Plant Protection Service
Ministry of Economic Affairs, Agriculture and Innovation

Overview:

Activities of NVWA-NRC, NL

Molecular test methods for CLso* evaluated

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

Plant Protection Service
Ministry of Economic Affairs, Agriculture and Innovation

Overview

- Motivation for activities on CLso
- Policy against introduction/establishment of CLso
- Actions initiated
  - National Survey
  - Comparison/implementation of diagnostic tests
  - Initiation of CLso collection
- Aknowledgement

Initiation of activities NVWA NRC on CLso

2009: Addition of CLso to the EPPO Alert list

Quick scan on CLso and vectors performed.

- Potential disease impact: high (USA, NZ).

- Entry CLso:

  possible but likeliness low without vector

- Need for information on prescense of CLso and its vectors

Follow up actions upon Quick scan

- CLso Survey initially in tomato glasshouse crops,
- later (from 2010) also extend to carrot field crops

Inspection on visual symptoms

Suspect symptomatic samples tested; Outsourced, USDA.

- Vector Survey initially tomato glasshouse crops,
- later (from 2011) extended outdoors to carrot.

Other actions initiated by NVWA, NL (I)

Preparation of datasheet on CLso.

- Special attention to symptoms and appearance of vectors

Extension of knowledge on CLso in national work group.

- Members: Universities, Inspection services, seed companies,
- Other stakeholders in crop protection sector

Participation in EPPO Expert Work Group:

Pest Risk Analysis on CLso and its vectors .

- Resulting in: inclusion of CLso potato haplotypes and its vector
- B. Cockerelli on EPPO A1 list.

Other actions initiated by NVWA, NL (II)

Participation in EUPHRESCO – IVIA - Test Performance Study detection of "*Candidatus* Liberibacter solanacearum" CaLsol/100 complete kit of Plant Print Diagnostics S.L.

Evaluation of real-time PCR platforms using TPS-IVIA samples of 'Ca. Liberibacter spp'.

Implementation of molecular biological tests for the detection and identification of CLso in (carrot) plant tissue samples.

Establishment of a in vivo CLso collection in tomato and potato plants

Survey on CaLso and its vectors and diagnosis

In glasshouse tomato crops and carrot field crops

Inspection for suspect symptomatic plants and insects

Suspect insects are send to NVWA-NRC for determination

Suspect symptomatic plants are send to NVWA-NRC for testing on CLso infections, starting with a initial screening test

Eventually CLso identity verification of samples tested positive in the initial screening tests

Suspect samples in carrot survey 2010 - 2014

No carrot psyllid nor potato psyllid identified so far.

Suspect samples in carrot survey 2010 - 2014

Suspect samples in carrot survey 2010 - 2014

Survey results on Psyllids NL 2010 – 2014

Crops: Tomato and Carrot fields in NL

Focus on:

- Trioza apicalis
- Bactericera cockerelli
- Bactericera trigonica
- Bactericera nigricornis
- Bactericera tremblayi

No CLso vector insects found till to date.

Survey results on CLso in NL 2009 – 2011 (I)

Crops: Tomato and Carrot

Detection testing until 2011 by USDA-ARS,
Yakima Agricultural Research Lab – Wapato (USA).

inspecition sites: 2009, 9; 2010, 25; 2011; 75.

supect symptomatic samples:
total 26 over that period (no tomatoes)

samples tested positive in screening test: None

Survey on CLso in NL 2012 + 2013 (II)

Crops: Tomato and Carrot

Detection testing and identification by NVWA-NRC, after in home test implementations.

supect symptomatic samples:
total 83 over 2 year period (no tomatoes)

2 samples received from a company in another county where CLso infections are known present

samples tested positive: Two (foreign) samples.

Survey Stolbur phytoplasma in NL 2011 – 2014

Carrot survey samples CLso 2011 - 2014 are also tested on phytoplasma in general, and specific on stolbur phytoplasma.
phytoplasma was detected (>50%); No stolbur phytoplasma.

