Final Report

Project title (Acronym)
Harmonized protocol for monitoring and detection of *Xylella fastidiosa* in its host plants and its vectors (PROMODE)

Project duration:

Start date:	01-09-2016
End date:	21-08-2018

Contents

1. Research consortium partners 3
2. Short project report 9
2.1. Short executive summary 9
2.2. Project aims 9
2.3. Description of the main activities 10
2.3.1. Training courses 10
2.3.2. Interlaboratory comparison 10
2.3.3. Additional activities carried out at national level 11
2.4. Main results 13
2.4.1. Training courses 13
2.4.2. Interlaboratory comparison 14
2.4.3. Additional activities carried out at national level 14
2.5. Conclusions and recommendations to policy makers 17
2.6. Benefits from trans-national cooperation 19
3. Publications 20
3.1. Article(s) for publication in the EPPO Bulletin 20
3.2. Article for publication in the EPPO Reporting Service 20
3.3. Article(s) for publication in other journals 20
4. Open Euphresco data 21

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2. Short project report

2.1. Short executive summary

The PROMODE project workplan was developed to achieve two goals: (i) to strenghthen/build the capacity of the laboratories working on the diagnosis of plant pathogens and (ii) to improve the performance (such as reliability and sensitivity) of the diagnostic tests used for the detection and identification of *X. fastidiosa* in host plants and insect vectors. In order to avoid duplication of efforts, the PROMODE network had set relevant collaborations with the EU-funded consortia of POnTE (GA 635646) and XF-ACTORS (GA 727987), whose research activities were also focused on the same pathogen. Three main actions were undertaken during the PROMODE project lifespan:

- To support the organization of laboratory training courses through which trainees could get hands-on practise on different diagnostic protocols;
- To organize proficiency tests and test performance studies, to gather data on the proficiency of the laboratories involved in the official monitoring program as well as to assess the performance criteria of the different diagnostic tests available.
- To improve specific aspects of the diagnostic protocols: i.e. use of novel approaches like digital PCR, LAMP or HTS, and to validate the use of reliable internal controls.

Four distinct training courses were organized and three large interlaboratory validations were carried out. Useful information on the most suitable diagnostic tests for the rapid detection of the bacterium in different plant matrices was obtained, as well as data on the detection limit of the different tests. The test performance studies carried out within PROMODE implemented the availability of supporting validation data for the diagnostic tests nowadays included in the EPPO Diagnostic Protocol for *X. fastidiosa* (EPPO 7/24 3[1]). With regard to the development of novel diagnostic tests, partners of PROMODE successfully demonstrated the possibility to detect the bacterium in the host plants by digital PCR, HTS, and triplex real-time PCR.
The PROMODE network was actively involved in numerous dissemination events, among which the most relevant was the co-organization of the first European Conference on *Xylella fastidiosa*[2], held in November 2017 in Palma de Mallorca (Spain).

2.2. Project aims

The goal of the project was to enhance the scientific knowledge on the diagnosis of *X. fastidiosa* in order to support the development of harmonized sampling and diagnostic procedures, for an effective identification of *X. fastidiosa* in its host plants and vectors.
X. fastidiosa is a very complex plant pathogen bacterium:

(i) characterized by a wide host range (EFSA, 2018) and transmitted by several xylem-feeding Auchenorrhyncha;
(ii) characterized by high intraspecific genetic variability, with several subspecies have been either accepted or proposed;
(iii) strains may differ for their pathogenicity and host-range;
(iv) infections span from causing severe diseases to latent infections.

All together, these aspects delineate a complex scenario to be considered for setting up effective pathogen surveillance programs, for which the standardization of common procedures for reliable and sensitive detection is a crucial requirement.

