EU project POnTE 'Pest Organisms Threatening Europe'

Second European Conference on *Xylella fastidiosa:* how research can support solutions

Ajaccio, 29th – 30th October 2019

2015-2019 Main outcomes of WP4 POnTE research on *Diagnostic of Xylella fastidiosa*

Françoise Poliakoff

Anses – Plant Health Laboratory - France

WP4: Implementation and validation of diagnostic kits for early and rapid detection of target pathogens in host plants and vectors.

Partners: ANSES, CNR, UNIBA, CSIC, IVIA, AGRITEST, LOEWE, UCR_

Task 4a. Development of novel and high-throughput diagnostic procedures to detect *Xylella fastidiosa (Xf)* in plants and vectors

- New antisera (Agritest-Loewe-IVIA)
- Methods based on Print and cards
- Automatized protocols for DNA extractions
- Rapid biomolecular tests (Enbiotech, Agdia, etc)
- Method for Xf detection in *Philaenus spumarius*

Task 4b. Development of strain/species-specific assays

- Rapid screening tests for strain typing

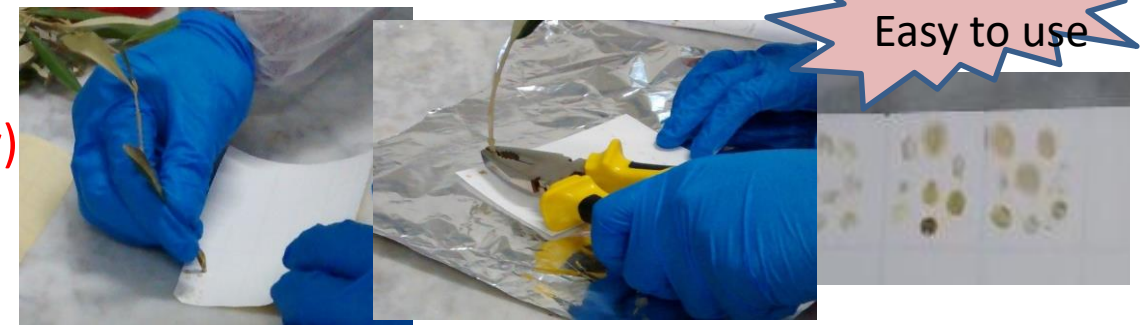
Deliverable D 4.1. Improved and newly developed protocols for the CoDiRO strain detection.

Task 4e. Validation of official procedures through performance studies and interlaboratory tests

Improvement of serological tests

➢ **Preparation of samples direct in the fields based on plant prints on cards/membrane to** *overcome the preparation of sap, extraction and purification of total nucleic acids*
(CNR, UNIBA, AGRITEST, LOEWE)

Low cost
Easy to use



- **DTBIA**

(Direct tissue blot immunoassay)

- **FTA card - ELISA**

- **Lateral flow test strips based on antiserum against the subsp pauca ST53 strain**
(Loewe)

	DAS ELISA	Lateral Flow
Analytical specificity	100%	100%
Analytical sensitivity	10^4 cells/mL	10^5 cells/mL
Diagnostic specificity	100%	85%

➢ **Production of New antibodies for ELISA as well as for Immunofluorescence (IF)**

- Recombinant Protein MopB (Agritest) + **Positive control on card for matrices**
- **Monoclonal** (IVIA)
- Specific subspecies antibodies for IF
  - **(X.f (Anses) X.f subsp fastidiosa Costa Rica (UCR)**

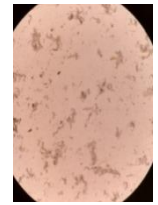
Improved sensitivity and specificity

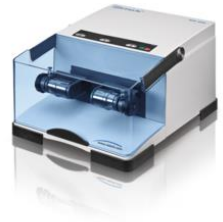
Development, optimization and validation of DNA extraction

- Different DNA extraction kits to be used with automatized equipment or platform at a large scale showed good performance for highly reliable diagnostic depending on matrices:
  - **Golden reference: CTAB home made DNA extraction, recommended on complex matrices (inhibitors)**
  - QuickPickTM SML Plant DNA Kit (Bio-Nobile) → Ornementals, vine, citrus
  - DNeasy® mericon™ Food kit (Qiagen) → Olive trees and other hosts
  - Maxwell® RSC PureFood GMO + Authentication Kit on an automated platform Maxwell® RSC (Promega)

- Preparation of samples direct in the fields for quick results overcoming the preparation of sap and DNA extraction:
  - **Whatman card followed by real time PCR**, **Lamp (Enbiotech**) or **RPA (Agdia**)
  - **The direct nucleic acid hybridization on nitrocellulose strip (lateral flow**) in a one tube nested PCR protocol (Loewe)

- Extraction on insect vectors (***Philaenus spum****arius)* crushing individuals heads or pools of 5; 10, 15 insects heads.
  - **CTAB home made DNA extraction**
  - QuickPickTM SML Plant DNA Kit (Bio-Nobile)





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New: ultrasonication step after samples crushing before DNA extraction improving the release of bacteria from biofilms for difficult matrices (Olea, Quercus) and sensitivity

- Preparation of sample direct in the fields for quick results overcoming the preparation of sap and DNA extraction:
  - **Whatman card followed by real time PCR**, **Lamp (Enbiotech**) or **RPA (Agdia**)
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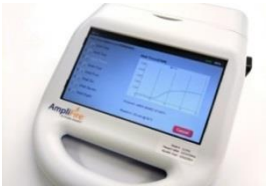

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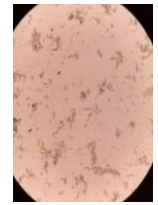
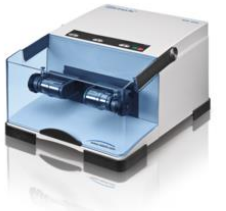
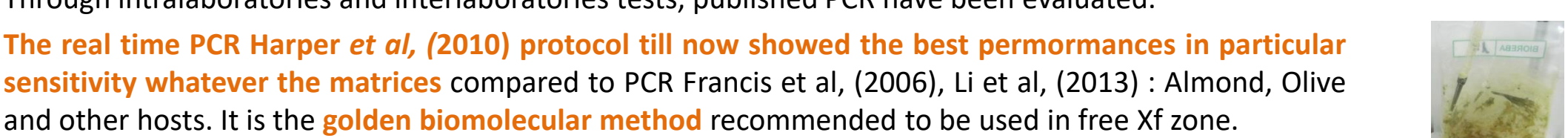
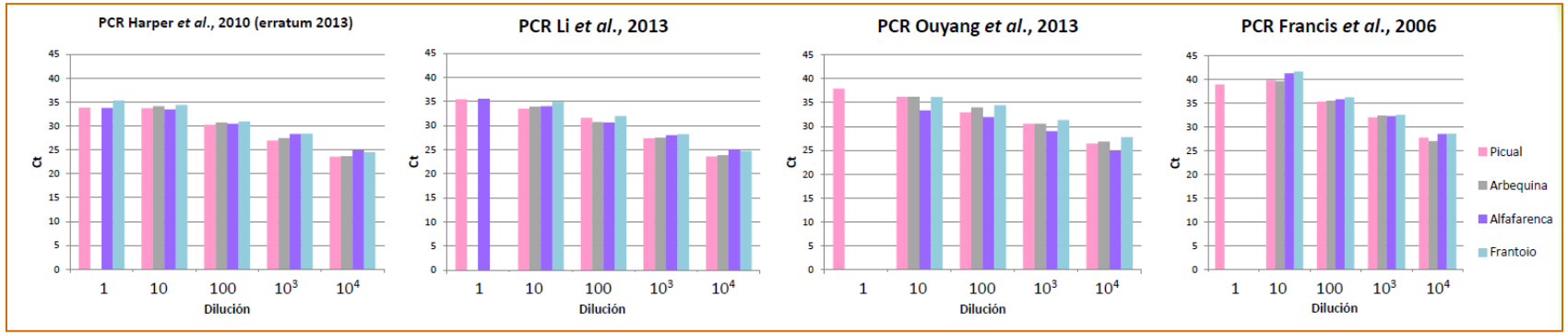