ID by sequence analysis (c. 20% of the positive samples)
Target fragments: 16S rDNA and ISR.

Blast results (NCBI):
all highly matching '*Candidatus* Phytoplasma asteris'

This diagnosis explains the symptomatic carrot plants.

For further details phytoplasma: Ko Verhoeven, NVWA-NRC

Evaluation of Realtime PCR Platforms using the EUPHRESCO - IVIA TPS samples

Applied Biosystems
7900 HT

Evaluation of Realtime PCR Platforms; in TPS

Student Internship Project

Joyce van Assen

Sep-Dec 2013

Bio-Rad
CFX 96

Applied Biosystems
7500

Roche
LC 480

Roche
LC 96

Experimental variety and evaluation criteria

- Besisdes CLso; also
- *Phytophthora ramorum*
- *Clavibacter michiganensis subsp. michiganensis*
- *Spodoptera* spp.
- *Bursaphelenchus xylophilus*
- Pospiviroid

  - limit of detection
  - Analytical specificity
  - User friendly
  - Platform specifications

EUPHRESCO – IVIA Test Performance Study Detection of CLso/100 complete kit of Plant Print Diagnostics S.L.

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	-	-	-	-	-	-	-	-	-	-	-

Evaluation of Realtime PCR Platforms using TPS

Since January 2013
Bio-Rad CFX 96

Implementation/Evaluation of tests for CLso

Seven detection test versions (4 references) selected from literature to be implemented for screening of survey samples on CLso.

Besides analytical performance criteria (sensitiviety, specificity, selectivity) comparison from literature data, also was taken into account:

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

Theoretical “Limit of Detection” (LOD) relative to Liefting

Implementation/Evaluation of tests for CLso

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

Implementation/Evaluation of tests for CLso

Liefting etal, 2009. Theoretical reference

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'

Implementation/Evaluation of tests for CLso

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	

Implementation/Evaluaton of tests for CLso

Choise of methods from literature comparison is mainly based on:

Analytical sensitivity, and

results from routine testing presented in different papers.

Chosen methods for further testing:

2x cPCR, both Ravindran et al, 2011 (adk and ITS)

Semi-nested (SYBR) Real-time from Beard et al 2011 (16S rDNA)

Real-time from Li et al 2012 (16S rDNA)

Chosen tests show 100% specificity (with chosen a-spec. organisms)

The four chosen PCRs (2 cPCRs, 2qPCRs) show highest sensitivity.

Implementation/Evaluation of tests for CLso

Specificity of both cPCRs (Ravindran) is determined with only one target strain.

Mainly potato and tomato plant parts were tested under the fowler of selectivity.

In the carrot surveys (moreover combined with Stolbur Phytoplasma as target) the automated QuickPick Plant DNA kit (Bionobile)(with additionally PVPP purification) has proven itself fit for purpose.

Implementation/Evaluation of tests for CLso

- Optimization before further practical comparison.

cPCR reactions were performed on a C1000 thermocycler from Bio-Rad

qPCR reactions were performed on a CFX 96 (Bio-Rad)

Implementation/Evaluation of tests for CLso

First aspect optimized

Conventional adk PCR (Ravindran):