[1] https://onlinelibrary.wiley.com/doi/epdf/10.1111/epp.12469
[2] https://www.efsa.europa.eu/en/events/event/171113

In the case of *X. fastidiosa*, diagnostic protocols need to be adapted for a wide range of plant matrices, for the broad-spectrum detection of the different strains/subspecies, for testing single or composite samples.
Detection of *X. fastidiosa* in insects is also paramount for the effective management of newly discovered *X. fastidiosa* outbreaks. Identification of potential vector(s) (through transmission tests and diagnostic tests) or identification of “spy insects” is a critical aspect for limiting the spread of the bacterium.

2.3. Description of the main activities

To accomplish the project objectives the network involved more than 20 organizations, some of which having strong experience in the detection of this bacterium in different host plants, thus ensuring an effective exchange of knowledge and expertise with teams from countries where the bacterium was not present and as such having more limited experience in processing infected samples under “real conditions”.

2.3.1. Training courses

Four training courses were organized in: the United Kingdom (event held in the premises of SASA), in France (event held in the premises of ANSES), and in Italy (event held in the premises of CNR-IPSP and UNIBA). The events followed a common format: (i) one-day session of lectures to illustrate the main host plants, bacterial subspecies and strains, the main features of the insect vectors, and the various diagnostic protocols available; (ii) two-day practical laboratory session. When organized in the area close to an infected zone, a field visit was planned to show the symptoms associated to the infections, to demonstrate the tools and procedures for sampling, and to collect insects. A maximum of 25 participants were admitted to each course, to maximize the practical involvement of the trainees; also, the laboratory sessions were organized in small working groups. A set of blind samples was processed by each group using different methods, and results were used for a comparative analysis and for critical discussions on the advantages, weaknesses or disadvantages of each method.
Training material was made available for each specific training course. Booklets containing the detailed diagnostic protocols used during the practical sessions were produced. The tutor made available detailed explanatory presentations.

2.3.2. Interlaboratory comparison

Two types of interlaboratory comparisons were organized in the framework of PROMODE, with the support of the H2020 consortia POnTE and XF-ACTORS.

1) The proficiency test (EU-XF- PT-2017-02), organized by partners CNR-IPSP, UNIBA and ANSES. The main objective of the proficiency test (PT) was to check the ability of laboratories to deliver accurate results for the detection and identification of *X. fastidiosa* in plant samples, by using serological (ELISA – enzyme-linked immunosorbent assay) and molecular tests (PCR, real-time PCR). The performance of the participating laboratories was determined based on the accuracy of the results obtained on a panel of blind plant samples.
A total of 35 laboratories participated in the validation and the following performance criteria were evaluated:
- sensitivity: based on the results obtained on 9 Xf-contaminated samples;
- specificity: based on the results obtained on 3 Xf-free samples;
- repeatability: based on the results obtained on 3 replicates for each Xf-contaminated and each Xf-free sample;
- accuracy: determined based on the results of three aforementioned performance criteria.

2) the test performance study (TPS) for the evaluation of molecular methods to detect *X. fastidiosa* in the vector *Philaenus spumarius* (17-XFAST-EU), organized by ANSES. This test performance study aimed to assess the efficiency and accurateness of different molecular methods used for the detection of the bacterium in the insect vector *Philaenus spumarius*. The samples consisted of both, artificially spiked samples and naturally infected specimens collected in the demarcated infected area of Apulia (southern Italy). The methods compared included:

- DNA extraction using the QuickPick™ Plant DNA kit (Bio-Nobile) and the CTAB DNA extraction protocol (EPPO PM7/24)
- LAMP assay based on the protocol of Yaseen *et al*., 2015
- Amplification: real time PCR Harper *et al*., 2010 (erratum 2013) either in single reaction or coupled with the amplification of the internal control (Ioos *et al*., 2009); real time PCR Francis *et al*., 2006 (both using the TaqMan or the Sybr green)

3) the test performance study (TPS) for the molecular detection of *X. fastidiosa* through quantitative real-time tests, organized by CNR-IPSP and UNIBA. This interlaboratory validation followed the proficiency test (PT) EU-XF- PT-2017-02 carried out in early 2017 and aimed to assess the performance and the accuracy of different real-time PCR tests to detect *X. fastidiosa* in host plants. In the framework of this TPS, five different real-time PCR tests were compared using a common panel of DNA templates. The DNA extracts used were those 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, at known concentrations (CFU/ml). A total of 13 laboratories participated and 5 different formats of real-time PCR compared:

- Harper *et al*., 2010;
- Li *et al*., 2013 (using both a standard TaqMan probe or an MGB-TaqMan probe);
- Francis *et al*., 2006 (including a modified protocol using SYBR green).

