positive samples)

Target fragments: 16S rDNA and ISR.

Blast results (NCBI):

all highly matching '*Candidatus* Phytoplasma asteris'

Those findings support earlier concluded effective DNA extraction using the implemented: automated QuickPick Plant DNA kit (Bionobile) (with additionally PVPP purification)

Implementation of identity verification test

Indentity verification on species level. After screening: Li et al, 2009.

Firstly: cPCR confirmation by Ravindran et al, 2011 (targeting ITS, adk or both), and

Partial sequence analysis of the ITS region (Amplified according Ravindran et al, 2011).

The 2012 and 2013 routine (foreign) samples positve in Li et al (and Ravindran et al) detection test were matched in Genbank.

Respective similarity:

99.3% (2 SNPs) with CLso Genbank accession FJ8305931, and

99.7% (1 SNP) with CLso Genbank accession JX308304.

Both samples assigned to a CLso specific clade by cluster analysis.

Additionally the first screening test results were confirmed by the other implemented qPCR from Beard et al.

Conclusions

The better options to include in diagnostic protocol for Clso (NVWA):

The NVWA-NRC optimized subsampling and crude plant sap extraction (based on Munyaneza, 2010).

Use of the automated QuickPick Plant Kit in combination with additional PVPP purification for DNA extraction from carrot tissue crude sap extract.

Real-time PCR from Li et al 2009 as (first) screening test using TaqMan Universal Master Mix chemistry

Sequence analysis of the ITS region, targeting aplicons obtained with the ITS test described by Ravindran et al, 2011 is a reliable method for identity verification up to species level.

Follow up

Further implementation up next:

Indentity verification on Haplotype level. Discrimination based on SNPs in 16S, ISR-23S, 50S (rplJ and rplL), Nelson *et al.,* 2011.

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Thank you

- Questions?