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

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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)
  - DTBIA (Direct tissue blot immunoassay)
  - FTA card - ELISA

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

- 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)

<table>
<thead>
<tr>
<th></th>
<th>DAS ELISA</th>
<th>Lateral Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical specificity</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Analytical sensitivity</td>
<td>10^4 cells/mL</td>
<td>10^6 cells/mL</td>
</tr>
<tr>
<td>Diagnostic specificity</td>
<td>100%</td>
<td>85%</td>
</tr>
</tbody>
</table>

Low cost
Easy to use
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)
  - QuickPick™ SML Plant DNA Kit (Bio-Nobile) for Ormentals, vine, citrus
  - DNeasy® mericon™ Food kit (Qiagen) for 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 spumarius*) 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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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 performances 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.


- 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 technique* (RPA) (Agdia) (sensitivity improved 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)

<table>
<thead>
<tr>
<th>Dneasy Mericon food kit</th>
<th>Whatman +qPCR</th>
<th>FTA-ELISA</th>
<th>DTBIA</th>
<th>RPA (Agdia)</th>
<th>LAMP Enbiotech</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Accuracy ((N_pA+N_{NA})/N)</td>
<td>84</td>
<td>88</td>
<td>87</td>
<td>98</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>% Sensitivity ((N_{PA}/N+))</td>
<td>74</td>
<td>78</td>
<td>80</td>
<td>96</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>% Specificity ((N_{NA}/N-))</td>
<td>97.5</td>
<td>100</td>
<td>95</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
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the inprint techniques show lower sensitivity and accuracy compared to quick ready PCR (LAMP – RPA)
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<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

The imprint 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)

<table>
<thead>
<tr>
<th>Data</th>
<th>ELISA MAbs (IVIA)</th>
<th>ELISA Loewe (IVIA)</th>
<th>Real time Francis (IVIA)</th>
<th>Real time Francis (CSIC)</th>
<th>RPA (Agdia) (CSIC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Accuracy ((N_{PA}+N_{NA})/N)</td>
<td>89.3</td>
<td>81.3</td>
<td>98.2</td>
<td>82</td>
<td>78</td>
</tr>
<tr>
<td>% Sensitivity ((N_{PA}/N+))</td>
<td>81</td>
<td>81</td>
<td>96.8</td>
<td>75</td>
<td>70</td>
</tr>
<tr>
<td>% Specificity ((N_{NA}/N-))</td>
<td>100</td>
<td>81.6</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>qPCR protocol</th>
<th>DNA extracts</th>
<th>Accuracy %</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
<th>Repeatability %</th>
<th>Reproducibility %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harper</td>
<td>CTAB</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Mericon</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Li-SP</td>
<td>CTAB</td>
<td>96</td>
<td>100</td>
<td>92</td>
<td>97</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>Mericon</td>
<td>99</td>
<td>100</td>
<td>97</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Li-MGB</td>
<td>CTAB</td>
<td>92</td>
<td>100</td>
<td>67</td>
<td>97</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Mericon</td>
<td>99</td>
<td>100</td>
<td>97</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Francis-SP</td>
<td>CTAB</td>
<td>98</td>
<td>97</td>
<td>100</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Mericon</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Francis-SG</td>
<td>CTAB</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Mericon</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

- 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
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)

TEST PERFORMANCE STUDY (Anses)
Evaluation of detection methods of X. fastidiosa on Philaenus spumarius

<table>
<thead>
<tr>
<th>Type of spiked samples</th>
<th>Number of replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.10⁵ bact./head</td>
<td>2</td>
</tr>
<tr>
<td>5.10⁴ bact./head</td>
<td>2</td>
</tr>
<tr>
<td>5.10³ bact./head</td>
<td>4</td>
</tr>
<tr>
<td>Healthy sample</td>
<td>4</td>
</tr>
<tr>
<td>Positive Amplification</td>
<td>1</td>
</tr>
<tr>
<td>Control (PAC)</td>
<td></td>
</tr>
</tbody>
</table>

20 laboratories participating
Equivalent and reliable results for:
Combination of DNA extraction
Quickpick /CTAB
with
• real time Harper et al, (2010)
• LAMP (Yaseen et al, 2015)

Combination of Quickpick and Francis is less reliable.
Standardization and Validation of HRM analysis-procedure

Step 1: Primers used (CNR, UNIBA)

<table>
<thead>
<tr>
<th>Primers</th>
<th>Target region</th>
<th>Amplicon Size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL5/HL6</td>
<td>HL protein</td>
<td>221 bp</td>
<td>Francis et al., 2006</td>
</tr>
<tr>
<td>1Fw-HRM gyrB</td>
<td>gyrB gene</td>
<td>120</td>
<td>Designed by IPSP-CNR/DiSSPA</td>
</tr>
<tr>
<td>1Rev-HRM gyrB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nuoLFw-HRM</td>
<td>nuoL gene</td>
<td>146</td>
<td>Designed by IPSP-CNR/DiSSPA</td>
</tr>
<tr>
<td>nuoLRev-HRM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Standardization and validation on DNA purified from bacterial pure culture of 36 strains (USA, Brazil, France, Italy)