2.3.3. Additional activities carried out at national level

ANSES

ANSES undertook the evaluation of different methods for detection of *X. fastidiosa* in vectors. Different approaches to crush the heads of the insects and different DNA purification methods were tested using artificially contaminated preparations. Indeed, different real-time tests were compared to detect the bacterium and a target gene of the insect as internal control. Briefly, maceration was performed either by using pestles or by using steel/glass beads; purification of the DNA was accomplished by using different commercial kits: QuickPick™ kit, DNeasy® Blood&Tissue kit, DNeasy® Plant Mini kit and NucleoSpin®. Indeed, as internal reference protocol, DNA was purified also using CTAB. For the amplification of *X. fastidiosa* the primers/probe developed by Harper were used, along with the amplification of two different internal controls: (i) Papayiannis *et al*. 2011 (targeting the cytochrome oxidase gene present in plants, that could be traced on the insects that fed on plants); (ii) Ioos *et al*. 2009, targeting the universal eucaryote internal control 18S.

ANSES participated in the interlaboratory test for the Triplex real-time PCR proposed by the partner WU.

INRA

INRA undertook the following activities:

- Building a collection of *X. fastidiosa* strains

- Identification of targets through genome analysis in order to be used as an identification method.
- Development of epidemio-surveillance tools for *X. fastidiosa*

WU

WU developed a triplex TaqMan real-time PCR for the detection of *X. fastidiosa*, by combining two previously described TaqMan real-time PCR tests specific for the pathogen (Harper *et al*. 2010, Ouyang *et al*. 2013) and an assay as (internal) control for DNA-extraction and amplification, to improve the diagnostic specificity of the triplex assay.
WU also demonstrated the use of HTS technologies for the analysis of 24 DNA extracts obtained from various infected sources and subjected to Illumina HiSeq sequencing, for testing the possibility to use this high-throughput approach for the detection and subspecies assignment and avoid bacterial isolation.

AGES

AGES tested the possibility to use multilocus sequence typing (MLST) directly on plant DNA and to assess if the use of a single gene (i.e. pilU) could have the same discriminatory power of the MLST protocol based on 7 genes.
AGES was also involved in the monitoring of insect vectors in grapevines and stone fruit orchards, using sticky traps. AGES team actively contributed to the capacity building, training and dissemination actions.

NIB

NIB implemented digital PCR for the detection of *X. fastidiosa* in various plants, to assess the efficiency of DNA extraction of *X. fastidiosa* from various plant matrices. In addition to the aforementioned interlaboratory validations, NIB took part in the international proficiency test for molecular detection of *Xylella fastidiosa* NIB-PT-2016-02 (Dreo, Tanja, Pirc, Manca and Matičič, Lidija. 2017. Final Report on the 'NIB Proficiency Test Round 2016-02': Proficiency Test for Molecular Detection of Xylella fastidiosa (No. 2017/001), Proficiency Test Reports. National Institute of Biology, Ljubljana.)

NVWA

NVWA tested two sampling approaches: use of composite samples and use of different portions of the leaves (midrib including petiole versus basal part of the leaves). NVWA was also involved in an extensive validation of the real time PCR assay described by Quyang *et al*. (2013).