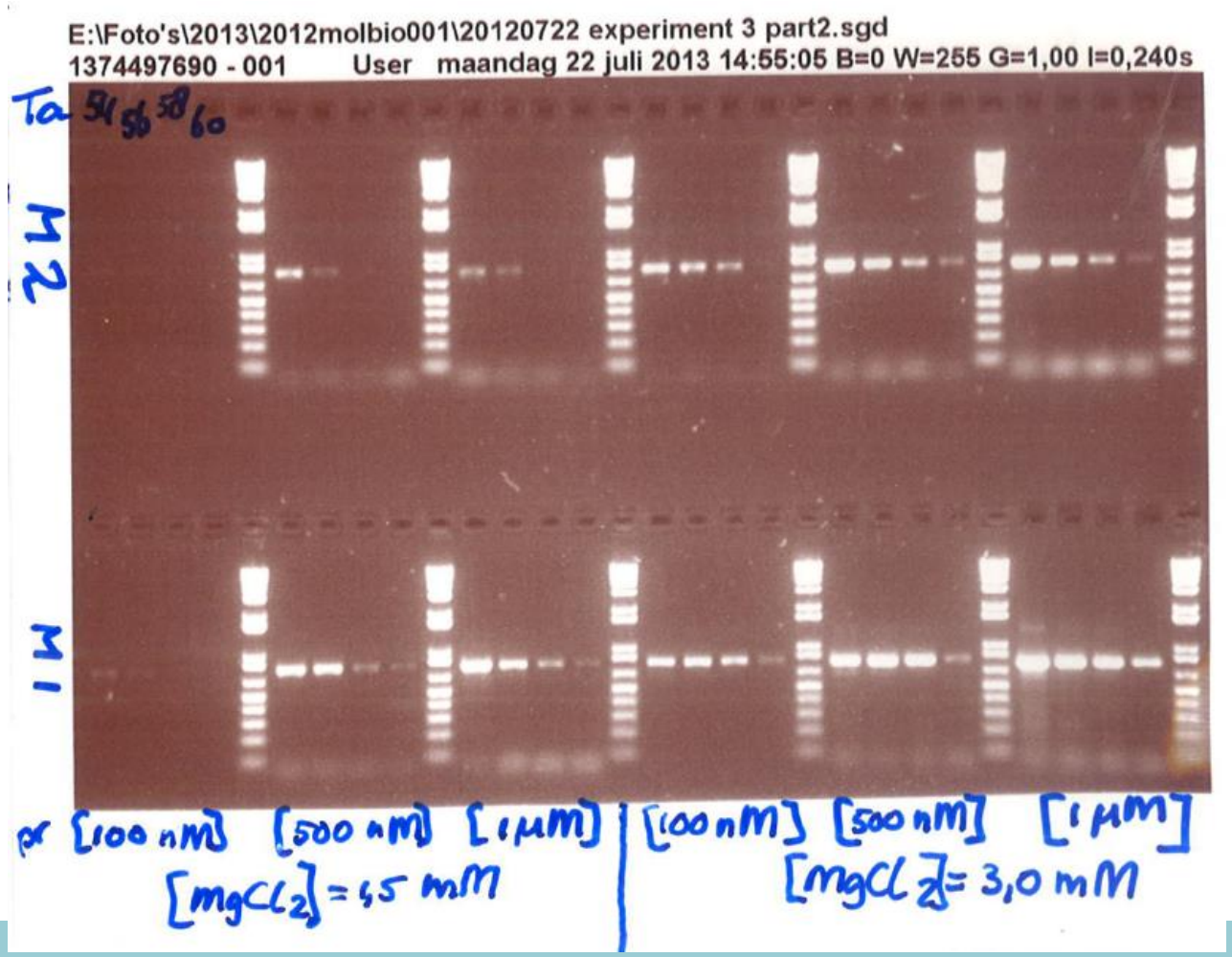
Annealing temperature gradient (54, 56, 58, 60° C),

$MgCl_2$ (1.5 mM and 3.0 mM) and

Primer (100 nM; 0.5 µM; 0.5 µM)

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

Implementation/Evaluation of tests for CLso



Implementation/Evaluation of tests for CLso

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 stem)						
	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+

Implementation/Evaluation of tests for CLso

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

Initially 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 conform i.

Implementation/Evaluation of tests for CLso

Effect on maceration of carrot material using a Mixer Mill MM301 (Retsch); Left: effet of one large bead (4 mm).

Right: 6-8 small (2 mm) beads.

