REPORT

External Quality Assessment studies for laboratory performance

EU-XF- PT-2017-02: Proficiency testing for the evaluation of molecular and serological diagnosis of *Xylella fastidiosa*

FEBRUARY-APRIL 2017

Sommario

This report was prepared based on the analyses of the results received by all 35 participant laboratories: https://www.ponteproject.eu/wp-content/uploads/2017/07/EU-XF-PT-2017-02-results.zip

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1. GENERAL INFORMATION

1.1 Background

Proficiency test (PT) is a way in which the performance and competence of laboratories are evaluated and assessed. In proficiency testing, standardized samples are prepared with known status regarding the presence of the target pathogen(s). These are sent out to participating laboratories that analyze them using their own methods, equipment and reagents, and send results back to the Organizer(s). The Organizer(s) analyzes the results and provides a report detailing all participants' results in confidential manner together with actual sample status.

The current proficiency test aimed to evaluate the performance (efficiency and accuracy) of laboratories involved in the diagnosis of *Xylella fastidiosa* by using molecular and serological methods. This interlaboratory comparison is part of the research activities on the implementation of surveillance and monitoring program for *X. fastidiosa*, within the framework of the following ongoing European projects:

- EUPHRESCO project (2015-F-146) "Harmonized protocol for monitoring and detection of *Xylella fastidiosa* in its host plants and its vectors"
- H2020 "POnTE – Pest Organisms Threatening Europe (635646)"
- H2020 "XF-ACTORS - *Xylella fastidiosa* Active Containment Through a multidisciplinary-Oriented Research Strategy (727987)".

1.2 Objectives

The main objective of this interlaboratory comparison was to check the ability of laboratories (i.e. proficiency test - PT) to deliver accurate testing results for the identification of *Xylella fastidiosa* in plant samples, by using serological (ELISA – enzyme-linked immunosorbent assay) and molecular assays (PCR, qPCR). This PT was organized in accordance with the EPPO 7/122 and ISO/IEC 17043 guidelines, and the performance of the participating laboratories was determined based on the accuracy of the results obtained on a panel of blind plant samples. More specifically, the accuracy was determined as the closeness of agreement between a test result and the accepted reference value and it included both trueness and precision. The trueness was evaluated through the capacity to obtain positive results from positive samples ("**sensitivity**") and negative results from negative samples ("**specificity**"); whereas, the precision was

evaluated through the capacity to obtain the same qualitative result from identical samples (replicates) analyzed under conditions of "**repeatability**".

The diagnostic methods used in this PT included the majority of the protocols described in the EPPO diagnostic protocol 7/24 (2).

Besides its main scope, this PT gave also the following opportunities:

- To perform a test performance studies (TPS) for the molecular protocols reported in the EPPO diagnostic protocol 7/24 (2), using the data recovered from the laboratories that were scored as "proficient" (i.e. assessed as "conform and satisfactory laboratories"). The low number of laboratories performing the ELISA tests did not allow a sufficient and suitable dataset for a TPS on the serological assays.
- An educational training for those laboratories that had never approached the detection of *X. fastidiosa* using some of the protocols tested in this PT.

1.3 Organizers

This PT was conceived and promoted by the Institute for Sustainable Plant Protection, CNR, Bari (Italy) and Department of Soil, Plant and Food Science of the University of Bari (Italy), and implemented in collaboration with Anses - Plant health laboratory, UBVO, Angers and RAPT, Saint Pierre de La Réunion, France.

1.4 Participating Laboratories

The laboratories participating to the PT are listed below; each laboratory was identified by an anonymous alphanumeric code to ensure results confidentiality.

INSTITUTION	COUNTRY
UBT - Agricultural University of Tirana	ALBANIA
AGES - Austrian Agency for Health and Food Safety	AUSTRIA
BFW - Federal Research and Training Centre	AUSTRIA
ILVO - Plant health Institute of Agricultural, Fisheries and Food research	BELGIUM
BFSA - Bulgarian Food Safety Agency	BULGARIA
HCPHS - Croatian Centre for Agriculture, Food and Rural Affairs	CROATIA
CISTA - Central Institute for Supervising and Testing in Agriculture	CZECH REPUBLIC
Anses - Laboratoire de la santé des végétaux	FRANCE
INRA	FRANCE
JKI - Julius Kuehn Institute	GERMANY
LOEWE - Loewe Biochemica GmbH	GERMANY
BPI - Benaki Phytopathological Institute	GREECE
Genlogs - Genlogs Biodiagnostics Ltd	HUNGARY
NEBIH - National Food Chain Safety Office	HUNGARY

