EUPHRESCO Final Report (NC)

for Non-Competitive research projects

Project Title and Acronym
Interlaboratory comparison and validation of detection methods for phytoplasmas of phytosanitary concern in European orchards FRUITPHYTOINTERLAB

Project Duration:

Start date:	01/02/10
End date:	29/04/11

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2. Executive Summary

Project Summary

Please provide a summary suitable for web publication and which is understandable to the intelligent non-scientist.
Include: Title, main objectives, appropriate Methods, Results and Conclusions. (max. 2 pages)

Inter-laboratory comparison and validation of detection methods for phytoplasmas of phytosanitary concern in European orchards

Introduction

Phytoplasmas are specialised plant pathogenic bacteria, colonizing the plant phloem tissue. They lack a cell wall, are non-culturable and are transmitted by insect vectors and by vegetatively propagated plant material. These plant pathogens are increasingly spreading and causing economical losses especially on fruit tree production. Molecular detection methods are already available; however sampling, extraction and detection require harmonisation and validation throughout European laboratories. This process would improve the reliability of results enabling the development of improved management strategies to prevent the spread of these diseases.

Main objectives

To carry out inter-laboratory ring-testing to validate test methods, on 16S and 23S ribosomal gene, for the detection of phytoplasmas ('*Candidatus* Phytoplasma mali','*Ca*. P. pyri', '*Ca*. P. prunorum') in symptomatic and asymptomatic plants from infected orchards, including nested-PCR and real time PCR.

Material

The 22 participant laboratories analysed a series of 30 blind samples, target (symptomatic and asymptomatic infected plants) and non-target (healthy plants and closely related bacteria) using protocols provided.
The total DNA was extracted from midribs using a CTAB protocol (Doyle and Doyle, 1990) and was sent to each partner as dried DNA. These samples came from one of four laboratories. All samples were assayed as 'undiluted' and 'tenfold diluted' extracts. Standards of a cloned P1/P7 fragments from '*Ca*. P. mali' and '*Ca*. P. pyri' in concentration from 10^7 to 10^1 were also included in the trials.

Methods

Four molecular protocols were submitted for ring testing:

1. AP group specific nested PCR, with the primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995), followed by a group specific PCR with fO1/rO1 (Lorenz *et al*., 1995) (nested PCR);
2. Real time for the specific detection of *'Ca.* P. mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum' (Nikolic *et al*., 2010) (specific qPCR);
3. Real time PCR for the universal detection of phytoplasmas (Christensen *et al*., 2004) (universal qPCR-C);
4. Real time PCR for the universal detection of phytoplasmas (Hodgetts *et al*., 2009) (universal qPCR-H).

Following discussion between laboratories, diagnostic sensitivity and diagnostic specificity were calculated using the R statistical framework.

Results

Analytical sensitivity obtained from standards of the cloned P1/P7 fragments from '*Ca*. P. mali' and '*Ca*. P. pyri' is almost 10^1 for all tested methods, except universal qPCR-H that amplifies a different DNA fragment.
The analysis of agreement between laboratories, diagnostic sensitivity (SE) and diagnostic specificity (SP) was calculated for each protocol and for diluted and undiluted samples. Fleiss' kappa index showed an 'almost perfect' agreement for all tested protocols, p-values were almost 0, and bootstrap

95 % confidence intervals gave an accurate correlation following Landis and Koch (1977) interpretation table. The mean values of sensitivity and specificity were high for all protocols, ranging from 98.2% to 100% for SE, whereas the SP ranged from 93.8% to 99.7%.

Conclusions

The results obtained in the interlaboratory trials showed that all four tested protocols were sensitive. The robustness of the protocols was also supported by the agreement levels for the different participants using different thermocyclers.

3. Report

Objectives and tasks of the project

The main objective was to run inter-laboratory ring-tests to validate test methods, on 16S and 23S ribosomal gene, for the detection of phytoplasmas ('C*andidatus* Phytoplasma mali','*Ca*. P. pyri', '*Ca*. P. prunorum') in symptomatic and asymptomatic plants from infected orchards, including:

- nested-PCR
- real-time PCR

The objectives of this project were set by to the COST action "Integrated Management of Phytoplasma Epidemics in Different Crop Systems FA0807".