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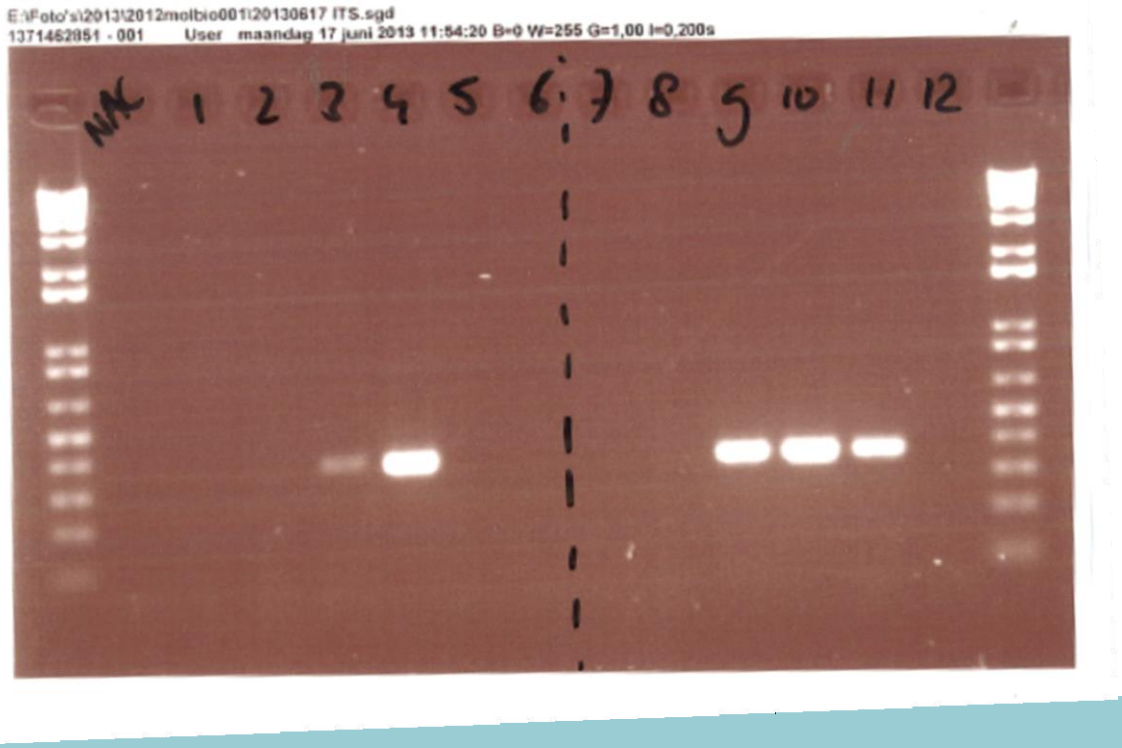
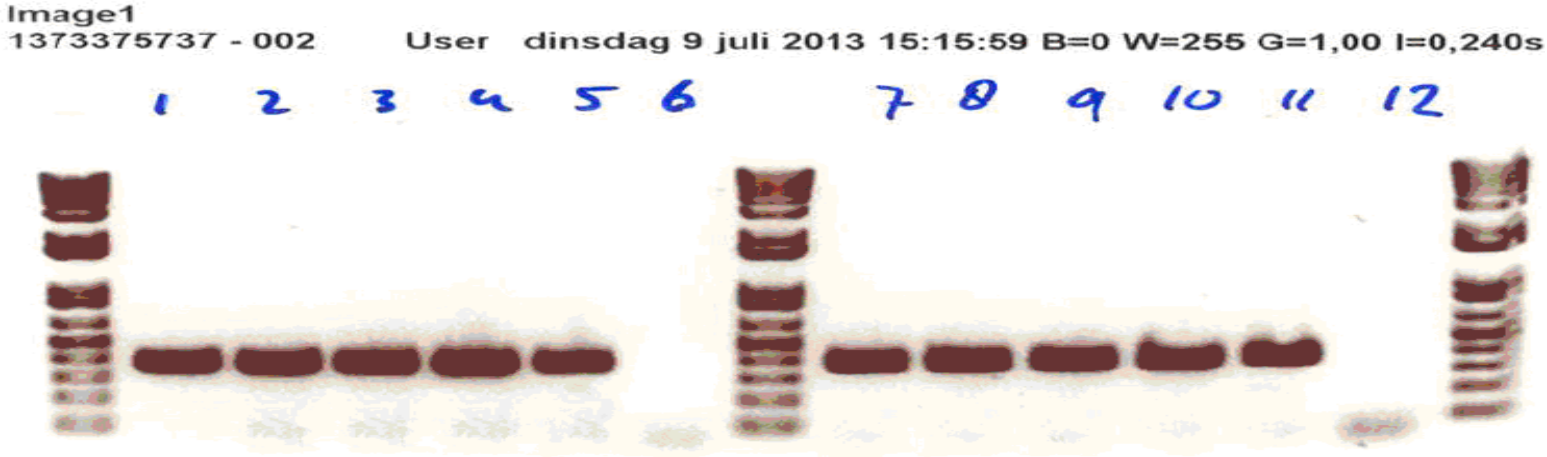