- Testing composite samples

Naturally infected leaves of *Olea europaea*, *Nerium oleander*, *Polygala myrtifolia*, *Coffea arabica* cv Nana and *Prunus avium*, were used to artificially contaminate pools of healthy leaves. The infection level of the samples was initially determined by testing single leaves with real-time PCR (Harper *et al*., 2010, erratum 2013). Then, to simulate the composite samples, either the sap recovered from the infected single leaf was mixed with the sap of the healthy leaves or the petioles from the infected samples were directly mixed with the pool of healthy leaves and macerated afterwards. The different ratios used ranged from 19 up to 349 healthy leaves (19, 49, 99, 199 and 349). Four replicates were performed for each combination and the experiments were repeated twice. A centrifugation to concentrate the extracts was performed in comparison with the so called "non-concentrated extracts" recovered without any step of concentration.

- Testing midribs vs basal part of the leaves

To speed up the process of sample preparation, a comparison was made to test the basal part of the leaf (including the petiole) or the midribs excised from the leaves. The experiments were performed using leaves of *Olea europaea*, *Nerium oleander*, and *Coffea arabica* cv Nana.

- Validating real-time PCR test of Quyang *et al*. 2013

The following criteria were evaluated: repeatability, analytical sensitivity, analytical specificity, diagnostic sensitivity, diagnostic specificity, reproducibility, robustness, as well as inhibition effects. The analytical sensitivity was evaluated in the following plant matrices: *Coffea arabica, Nerium oleander, Polygala myrtifolia, Olea europaea, Lavandula stoechas* and *Prunus avium*, using the four subspecies (LMG17159 - subsp *fastidiosa*, CFBP8173 - subsp. *multiplex*, CFBP8402 - subsp. *pauca* and CFBP8077 - subsp. *sandyi*) to prepare the artificially contaminated samples containing known bacterial concentrations.

ILVO

ILVO focussed on culturing of *Xylella fastidiosa,* inoculum preparation and validation of TaqMan real-time PCR of Harper *et al*. 2010.

- Based on the composition of existing culture media, two modified but fully autoclavable media were developed. They were evaluated for the three *X. fastidiosa* subspecies which differ slightly in growth rate, growth form and metabolically needs. The preference of each subspecies was determined.
- A culture protocol was elaborated to recover highly concentrated suspensions of planktonic cells. A softgel (SG) medium in which a liquid SG is poured on the preferred solid medium of the strain and the bacterial cells are then added to the SG. The nutrients of the medium gradually seep into the SG which is then scraped off the solid medium as a thin film and further used as highly concentrated *X. fastidiosa* inoculum with mainly planktonic cells.
- Filter sterilized xylem sap from the Semillon grapevine was also tested as growth medium in which *X. fastidiosa* formed dense biofilms.
- The performance of the *rim*M TaqMan PCR was evaluated on leaves from 12 woody plant species. The tests were done on CTAB extracts spiked with cells of *X. fastidiosa* at 4 levels of contaminations (10^3-10^6 CFU/ml) and using different master mixes.

2.4. Main results

2.4.1. Training courses

Training courses were successfully organised with the active participation and interest of laboratory staff and inspectors from a wide number of countries. The courses were an important opportunity to gather insights from the more experienced laboratories working at the forefront of European outbreaks (i.e. the courses held in southern Italy and France). More information is available at:

- https://www.ponteproject.eu/technical-workshops/the-successful-training-workshop-on-the-diagnosis-of-xylella-fastidiosa-and-identification-of-insect-vectors-held-in-locorotondo/
- https://www.ponteproject.eu/technical-workshops/achievements-successful-training-workshop-detection-identification-xylella-fastidiosa-held-angers/
- https://www.ponteproject.eu/technical-workshops/diagnostic-training-xylella-fastidiosa-19%E2%80%9222-september-2017-locorotondo-bari-italy/
- https://www.ponteproject.eu/technical-workshops/great-achievement-in-edinburgh-for-the-training-workshop-on-xylella-fastidiosa-detection/

2.4.2. Interlaboratory comparison

Proficiency test EU-XF- PT-2017-02. The proficiency test provided a good overview of the proficiency of laboratories and on the performance of the diagnostic tests currently used in EU/Mediterranean countries for the detection of *X. fastidiosa.* Briefly, the results indicated that when using the most sensitive and the most widely adopted diagnostic protocol (i.e. real-time PCR) the performance of the laboratories was very satisfactory in most of the cases; at the same time useful insights were obtained to achieve better results for the non-proficient laboratories, i.e. select different protocols for DNA extraction, different reagents and amplification conditions, use of automatized platform for the extraction of the DNA.