All proposed tasks were completed:

Task	**subtask**	**Partners involved**	**degree of achievement**
Task 1 Exchange of procedures for identification of phytoplasmas using nested-PCR or real time PCR.	Exchange two protocols on real-time PCR for the universal identification of all phytoplasmas. - Christensen *et al*., 2004 - Hodgets *et al*., 2009.	Aarhus University	ACHIEVED April 2010
	Exchange the protocol on nested PCR for the detection of phytoplasmas belonging to 16SrX group. Based on: - Deng & Hiruki, 1991 (primer P1) - Schneider *et al*., 1995 (primer P7) - Lorenz *et al*., 1995 (primers fO1/rO1)	NIB; CRA-PAV	
	Exchange 3 protocols on real-time PCR for the specific detection of '*Ca*. Phytoplasma pyri', '*Ca*. P. mali' and '*Ca*. P. prunorum'. These methods have been developed by NIB (Nikolic et al., 2010).	NIB	

Task	subtask	Partners involved	degree of achievement
Task 2 Select 30 samples to be included in the study. Extract the DNA and prepare uniform aliquots for all the partners.	Selection of plant material infected with '*Ca*. Phytoplasma pyri' and '*Ca*. P. mali' **- 5** of '*Ca*. Phytoplasma pyri'. **- 5** of '*Ca*. P. mali'.	JKI	ACHIEVED From February to May 2010
	Selection of plant material infected with '*Ca*. P. prunorum' (**5** samples).	DAAM; CRA-PAV	
	Selection of **6** negative controls of closely related bacteria	FERA	
	Selection of negative plant material controls (**9** samples)	JKI; IRTA; CRA-PAV	
Task 3 Send out material	Send 30 blind DNA extracts.	DAAM	ACHIEVED September 2010
Task 4 Ring test to detect '*Ca*. Phytoplasma pyri', '*Ca*. P. mali' and '*Ca*. P. prunorum' using PCR based methods.	nested PCR for the detection of phytoplasmas belonging to 16SrX group	All	ACHIEVED December 2010
	real-time PCR for the universal identification of all phytoplasmas. - Christensen *et al*., 2004 - Hodgets *et al*., 2009.	All partners with real-time instruments.	
	Specific detection of '*Ca*. Phytoplasma pyri', '*Ca*. P. mali' and '*Ca*. P. prunorum', using the methods developed by NIB.	All partners with real-time instruments.	
Task 5 Meeting of ring test participants.	Presentation and discussion of results	All	ACHIEVED April 2011
	Agree publication of results		
Task 6 Write final report		DAAM CRA-PAV	ACHIEVED June 2011

Material

30 samples (s1 – s30 sample 1 – sample 30), target (symptomatic and asymptomatic infected plants) and non-target (healthy plants and closely related bacteria) were selected (table 1) for all experiments in 22 European laboratories. As phytoplasmas are living quarantine organisms, the samples to be tested were sent out as dried DNA extracts. Standards of a cloned P1/P7 fragments from '*Ca*. P. mali' and '*Ca*. P. pyri' in concentration from 10^7 to 10^1 were also included in the trials. The total DNA was extracted from midribs using a CTAB protocol (Doyle and Doyle, 1990) in four laboratories (DAAM, JKI, CRA-PAV, FERA) and was sent to each partner as dried DNA. All the laboratories were asked to resuspend it in 500 µl of distilled water and to test the DNAs as undiluted and tenfold diluted samples.

Table 1. List of tested samples and their origin.

N°	Species	Sample status	Origin
1	apple	healthy	JKI
2	apple	'*Ca*. P. mali'	JKI
3	apple	'*Ca*. P. mali'	JKI
4	apricot	'*Ca*. P. prunorum'	CRA-PAV
5	apricot	'*Ca*. P. prunorum'	CRA-PAV
6	plum	healthy	DAAM
7	pear	healthy	JKI
8	plum	healthy	DAAM
9	pear	'*Ca*. P. pyri'	JKI
10	extracted DNA	bacteria	FERA
11	extracted DNA	bacteria	FERA
12	apple	'*Ca*. P. mali'	JKI
13	pear	'*Ca*. P. pyri'	JKI
14	extracted DNA	bacteria	FERA
15	apple	'*Ca*. P. mali'	JKI
16	apple	'*Ca*. P. mali'	JKI
17	extracted DNA	bacteria	FERA
18	pear	'*Ca*. P. pyri'	JKI
19	apple	healthy	JKI
20	pear	'*Ca*. P. pyri'	JKI
21	plum	healthy	DAAM
22	pear	'*Ca*. P. pyri'	JKI
23	extracted DNA	bacteria	FERA
24	extracted DNA	bacteria	FERA
25	pear	healthy	JKI
26	apricot	'*Ca*. P. prunorum'	CRA-PAV
27	pear	healthy	JKI
28	apricot	'*Ca*. P prunorum'	CRA-PAV
29	pear	healthy	JKI
30	plum	'*Ca*. P. prunorum'	DAAM

Methods

Four molecular protocols were submitted to the inter-laboratory ring test (see a partial reproduction of the submitted documents from Appendix1 to Appendix 4):

1. AP group specific nested PCR, (nested PCR);
2. real time PCR for the specific detection of '*Ca*. P. mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum' (Nikolic *et al*., 2010) (specific qPCR);
3. real time PCR for the universal detection of phytoplasmas (Christensen *et al*., 2004) (universal qPCR-C);
4. real time PCR for the universal detection of phytoplasmas (Hodgetts *et al*., 2009) (universal qPCR-H).

