European Union Reference Laboratory for Pest of Plants on Bacteria

Co-funded by the European Union

EU Reference Laboratory for pests of plants on bacteria and a proficiency test for molecular detection of *Xylella fastidiosa*

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European conference on Xylella 2021

efsa European Food Safety Authority

XF ACTORS

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- **five reference laboratories in plant health established in 2019** (Commission Implementing Regulation (EU) 2019/530 of 27 March 2019)
- European Union Reference Laboratory for pests of plants **on bacteria**
- **tasks** include **organization of proficiency tests for NRLs** of EU MSs

Netherlands Food and Consumer Product Safety Authority
Ministry of Agriculture, Nature and Food Quality

ILVO
Flanders research institute for agriculture, fisheries and food

crea
Council for Agricultural Research and Economics - Research Centre for Plant Protection and Certification of Rome

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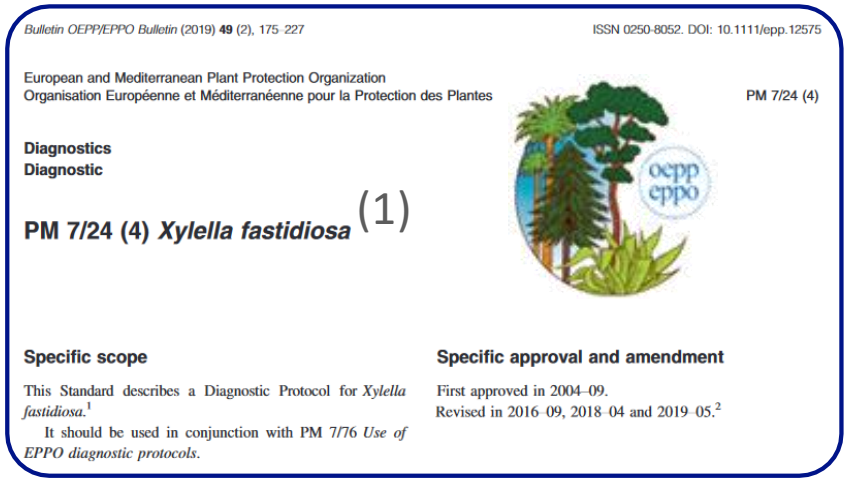
About *Xylella fastidiosa*

- sudden **increase in sample numbers and varied host plants**
- necessity to implement (more) **molecular tests**
- **building experience**(1,2,3)



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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

PM 7/24 (4)

Diagnostics
Diagnostic

PM 7/24 (4) *Xylella fastidiosa* (1)

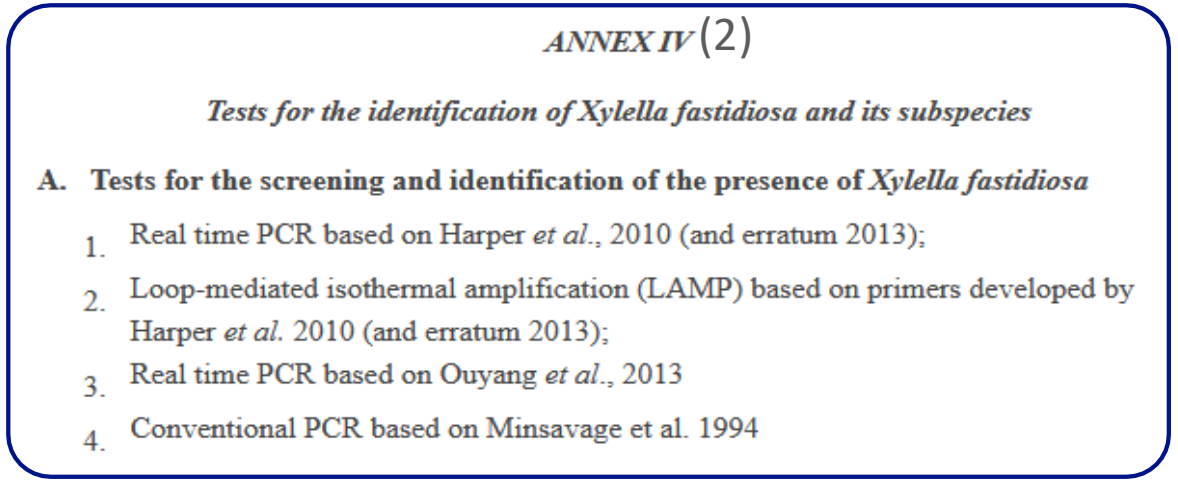
Specific scope

This Standard describes a Diagnostic Protocol for *Xylella fastidiosa*.[1]

It should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Specific approval and amendment

First approved in 2004–09.
Revised in 2016–09, 2018–04 and 2019–05.[2]



ANNEX IV (2)