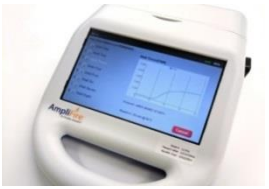
Development, optimization and validation of biomolecular methods

- Through intralaboratories and interlaboratories tests, published PCR have been evaluated.
- **The real time PCR Harper *et al, (*2010) protocol till now showed the best permormances in particular sensitivity whatever the matrices** compared to PCR Francis et al, (2006), Li et al, (2013) : Almond, Olive and other hosts. It is the **golden biomolecular method** recommended to be used in free Xf zone.





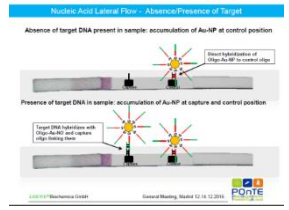
Differences in inhibitors depending on the cultivar (IVIA)



- On ***Philaenus spum****arius,* real time PCR Harper *et al*; (2010) alone or associated with (IOOS et al, 2009) in a duplex PCR for internal control showed good efficiency.

- Portable devices that can be used by the fields (or near the fields) **without moving the infected material from the infected to *Xf-free areas.*** ***(40 minutes for the results)***
  - **PCR Lamp (Enbiotech)** (*isothermal amplification reaction)*
  - ***AmplifyRP kit based on*** **Recombinant Polymerase Amplification techn A (Agdia)** (sensitivity ed with the detection chamber)

- The **lateral flow** based on direct nucleic acid hybridization on nitrocellulose strip in a one tube nested PCR protocol gave a sensitivity (10-100 Xf cells) matching with other lateral flow devices. (Loewe)



Development, optimization and validation of biomolecular methods

On 90 olive samples assessed for Xf by ELISA and Dneasy Mericon food kit + real time PCR by Harper (CNR, UNIBA, AGRITEST)

Dneasy Mericon food kit	Whatman +qPCR	FTA-ELISA	DTBIA	RPA (Agdia)	LAMP Enbiotech	ELISA
% Accuracy ($N_{PA}+N_{NA}$)/N	**84**	**88**	**87**	**98**	**98**	**98**
% Sensitivity (N_{PA}/N+)	74	78	80	96	96	96
% Specificity (N_{NA}/N-)	97.5	100	95	100	100	100

⇒ the inprint techniques show lower sensitivity and accuracy compared to quick ready PCR (LAMP – RPA)

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⇒ the inprint techniques show lower sensitivity and accuracy compared to quick ready PCR (LAMP – RPA)

On 80 almond trees samples collected in the field assessed for Xf using the CTAB DNA extraction protocol + qPCR by Harper (IVIA, CSIC)

Data	ELISA MAbs (IVIA)	ELISA Loewe (IVIA)	Real time Francis (IVIA)	Real time Francis (CSIC)	RPA (Agdia) (CSIC)
% Accuracy ($N_{PA}+N_{NA}$)/N	**89.3**	**81.3**	**98.2**	**82**	**78**
% Sensitivity (N_{PA}/N+)	81	81	96.8	75	70
% Specificity (N_{NA}/N-)	100	81.6	100	100	100

⇒ a lower sensitivity and accuracy are associated with serological test or RPA compared to real time PCR Francis

TEST PERFORMANCE STUDY (CNR, UNIBA)

XF ACTORS Euphresco
Xylella Fastidiosa Active Containment Through a multidisciplinary-Oriented Research Strategy

Molecular detection of Xylella fastidiosa through real time assays.

Implementation of the Proficiency test EU-XF- PT-2017-02

November 2017 – January 2018

DNA TEMPLATES

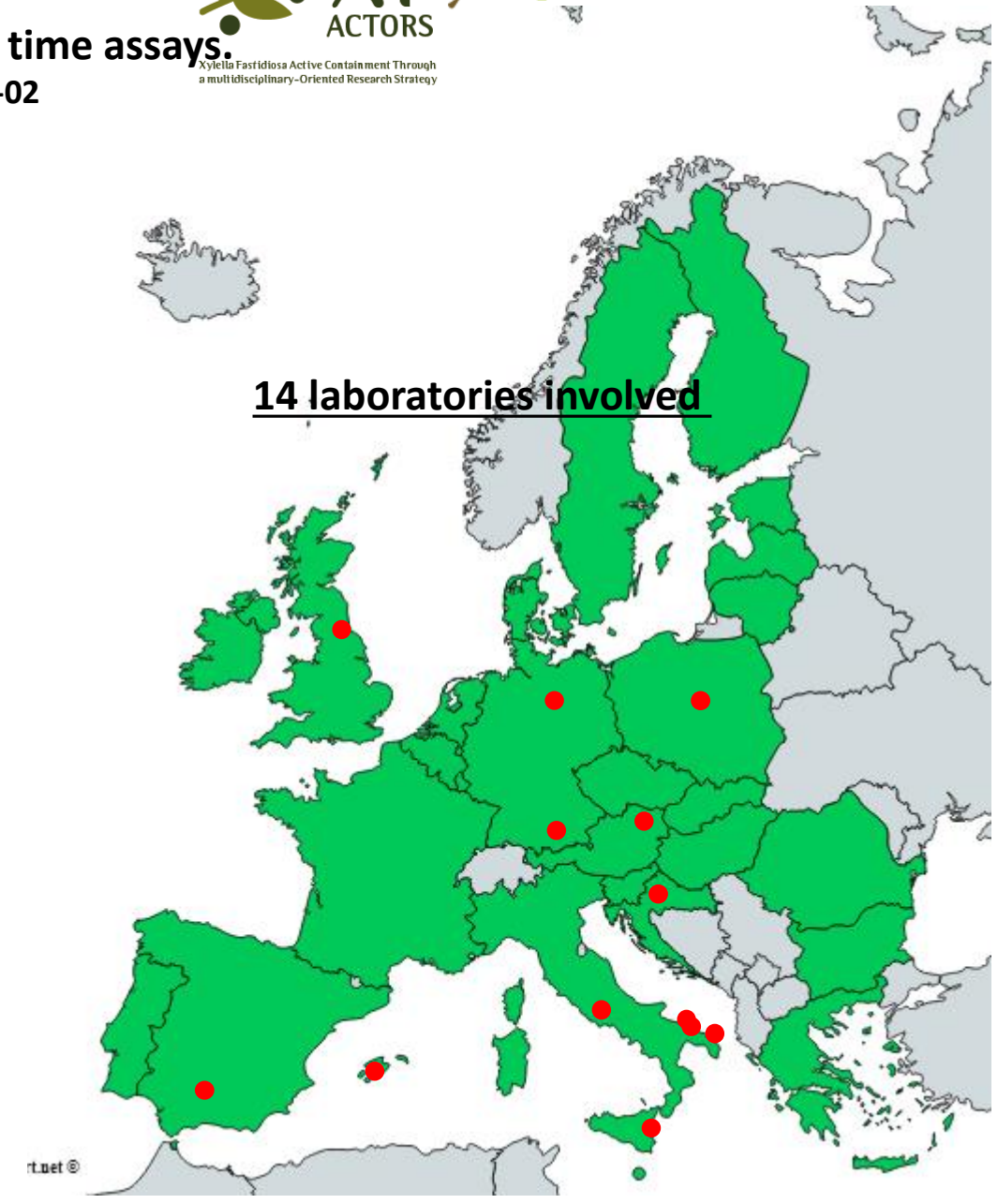
- DNA extracts previously recovered in the framework of the PT EU-XF- PT-2017-02, from olive tissues spiked with bacterial suspension of *X. fastidiosa* subsp. *pauca* strain De Donno

  of 10^6 cfu/mL to 10^4 cfu/mL.