In order to standardize the experiments Taq DNA polymerase and real time master mix brand were specified and TaqMan probes were supplied by some partners.

Some controls were used for each method:

- positive (DNA from known AP, PD and ESFY-infected plants)
- NTC (no DNA, add water; 'no template control') (For every reaction mix two NTCs are prepared– one at the start of pipetting (NTC1) and one at the end (NTC2).)

For each Real Time method, samples were run direct and diluted:

- s1 – s30 (unknown sample 1 – unknown sample 30)
- s1 10x – s30 10x (unknown sample 1 diluted 10 times - unknown sample 30 diluted 10 times) (Each DNA extract should be diluted 10 times: 5 µl of DNA + 45 µl of sterile nuclease free water. Mix all dilutions well and spin briefly (~5 s) in a centrifuge.)

Nested PCR (Appendix 1)

The protocol is based on a direct PCR using the universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995), followed by a group specific nested PCR performed with the primer pairs fO1/rO1 (Lorentz et al., 1995). Direct and nested PCR were performed in a 25 µl mixture containing: 1X Green GoTaq reaction buffer, 200 µM dNTPs (Promega), 0.4 µM of each primer, 0.625 U of GoTaq DNA polymerase (Promega), 1 µl of extracted DNA for direct PCR and 1 µl of P1/P7 amplicons diluted 1:30 for nested PCR (for 50 µl mixture: 2 µl DNA/diluted PCR product).
The cycling parameters included an initial denaturation step at 94°C for 2 min, followed by 36 and 38 cycles, for direct and nested PCR, respectively: 1 min at 94°C (denaturation), 1 min at 55°C and 50°C, for direct and nested PCR, respectively (annealing), 2 min at 72°C (extension) and a final extension step at 72°C for 8 min.

Specific qPCR (Appendix 2)

The method is a TaqMan real time PCR (qPCR) using three specific FAM-MGB probes (AP-P, ESFY-P and PD-P) in separate reactions, using chemicals and amplification conditions reported by Nikolic et al. (2010).

Universal qPCR-C (Appendix 3)
The method is a TaqMan qPCR using a FAM-TAMRA phytoplasma universal probe using chemicals and amplification conditions reported by Christensen et al. (2004).

Universal qPCR-H (Appendix 4)
The method is a TaqMan qPCR using a VIC-TAMRA phytoplasma universal probe using chemicals and amplification conditions reported by Hodgetts et al. (2009).

All the participants included their own positive and negative template controls. By qPCR, all samples were tested in duplicate. COX or human 18S rRNA (Applied Biosystems) was used as endogenous quality control of DNA extraction. qPCR were performed in 10 or 25 µl reactions.

Processing of the results data

The participants were asked to provide only '+' or '-' results for nested-PCR. Ct values for each replicate were asked for qPCR protocols, specifying threshold and baseline (manual or automatic). A template for the results was distributed to all participants.

The following parameters were calculated, using the R statistical framework (2010), to analyze the result:

1. **Agreement between laboratories**
   Measured by calculation of the Kappa coefficient and interpreted as reported in: The index of agreement between the considered laboratories is measured by the Fleiss' kappa index (Fleiss *et al*., 2003). R output refers the laboratories as raters, the samples as subjects.
   Landis and Koch (1977) gave the following table for interpreting kappa values

   - < 0 Poor agreement
   - 0.00 to 0.20 Slight agreement
   - 0.21 to 0.40 Fair agreement
   - 0.41 to 0.60 Moderate agreement
   - 0.61 to 0.80 Substantial agreement
   - 0.81 to 1.00 Almost perfect agreement

   Notice that the values referred by Landis are only an approximate guideline.

   The output includes also significance tests for the kappa index (the null hypothesis is a zero kappa value) and two bootstrap confidence intervals at 95% confidence level.

2. **Diagnostic sensitivity (SE)**
   An estimation of the ability of the method to detect the target.
   SE=100xTP/(TP+FN) (table 2)

3. **Diagnostic specificity (SP)**
   An estimation of the ability of the method not to detect the non-target.
   SP= 100xTN/(FP+TN) (table 2).

Table 2. Parameters for calculation of diagnostic specificity and sensitivity.

TP – true positive	positive detected from positive expected
FN - false negative	negative detected from positive expected
FP - false positive	positive detected from negative expected
TN - true negative	negative detected from negative expected

Results

All the participant laboratories carried out analysis on all DNA samples according to the provided protocols. The involvement of the participants in each protocol is summarized in Table 3.

Table 3. Number of laboratories involved in each tested protocol.