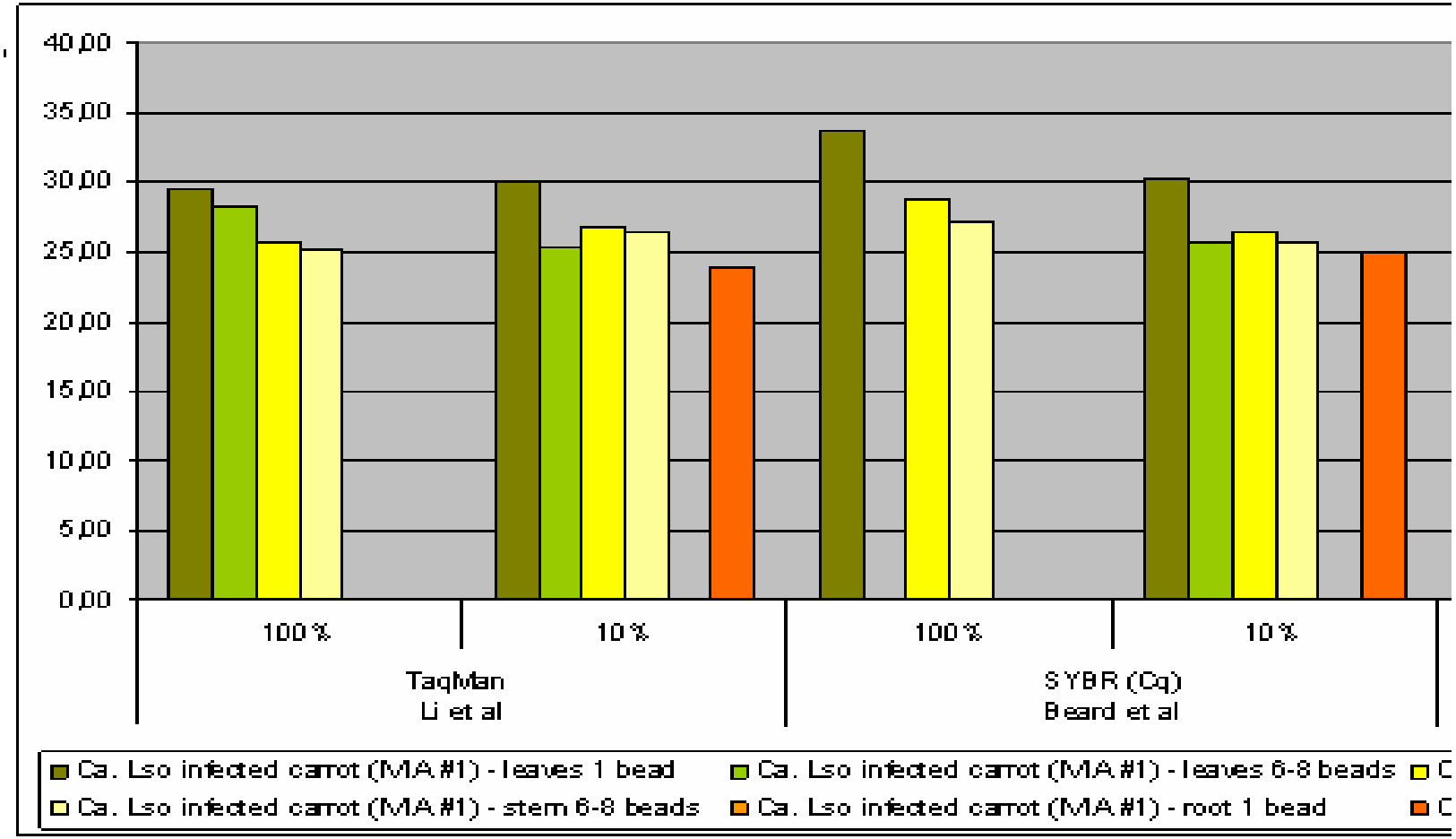
Implementation/Evaluation of tests for CLso