Data were also recovered on the performance of the molecular tests. TPS results showed optimal performance values (ranging from 97 to 100%) for all methods and for all performance criteria. Thus, although carried out under different amplification conditions, the high reproducibility and accuracy values obtained within this TPS, underline the robustness (PM 7/76) of the molecular diagnostic tests (extraction procedures and amplification protocols) evaluated in this TPS, and currently being the most common used protocols, confirming their suitability for the diagnosis of *X. fastidiosa* in plant materials.

Test performance study 17-XFAST-EU. When comparing the different real-time PCR tests, results indicated that the real-time PCR of Francis *et al*., 2006 using SYBR green was less sensitive than the real-time PCR of Harper *et al*., 2010 (in simplex or duplex). No difference appeared between the results obtained with the real-time PCR of Harper *et al*., 2010 in duplex and simplex, so it is recommended to use the duplex integrating an internal control. No differences were recorded between processing the entire head or the head after removing the eyes of the insects. Results obtained with the LAMP method were very similar to those obtained with real-time PCR for the different performance criteria. For the QuickPick™ DNA extraction method, this TPS showed some significant differences between the results obtained by the laboratories using a robot and laboratories using a 'by hand' protocol, especially in term of sensitivity and reproducibility. It is recommended the use of the QuickPick™ kit preferably in association with the use of a robot.

Test performance study EU-XF- PT-2017-02. The accuracy, repeatability and reproducibility of the real-time PCR protocols assessed were in the range of 92-100%, thus the molecular diagnostic tests (including both the plant DNA extraction procedures and the real-time PCR protocols) showed robustness (PM 7/76) and proved to be suitable for the diagnosis of *X. fastidiosa* in plant materials. The real-time PCR protocols designed by Harper *et al*. 2010 and Francis *et al*. 2006 (this latter using both the TaqMan probe or SYBR Green), produced the best performance values, regardless the method used for the extraction of the DNA, reaching values of 98-100% for the different performance criteria analyzed. The real-time PCR protocols designed by Li *et al*. 2013, either using a standard TaqMan probe or a MGB-TaqMan probe, produced the lowest Ct values, and although they showed general good performances (values >92%) these were the lowest among the five protocols compared.

2.4.3. Additional activities carried out at national level

ANSES

Evaluation of different methods for the detection of *X. fastidiosa* in vectors. When different methods of extraction and DNA purification were compared the following conditions produced the best results (for the evaluated performance criteria): (i) use of 10 steel balls of 3 mm diameter and grinding for 2 min at 30 Hz; (ii) purification of the DNA using the QuickPick™ kit.

With regard to the real-time PCR, the use of the tests developed by Harper *et al*. 2010 and Li *et al*. 2013 produced similar results in term of sensitivity; although the latter yielded lower

diagnostic specificity values. For the detection of the internal control, inconsistent results were obtained when the primers/probe designed by Papayiannis *et al*. 2011 were used; conversely, consistent amplifications were recovered using the primers designed by Ioos *et al*. 2009. When this test was coupled with the detection of the bacterium (multiplex test for the detection of *X. fastidiosa* and the 18S internal control), no interference was recorded, i.e. the quantitation cycles recovered for the real-time PCR of Harper *et al*. 2010 in duplex test gave similar results to those recovered when a singleplex test was performed. The detection limit was defined as low as 10^3 CFU/head.

Triplex real-time PCR test. Similar Ct values were obtained for *X. fastidiosa* for both targets included in the triplex test (Harper *et al*., 2010 and Ouyang *et al*., 2013) indicating that this test has the valuable advantage of providing double confirmation of the presence of the bacterium in a single reaction. Under the conditions tested at ANSES, the detection of the internal control Acat (added to the samples) did not produce satisfactory results, as amplification curves were not optimal.