Number of labs involved	Nested PCR	Specific qPCR (Nikolic *et al.*, 2010)	Universal qPCR-C (Christensen *et al.*, 2004)	Universal qPCR-H (Hodgetts *et al.*, 2009)	Plasmid
6	X	X	X	X	X
8	X	X	X	X	
2	X	X	X		
4	X				
1	X		X	X	
1	X	X		X	

High quality DNA extractions were confirmed by the results of the endogenous controls. Analytical sensitivity obtained from standards of the cloned P1/P7 fragments from '*Ca*. P. mali' and '*Ca*. P. pyri' is reported in table 4. No relevant differences were observed among the protocols.

Table 4. Analytical sensitivity of the tested protocols calculated using serial dilutions of P1/P7 cloned fragment from '*Ca.* P. mali' and '*Ca.* P. pyri'. The analysis was not performed for Universal qPCR-H,that amplifies a region of 23S gene not included in P1/P7 amplicons.

Laboratories(*)	Nested-PCR		Specific qPCR		Universal qPCR-C	
	'*Ca*. P. mali'	'*Ca*. P. pyri'	'*Ca*. P. mali'	'*Ca*. P. pyri'	'*Ca*. P. mali'	'*Ca*. P. pyri'
1	n.t.	n.t.	10^1	10^1	10^1	10^2
8	n.t	n.t	10^1	10^1	10^1	10^1
11	n.t.	n.t.	n.t.	10^1	10^1	10^1
12	10^3	10^1	n.t.	n.t.	10^1	10^2
13	n.t	n.t	10^1	10^1	10^1	10^1
16	10^1	10^1	n.t	n.t	n.t	n.t

(*)In the analysis of the results all the participant laboratories were reported with anonymous number.

If a laboratory had more than 10% of unexpected differences between two replicates (Table 5), or more than 10% of unexpected differences between the undiluted and diluted sample (Table 6), for one method, all the results obtained for that method and laboratory were omitted in the statistical analysis. One lab (lab 11) had problems with their mastermix when specific qPCR-ESFY and –PD was performed (negative results also for positive qPCR control); therefore these results were also omitted.

Table 5. Analysis of high differences between two parallels (more than 3 Ct). Differences between 3 and 6 Ct which were observed at the end (for example one replicate undetermined, the other with Ct 36) are not included. Specific qPCR is shown for each probe as NIB-AP, NIB-ESFY and NIB-PD.

Laboratory(*)	Universal qPCR-H	Universal qPCR-C	Specific qPCR -AP	Specific qPCR-ESFY	Specific qPCR -PD
1	0%	0%	0%	0%	0%
2	48%	7%	10%	15%	33%
3	0%	0%	0%	0%	0%
4		0%	0%	2%	0%
5	0%	0%	0%	2%	0%
6					
7	13%	13%	0%	3%	3%
8	0%	0%	0%	0%	0%
9	2%	0%	3%	0%	0%
10	3%	0%			
11	0%	0%	3%	0%	2%
12	0%	2%	0%	0%	0%
13	3%	12%	0%	0%	0%
14					
15	0%	0%	0%	0%	0%
16					
17	12%	12%	3%	2%	5%
18					
19	0%	0%	0%	0%	0%
20	0%		0%	0%	0%
21		3%	5%	5%	5%
22	0%	2%	0%	0%	0%

more than 20%
between 15 and 20%
between 10 and 15%
between 5 and 10%

(*)In the analysis of the results all the participant laboratories were reported with anonymous number.

Table 6. Unexpected differences between undiluted and diluted samples. Specific qPCR is shown for each probe as NIB-AP, NIB-ESFY and NIB-PD

Laboratory(*)	Universal qPCR-H	Universal qPCR-C	Specific qPCR -AP	Specific qPCR-ESFY	Specific qPCR -PD
1	0%	0%	0%	0%	0%
2	90%	53%	27%	37%	47%
3	0%	0%	0%	0%	0%
4		0%	0%	0%	0%
5	7%	3%	0%	3%	3%
6					
7					
8	0%	0%	3%	0%	3%
9	0%	0%	20%	0%	3%
10	0%	3%			
11	0%	17%	7%	0%	3%
12	0%	0%	0%	0%	7%
13	7%	10%	43%	27%	20%
14					
15	37%	20%	17%	13%	0%
16					
17	20%	30%	20%	7%	17%
18					
19	0%	0%			
20	3%		0%	0%	0%
21		13%	7%	7%	13%
22	0%	10%	0%	3%	3%

more than 20%
between 15 and 20%
between 10 and 15%
between 5 and 10%

(*)In the analysis of the results all the participant laboratories were reported with anonymous number.