INSTITUTION	COUNTRY
DAFM- Department of Agriculture Food and the Marine	Ireland
AGRITEST SRL	ITALY
CIHEAM-IAMB Istituto Agronomico Mediterraneo	ITALY
CREA-PAV - Consiglio per la ricerca e la sperimentazione in agricoltura, Centro di ricerca per la Patologia Vegetale	ITALY
CRSFA - Centro di Ricerca, Sperimentazione e Formazione in Agricoltura	ITALY
Di3A - Dipartimento di Agricoltura, Alimentazione e Ambiente, Università degli Studi di Catania	ITALY
Osservatorio per le malattie delle piante di Acireale – Regione Sicilia	ITALY
SAFE - Dipartimento di Scienze Agrarie, degli Alimenti e dell'Ambiente	ITALY
SELGE- Institute for Sustainable Plant Protection, CNR and Department of Soil, Plant and Food Science, University of Bari	ITALY
Unisalento - Dipartimento di Scienze e Tecnologie	ITALY
PIORIN - Main Inspectorate of Plant Health and Seed Inspection	POLAND
RIH - Research Institute of Horticulture	POLAND
INIAV - Instituto Nacional de Investigação Agrária e Veterinaria	PORTUGAL

INSTITUTION	COUNTRY
UB - University of Belgrade, Faculty of Agriculture, Laboratory for Phytobacteriology	SERBIA
NIB - National Institute of Biology	SLOVENIA
CSIC - Institute for Sustainable Agriculture	SPAIN
IVIA - Instituto Valenciano de Investigaciones Agrarias	SPAIN
LOSVIB - Laboratorio Oficial de Sanidad Vegetal de las Islas Baleares	SPAIN

INSTITUTION	COUNTRY
Fera	UK
Forest Research	UK
SASA - Science and Advice for Scottish Agriculture	UK

1.5 Timeline of the Proficiency Test

The panel of samples, including those used for the homogeneity and stability, were prepared during the week of 13-17 February 2017 and kept at -20°C prior to be shipped. The shipment was organized for the majority of the laboratories on February 20, 2017. With the exception of two laboratories for which the shipment was made on the 22nd and 24th of February, 2017.

The **homogeneity** tests for all diagnostic methods were performed between February 13 and 15, soon after preparing the different batches of artificially spiked samples.

The **stability** tests were performed, for the molecular tests, during the week of 10-14 April 2017, and for the ELISA tests on April 27, 2017.

Participants were requested to perform the selected diagnostic tests and send the results to the Organizers, by March 27, 2017.

With the results provided by all the participant laboratories, a preliminary report was drafted on May 5, 2017, and shared with the member of the EPPO Panel on Diagnostic in Bacteriology, with the aim of providing useful information for the revision of the diagnostic protocol EPPO protocol during the Panel meeting held at the end of May, 2017.

All the raw data (qualitative results: positive, negative, undetermined) received from the different participating laboratories were collected in separate excel files and send to all participants, with the corresponding decrypted sample codes.

This final report includes the suggestions raised during the discussion of the EPPO Panel on Diagnostic in Bacteriology, and it was delivered by the end of July 2017.

2. PANEL OF EXPERIMENTAL SAMPLES

The movement and manipulation of *Xylella*-infected plant materials must comply with the requirements of the EU Directive 2000/29, and only authorized laboratories are allowed to manipulate and process infectious materials. To overcome this limitation and to avoid any phytosanitary risk, the panel of experimental blind samples consisted of plant sap spiked with heat- inactivated bacterial suspensions of *Xylella fastidiosa* subsp *pauca* strain De Donno (CFBP 8402). More specifically, the samples consisted of crude sap prepared from olive leaf petioles (collected from *Xylella*-free certified plants) macerated using the specific extraction buffers according to the protocols under evaluation. The panel of samples included randomized *Xylella*-free preparations and samples spiked with heat-inactivated (incubation at 70°C for 15min) bacterial suspensions at three different concentrations (10^6, 10^5 and 10^4 cells/ml). Bacterial suspensions were prepared by scraping 10-days old colonies of *X. fastidiosa* subsp. *pauca* strain De Donno (CFBP 8402).