- processed using either the DNeasy® mericon™ Food kit (Qiagen) or the CTAB-based extraction protocol

5 different real time PCR assay formats tested:

-Harper et al., 2010
- Li et al., 2013, using standard TaqMan probe
-Li et al., 2013, using MGB-TaqMan probe
-Francis et al., 2006
- Francis et al., 2006, modified using Sybr green



TEST PERFORMANCE STUDY (CNR, UNIBA)

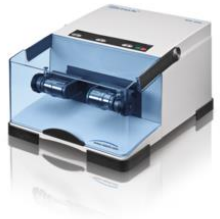
Values of **performance criteria** obtained for the **5 different qPCR** on **CTAB-based and DNeasy® mericon™ Food kit (Qiagen)extracts**. Percentage below 100% are shaded

qPCR protocol	DNA extracts	Accuracy %	Sensitivity %	Specificity %	Repeatability %	Reproducibility %
Harper	CTAB	100	100	100	100	100
	Mericon	100	100	100	100	100
Li-SP	CTAB	96	100	92	97	96
	Mericon	99	100	97	99	99
Li-MGB	CTAB	92	100	67	97	92
	Mericon	99	100	97	99	99
Francis-SP	CTAB	98	97	100	100	98
	Mericon	100	100	100	100	100
Francis-SG	CTAB	100	100	100	100	100
	Mericon	100	100	100	100	100

- ❑ Accuracy, repeatability and reproducibility of 5 qPCR protocols: in the range of 92-100% **Harper *et al* (2011), is the golden reference of real time PCR with the lowest Cq values.**
- ❑qPCR Li-SP and Li-MGB: produced false positives results
- ❑TaqMan probe-MGB (Li et al. 2013): do not improved any of the performance parameters

TEST PERFORMANCE STUDY (Anses)

Evaluation of detection methods of X. fastidiosa on *Philaenus spumarius*



DIAGNOSTIC PROCEDURES EVALUATED

DNA extraction:

- QuickPick™ Plant DNA kit (Bio-Nobile)
- CTAB DNA extraction protocol (EPPO PM7/24)
- Yaseen *et al.*, (2015) DNA extraction
- (for natural insects only)

Amplification:

- Real time PCR Harper *et al.*, (2010 - erratum 2013)
- Real time PCR Harper *et al.*, (2010 - erratum 2013)

in duplex with primers Ioos *et al.*, (2009) (internal control)

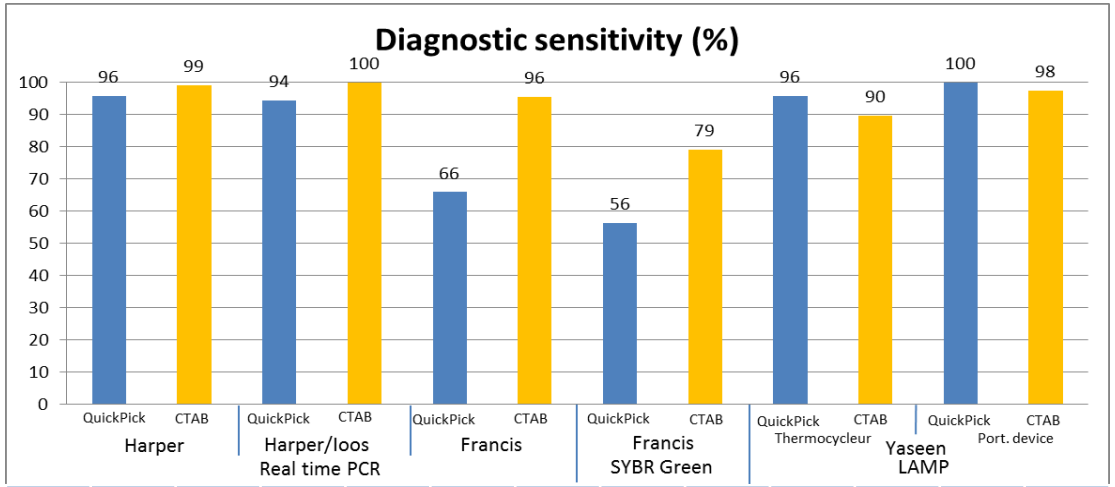
- Real time PCR Francis *et al.*, (2006)
- PCR Francis *et al.*, (2006) using SYBR dying
- LAMP Harper *et al.*, 2010 modified by Yaseen *et al.*, (2015)

Type of spiked samples	Number of replicates
5.10^5 bact./head	2
5.10^4 bact./head	2
5.10^3 bact./head	4
Healthy sample	4
Positive Amplification Control (PAC)	1

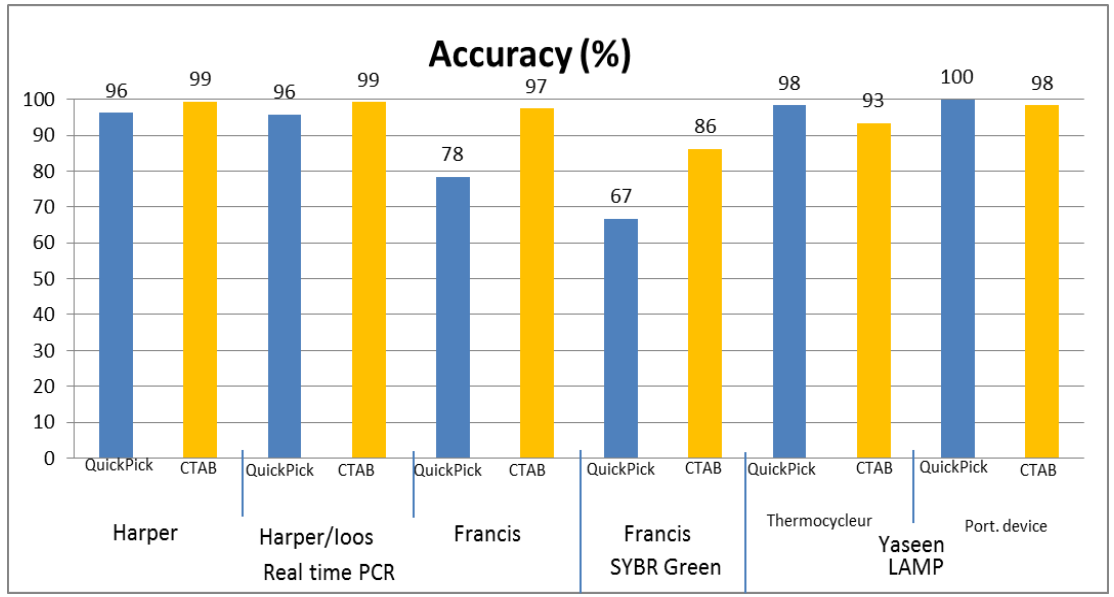
20 laboratories participating

TEST PERFORMANCE STUDY (Anses)