Tests for the identification of Xylella fastidiosa and its subspecies

A. Tests for the screening and identification of the presence of *Xylella fastidiosa*

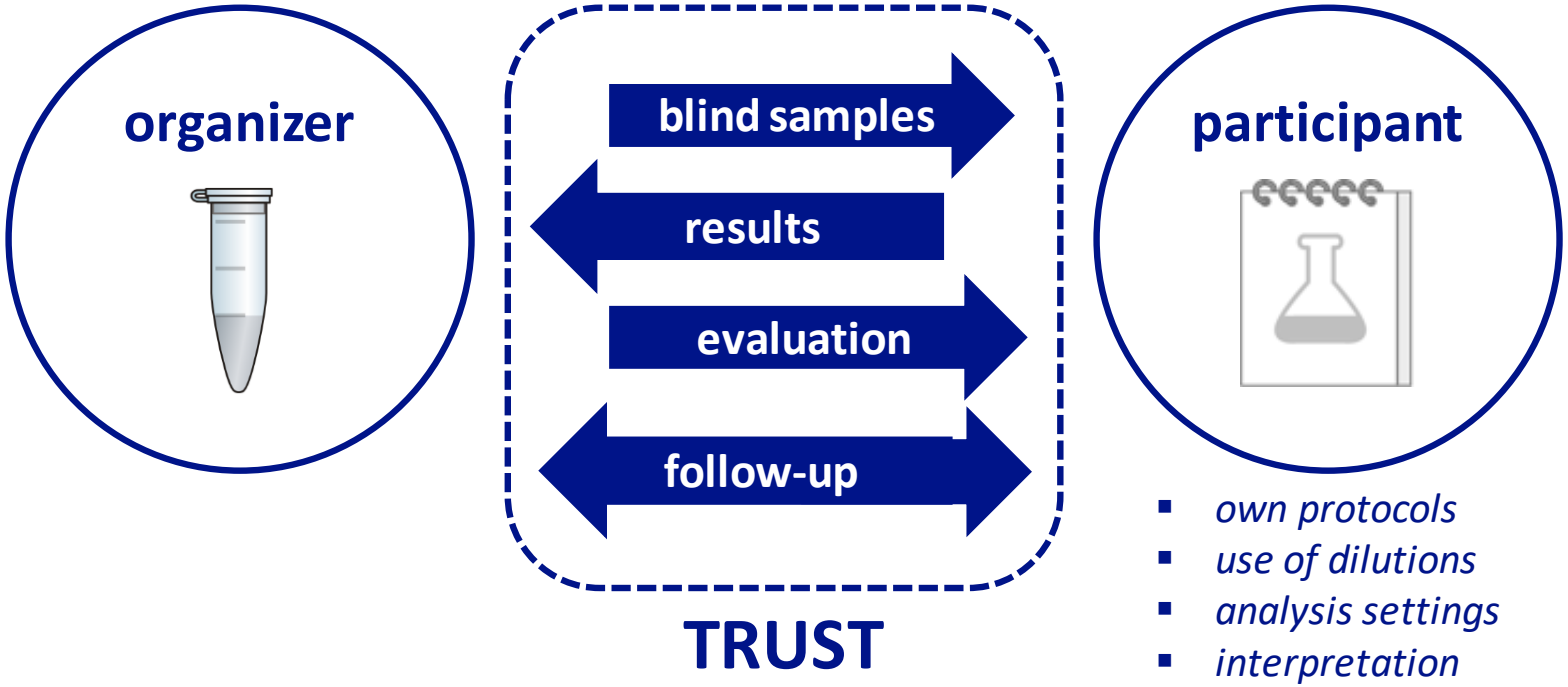
1. Real time PCR based on Harper *et al.*, 2010 (and erratum 2013);
2. Loop-mediated isothermal amplification (LAMP) based on primers developed by Harper *et al.* 2010 (and erratum 2013);
3. Real time PCR based on Ouyang *et al.*, 2013
4. Conventional PCR based on Minsavage et al. 1994

- **priority pest** in the **work programme of the EURL on bacteria in 2019:** to assess proficiency of the EU national reference laboratories (NRLs) to detect *Xylella fastidiosa* in plant samples using molecular methods.

(1)EPPO PM7/24 (4); (2)Commission Implementing Regulation (EU) 2020/1201 of 14 August 2020 as regards measures to prevent the introduction into and the spread within the Union of Xylella fastidiosa (Wells et al.); (3) EFSA pest survey card https://www.efsa.europa.eu/en/supporting/pub/en-1667

Proficiency tests – what are they?

- a way of assessing and demonstrating **competence of laboratories**
- a crucial **element of quality assurance system** (ISO 17025)



- *own protocols*
- *use of dilutions*
- *analysis settings*
- *interpretation*
- *own reagents*
- *treat it as a regular sample*

Our experience[4]

Proficiency test	Type of material	Participants
2018 Molecular detection of *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) (small ILC)	DNA from spiked plant extracts	4
2018 Molecular and/or serological detection of *Erwinia amylovora*	spiked plant extracts	27
2018 Molecular detection of *Ralstonia solanacearum* (RSSC)	DNA	29
2017 Molecular detection of *Pantoea stewartii*	spiked plant extracts	20
2017 Molecular detection and phylotyping of *Ralstonia solanacearum (RSSC)*	DNA	29
2016 Molecular detection of '*Candidatus* Liberibacter solanacearum'	synthetic DNA	20
2016 Molecular detection *of Xylella fastidiosa*	DNA	29
2016 Molecular detection of *Ralstonia solanacearum*	DNA	2
2015 Molecular detection of *Erwinia amylovora*	DNA	17
2015 Molecular detection of *Ralstonia solanacearum*	DNA	18
2012 Molecular and serological identification of *Clavibacter michiganesis* subsp. *sepedonicus*	isolates	2
2012 Molecular and serological identification of *Ralstonia solanacearum*	isolates	2

(4) Dreo, International proficiency testing schemes in plant health supported by digital PCR, presented at ICPP 2018, https://www.apsnet.org/meetings/annual/meetingarchives/Pages/2018videos.aspx?LID=Paper10949.mp4