INRA

The *X. fastidiosa* strains collection was built and made available to the entire scientific community through the website of the CIRM-CFBP (http://catalogue-cfbp.inra.fr/recherche_e.php). 34 strains are publicly available (as of 2019-03). These strains represent the known genetic diversity of the *X. fastidiosa* species, with strains from subspecies *fastidiosa, sandyi, multiplex, morus* and *pauca*, including the type strains of the *fastidiosa, sandyi,* and *multiplex* subspecies (CFBP 7970, CFBP 8077, and CFBP 8173, respectively). This collection also hosts strains representing the *X. fastidiosa* diversity isolated in Europe, *i.e.* the CoDiRO strain (CFBP 8402), three multiplex strains isolated in France (CFBP 8416 [ST7], CFBP 8417 [ST6], and CFBP 8418[ST6]), and strains from plant material intercepted in France (CFBP 8072, CFBP 8073, CFBP 8074, CFBP 8429, and CFBP 8478).

A software was developed to mine genome sequences in order to search for signatures that are specific of groups of strains of interest (Denancé *et al*., 2019). This tool was specifically used to identify fragments specific of the *fastidiosa, sandyi, multiplex,* and *pauca* subspecies and of the *X. fastidiosa* species. Primers were designed and real-time PCR multiplex tests were developed and tested on bacterial strains and infected plant materials. Inclusivity, exclusivity, and efficiency were determined as being at least equal to those of the Harper detection test (Harper *et al*., 2010), but these tests allow simultaneously detection and identification at the subspecies level (Dupas *et al*., in prep).

Symptomatic samples from more than 30 plant species were collected in June 2017 and September 2018 in Corsica, France. Modified CTAB and Quick-Pick protocols including sonication of the samples and a modified BSA concentration were used to extract the DNA from these plant samples. Harper's real-time PCR detection test (Harper *et al*., 2010) and the newly developed nested MLST protocol (Cesbron *et al*., in prep) were used to ensure detection and identification of *X. fastidiosa* in these samples.

WU

Results showed that the triplex TaqMan real-time PCR was as sensitive as the simplex Harper TaqMan real-time PCR, commonly used for the detection of *X. fastidiosa*. The gBlocks gave the possibility to accurately determine the analytical sensitivity of individual tests in a multiplex setting. The specificity of the triplex PCR test substantially improved as the test targets two instead of only one locus, and the loci are clearly separated on the *X. fastidiosa* genome.

HTS analysis showed that the reads of the infected samples from Italy mapped clearly on the CoDiRO strain genome sequence. Read mapping of the infected samples from Spain resulted in a tentative classification of the pathogen in three different subspecies (*multiplex*, *fastidiosa* and *pauca*). Reads in the three infected samples from France mapped on the multiplex reference genome. In the majority of the 31 samples analysed, the pathogen could be identified up to the subspecies level and for one sample even the whole genome could be assembled and the sequence type could be determined.

NIB

Digital PCR was implemented for the detection of *X. fastidiosa* in various plants and to assess the efficiency of DNA extraction from various plant matrices, using the Quick Pick DNA extraction kit (BioNobile). Results of the real-time PCR can be affected by the presence of contaminants/inhibitors and, in general, diluting the extracts may reduce this effect (i.e. 1:10). Conversely, digital PCR is less affected by the plant matrices, exhibiting comparable or better analytical sensitivity event when testing undiluted samples. Using spiked controls and analysing samples undiluted and in one reaction, 128/129 of the positive controls tested positive, corresponding to 99,2 % diagnostic sensitivity. LAMP test described by Harper *et al*. 2010 was adapted to fluorescent detection and used to analyse vectors and plants, including naturally infested samples provided by the laboratory of CNR, Bari. The same samples were used for training and educational purposes.