Detection methods of X. fastidiosa on *Philaenus spumarius*: Results



N	13	14	10	12	12	14	2	3	7	6	2	5



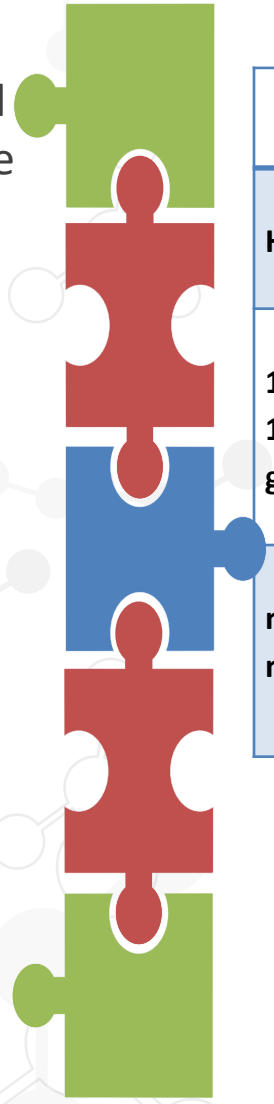
Equivalent and reliable results for:
Combination of DNA extraction Quickpick /CTAB
with

- **real time Harper *et al*, (2010)**
- **duplex PCR (Harper *et al*, (2010), Ioos *et al* (2009))**
- **LAMP (Yaseen et al, 2015)**

Combination of Quickpick and Francis is less reliable.

Standardization and Validation of HRM analysis-procedure

1. Standardization and validation on DNA purified from bacterial pure culture of 36 strains (USA, Brazil, France, Italy)
2. Validation on 30 field samples infected by De Donno strain, genotype ST53 subsp *pauca*
3. Data compared with phylogenetic analysis



Step 1 : Primers used (CNR, UNIBA)

Primers	Target region	Amplicon Size (bp)	References
HL5/HL6	HL protein	221 bp	Francis *et al.*, 2006
1Fw-HRM gyrB 1Rev-HRM gyrB	gyrb gene	120	Designed by IPSP-CNR/DiSSPA
nuoLFw-HRM nuoLRev-HRM	nuoL gene	146	Designed by IPSP-CNR/DiSSPA

Step 2:
qPCR with SYBRGreen PCR Master Mix

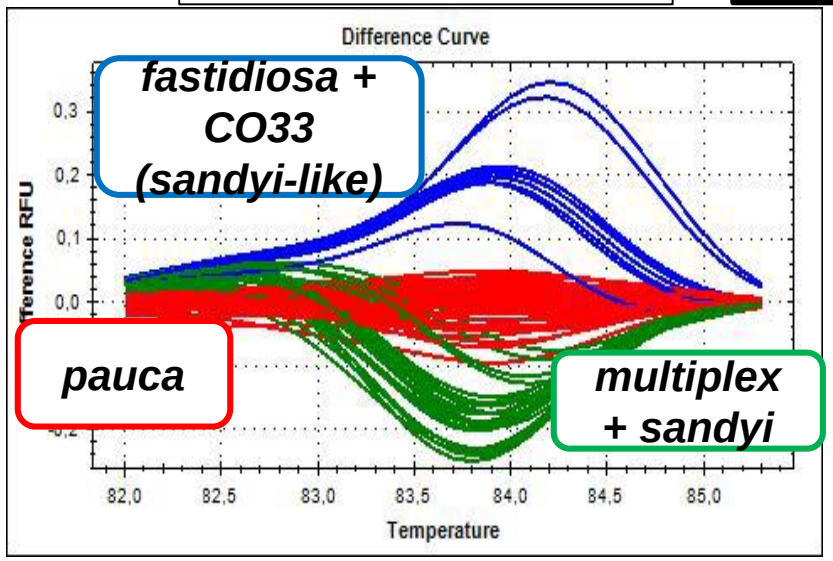
Step 3:
Melt-curve analysis after amplification by collecting data

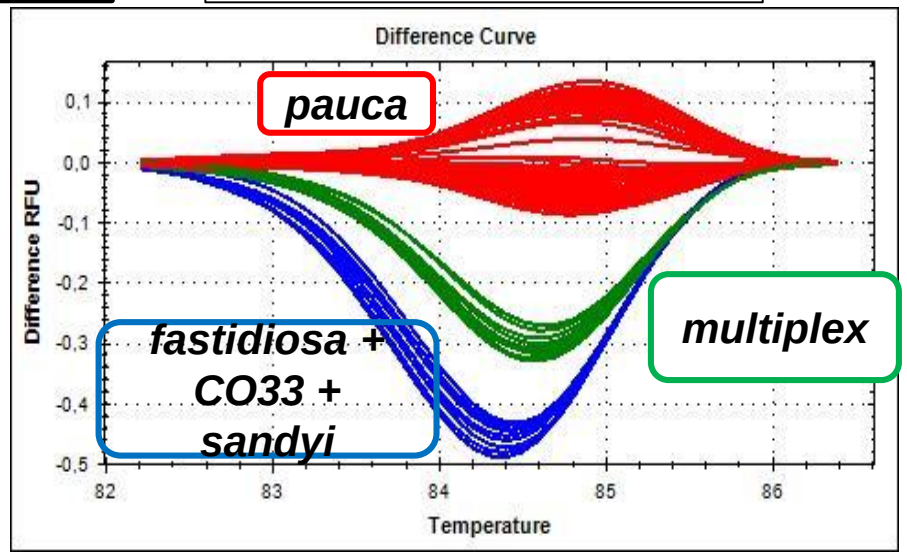
Rapid screening test for strain typing: HRM