- Need expressed to organize an interlaboratory comparison
- Capability of the organizer
- Planning:
  Design of the plan
  Preparation of instructions to participants
- Preparation of samples
  homogeneity and stability testing
  Assign values for samples
- Dispatch of samples
- Analysis of results
- Reporting

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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

PM 7/122 (1)

Diagnostics
Diagnostic

Adobe Acrobat Document

PM 7/122 (1) Guidelines for the organization of interlaboratory comparisons by plant pest diagnostic laboratories*

INTERNATIONAL STANDARD

ISO/IEC 17043

First edition
2010-02-01

Conformity assessment — General requirements for proficiency testing

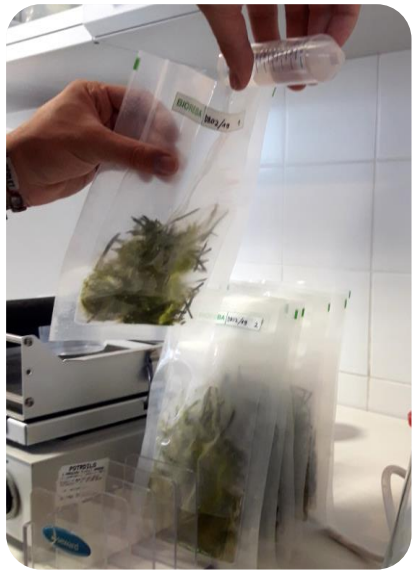
Évaluation de la conformité — Exigences générales concernant les essais d'aptitude

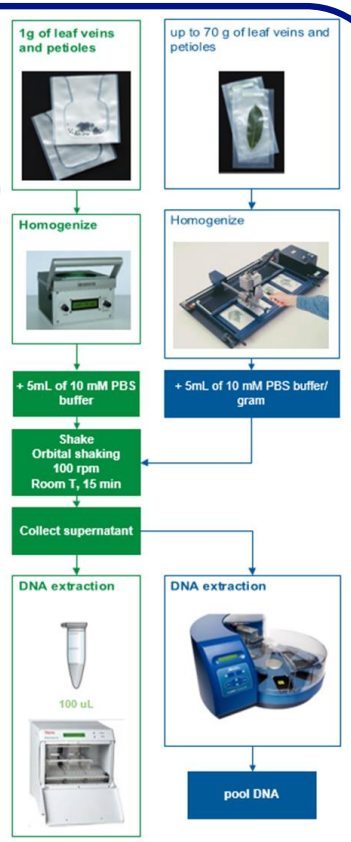
- simple
- requires a significant amount of time, effort, attention to detail and knowledge

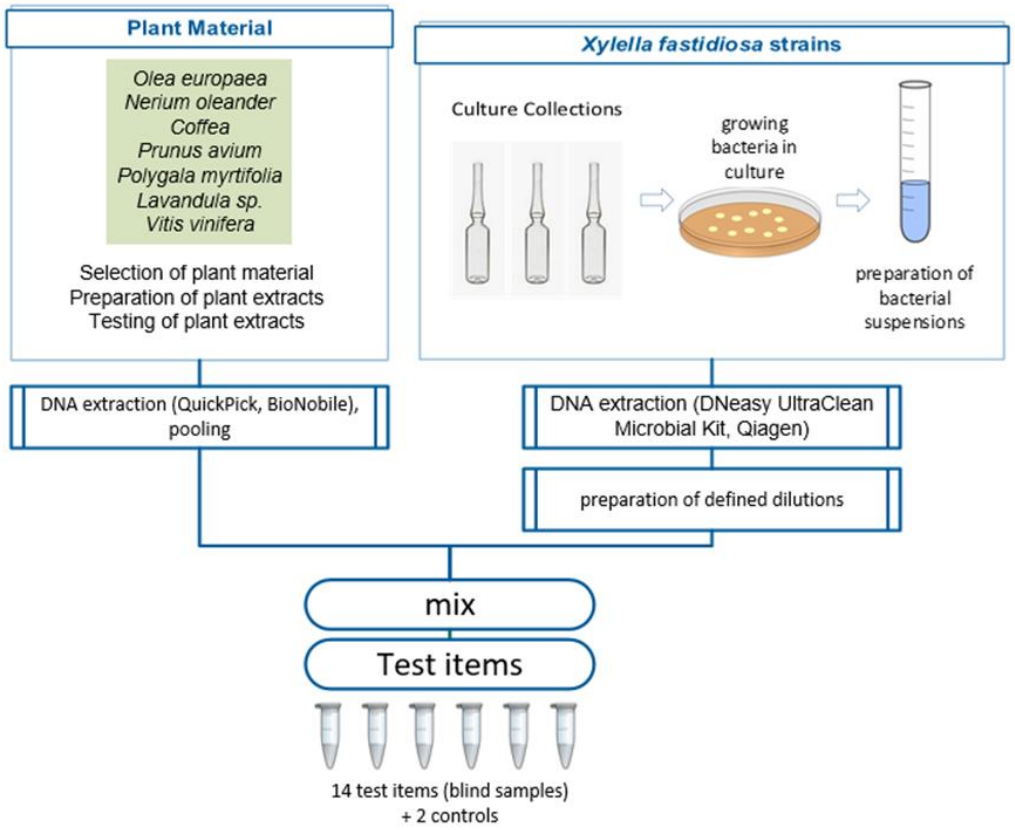
Test items: DNA + DNA

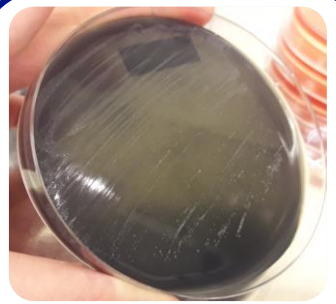
- time constraints for preparation and obtaining LOAs