Homogenization for crude plant extract preparation according respectively NVWA-NRC (left) and Munyaneza (right), and amount of starting plant material



No qualitative effects on PCR efficiëncy observed; not by method nor by homogenate (sap) volume.

Evaluation CLso tests; Real-time PCR versions



Implementation/Evaluation of tests for CLso

Remark on Real-time protocol of Beard et al, 2012. It missed one out of four undiluted DNA extract of infected carrot stem.

Using DNeasy Plant Mini Kit (Qiagen) for both Real-time PCRs (Li et al, 2009 and Beard et al, 2011) failed detection of undiluted carrot root extract.

10x dilutions of those difficult extracts gives the same qualitative results as the conventional PCRs.

Those positive results only after dilution suggests inhibition plays a role in the DNA extracts obtained with the DNeasy Plant Mini Kit.

Implementation/Evaluation of tests for CLso

Curves:

The TaqMan assay described by Li et al, 2009 produces nice curves with almost no background.

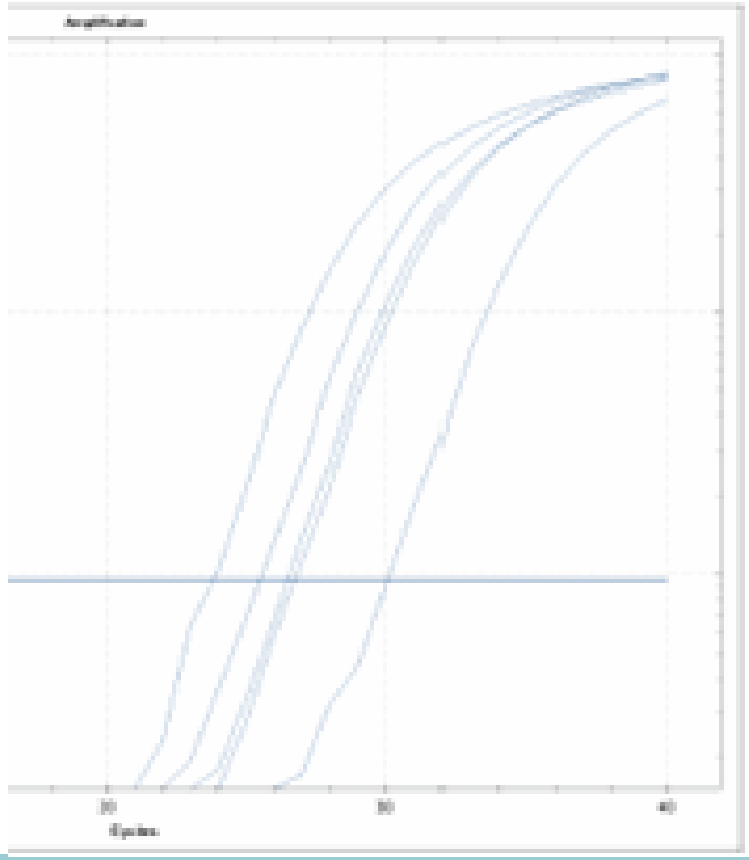
In contrary to the SYBR green assay described by Beard et al, 2012.