The analysis of agreement between laboratories, diagnostic sensitivity (SE) and diagnostic specificity (SP) was calculated for each protocol and for diluted and undiluted samples (Table 7 and 8). Fleiss' kappa index showed an 'almost perfect' agreement for all tested protocols, p-values were almost 0, and bootstrap 95 % confidence intervals gave an accurate concordance following Landis and Koch (1977) interpretation table. The mean values of sensitivity and specificity were high for all protocols, ranging from 98.2% to 100% for SE, whereas the SP ranged from 93.8% to 99.7%.

Table 7. Diagnostic sensitivity (SE), Diagnostic specificity (SP) and agreement values calculated by the Fleiss' kappa index for each tested protocol with original samples.

Method	Number of laboratories considered	Mean		Kappa index	Kappa bootstrap confidence intervals
		SE	SP		
Nested-PCR	20	99.3	97.7	0.94	0.993, 0.977
Universal qPCR-C	10	100	96	0.926	0.856, 1.002
Universal qPCR-H	12	99.4	97.2	0.945	0.874, 1.025
Specific qPCR – '*Ca*. P. pyri'	12	100	99.7	0.98	0.832,1.147
Specific qPCR – '*Ca*. P. mali'	12	100	98.7	0.924	0.788,1.09
Specific qPCR – '*Ca*. P. prunorum'	13	100	93.8	0.84	0.699,1.008

Table 8. Diagnostic sensitivity (SE), Diagnostic specificity (SP) and agreement values calculated by the Fleiss' kappa index for each tested protocol with 10 fold diluted samples.

Method	Number of laboratories considered	Mean		Kappa index	Kappa bootstrap confidence intervals
		SE	SP		
Universal qPCR-C	10	100	98.7	0.973	0.937,1.011
Universal qPCR-H	12	98.3	98.9	0.944	0.899,0.991
Specific qPCR – '*Ca*. P. pyri'	10	100	100	1.0	
Specific qPCR – '*Ca*. P. mali'	10	100	99.6	0.976	0.825,1.15
Specific qPCR – '*Ca*. P. prunorum'	11	98.2	100	0.978	0.935,1.019

It is interesting to note that two healthy plum samples, sample 6 and sample 21, gave unexpected results when analyzed by some real-time PCR methods. Only one laboratory detected the presence of phytoplasma in samples 6 and 21 by nested or Universal qPCR-C. But sample 6 was detected positive by 4 of 12 laboratories and by 10 of 13 when it was analyzed by Universal qPCR-H and Specific qPCR- ESFY respectively. Sample 21 was detected positive by Specific qPCR- ESFY in 5 of 13 laboratories. It is possible that some methods were more sensitive than others and that the expected healthy samples were actually plums with a low titre of phytoplasma, just near the limit of detection of some methods and not detectable by the others.

Discussion

The results obtained in the inter-laboratory trials showed that all four methods tested were sensitive. The robustness of the protocols was also supported by the agreement levels for the different participants using different thermo cyclers. Nevertheless, the diagnostic specificity values were affected by some unexpected results that leave important questions and make it necessary for further investigations. In particular, some non-target samples (plants assumed as healthy) gave positive results in different experiments and laboratories, this result indicates

the necessity to establish if the samples are 'true negative' or if they have a low titre of phytoplasma, detectable only by highly sensitive techniques.
Finally, in this ringtest the DNA extraction steps have not been evaluated as phytoplasmas are living quarantine pathogens (see CE 95/44 directive). These results therefore are only related to the reliability of the amplification procedures, even though the nucleic acid extraction should be considered a critical step in phytoplasmas detection.
Questions that have arisen from the preliminary analysis of these results have led to further suggested work.

Main conclusions

From the results of this project reliable and validated laboratory protocols are available. These protocols complement each other (nested and real time PCR), and can be used to comply with the official testing programs (routine surveys).

The harmonization of protocols by ring testing is a great way to achieve greater uniformity among laboratories making them more efficient in the detection of the phytoplasmas studied in this project.

This is important because they are quarantine phytoplasmas that have a very uneven distribution in the tree, variations occur in their concentration throughout the seasons and also vary from year to year with climatic conditions. The host plant material can also contain a large number of inhibitors, which when not properly disposed of during the extraction of nucleic acid may inhibit the PCR reaction. The quality of the DNA extractions is crucial for use in nested PCR and an even more limiting factor n real-time PCR.

Therefore, further work needs to be carried out on aspects of field sampling and also on finding quick and easy extraction methods. In field samples where symptoms are not evident, the DNA extraction by Mini-prep does not give a big enough representative sample, especially in the case of mother plants. The long nucleic acid extraction methods take a lot of time and are not practical when there are a lot of samples.

A possible research line could be to evaluate detection methods for other phytoplasmas of great interest, for example the grapevine phytoplasmas. The possibility of evaluating different extraction methods is also proposed.