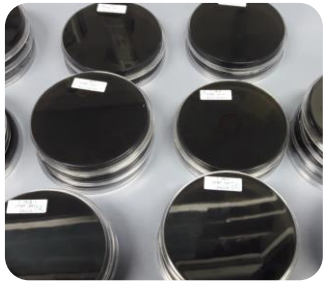
up-scaled preparation of plant extracts (5)











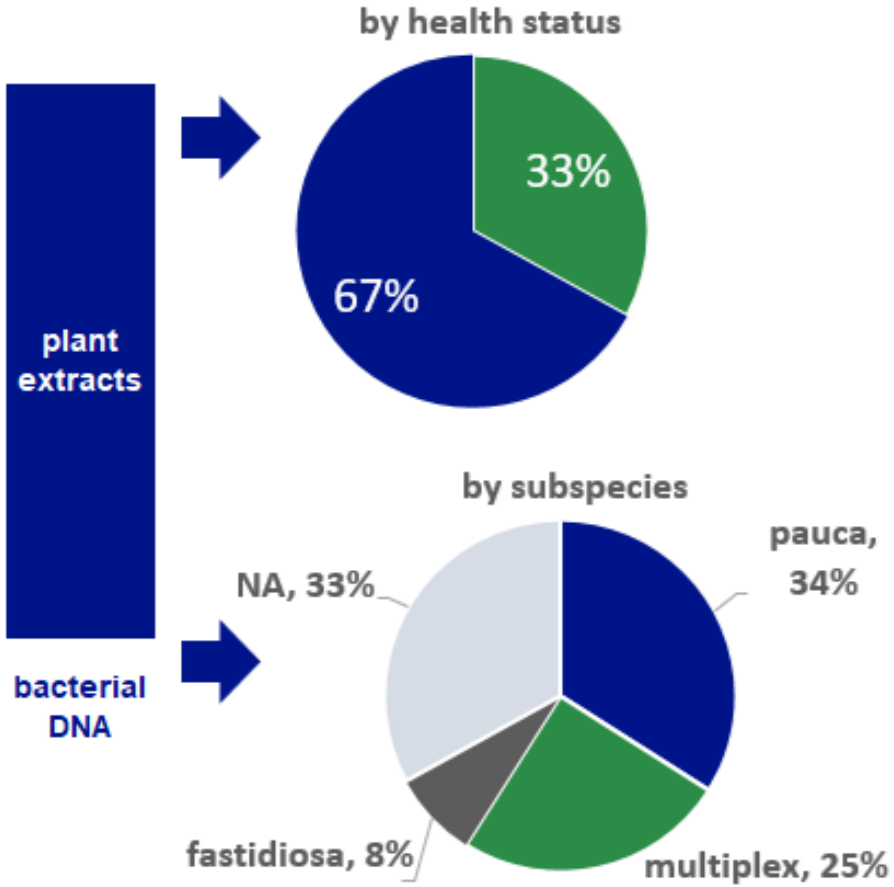
various Xf subspecies (6)

(5) 40-120 individual DNA extractions/test item → pooling; (6) X. fastidiosa isolates of subsp. pauca, multiplex and fastidiosa provided by and used with permission of the CFBP, use limted to non-commercial use only (MTA).

Panel of test items

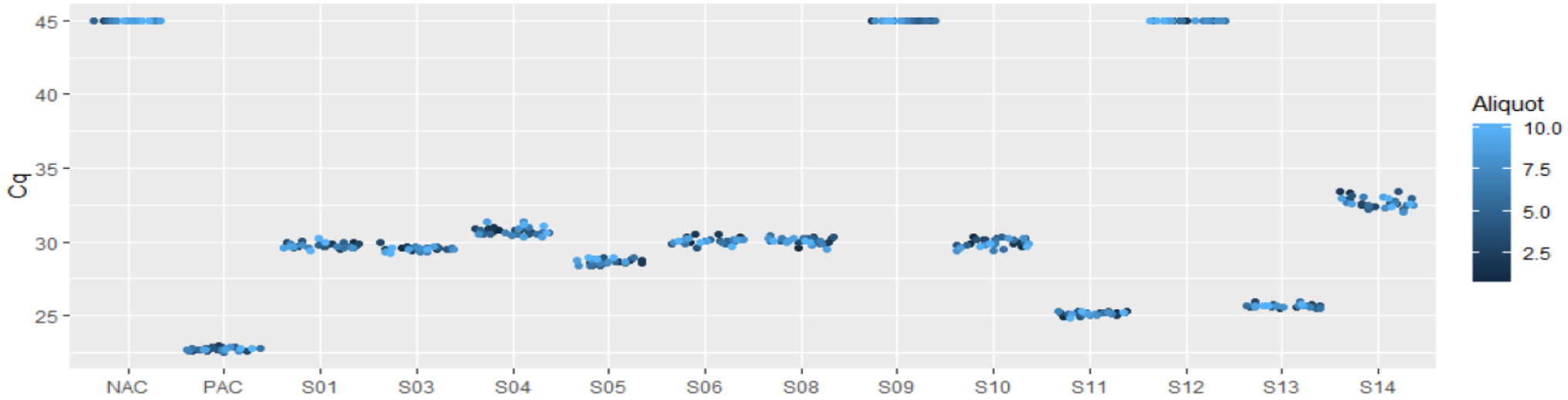
- DNA (host plants) + DNA (Xf, relevant subspecies), target Cq from literature

