COST STSM - Final Report

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COST Action: FA0807

STSM Topic: Integrated Management of Phytoplasma Epidemics in Different Crop Systems

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Development of rapid in-field diagnostic techniques for phytoplasmas

1. Background

Phytoplasmas are minute bacteria without cell walls that inhabit phloem sieve elements in infected plants. In nature they are transmitted from plant to plant by phloem-feeding insect vectors and are associated with diseases in about 1000 plant species, in which they can cause major yield losses. Due to the fact that they are not able to exist without their host, phytoplasmas can only be diagnosed and distinguished by using molecular biology based methods.

In today's diagnostics the primary demands are not only for sensitivity and reliability but also for rapid result acquirement and simplicity with minimal equipment requirements, which would allow in-field or 'point-of-care' diagnostics. Rapid DNA extraction methods in combination with the highly specific loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000) procedure meet the criteria of being sensitive, fast, simple and portable.

2. Purpose of the visit

The aim of this scientific mission was to (i) compare a widely used DNA extraction method (CTAB) with two more rapid and simpler DNA extraction methods, (ii) develop phytoplasma specific primers for LAMP, (iii) test the existing phytoplasma-specific LAMP primers (16SrXII) for their specificity and sensitivity with a wide range of phytoplasma isolates and (iv) multiplex different phytoplasma-specific LAMP primers.

3. Description of the work carried out during the visit

Plant and DNA material

All plant and DNA material is specified in Table 1.

Table 1: Plant and DNA material

Plant	Phytoplasma	Plant material - leaves (No. of samples)	CTAB extracted DNA material (No. of samples)
INFECTED MATERIAL			
Catharanthus roseus	Rehmannia glutinosa (RG) I-B	4	1
	Strawberry green petal (SGP) I-C	4	1
	Ribes in vinca (RIV) I-C	4	1
	Sweet potato little leaf (SPLL) II-D	5	1
	Vinca coconut phyllody (VCP) II-?	4	1
	Faba bean phyllody (FBP) II-C	4	1
	Crotalaria saltiana phyllody (FBPSA) II-C	4	1
	Soybean phyllody (SOYP) II-C	4	1
	Western X disease (WX) III	0	1
	Peach X disease (CK) III-A	2	1
	Euscelidius variegatus (API) III-B	0	1
	Poinsettia branching factor (JRI) III-H	0	2
	Lethal yellows (LYD) IV	0	1
	Elm witches broom (ULW) V	0	1
	Brinyal little leaf (BLL) VI	0	1
	Ash yellows (ASHY) VII	0	1
	Pigeonpea witches broom (PPWB) IX	0	1
	Apple proliferation (AP) X-A	0	1
	European stone fruit yellows (ESFY) X-B	0	1
	Pear decline (PD) X-C	0	1
	Stolbur (STOL) XII	0	1
	Mexican periwinkle virescence (MPV) XIII	0	1
Cynodon dactylon	(BGWL) XIV	0	1
Pennisetum purpureum	Napier grass stunt (NGS) XI	3	1
Digitaria	XI or XIV	5	3
Setaria	XI or XIV	1	0
Cynodon dactylon	XI or XIV	1	0
Clematis	V	0	1
Grapevine	V	3	4
	XII-A	6	17
Papaya	XII	0	9
Thistle	I	1	0
Tomato	I	2	0
Parthenium	II-B	0	4
Linseed	II	1	0
Poinsetia	III	3	0
Coconut palm	IV	0	38
Date palm	IV	3	0

HEALTHY MATERIAL			
Catharanthus roseus	/	3	0
Grapevine	/	2	5
Clematis	/	0	1
Coconut palm	/	0	75
Citrus	/	6	15
Thistle	/	3	0
Tomato	/	3	0
Linseed	/	4	0
Parthenium	/	0	1
Papaya	/	0	21
Unknown grass weed	/	6	2
Coffee	/	0	1
Brassica sp.	/	0	1
Elephant grass	/	0	1
Solanaceous	/	0	1
Sorghum helepemse	/	1	0
Comolina	/	0	1
Neem	/	0	1
Seweet potato	/	1	0
Setaria	/	1	0
Passion fruit	/	0	1

3.1 Comparison of DNA extraction methods

Most commonly, the CTAB-based method is used for DNA extraction (Ahrens & Seemüller, 1992), but it is time-consuming and therefore not suitable for in-field diagnostics. For that reason two other methods were tested: extraction of DNA using lateral flow devises (LFD) (Tomlinson et al., 2010) and alkaline polyethylene glycol-based DNA extraction method (PEG) (Chomczynski et al., 2006). The latter turned out to be more suitable for in-field diagnostics. It excels in speed and simplicity. It takes only 15 min of plant tissue incubation in PEG solution for DNA to be extracted. However, before fully taking it to in-field use, we recommend that it should be validated for the type/species of plant tissue that you intend to use it for. It is possible that it may not work for all tissues equally, and there may also be problems with storage of the extracted DNA. In other respects, the LFD method is very useful when dealing with high-titer phytoplasma infected plant hosts such as *C. roseus*.