Papers, other publications and dissemination activities

Meeting to discuss results between partners
Meeting Dates: April, 27th 2011
Location: European and Mediterranean Plant Protection Organization, Paris, France
With the economic support of ECOST-MEETING-FA0807-270411-007301

List of assistants:

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Ana O. Alfaro Fernández	Instituto Agroforestal Mediterráneo-Universidad Politécnica de Valencia	Spain
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Assunta Bertaccini	DiSTA, Patologia Vegetale, University of Bologna	Italy
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Camilo Gianinazzi	International Plant Analysis and Diagnostics	Italy
De Jonghe Kris	Institute for Agricultural and Fisheries Research	Belgium
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Stéphan Steyer	CRA-W Walloon Agricultural Research Centre, Life Sciences Department	Belgium
Zhelju Georgiev Avramov	Central Laboratory for Plant Quarantine	Bulgaria

Papers

1. European interlaboratory comparison and validation of detection methods for '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma prunorum' and '*Candidatus* Phytoplasma pyri': preliminary results. Bulletin of Insectology 64 (Supplement): Sxx-Sxx, 2011. ISSN 1721-8861
   Presentation in the 2nd International Phytoplasma Working Group (IPWG)Meeting that will take place the 12th-16th September of 2011 in Neustadt (Germany)
2. It was decided to publish a complete article in the EPPO bulletin.

Acknowledgements

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Appendix 1: Nested PCR

This protocol provides a detailed description of nested PCR that can be used for AP group (16SrX-group) specific detection. This was prepared by Nataša Mehle, National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia. The details about the protocol (reaction compositions, cycling parameters) were obtained from Graziella Pasquini.

Reagents:

- Primers:

primers	sequence	specificity	reference
P1	5'-AAG-AGT-TTG-ATC-CTG-GCT-CAG-GAT-T-3'	phytoplasma	Deng and Hiruki, 1991 Schneider et al.,1995
P7	5'-CGT-CCT-TCA-TCG-GCT-CTT-3'		
fO1	5'-CGG-AAA-CTT-TTA-GTT-TCA-GT-3'	AP group	Lorenz et al., 1995
rO1	5'-AAG-TGC-CCA-ACT-AAA-TGA-T-3'		

- GoTaq DNA Polymerase (Promega, Cat. no. M3171) (includes: 5U/µl GoTaq DNA Polymerase (M830A), 5x Green GoTaq Reaction Buffer (M791A))
- PCR nucleotide mix (Promega, Cat. No. C1141) (Conc.: 10mM each)
- Nuclease – free water
- Chemicals for agarose gel electrophoresis + size marker

Reaction Composition:

Detection assays:

- if the reaction volume is 25 µl:

mix for PCR P1/P7	**final conc. in 25µl**	**volume for 1 reaction (µl)**	**volume for 36 reactions (µl)**
Sterile, nuclease-free water		16.375	589.50
5x Green GoTaq Reaction Buffer	1 x	5.0	180.0
10 mM PCR Nucleotide mix (dNTP)	200 µM	0.5	18.0
primer: P1 (10 µM)	0.4 µM	1.0	36.0
primer: P7 (10 µM)	0.4 µM	1.0	36.0
5U/µl GoTaq DNA polymerase	0.625 U	0.125	4.50
TOTAL		24.0 µl	864.0

Add 1 µl of each DNA extract or control to each test tube, as required.

mix for nested PCR f01/r01	final conc. in 25µl	volume for 1 reaction (µl)	volume for 36 reactions (µl)
Sterile, nuclease-free water		16.375	589.50
5x Green GoTaq Reaction Buffer	1 x	5.0	180.0
10 mM PCR Nucleotide mix (dNTP)	200 µM	0.5	18.0
primer: f01 (10 µM)	0.4 µM	1.0	36.0
primer: r01 (10 µM)	0.4 µM	1.0	36.0
5U/µl GoTaq DNA polymerase	0.625 U	0.125	4.50
TOTAL		24.0 µl	864.0

Add 1 µl of each diluted P1/P7 PCR product to each test tube.

Cycling conditions:

Switch on the thermal cycler in advance as per producer's instructions to warm it up and stabilize conditions.

Set cycling parameters:

- For PCR P1/P7:

2 min at 94 °C	denaturation
36 cycles of 1 min at 94 °C 1 min at 55 °C 2 min at 72 °C	 denaturation annealing extension
8 min at 72 °C	final extension
∞ at 4 °C	stop

- For nested PCR f01/r01:

2 min at 94 °C	denaturation
38 cycles of 1 min at 94 °C 1 min at 50 °C 2 min at 72 °C	 denaturation annealing extension
8 min at 72 °C	final extension
∞ at 4 °C	stop

Adjust reaction volume.