ID	Type	Host Plant	*Xylella fastidiosa* subsp. (strain)	Status	Target Cq
1	Test item	*Nerium oleander*	*pauca* (3025)	pos	31
2	Test item	*Nerium oleander*	NA	neg	NA
3	Test item	*Lavandula angustifolia*	*multiplex* (3019)	pos	31
4	Test item	*Coffea*	*pauca* (2923)	pos	32
5	Test item	*Polygala myrtifolia*	*multiplex* (3018)	pos	29
6	Test item	*Prunus avium*	*multiplex* (3019)	pos	31
7	Test item	*Prunus avium*	NA	neg	NA
8	Test item	*Vitis vinifera*	*fastidiosa* (3028)	pos	31
9	Test item	*Vitis vinifera*	NA	neg	NA
10	Test item	*Olea europaea*	*pauca* (3023)	pos	30
11	Test item	*Olea europaea*	*pauca* (3023)	pos	26
12	Test item	*Olea europaea*	NA	neg	NA
13	Test item	bacterial DNA	*fastidiosa* (3029)	pos	26
14	Test item	bacterial DNA	*fastdiosa* (3029)	pos	32
PAC	Control	bacterial DNA	*pauca* (3023)	pos	23
NAC	Control	water	NA	neg	NA



Homogeneity

- **10 aliquotes / each test item and control**, analyzed in 3 replicates with real-time PCR (Harper et al., 2010, erratum 2013) and PCR (Minsvage et al., 1994)



Qualitative result

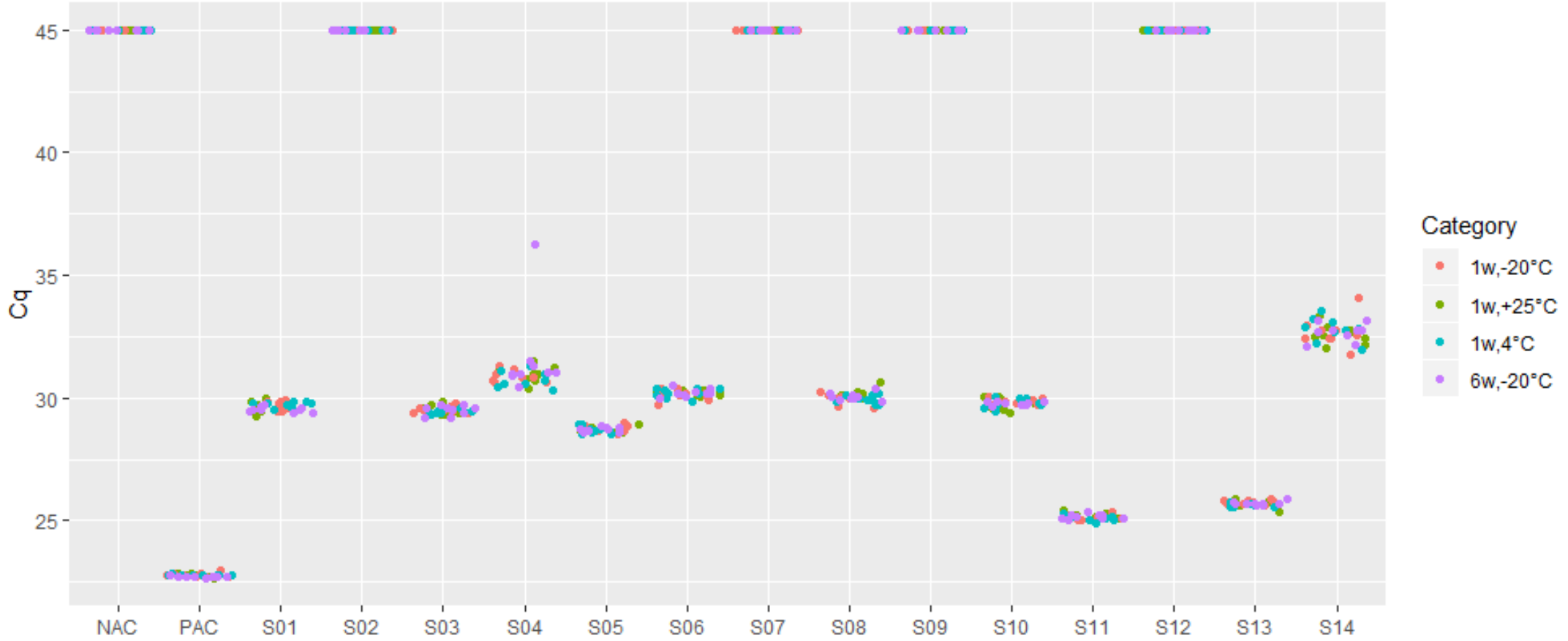
✓ 100 % concordance
✓ no amplification in negative test items