3.2 LAMP

All singleplex LAMP reactions were performed on a Genie I (OptiGene) in optical 8-well strips using the following conditions: 30 min at 63 °C, followed by gradual temperature increase at 1.0 °C/s ramp rate to 99 °C. Specific LAMP assays were performed in final 21 µl reaction volumes containing 1 µl of sample DNA , 0,2 µM of each F3 and B3 primers, 1 µM of each FIB, BIP, FL and BL primers and 1x Isothermal Master Mix – 400rxns; (Genesys Ltd.). The software Genie II (OptiGene) was used for fluorescence acquisition and melting curve analysis.

3.2.1 Primer design

The sequences of 16SrIII phytoplasmas were obtained from the NCBI (National Center for Biotechnology Information) Nucleotide database using the Basic Local Alignment Search Tool (BLAST) and aligned in Vector NTI 9.0 (Invitrogen) to find suitable regions for the amplicon design. (Accession numbers: EU168766, EU168768 and EU168768.) Primers were designed in PrimerExplorer V4 (Eiken Chemical Co., Ltd.) within the 23S rRNA region.

3.2.2 Specificity and sensitivity of LAMP

The specificity of LAMP primers (which had been designed in previous work) for 16SrXII was determined by testing the cross-reactivity with: (i) DNA samples of phytoplasma reference strains cultivated in *C. roseus and P. purpureum,* (ii) DNA from the field samples of grapevine and papaya, which were diagnostically predetermined to be infected with Stolbur phytoplasma - 16SrXII and (iii) healthy plant DNA material (Table 1).

No non-specific signals were observed from extensive specificity tests showing that the 16SrXII LAMP assay is highly specific. Sensitivity tests on grapevine samples which had been subjected to qPCR analysis in Slovenia confirmed the fact that the sensitivity of LAMP assays in general is one log order lower in comparison to qPCR assays (personal consultation with Dr. M. Dickinson). In addition, a new assay was developed for 16SrIII phytoplasmas. The specificity of the LAMP primers for 16SrIII designed during the short scientific mission was determined by testing the

cross-reactivity with DNA samples of phytoplasma reference strains cultivated in *C. roseus and P. purpureum* (Table 1.).

The sensitivity of LAMP primers for 16SrIII was performed on 10-fold serial dilutions of DNA in water ranging from 10^0 to a 10^5 from 16SrIII. Assay sensitivity was confirmed by preliminary specificity and sensitivity tests. First positive results started to emerge as early as in the 10th min of the run. A positive signal was obtained with one other phytoplasma from 16SrX-B group. Further work is now being undertaken to continue the evaluation of this new 16SrIII test.

3.2.3 Multiplexing

The aim of multiplexing was to design tests that could be used for diagnosis of more than one phytoplasma type in a single assay, using differences in the melting temperatures (Tm) as a means of determining which phytoplasma is present in a sample. After looking at the melting temperatures for the different LAMP assays and the frequency of two phytoplasmas appearing in the same plant host, we determined that the following multiplex LAMP assays would be possible: 16SrI coupled with 16SrII, and 16SrI coupled with 16SrXII. Singleplex reaction results showed the difference in Tm within the first couple would be 2°C, and the difference within the second couple would be only 1°C, but 16SrXII has a distinctive peak shape (Figure 1), which allows it to be distinguished.

Different combinations of primer concentrations were used to assess the optimal primer concentration (see Table 2) in the multiplex assays. So far, only the 16SrI and 16SrII multiplex LAMP assay with 1:2 primer set ratio has been successful.

Table 2: Combinations of LAMP assays for multiplex reactions and tested primer set concentrations.