This type of artificially contaminated samples, thus different from the naturally infected plant sample, may have reduced the yield of bacterial target DNA using some extraction methods, especially those that include an initial precipitation of the bacterial cells (i.e. the Quickpick (Bionobile) or the DNeasy plant mini kit (Qiagen). Thus, the results obtained may slightly differ from those that could be achieved using fresh infected samples.

The panel of samples tested using the molecular diagnostic methods included:

Samples	Assigned Value	Used for laboratory performance assessment
3 replicates of Xylella-free olive sap (non-infected)	negative	X
3 replicates containing 10^6 cells/ml of *X. fastidiosa*	positive	X
3 replicates containing 10^5 cells/ml of *X. fastidiosa*	positive	X
3 replicates containing 10^4 cells/m of *X. fastidiosa*	positive	X
1 lure sample	Positive/negative randomly chosen	
1 tube of Positive Amplification Control (PAC) for qPCR and PCR assays consisting in purified bacterial DNA.	positive	

The panel of samples tested by ELISA included:

Samples	Assigned Value*	Used for laboratory performance assessment
3 replicates of Xylella-free olive sap (non-infected)	negative	X
3 replicates containing 5x10^6 cells/ml of *X. fastidiosa*	positive	X
3 replicates containing 5x10^5 cells/ml of *X. fastidiosa*	positive	X
3 replicates containing 5x10^4 cells/m of *X. fastidiosa*	positive	X
1 lure sample	Positive/negative randomly chosen	

For each laboratory, these panel of samples was used to assess the following performance criteria:

- **sensitivity**: based on the results obtained for the 9 Xf-contaminated samples;
- **specificity**: based on the results obtained for the 3 Xf-free samples;
- **repeatability**: based on the results obtained on the 3 replicates for each Xf-contaminated and each Xf-free sample;
- **accuracy**: determined based on the results of three aforementioned performance criteria.

The inclusion of a **lure** sample ensured that each laboratory received a variable number of Xf-contaminated and Xf-free samples, avoiding risks of result comparison among the laboratories. A positive amplification control (PAC) for molecular test was also provided. The results of the lure and PAC were not used to assess the performance criteria.

Samples consisted of frozen olive sap aliquots, shipped in microcentrifuge safe lock tubes (2 ml), of 0.5-1.2ml. All laboratories received the samples at cold temperature, and none reported any sample degradation or apparent alteration. Only in one laboratory, it was reported that the volume of one sample was lower than expected.

3. DIAGNOSTIC PROCEDURES PERFORMED

The protocols selected by the participant laboratories included molecular and serological tests (Tables 1-3), with molecular tests being used in the majority of the laboratories.

Molecular tests consisted of quantitative real time PCR (qPCR) assay based on the primers and the TaqMan probe developed by Harper et al. (2010), whereas conventional PCR was based on the primers reported by Minsavage et al. (1994).

Each laboratory performed the tests using their own instruments, amplification conditions and reagents (master mix, reaction volumes, primers/probe concentrations) (Table 1-2). Whereas, the preparation of the DNA extracts was carried out according to the protocol(s) routinely adopted by each laboratory and/or listed in the EPPO diagnostic protocol 7/24 (2). To this end, the Organizers provided reference protocols, in order to ensure that the plant sap provided would be properly processed. In the case of PT, the ISO/IEC 17043 standards reports that participants shall normally be expected to use the test method, calibration or measurement procedure of their choice, which should be consistent with their routine procedures; however, as in the case of this PT, there is also the possibility that the Organizer may instruct participants to use a specified method in accordance with the planned scheme of the proficiency test.

In Table 3 are reported the experimental conditions used in the different laboratories for carrying out the ELISA tests.

Table 1. Laboratories performing real-time PCR (qPCR) (Harper et al., 2010). Specifications are provided on the selected method(s) to extract the DNA, on the qPCR master mixes and instruments used.