Quantitative (Cq) result

✓ expected Cq (from 22.51 to 33.40 for positive samples)
✓ CV of Cq(Xyf DNA, 32) = 1.1%
✓ all other pos test items CV < 1 %

Stability

- ✓ short term and long-term



- 100 % concordance, Cv(Cq) < 1 % except for S4 (*Coffea* + *pauca*, max 1.8 %) and S14 (*fastidiosa* DNA, max 5 %)

Assigned values

ID	Type	Host Plant	*Xylella fastidiosa* subsp. (strain)	Status	qPCR (Harper et al., 2010, erratum 2013)		PCR (Minsavage *et al*., result (result/three reps)		LAMP (Harper *et al*., 2010) 0x			LAMP (Harper *et al*., 2010) 10x		
					min(Cq) - max(Cq)	average(Cq) ± CV	0x	10x	result	Tp	Tm	result	Tp	Tm
1	Test item	*Nerium oleander*	*pauca* (3025)	pos	29.4 - 30.2	29.73 ± 0.006	pos (3/3)	pos (3/3)	pos	15.8	89.0	neg	NA	NA
2	Test item	*Nerium oleander*	NA	neg	neg	NA	neg (3/3)	neg (3/3)	neg	NA	NA	neg	NA	NA
3	Test item	*Lavandula angustifolia*	*multiplex* (3019)	pos	29.2 - 30.0	29.51 ± 0.005	neg (3/3)	pos(3/3)	neg	NA	NA	pos	14.2	88.6
4	Test item	*Coffea*	*pauca* (2923)	pos	30.3 - 31.3	30.75 ± 0.009	pos (3/3)	pos (2/3)	pos	16.5	89.0	neg	NA	NA
5	Test item	*Polygala myrtifolia*	*multiplex* (3018)	pos	28.3 - 29.0	28.67 ± 0.006	pos (3/3)	pos (3/3)	pos	14.0	89.1	neg	NA	NA
6	Test item	*Prunus avium*	*multiplex* (3019)	pos	29.6 - 30.6	30.06 ± 0.007	pos (3/3)	pos (3/3)	pos	16.0	89.0	neg	NA	NA
7	Test item	*Prunus avium*	NA	neg	neg	NA	neg (3/3)	neg (3/3)	neg	NA	NA	neg	NA	NA
8	Test item	*Vitis vinifera*	*fastidiosa* (3028)	pos	29.5 - 30.4	30.04 ± 0.007	neg (3/3)	pos (3/3)	pos	15.8	89.0	neg	NA	NA
9	Test item	*Vitis vinifera*	NA	neg	neg	NA	neg (3/3)	neg (3/3)	neg	NA	NA	neg	NA	NA
10	Test item	*Olea europaea*	*pauca* (3023)	pos	29.4 - 30.3	29.92 ± 0.008	pos (3/3)	pos (3/3)	pos	17.0	88.9	neg	NA	NA
11	Test item	*Olea europaea*	*pauca* (3023)	pos	24.8 - 25.3	25.14 ± 0.005	pos (3/3)	pos (3/3)	pos	11.2	89.1	pos	11.6	88.6
12	Test item	*Olea europaea*	NA	neg	neg	NA	neg (3/3)	neg (3/3)	neg	NA	NA	neg	NA	NA
13	Test item	bacterial DNA	*fastidiosa* (3029)	pos	25.5 - 25.9	25.67 ± 0.004	pos (3/3)	pos (3/3)	pos	10.4	88.5	pos	12.1	88.4
14	Test item	bacterial DNA	*fastdiosa* (3029)	pos	32.0 - 33.4	32.68 ± 0.011	pos (3/3)	pos (3/3)	pos	16.2	88.5	neg	NA	NA
PAC	Control	bacterial DNA	*pauca* (3023)	pos	22.5 - 23.0	22.75 ± 0.005	pos (3/3)	NA	pos	10.1	88.4	NA	NA	NA
NAC	Control	water	NA	neg	neg	NA	neg (3/3)	NA	neg	NA	NA	NA	NA	NA

Expected concordance	100 % concordance (undiluted)	86 % concordance (undiluted samples only)	93 % concordance (undiluted samples only)
		100 % concordance (undiluted and 10x diluted)	100 % concordance (undiluted and 10x diluted)

- ✓ all three tests expected toallow for 100 % concordance

Assigned values

ID	Type	Host Plant	*Xylella fastidiosa* subsp. (strain)	Status	dPCR (Harper *et al*., 2010, erratum 2013) average concentration	dPCR (Harper *et al*., 2010, erratum 2013) CV [%]
1	Test item	*Nerium oleander*	*pauca* (3025)	pos	$4.2 \cdot 10^4$	6.9 %
2	Test item	*Nerium oleander*	NA	neg	neg	NA
3	Test item	*Lavandula angustifolia*	*multiplex* (3019)	pos	$5.0 \cdot 10^4$	4.5 %
4	Test item	*Coffea*	*pauca* (2923)	pos	$1.6 \cdot 10^4$	6.3 %
5	Test item	*Polygala myrtifolia*	*multiplex* (3018)	pos	$8.3 \cdot 10^4$	6.3 %
6	Test item	*Prunus avium*	*multiplex* (3019)	pos	$3.5 \cdot 10^4$	12.0 %
7	Test item	*Prunus avium*	NA	neg	neg	NA
8	Test item	*Vitis vinifera*	*fastidiosa* (3028)	pos	$2.5 \cdot 10^4$	6.7 %
9	Test item	*Vitis vinifera*	NA	neg	neg	NA
10	Test item	*Olea europaea*	*pauca* (3023)	pos	$3.1 \cdot 10^4$	8.0 %
11	Test item	*Olea europaea*	*pauca* (3023)	pos	$6.7 \cdot 10^5$	5.8 %
12	Test item	*Olea europaea*	NA	neg	neg	NA
13	Test item	bacterial DNA	*fastidiosa* (3029)	pos	$7.2 \cdot 10^5$	6.9 %
14	Test item	bacterial DNA	*fastdiosa* (3029)	pos	$6.1 \cdot 10^3$	9.6 %
PAC	Control	bacterial DNA	*pauca* (3023)	pos	$4.8 \cdot 10^6$	3.3 %
NAC	Control	water	NA	neg	neg	NA