1. HL primer set

CLUSTERS

2. nuoL primer set

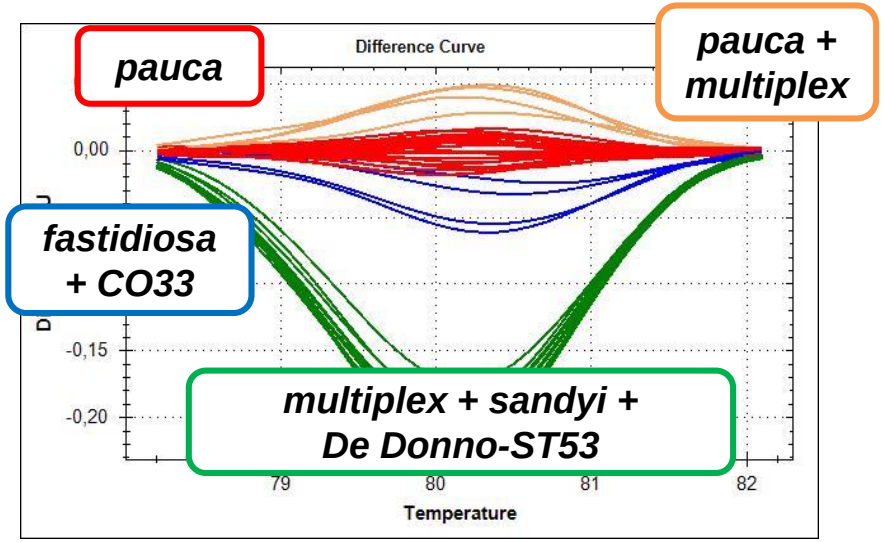




3 clusters, supported also by phylogenetic analysis
Validated on field samples

3. gyrB primer set

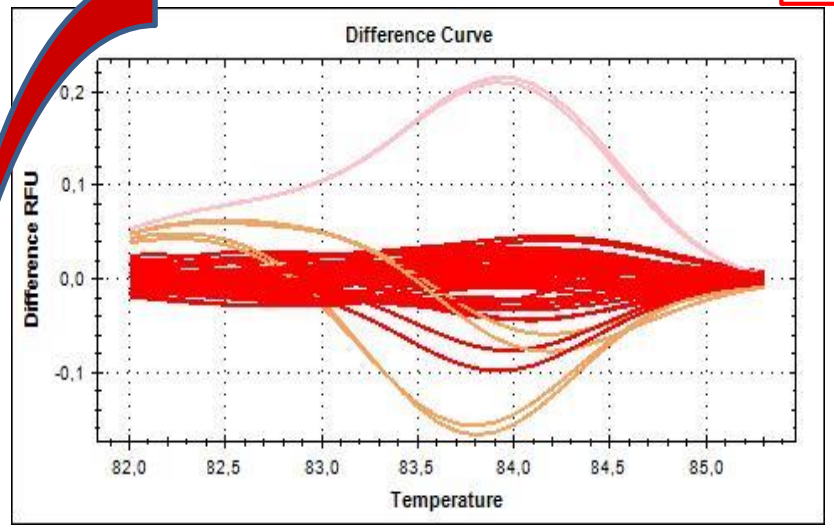
Incongruent results: no validated on field samples



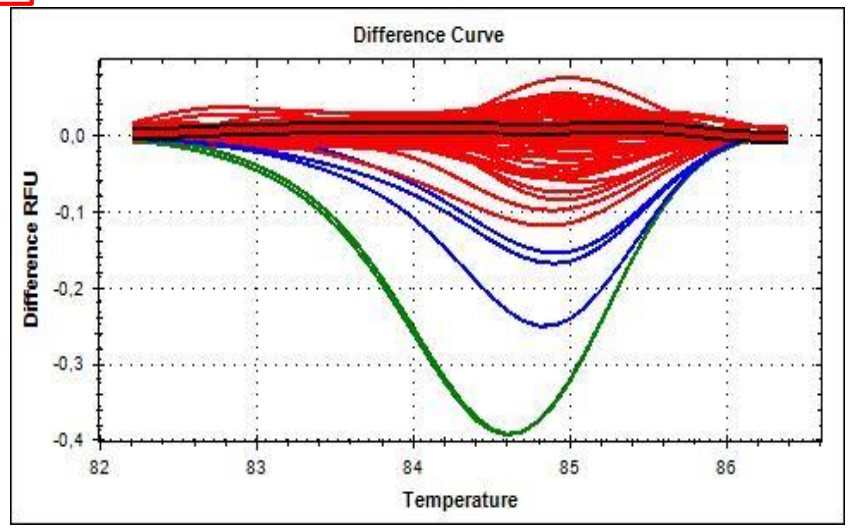
Rapid screening test for strain typing: HRM

HL primer set

pauca



nuoL primer set



HRM analysis on field samples

- HRM analysis by both primer sets correctly grouped all the field samples into the cluster of the subspecies *pauca*
- Useful tool to assign genotypes to a subsps. cluster for pre-screening infected samples to be further analyzed by MLST (more complex, time consuming and costly)

Reported in:

Eur J Plant Pathol (2019) 154:1195–1200
https://doi.org/10.1007/s10658-019-01736-9

A new variant of *Xylella fastidiosa* subspecies *multiplex* detected in different host plants in the recently emerged outbreak in the region of Tuscany, Italy

Maria Saponari · Giusy D'Attoma · Raied Abou Kubaa · Giuliana Loconsole · Giuseppe Altamura · Stefania Zicca · Domenico Rizzo · Donato Boscia

Rapid screening test for strain typing: multiplex minisequencing

The single-nucleotide primer extension **(SNuPE)** method using a multiplex amplification of ***gyr*B gene** to differentiate all subspecies and genotypes was on validation by CSIC.

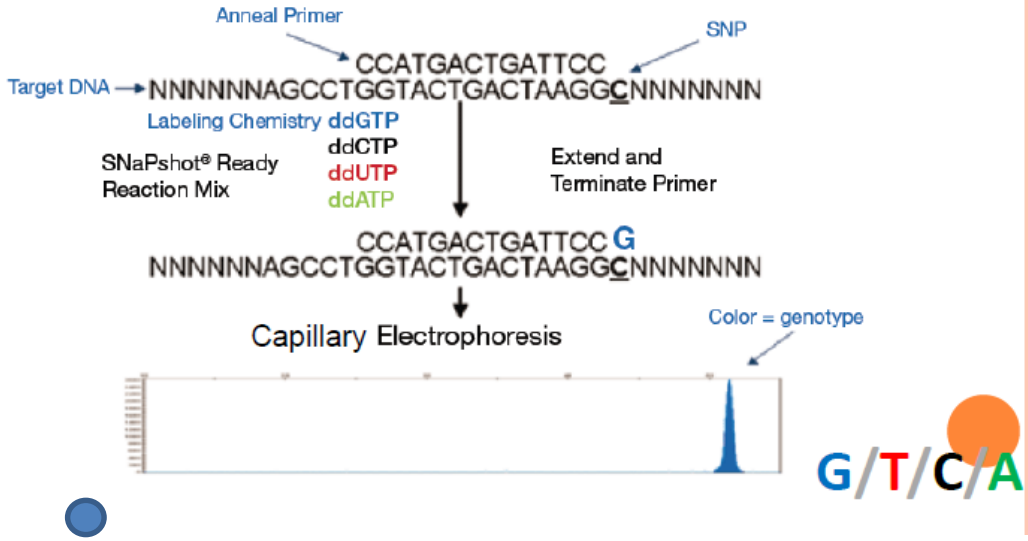
(1) approach:

Multiplex minisequencing based on fast Single-Nucleotide Primer Extension

used to differentiate between strains (haplotypes) in samples of total DNA extracted from bacteria, infected plants, and insect vectors

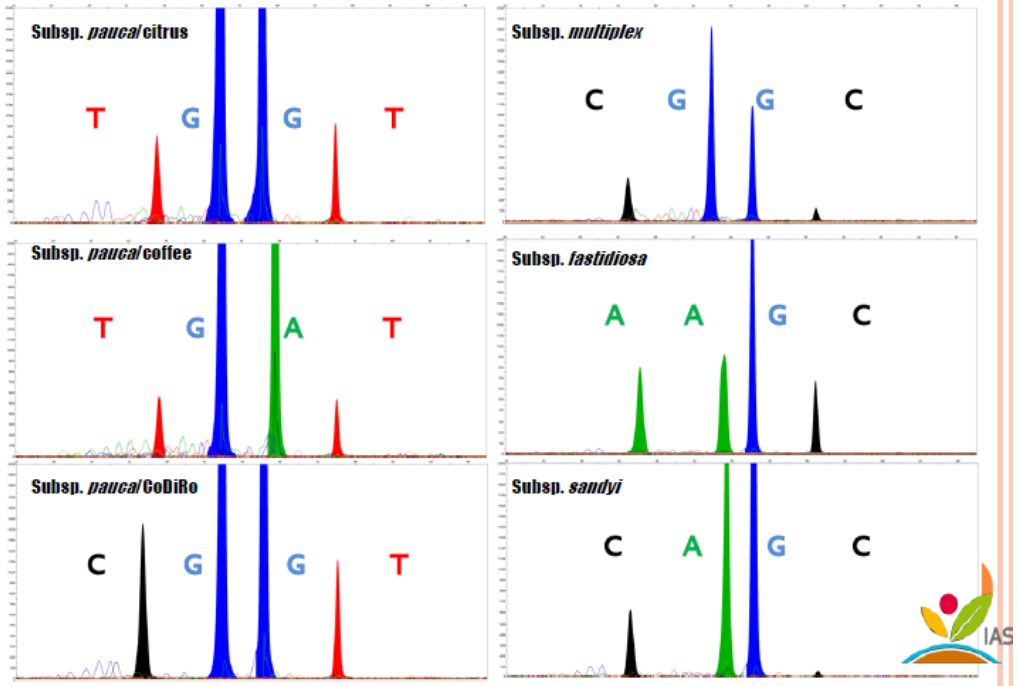
The SNaPshot® Multiplex Kit, based on a single nucleotide primer extension