NVWA

Testing composite samples. When testing olives, the bacterium could be detected in all composite asymptomatic samples up to 350 leaves, although the resultant Ct values were in the range of 32,34 - 39,36 for the non-concentrated extracts and 30,78 - 38,08 for the concentrated extracts. Detection failed in some samples containing 200 and 350 leaves when the concentrated extracts was used. Comparable results have been obtained for other plant species, with the substantial exception of *Prunus avium* where much higher Ct values were obtained. These results refer to the use of the pre-homogenized infected leaves added to the sap recovered from healthy leaves; when the infected leaves were added directly to the mix of healthy leaves the detection consistently failed, indicating that the homogenization step is critical to allow the release of the bacterium from the plant tissues and gather consistent detection.

Testing midribs vs basal part of the leaves. The comparison of the results obtained by testing the basal part of the leaves vs the leaf midribs, did not result in major differences in terms of Ct values.

Validation of the real-time PCR test Ouyang *et al*. 2013. The real time PCR test of Quyang *et al,* 2013 was found to be less sensitive than the real time PCR of Harper *et al*, 2010 (erratum 2013), with the exception of *Lavandula stoechas*; this plant matrix gave major inhibition in both real-time PCR tests. When 45 *X. fastidiosa* isolates representing the different subspecies and strains and 96 potentially related bacterial species, including *Xylella taiwanensis* and a number of *Xanthomonas* spp. were used to assess the analitycal specificity, values of 100% were recovered for both real-time PCR tests, as inclusivity and exclusivity.

ILVO

Improvement of the culture media. Active charcoal and hemin chloride proved to have a very important role in the growth as attachment structure for development of microcolonies or as

scavenger for oxidative stress components. Yeast extract can also promote growth, specifically of *X. multiplex* and *pauca* strains, but had no effect on growth of *fastidiosa* strains. Phytagel was the preferred solidifying agent compared to bacto agar and gelrite. Addition of sucrose or glycerol did not enhance growth, whereas addition of chitin stimulated growth. Some media such as PW and MYX showed the tendency to stimulate separate single colonies.

Using the xylem sap from the Semillon grapevine as substrate for the growth requires cells from solid media to adapt their growth gradually by increasing the concentrations of xylem sap. This may require several weeks which increases the risk of contamination in the cultures, reducing the suitability of this alternative approach as efficient protocol for growing *X. fastidiosa*.

When the performance of the *rim*M TaqMan real-time PCR was evaluated on different woody plant species, significant differences between the various mixes were observed, and the PerfeCTa ToughMix (Quanta Biosciences) yielded the best results.

2.5. Conclusions and recommendations to policy makers

Important information on the performance of different diagnostic protocols and on the proficiency of laboratories was gathered from the interlaboratory comparison carried out in the framework of PROMODE with the collaboration of the H2020 consortia POnTE and XF-ACTORS.

- The real-time PCR tests currently part of the EPPO standard PM 7/24 (3) resulted the most sensitive among the molecular tests compared
- When using the molecular approaches, the majority of the participating laboratories were assessed as “conform - highly proficient”, i.e. they reached a level of accuracy higher than 90%. Conversely, lower values of accuracy were obtained when the laboratories used ELISA tests.
- CTAB-based extraction protocol and the Qiagen Mericon Food Lysis produced the lowest values of quantitation cycles (i.e. higher sensitivity) among the methods compared for the purification of the total DNA from different plant matrices.
- Similarly, CTAB-based extraction protocol turned to be the most efficient method for the preparation of the DNA extracts from the insect vectors. However, extraction with the QuickPick kit represents a good alternative.
- When processing the specimens of *Philaenus spumarius*, the predominant European vector, the removal of the eyes did not improve the diagnostic and analytical sensitivity.
- The amplification of internal control, either for the plant or for the insects, did not affect the analytical sensitivity of the tests targeting *X. fastidiosa*.