European Union Reference Laboratory for Pest of Plants on Bacteria

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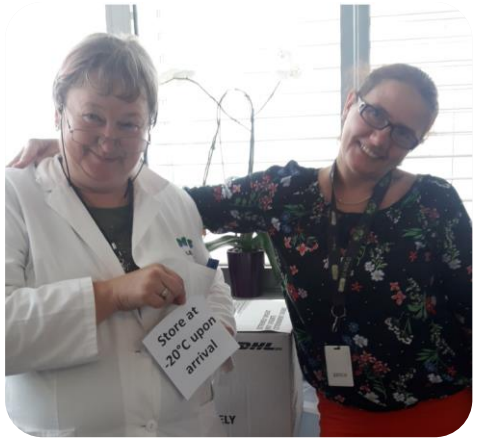
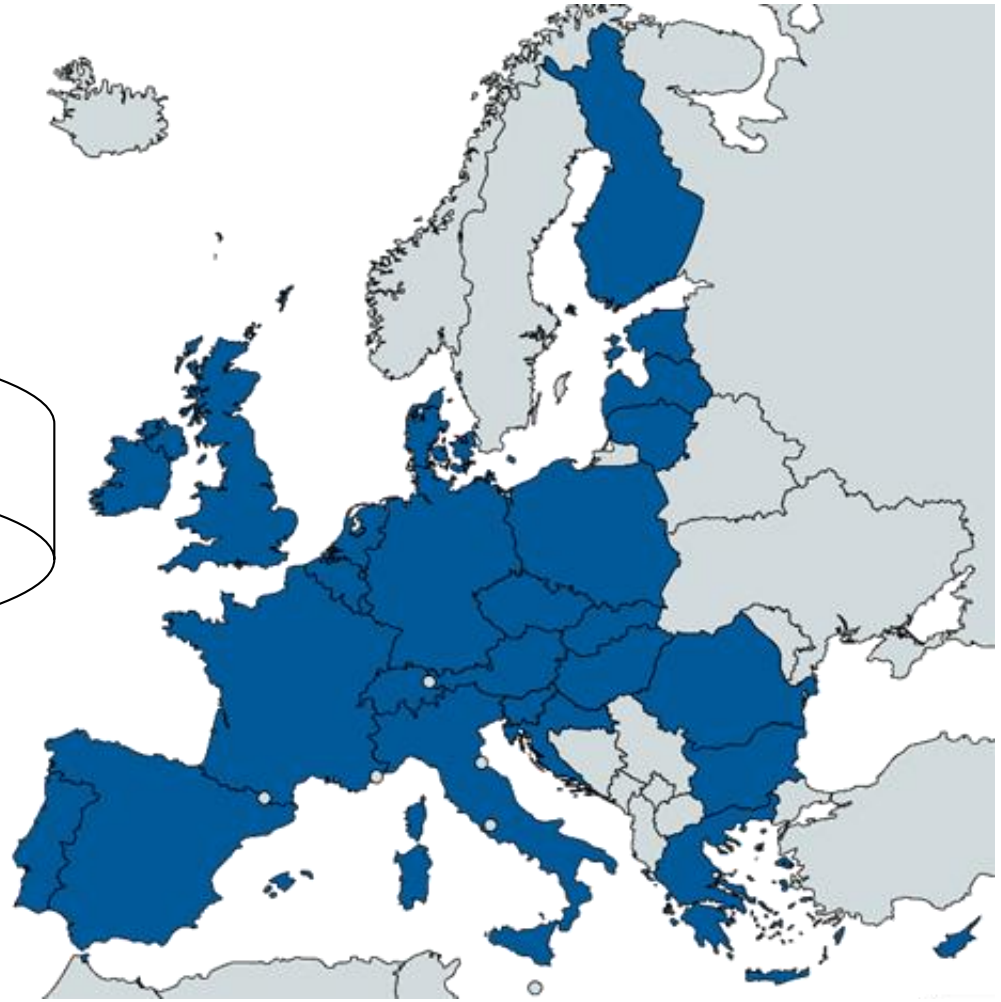


- Invitation August 2nd, 2019
- Registraton
- Registration by September 6th, 2019
- Samples sent out September 24th, 2019
- Analysis
- Deadline for results submission October 16th, 2019
- Reports October 31st, 2019