Validation on 30 field samples infected by De Donno strain, genotype ST53 subsp pauca

Data compared with phylogenetic analysis

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**
  - fastidiosa + CO33 (sandyi-like)
  - pauca
  - multiplex + sandyi

2. **nuoL primer set**

- **CLUSTERS**
  - pauca
  - fastidiosa + CO33 + sandyi
  - multiplex

3. **gyrB primer set**

- **CLUSTERS**
  - pauca
  - fastidiosa + CO33
  - multiplex + sandyi + De Donno-ST53

3 clusters, supported also by phylogenetic analysis

Validated on field samples

Incongruent results: no validated 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).
The single-nucleotide primer extension (SNeuPE) method using a multiplex amplification of *gyrB* gene to differentiate all subspecies and genotypes was on validation by CSIC.

**Rapid screening test for strain typing: multiplex minisequencing**

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

**Multiplex minisequencing: Results**

Minisequencing obtained independently by each primer using DNA from type strains (four SNeuPE reactions and one sequencing run per sample mixing them).
Validation of the technique and diagnostic usefulness using blind samples: Bacteria, insects and plant DNA

**Philaenus spumarius** (Direct PCR)

**Philaenus spumarius** (2-rounds PCR)

Rapid screening test for strain typing: multiplex minisequencing

Limit
\[ Cq = 32 \]
Rapid screening test for strain typing: multiplex minisequencing

**Subsp. pauca/CoDiRo**

- Grevillea juniperina: Cq = 23.79
- Lavandula stoechas: Cq = 18.08
- Westringia glabra: Cq = 20.88
- Cistus creticus: Cq = 28.85

- Chenopodium sp.: Cq = 31.25
- Poacea sp.: Cq = 30.20
- Carissa macrocarpa: Cq = 24.69
- Laurus nobilis: Cq = 16.43

- Myoporum: Cq = 25.14
- Acacia saligna: Cq = 26.82
- Spartium: Cq = 22.26
- Lavandula angustifolia: Cq = 20.39

- Asparagus acutifolius: Cq = 19.85
- Dodonaea viscosa pur: Cq = 18.32
- Olive: Cq = 24.11
- P. spumarius
Deliverable D 4.4 – Official diagnostic protocol for Xylella fastidiosa

EPPO PM7/24 (4)

Abstract: Xylella fastidiosa is a bacterium that causes disease in plants, including Xylella fastidiosa epiphyllae. The bacterium is an important vector for Xylella fastidiosa, which is a major threat to agriculture and horticulture. The EPPO PM7/24 diagnostic protocol is a standardized method for the detection and identification of Xylella fastidiosa. The protocol includes a range of diagnostic tools, such as polymerase chain reaction (PCR) and nucleic acid hybridization, to facilitate the detection of Xylella fastidiosa in different plant species. The protocol is designed to be user-friendly and accessible to practitioners in plant health and disease management.
Deliverable D 4.4 – Official diagnostic protocol for Xylella fastidiosa

EPPO PM7/24 (4)

PM 7/24 (4) Xylella fastidiosa

Specific scope
This Standard describes a Diagnostic Protocol for Xylella fastidiosa It is used in conjunction with PM 576 Use of EPPO diagnostic protocols.

1. Introduction

Xylella fastidiosa causes many important plant diseases such as Pierce’s disease of grape, phony peach disease, plum rust and citrus tartrazine (citrus disease and citrus rust). The pathogen is transmitted by many species of plant phloem feeding insects, typically members of the family Cicadellidae. Most commonly it is associated with the European green shield bug (Acrosternum hilare Linnaeus, 1758) and the brown apple moth (Epiphyas postvittana (Zeller, 1846)). The presence of X. fastidiosa has been confirmed in many other species such as Acrosternum detritum (Burmeister, 1840), Acrorhynchus bilineatus (Linnaeus, 1771), Edessa incisata (Linnaeus, 1758) etc. (Cointreau et al., 2014). In Italy, X. fastidiosa has been confirmed in 70% of the vine population (Roncaglia et al., 2014), in citrus trees and in olive trees (Papi et al., 2015; Parenti et al., 2015). The presence of X. fastidiosa has also been confirmed in other species such as Acrosternum detritum (Burmeister, 1840), Acrorhynchus bilineatus (Linnaeus, 1771), Edessa incisata (Linnaeus, 1758) etc. (Cointreau et al., 2014). In Italy, X. fastidiosa has been confirmed in 70% of the vine population (Roncaglia et al., 2014), in citrus trees and in olive trees (Papi et al., 2015; Parenti et al., 2015).
Thank you for your attention!

SUSTAINABLE FOOD SECURITY
SFS-03a-2014: Native and alien pests in agriculture and forestry POnTE (Pest Organisms Threatening Europe) cod. 635646
web: www.ponteproject.eu | mail: info@ponteproject.eu

We must not rest on our laurels
When you carry out your work redo it twenty times

(Nicolas Boileau) (1636, 1711).