Appendix 2 Specific qPCR

This protocol provides a detailed description of methods that can be used for specific detection of '*Ca.* Phytoplasma pyri', '*Ca.* P. mali' and '*Ca.* P. prunorum' by real time PCR. This protocol was prepared by Nataša Mehle, National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia. If you have specific questions about the TaqMan assay, please contact: Nataša Mehle [natasa.mehle@nib.si]

Reagents:

- Primer and probes:
  - forward primer: SAD-F (10 µM)
  - reverse primer: SAD-R (10 µM)
  - probe FAM-MGB: AP-P (2,5 µM)
  - probe FAM-MGB: ESFY-P (2,5 µM)
  - probe FAM-MGB: PD-P (2,5 µM)
- 2× Maxima™ qPCR Master Mix (Fermentas; Cat. no. K0232)
- 2× TaqMan Universal PCR Master Mix (Applied Biosystems; Cat. no. 4304437)

Reaction Composition:

Detection assays: if the reaction volume is 25 µl:

mastermix for AP	final conc. in 25µl	volume for 1 reaction (µl)	volume for 158	reactions (µl)
Sterile, nuclease-free water		2.10	331.80	
2× Maxima™ qPCR Master Mix (Fermentas)	1 x	12.50	1975.00	
forward primer: SAD-F (10 µM)	900 nM	2.25	355.50	
reverse primer: SAD-R (10 µM)	900 nM	2.25	355.50	
probe MGB: AP-P (2.5 µM)	90 nM	0.90	142.20	
TOTAL		20.0 µl	3160.0	

mastermix for ESFY	final conc. in 25µl	volume for 1 reaction (µl)	volume for 158	reactions (µl)
Sterile, nuclease-free water		2.10	331.80	
2× Maxima™ qPCR Master Mix (Fermentas)	1 x	12.50	1975.00	
forward primer: SAD-F (10 µM)	900 nM	2.25	355.50	
reverse primer: SAD-R (10 µM)	900 nM	2.25	355.50	
probe MGB: ESFY-P (2.5 µM)	90 nM	0.90	142.20	
TOTAL		20.0 µl	3160.0	

mastermix for PD	final conc. in 25µl	volume for 1 reaction (µl)	volume for 158	reactions (µl)
Sterile, nuclease-free water		2.10	331.80	
2× Maxima™ qPCR Master Mix (Fermentas)	1 x	12.50	1975.00	
forward primer: SAD-F (10 µM)	900 nM	2.25	355.50	
reverse primer: SAD-R (10 µM)	900 nM	2.25	355.50	
probe MGB: PD-P (2.5 µM)	90 nM	0.90	142.20	
TOTAL		20.0 µl	3160.0	

Add 5 µl of each DNA extract (undiluted and 10x diluted) or control to each test well, as required.

Cycling conditions:

Unless essential e.g. due to specific non-ABI kit requirements, please do not alter this.

Switch on the thermal cycler in advance as per producer's instructions to warm it up and stabilize conditions.

Set cycling parameters:

2 min at 50 °C	UNG activation step
10 min at 95 °C	polymerase activation
45 cycles of 15 s at 95 °C 1 min at 60 °C	 DNA denaturation annealing and extension

Adjust reaction volume if needed.

Analyzing data:

For analysis there are usually different options available with regard to setting signal and noise limits: automatic and manual.

The following are instructions for analysis of real-time PCR results for Applied Biosystems' analysis softwares, please adapt them as suitable to your instrument.

- use automatic baseline
- the threshold should be set manually crossing the exponential phase of control amplification curves
- Record Ct values on data-collection sheet
- In case of doubtful results check the multicomponent plot. In positive samples FAM should be increasing.

Interpretation of results:

The positive control should be positive, otherwise an error in the PCR reaction should be considered.

NTC should be negative, otherwise contamination of reaction mix should be considered.

Any sample giving a Ct of less than 40, should be scored as positive.

Appendix 3 Universal qPCR-C

This protocol provides a detailed description of methods that can be used for universal detection of phytoplasmas by real time PCR.

This protocol was prepared by Mogens Nicolaisen, Aarhus University on the basis of the paper:
Christensen et al (2004) Distribution of phytoplasmas in infected plants as revealed by real-time PCR and bioimaging. *Molecular Plant-Microbe Interactions* 17: 1175-1184.

Reagents:

- Phytoplasma forward primer: CGTACGCAAGTATGAAACTTAAAGGA (10 µM)
- Phytoplasma reverse primer: TCTTCGAATTAAACAACATGATCCA (10 µM)
- Phytoplasma probe FAM-TAMRA: TGACGGGACTCCGCACAAGCG (2,5 µM)

- TaqMan Universal PCR Master Mix (Applied Biosystems; Cat. no. 4304437)

Reaction Composition:

	10 µl reaction	25µl reaction
	µl	µl
TaqMan Universal PCR Master Mix	5	12.5
Forward phytoplasma primer (10 µM stock)	0.3	0.75
Reverse phytoplasma primer (10 µM stock)	0.9	2.25
Phytoplasma probe (2.5 µM stock)	0.4	1
Water	1.4	3.5
Total	8	20

aliquot 8 (20) µl into each well

Add 2 (5) µl template DNA

Cycling conditions:

Unless essential e.g. due to specific non-ABI kit requirements, please do not alter this.