Laboratory	Extraction protocol/kit	Mastermix Real-time PCR/ reaction volume	Thermocycler
L01	CTAB DNeasy® mericon™ Food-Qiagen QuickPick™ Plant DNA Bio-Nobile + magnet pipet	PerfeCTa FastMix II (Quantabio)/ 10µl	Eppendorf Realplex 4 Mastercycler S
L03	QuickPick™ Plant DNA Bio-Nobile + KingFisher™ isolation robot	Applied Biosystems TaqMan® Fast universal master mix/ 20µl	CFX96 BioRad
L04	CTAB DNeasy® mericon™Food-Qiagen DNeasy® Plant Mini kit -Qiagen	Applied Biosystems, TaqMan® Universal Master Mix II, no UNG, + MgCl2 added at a final concentration of 1,5 mM/ 20µl	Bioneer, Exicycler™ 96
L09	CTAB DNeasy® mericon™ Food-Qiagen	Taqman fast advanced master mix Applied Biosystems/ 20µl	Bio-Rad CF-X96

Laboratory	Extraction protocol/kit	Mastermix Real-time PCR/ reaction volume	Thermocycler
L10	CTAB DNeasy® mericon™ Food-Qiagen	BIORAD SsoAdvanced™ Universal Probes Supermix, no BSA/ 20µl	CFX96 Touch™ Real-Time PCR Detection System
L11	CTAB DNeasy® mericon™ Food-Qiagen QuickPick™ Plant DNA Bio-Nobile + magnet pipet	5x HOT FIREPol® Probe Universal qPCR Mix (with/without uracil-DNA-glycosylase and dUTP/ 20 µl 2x FastStart Essential DNA Probes Master Roche (with/without uracil-DNA-glycosylase and dUTP) / 20µl	LIGHTCYCLER 480 I
L12	CTAB, DNeasy® mericon™ Food-Qiagen	Quantinova PROBE PCR KIT, Qiagen (activation at 95°C 5 min), no BSA/ 15 µl	Biorad iQ Cycler
L13	CTAB, DNeasy® mericon™ Food-Qiagen QuickPick™ Plant DNA Bio-Nobile + KingFisher™ isolation robot	TaqMan Fast Universal PCR Master Mix, Applied Biosystems/ 20µl	Applied Biosystems 7900HT
L14	QuickPick™ Plant DNA Bio-Nobile + magnet rack	TaqMan Fast universal PCR MasterMix (Applied Biosystems)/ 20µl	Roche Ligthcycler 480
L16	CTAB	TaqMan Universal PCR Master Mix (AB) , no BSA/ 20µl	Stratagene Mx3005P
L17	CTAB	Promega Go Taq OCR Master mix 2x/20 µl	BioRad CFX96 qPCR
L18	CTAB, DNeasy® mericon™ Food-Qiagen DNeasy® Plant Mini kit-Qiagen	Diagenod/ 20µl	iCycler BioRad
L19	CTAB DNeasy® mericon™ Food - Qiagen QuickPick™ Plant DNA Bio-Nobile + magnet pipet	GoTaq Probe qPCR Master mix – Promega and ROX reference Dye - Invitrogen/ 20µl	Applied Biosystems StepOnePlus
L20	CTAB DNeasy® mericon™ Food – Qiagen QuickPick™ Plant DNA Bio-Nobile + magnet pipet	TaqMan® Universal PCR Master Mix Applied Biosystem/ 20µl	StepOnePlus Applied Biosystems
L21	CTAB, DNeasy® mericon™ Food-Qiagen	Solis Biodyne, CyGreen (unspecific dsDNA dye) 5xMasterMix, 63.5°C as annealing/elongation T, 0.125µM final Primer concentration, no probe/ 20µl	ABI QuantStudio 3
L22	DNeasy® mericon™ Food-Qiagen DNeasy® Plant Mini kit-Qiagen	TaKaRa/ 10µl	ECO ILLUMINA
L23	CTAB	Bioline SensiFast Probe No-Rox /20µl	Corbett Research RG-3000

Laboratory	Extraction protocol/kit	Mastermix Real-time PCR/ reaction volume	Thermocycler
	DNeasy® mericon™ Food- Qiagen		
L24	QuickPick™ Plant DNA Bio-Nobile + KingFisher™ isolation robot	TaqMan™ Fast Universal PCR Master Mix (Applied Biosystems)/ 20µl	ViiA 7 (Applied Biosystems)
L26	CTAB, DNeasy® mericon™ Food- Qiagen	Applied Biosystems/ 20µl	Roche LC-480
L27	CTAB	Applied Biosystems, no BSA. When BSA was added according to protocol, all samples including PAC were negative/ 20µl	Bio-Rad CFX96
L28	CTAB DNeasy® mericon™ Food- Qiagen	SSo Universal probe, Biorad/20µl	Biorad, CFX96
L29	CTAB DNeasy® mericon™ Food- Qiagen QuickPick™ Plant DNA Bio-Nobile + BioSprint15-Qiagen	Sigma ReadyMix for Quantative PCR/ 20µl	Applied Biosystems 7900
L30	CTAB, DNeasy® mericon™ Food- Qiagen DNeasy® Plant Mini kit- Qiagen QuickPick™ Plant DNA Bio-Nobile + magnet pipet	Taqman fast advanced master mix (Applied Biosystems) /20µl	CFX 96 Bio Rad
L33	CTAB	TaqMan® Environmental Master Mix 2.0 (Applied Biosystems), final concentration of primers 0.4 µM, of probe 0.2 µM /25µl	Applied Biosystems 7900 HT Fast Real-time PCR System
L34	QuickPick™ Plant DNA Bio-Nobile + magnet rack	TaqMan fast universal PCR master mix (Applied Biosystems), BSA (10µg/µl) 0.6µl/ 20µl	Biorad
L35	QuickPick™ Plant DNA Bio-Nobile + magnet pipet	Applied Biosystems	Applied Biosystems