- post carrier
- dry ice

- next day delivery
- max 2d



- all participants received the same panel with randomized samples

Map created with mapchart.net

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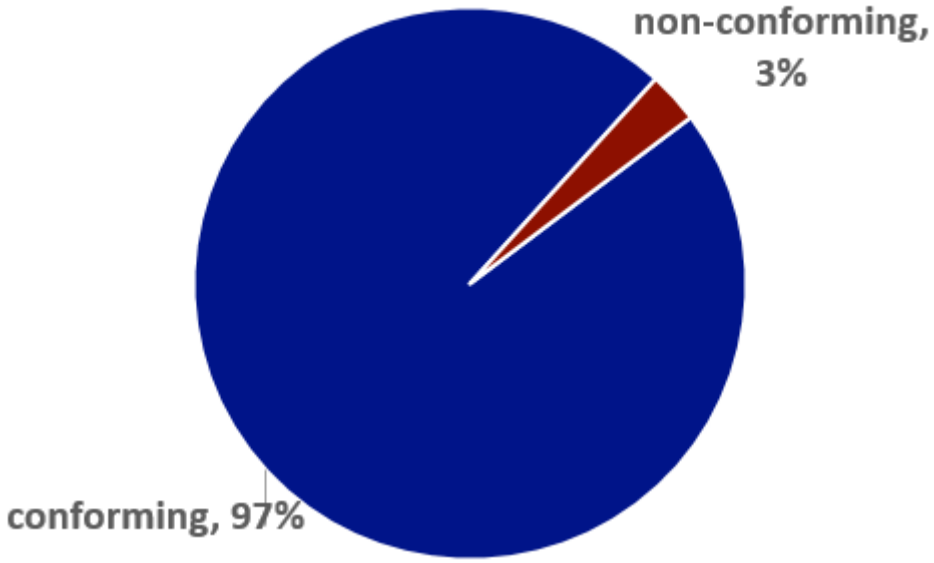
Overall conformity

- (a participant excluded from the analysis)
- 26 participants x 14 test items = 364 data points, of these:



Conformity by participant

Lab ID	Number of		Percent of	
	con	non-con	con	non-con
1	14	0	100%	0%
2	14	0	100%	0%
3	14	0	100%	0%
4	14	0	100%	0%
5	12	2	86%	14%
6	13	1	93%	7%
7	14	0	100%	0%
8	14	0	100%	0%
9	14	0	100%	0%
10	14	0	100%	0%
11	14	0	100%	0%
12	14	0	100%	0%
13	13	1	93%	7%
14	13	1	93%	7%
15	14	0	100%	0%
16	12	2	86%	14%
17	13	1	93%	7%
18	14	0	100%	0%
19	14	0	100%	0%
20	14	0	100%	0%
21	14	0	100%	0%
22	14	0	100%	0%
23	14	0	100%	0%
24	12	2	86%	14%
25	14	0	100%	0%
26	14	0	100%	0%

- seven (7) labs < 100 %

Conformity by host plants

Host plant / bacterial DNA	Number of test items		Number of results		Percentage of results	
	pos	neg	conforming	non-conforming	conforming	non-conforming
Nerium oleander	1	1	52	0	100%	0%
Lavandula angustifolia	1	0	21	5	81%	19%
Coffea	1	0	26	0	100%	0%
Polygala myrtifolia	1	0	26	0	100%	0%
Prunus avium	1	1	52	0	100%	0%
Vitis vinifera	1	1	48	4	92%	8%
Olea europaea	2	1	78	0	100%	0%
X. fastidiosa DNA	2	0	51	1	98%	2%

Conformity by Xf subspecies

Xylella fastidiosa subsp.	Number of test items	Number of results		Percentage of results	
		conforming	non-conforming	conforming	non-conforming
pauca	4	104	0	100%	0%
multiplex	3	73	5	94%	6%
fastidiosa	3	73	5	94%	6%

Differences among labs: tests and use of dilutions

Test(s)	Participants reporting 100% conforming results			Participants reporting any non-conforming results			Total (number / %)	
	diluted	diluted, sequentially	undiluted	diluted	diluted, sequentially	undiluted		
Harper & Minsavage	2	1	2			3	8	30.8%
Harper	1	1	2			1	5	19.2%
Harper & Ouyang & Minsavage	3						3	11.5%
Harper & Francis & Li & Ouyang	1		1				2	7.7%
Harper & Francis & LAMP			1		1		2	7.7%
Harper & LAMP	1						1	3.8%
Harper & Francis & Schaad	1						1	3.8%
Harper & Francis & Li		1					1	3.8%
Harper & Francis & Minsavage			1				1	3.8%
Minsavage						1	1	3.8%
Harper & Ouyang					1		1	3.8%
Total (number / %)	9	3	7	0	2	5	26	
	34.6%	11.5%	26.9%	0.0%	7.7%	19.2%	100.0%	100.0%

- **non-conforming results only with no dilutions or sequential dilutions**
- at the same time, 100 % conforming results were obtained with either approach
- we can conclude that **non-conformities were a consequence of inappropriate use of dilutions or sub-optimal tests** → individual recommendations

Conclusions

- **73 % participants 100 % conforming results**
- **the concentrations of *X. fastidiosa* as expected** in plant material of tested host plants **are readily detected by**:
  - ✓ **different methods and tests** and their combinations
  - ✓ with **different approaches to dilutions of samples**
  - ✓ using **different core reagents and instruments**
- no urgent need to revise the EPPO diagnostic protocol

Knowledge is more important than standardization

- DNA level → identification of improvements

Acknowledgements

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oepp eppo

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Co-funded by the European Union

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MINISTRY OF AGRICULTURE, FORESTRY AND FOOD

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