SNaPshot® Kit Single-Base Extension Labeling Chemistry



Multiplex minisequencing: Results

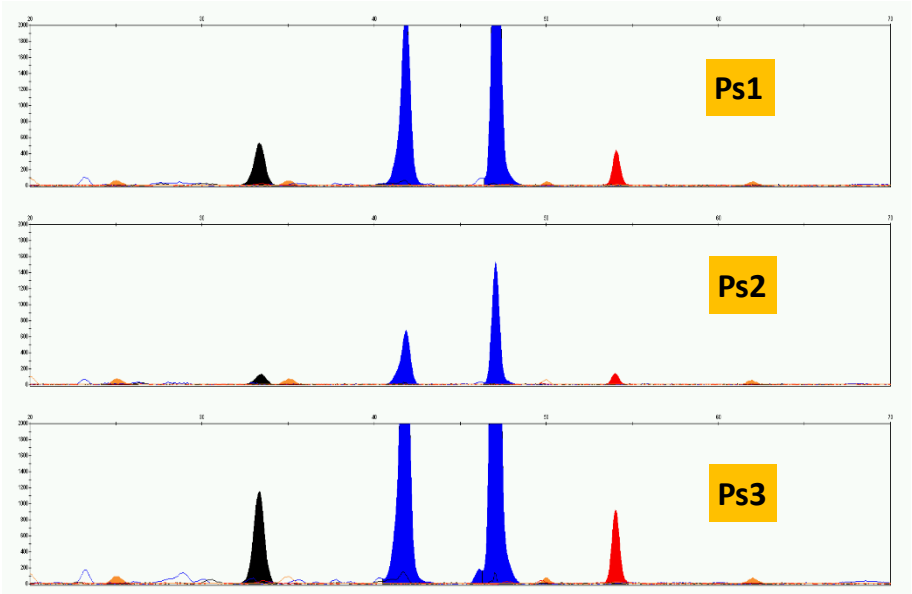
Minisequencing obtained independently by each primer using DNA from type strains (four SNuPE reactions and one sequencing run per sample mixing them)



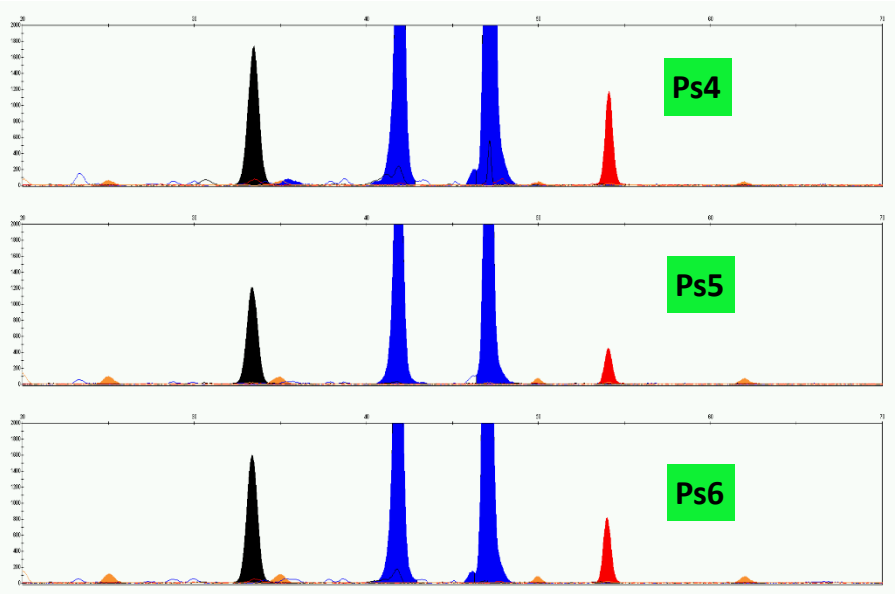
Rapid screening test for strain typing: multiplex minisequencing

Validation of the technique and diagnostic usefulness using blind samples: Bacteria, insects and plant DNA

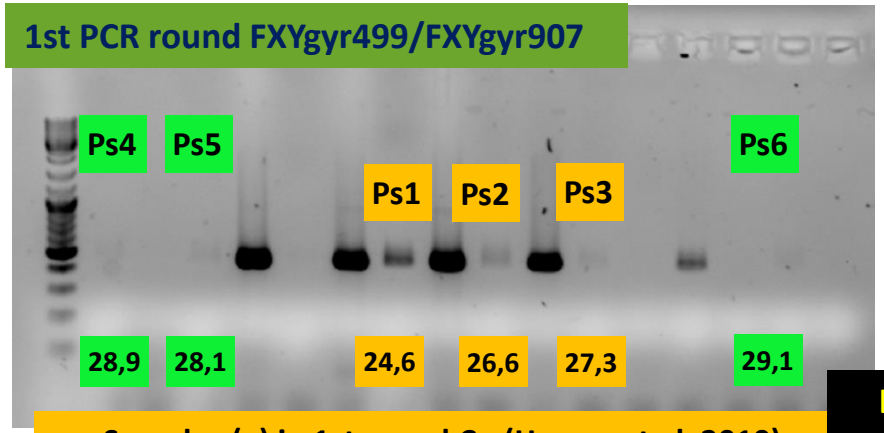
Philaenus spumarius (Direct PCR)



Philaenus spumarius (2-rounds PCR)



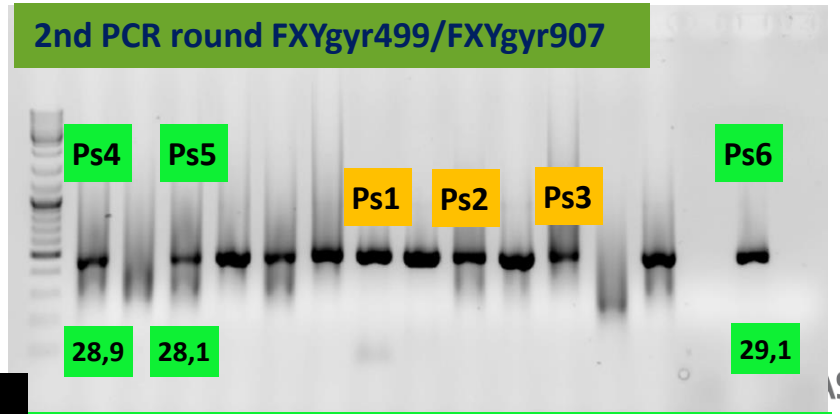
1st PCR round FXYgyr499/FXYgyr907



Samples (+) in 1st round Cq (Harper et al 2010)

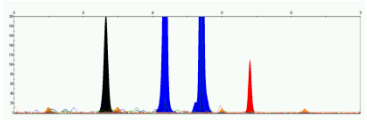
Limit Cq = 32

2nd PCR round FXYgyr499/FXYgyr907



Samples (-) in 1st round Cq (Harper et al 2010)