Switch on the thermal cycler in advance as per producer's instructions to warm it up and stabilize conditions.

Set cycling parameters:

2 min at 50 °C	UNG activation step
10 min at 95 °C	polymerase activation
45 cycles of 15 s at 95 °C 1 min at 60 °C	 DNA denaturation annealing and extension

Adjust reaction volume if needed.

Analyzing data:

For analysis there are usually different options available with regard to setting signal and noise limits: automatic and manual.

The following are instructions for analysis of real-time PCR results for Applied Biosystems' analysis softwares, please adapt them as suitable to your instrument.

- use automatic baseline
- the threshold should be set manually crossing the exponential phase of control amplification curves
- Record Ct values on data-collection sheet
- In case of doubtful results check the multicomponent plot. In positive samples FAM should be increasing.

Interpretation of results:

The positive control should be positive, otherwise an error in the PCR reaction should be considered.

NTC should be negative, otherwise contamination of reaction mix should be considered.

Any sample giving a Ct of less than 40, should be scored as positive.

Appendix 4 Universal qPCR-H

This protocol provides a detailed description of methods that can be used for universal detection of phytoplasmas by real time PCR. This booklet was prepared by Mogens Nicolaisen, Aarhus University on the basis of the paper:

Hodgetts et al. (2009) Panel of 23S rRNA Gene-Based Real-Time PCR Assays for Improved Universal and Group-Specific Detection of Phytoplasmas. *Applied and Environmental Microbiology* 75: 2945-2950.

Reagents

- Phytoplasma forward primer JH-F1: G**G**T**C**TCCGAATGGG**AA**AACC (10 µM)
- Phytoplasma forward primer JH-F all: **A**TT**T**TCCGAATGGG**GC**AACC (10 µM)
- Phytoplasma reverse primer JH-R: CTCGTCACTACTACC**R**GAATCGTTATTAC (10 µM)
- Phytoplasma probe JP-H uni FAM-MGB: AACTGAAA**T**ATCTAAGTA**A**C (2.5 µM)

Plant specific control assay:

Cox-F: CGT CGC ATT CCA GAT TAT CCA (10 µM)
Cox-R: CAA CTA CGG ATA TAT AAG AGC CAA AAC TG (10 µM)
Cox-P VIC-TAMRA: TGC TTA CGC TGG ATG GAA TGC CCT (2.5 µM)

- TaqMan Universal PCR Master Mix (Applied Biosystems; Cat. no. 4304437)

Reaction Composition:

Mastermix for universal phytoplasma detection:

	10 µl reaction	25µl reaction
	µl	µl
TaqMan Universal PCR Master Mix	5	12.5
Forward JH F1 (10 µM stock)	0.3	0.75
Forward JH F all (10 µM stock)	0.3	0.75
Reverse JH-R (10 µM stock)	0.3	0.75
Probe JH-P uni (2.5 µM stock)	0.4	1
Water	2	5
Total	8	20

Mastermix for plant control:

	10 µl reaction	25µl reaction
	µl	µl
TaqMan Universal PCR Master Mix	5	12.5
Cox-F (10 µM stock)	0.3	0.75
Cox-R (10 µM stock)	0.3	0.75
Cox-P (2.5 µM stock)	0.4	1
Water	2	5
Total	8	20

aliquot 8 (20) µl into each well
Add 2 (5) µl template DNA

Cycling conditions:

Unless essential e.g. due to specific non-ABI kit requirements, please do not alter this.

Switch on the thermal cycler in advance as per producer's instructions to warm it up and stabilize conditions.

Set cycling parameters:

2 min at 50 °C	UNG activation step
10 min at 95 °C	polymerase activation
40 cycles of 15 s at 95 °C 1 min at 60 °C	 DNA denaturation annealing and extension

Adjust reaction volume if needed.

Analyzing data:

For analysis there are usually different options available with regard to setting signal and noise limits: automatic and manual.

The following are instructions for analysis of real-time PCR results for Applied Biosystems' analysis softwares, please adapt them as suitable to your instrument.

- use automatic baseline
- threshold should be set manually crossing exponential phase of control amplification curves
- Record Ct values on data-collection sheet
- In case of doubtful results check the multicomponent plot. In positive samples FAM should be increasing.

Interpretation of results:

The positive control should be positive, otherwise an error in the PCR reaction should be considered.

NTC should be negative, otherwise contamination of reaction mix should be considered.

Any sample giving a Ct of less than 40, should be scored as positive.