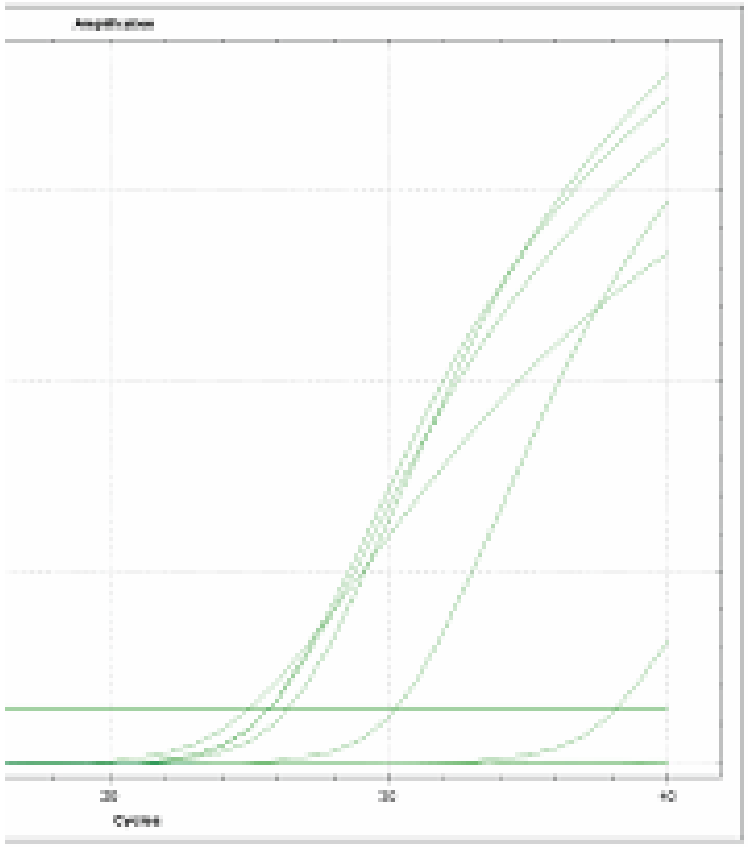
The plateau phases of the Li and Beard assays are sufficiently high.

Ct values for the Li and Beard assays are almost identical, and no significant difference was found.

Implementation/Evaluation of tests for CLso

Output cruve; Left: Li et al, 2009. Right: Beard et al, 2012.





Implementation of identity verification test

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

Indentity verification on species level.

The 2012 Foreign samples positve in Li 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 screening tests: Ravindran et al, and Li et al.

Conclusions on CLso test evaluations (1)

Data and Results from literature investigation, Survey and experiments yield the following for a CLso diagnostic protocol:

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 is a suitable test for initial screening of (carrot) samples, as shown from comparisons. No false positive nor false negatives resulted in this study from this test.

Conclusions on CLso test evaluations (2)

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

Additional confirmation of the positive initial screening test by the other implemented screening tests (Ravindran et al, Beard et al.) is not needed.

Beard et al has shown in the implementation to can even lead to introduction of errors since late Ct values can be obtained with non-target samples. These can however be filtered out using melting curve analysis.

Also generating of additional sequence data for the adk gene does not increase the reliability of the final diagnosis.

Conclusions on CLso test evaluations (3)

Further implementation up next:

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

Two options:

DNA barcoding approach, and

Real-time PCR with haplotype specific probes.

Collection items CLso; grafted tomato plants

Establishment of Collection;
Two accessions From USA.
First one Ceased.

Collection items CLso; grafted potato plants

Potato

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