Rapid screening test for strain typing: multiplex minisequencing

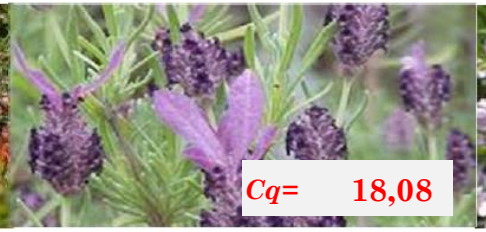


C G G T

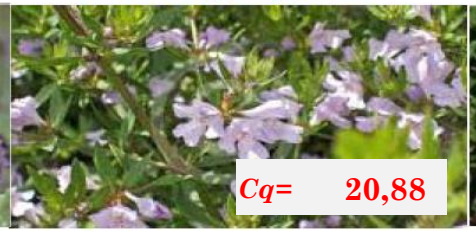
Subsp. pauca/CoDiRo

Grevillea juniperina

Lavandula stoechas

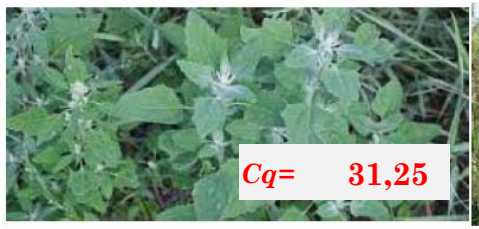


Westringia glabra

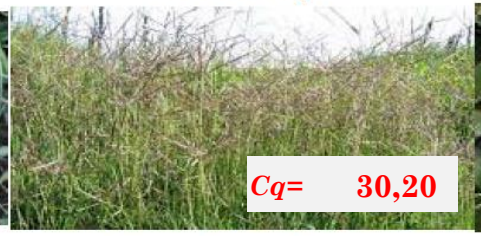


Cistus creticus

Chenopodium sp.



Poacea sp.



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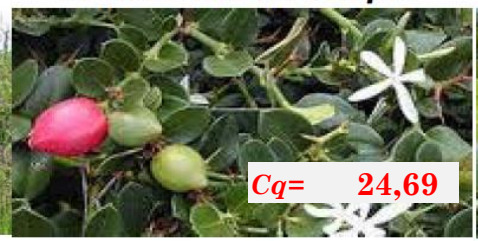

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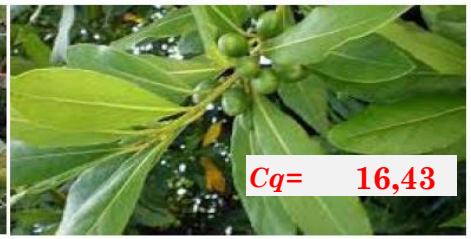
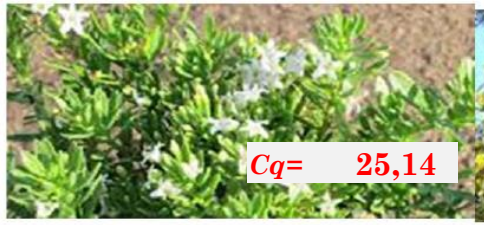


Myoporum



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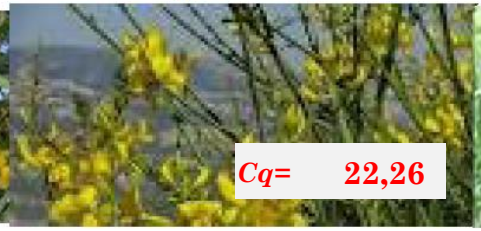
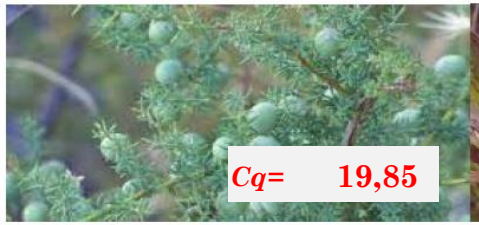
Spartium



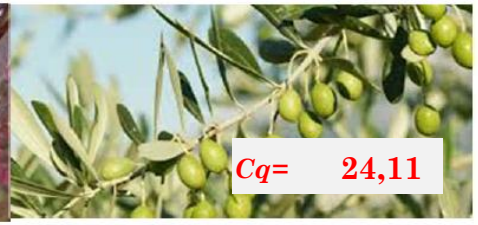
Lavandula angustifolia

Asparagus acutifolius



Dodonaea viscosa pur

Olive



P. spumarius

Deliverable D 4.4 – Official diagnostic protocol for *Xylella fastidiosa*

EPPO PM7/24 (4)

Bulletin OEPP/EPPO Bulletin (2019) **49** (2), 175–227 ISSN 0250-8052. DOI: 10.1111/epp.12575

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*

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]

1. Introduction

Xylella fastidiosa causes many important plant diseases such as Pierce's disease of grapevine, phony peach disease, plum leaf scald and citrus variegated chlorosis disease, and olive scorch disease, as well as leaf scorch on almond and on shade trees in urban landscapes, for example *Ulmus* spp. (elm), *Quercus* spp. (oak), *Platanus sycamore* (American sycamore), *Morus* spp. (mulberry) and *Acer* spp. (maple). Based on current knowledge, *X. fastidiosa* occurs primarily on the American continent (Almeida & Nunney, 2015). A distant relative found in Taiwan on Nashi pears (Leu & Su, 1993) is another species named *Xylella taiwanensis* (Su *et al*., 2016). However, *X. fastidio111sa* has also been confirmed on grapevine in Taiwan (Su *et al*., 2014). The presence of *X. fastidiosa* on almond and grapevine in Iran (Amanifar *et al*., 2014) was reported (based on isolation and pathogenicity tests), but so far strain(s) are not available. The reports from Turkey (Guldur *et al*., 2005; EPPO, 2018), Lebanon (Temsah *et al*., 2015; Habib *et al*., 2016) and Kosovo (Berisha *et al*., 1998; EPPO, 1998) are unconfirmed and are considered invalid. Since 2012, different European countries have reported interception of infected coffee plants from Latin America (Mexico, Ecuador, Costa Rica and Honduras) (Legendre *et al*., 2014; Bergsma-Vlami *et al*., 2015; Jacques *et al*., 2016). The outbreak of *X. fastidiosa* in olive trees in southern Italy (Saponari *et al*., 2013; Martelli *et al*., 2016) and the common presence of the bacterium in Mediterranean plant species, for example *Nerium oleander* and *Polygala myrtifolia*, in the natural and urban landscape of southern Italy, Corsica, along the Mediterranean coast in France and in the Balearic Islands and mainland Spain (EPPO, 2018) constitutes an important change to its geographical distribution and also adds new host plants. The EFSA database (EFSA, 2018) includes 563 plant species reported to be infected by *X. fastidiosa*, for 312 of which the infection has been determined with at least two different detection tests. These species cover hundreds of host plant genera in 82 botanical families (61 botanical families when considering only records with at least two different detection methods). The list of hosts in Europe is regularly updated with the results of surveys (EU, 2019).