In addition, research activities conducted by several project partners contributed to gain useful knowledge for the diagnosis of *X. fastidiosa*:
- A novel MLST protocol was implemented at INRA to increase the sensitivity of the PCR amplification when plant/insect total DNA is used as template. The work is currently under publication.
- A novel multiplex test was optimized and validated for the simultaneous detection of two target genes of *X. fastidiosa* and an internal control (Bonants *et al*., 2019). This test allows for a fast double confirmation of the presence/absence of the bacterium in the tested samples. The test will be included in the list of the tests recommended in the EPPO Diagnostic Protocol on *Xylella fastidiosa*.
- Digital PCR proved to be a promising tool for the accurate detection and quantitation of *X. fastidiosa*, however given the high number of plant species and genetically different bacterial strains, more data needs to be collected through specific testing and validations.

- Results obtained by testing pooled samples (multiple composite samples) were not consistent and highlighted the need to perform a more accurate homogenization/grinding of the samples prior to improve the detectability of the bacterium when testing samples with low incidence of positive tissues. The knowledge of this critical point allowed to suggest improvements for the preparation for composite samples to be collected from large lots of consignments or from nursery stock productions. Research is now ongoing to optimize a suitable protocol that can support the mandatory controls introduced with the Commission Implementing Decisions (EU) 2017/2352 and 2018/1511.

In conclusion, although relevant research efforts have been devoted in the last 5 years to improve the diagnostic tools available for *X. fastidiosa* and important achievements have been reached toward the optimization and harmonization of common and reliable detection procedures, the complexity of the infections caused by this quarantine pathogen still requires efforts that can help to better support early and sensitive detection approaches. To this end, more research is needed to better estimate the threshold of detectability in asymptomatic hosts as well as to better define the latency period in relation to the numerous host species and bacterial strains so far reported in the EU affected Countries.

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2.6. Benefits from trans-national cooperation

The large number of laboratories that cooperated in PROMODE allowed to gather solid and repeatable results. The results obtained were possible thanks to the collaboration of different research networks, that allowed an effective sharing of knowledge and exchange of expertise on this plant pathogen recently detected in the EPPO region.

3. Publications

3.1. Article(s) for publication in the EPPO Bulletin

None.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

Bonants P, Griekspoor Y, Houwers I, Krijger M, van der Zouwen P, van der Lee TAJ, van der Wolf J (2019). Development and Evaluation of a Triplex TaqMan Assay and Next-Generation Sequence Analysis for Improved Detection of Xylella in Plant Material. Plant Dis. doi: 10.1094/PDIS-08-18-1433-RE.

Cesbron *et al*., in preparation. Nested-MLST is a highly sensitive and specific method to identify *Xylella fastidiosa* subspecies directly in plant samples

Denancé N, Briand M, Gaborieau R, Gaillard S, Jacques MA (2019). Identification of genetic relationships and subspecies signatures in *Xylella fastidiosa*. BMC Genomics, *20*(1), 239.

Dupas *et al*., in preparation. New tetraplex qPCR assays for simultaneous detection and identification of *Xylella fastidiosa* subspecies in plant tissues.

Giovani B, Cellier G, McMullen M, Saponari M, Stefani E, Petter F (2019). From transnational research collaboration to regional standards. BASE 23(1): 30-35. doi: 10.25518/1780-4507.17642.

4. Open Euphresco data

Protocols for the detection of *Xylella fastidiosa*
https://www.ponteproject.eu/wp-content/uploads/2016/10/Protocols-for-detection-of-Xf_SASA-workshop-Edinburgh-2016.pdf

Reports on the External Quality Assessment studies for laboratory performance

- https://pdfs.semanticscholar.org/012a/a8febc9bbe2e0a3bc22f12f70ff968f1144e.pdf?_ga=2.212504924.931771943.1552994590-133780236.1519741782
- https://drive.google.com/drive/folders/1-eVa4sCr4DNrICAAPfPIi8FnMCObB5PM

First international proficiency testing for laboratory performance for detection of *Xylella fastidiosa*
https://www.efsa.europa.eu/sites/default/files/event/171113/171113-6.2_Loconsole.pdf