		Primer set concentration		
		A	B	C
LAMP assay combination (average Tm)	**16SrI*** (84.9±0.2) & **16SrII** (82.9±0.3)	o	o	•
	16SrI* (84.9±0.2) & **16SrXII** (86.6±0.1)	o	o	o

* – concentration of 16SrI was as described in section 3.2
A – cocncentration of 2nd primer set was the same as 16SrI
B – cocncentration of 2nd primer set was 1.5-fold higher than 16SrI
C – cocncentration of 2nd primer set was 2-fold higher than 16SrI
o – unsuccessful test
• – successful test

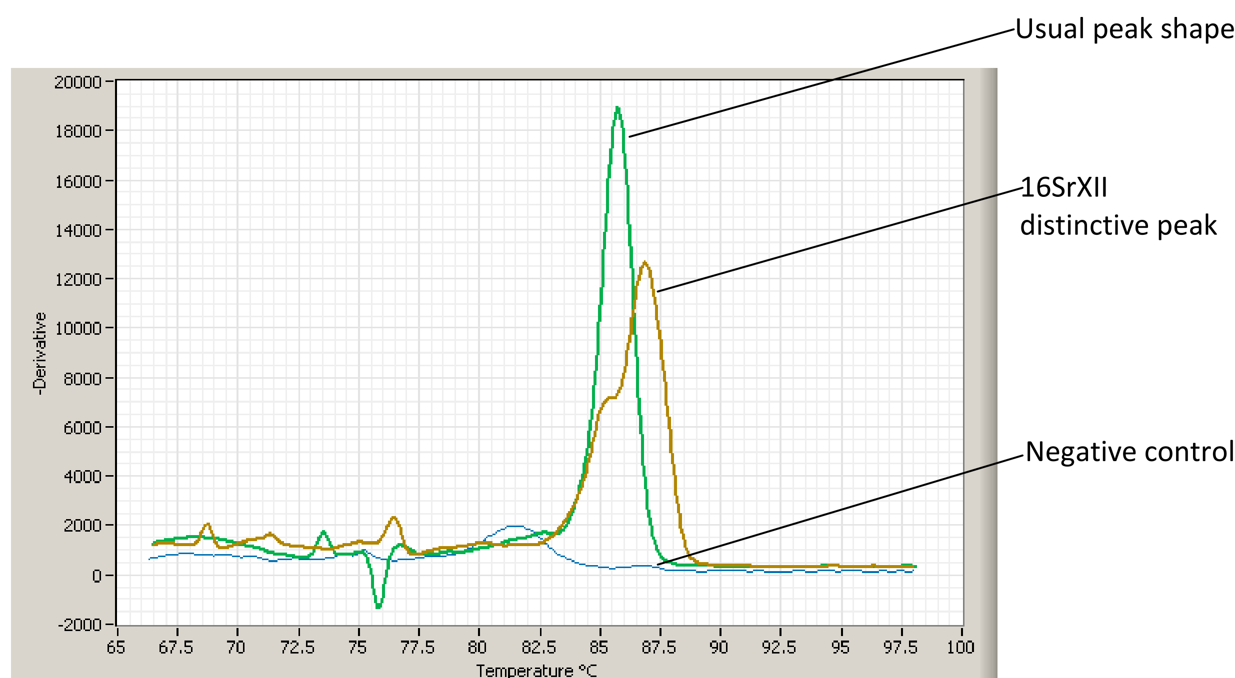

Figure 1: Comparison of usual Tm peak shape and 16SrXII distinctive Tm peak shape.

4. Future collaboration with host institution

Since the two institutions (University of Nottingham, School of Biosciences, UK and National Institute of Ljubljana, Slovenia) involved in this work have a common goal (exploring different aspects of the phytoplasma life in association with their hosts and vectors), the collaboration is likely to be enhanced and developed further in the future.

5. Projected publications

Title:

Use of a real-time LAMP isothermal assay for detecting phytoplasmas in fruit and weeds of the Ethiopian Rift Valley

Authors:

Berhanu Bekele, Jennifer Hodgetts, Jenny Tomlinson, Neil Boonham, Petra Nikolić, Philip Swarbrick and Matthew Dickinson

Journal:

Submitted to Plant Pathology

6. References

- Ahrens U. and Seemüller E. 1992. Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology 82: 828-832.

- Chomczynski P., Rymaszewski M. 2006. Alkaline polyethylene glycol-based method for direct PCR from bacteria, eukaryotic tissue samples and whole blood. BioTechniques 40:454-458.

- Notomi T., Okayama H., Masubuchi H., Yonekawa T., Watanabe K., Amino N., Hase T. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research 28 (12):e63.

- Tomlinson J.A., Boonham N., Dickinson M. 2010. Development and evaluation of a one-hour DNA extraction and loop-mediated isothermal amplification assay for rapid detection of phytoplasmas. Plant Pathology 59 (3): 465-471.