Xylella fastidiosa is a member of the family Xanthomonadaceae of the Gammaproteobacteria. The genus *Xylella* contains two species, *X. fastidiosa* and *X. taiwanensis*. There are three formally accepted subspecies of *X. fastidiosa*, i.e. *fastidiosa*, *pauca* and *multiplex* (Schaad *et al*., 2004), based on DNA–DNA hybridization data, although only two, *fastidiosa* and *multiplex*, are so far considered valid names by the International Society of Plant Pathology Committee on the Taxonomy of Plant Pathogenic Bacteria (ISPP-CTPPB) (Bull *et al*., 2012). Since that publication, several additional *X. fastidiosa* subspecies have been proposed based on multilocus sequence typing (MLST) analysis (Scally *et al*., 2005; Yuan *et al*., 2010), including subsp. *sandyi* (on *N. oleander*; Schuenzel *et al*., 2005), subsp. *tashke* (on *Chitalpa tashkentensis*; Randall *et al*., 2009) and subsp. *morus* (on mulberry; Nunney *et al*., 2014a,b). Recently, a revision of the *X. fastidiosa* subspecies has been proposed (Marceletti & Scortichini, 2016) based on comparative genomic analysis.

The bacterium colonizes two distinct habitats, i.e. the xylem network of plants and the foregut of insects belonging to the

[1]The use of names of chemicals or equipment in this EPPO Standard implies no approval of them to the exclusion of others that may also be suitable. Temperatures given for refrigeration, freezing, growth chambers etc. are usually approximate.

[2]As more experience with the diagnosis of *Xylella fastidiosa* will be gathered in the coming months, the EPPO Secretariat intends to schedule a new revision of this Protocol at the next Panel on Diagnostics in Bacteriology in 2019.

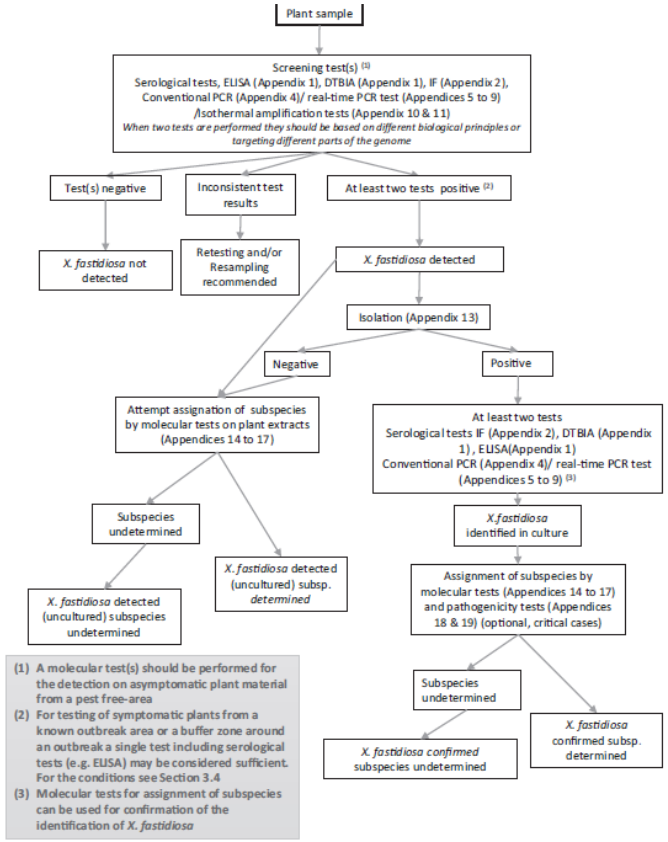


Fig. 1 Flow diagram for the diagnostic procedure for *Xylella fastidiosa* on plant material.

Deliverable D 4.4 – Official diagnostic protocol for *Xylella fastidiosa*

EPPO PM7/24 (4)

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European and Mediterranean Plant Protection Organization
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Diagnostics
Diagnostic

PM 7/24 (4) *Xylella fastidiosa*

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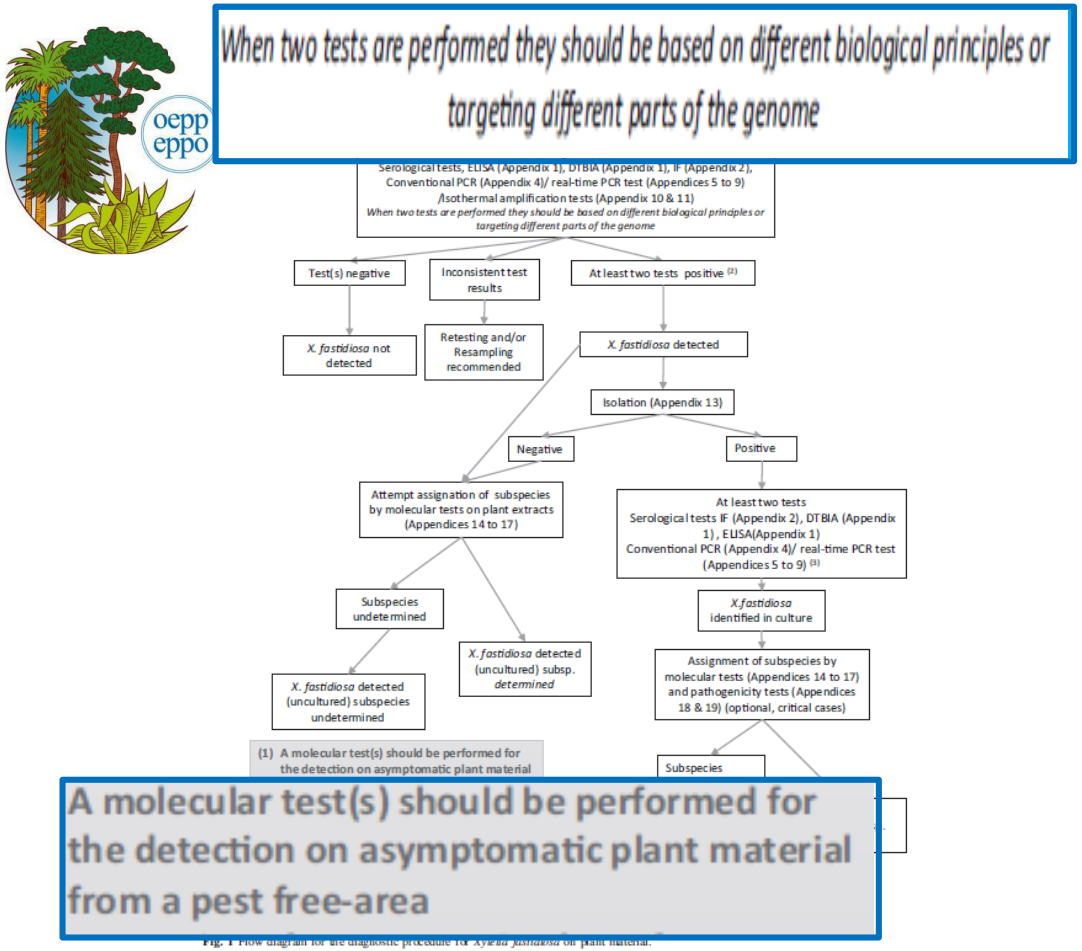

Fig. 1 Flow diagram for the diagnostic procedure for *Xylella fastidiosa* on plant material.

European Commission

Horizon 2020
European Union funding
for Research & Innovation

POnTE
PestOrganisms
ThreateningEurope

(Nicolas Boileau) **(1636, 1711).**

We must not rest on our laurels

When you carry out your work redo it twenty times

Thank you for your attention!

SUSTAINABLE FOOD SECURITY

H2020-SFS-2014-2 Sub call of: H2020-SFS-2014-2015

SFS-03a-2014: Native and alien pests in agriculture and forestry POnTE (Pest Organisms Threatening Europe) cod. 635646

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