Table 2. Laboratories performing PCR (Minsavage et al. 1994). Specifications are provided on the selected method(s) to extract the DNA, on the PCR master mixes and instruments used.

Laboratory	Extraction protocol/kit	PCR mix/ reaction volume	Thermocycler
L01	CTAB DNeasy® mericon™ Food-Qiagen QuickPick™ Plant DNA Bio-Nobile + magnet pipet	5x FIREPol® Master Mix Ready to Load, 7.5 mM MgCl2 (Solis Biodyne)/ 10µl	Biometra T3000
L03	QuickPick™ Plant DNA Bio-Nobile + KingFisher™ isolation robot	Platinum®Taq DNA Polymerase, Invitrogen/ 25µl	9700 Applied Biosystems
L04	CTAB DNeasy® mericon™Food-Qiagen DNeasy® Plant Mini kit - Qiagen	Platinum®Taq DNA Polymerase, Invitrogen/ 25µl	GeneAmp® PCR System 9700, Applied Biosystems
L05	CTAB	AccuStart II PCR Tough Mix, Quantabio/ 50µl	96 Universal Gradient, peqSTAR
L06	CTAB DNeasy® mericon™Food-Qiagen DNeasy® Plant Mini kit - Qiagen	Platinum®Taq DNA Polymerase, Invitrogen/ 25µl	Veriti Thermal Cycler of Applied Biosystems
L07	CTAB DNeasy® mericon™ Food-Qiagen	Promega/ 25µl	Biorad T100
L08	CTAB DNeasy® mericon™ Food-Qiagen	Go Taq G2 Flexi polymerase – Promega/ 12.5µl	XP cycler / Bioer
L09	CTAB DNeasy® mericon™ Food-Qiagen	AmpliTAQ Gold 360 Master mix (Applied Biosystems)/ 25µl	Biorad T100
L10	CTAB DNeasy® mericon™ Food-Qiagen	GoTaq® G2 DNA Polymerase-Promega/ 25µl	Biorad T100
L11	CTAB DNeasy® mericon™ Food-Qiagen QuickPick™ Plant DNA Bio-Nobile + magnet pipet	BIOLINE-MY TAQ / 20µl	Biorad T100
L12	CTAB, DNeasy® mericon™ Food-Qiagen	GoTaq Flexi DNA polymerase-Promega/ 25µl	mastercycler ep gradient/eppendorf
L13	CTAB, DNeasy® mericon™ Food-Qiagen QuickPick™ Plant DNA Bio-Nobile + KingFisher™ isolation robot	Platinum®Taq DNA Polymerase, Invitrogen/ 25µl	Applied Biosystems 2720

L15	CTAB	Platinum®Taq DNA Polymerase, Invitrogen/ 25µl	Kyratec SuperCycler Thermo Cycler SC 200
L16	CTAB	Promega 5X PCR GoTaq Flexi buffer and GoTaq G2 Colorless Master Mix/ 25µl	2720 Thermal Cycler (Applied Biosystems)
L17	CTAB	FastStart Taq DNA Polymerase, Roche/ 25µl	Sensoquest Labcycler
L18	CTAB DNeasy® mericon™ Food- Qiagen DNeasy® Plant Mini kit- Qiagen	GoTaq G2 Flexi, Promega / 25µl	Biometra - T Professional Basic, Thermocycler Gradient
L19	CTAB DNeasy® mericon™ Food - Qiagen QuickPick™ Plant DNA Bio-Nobile + magnet pipet	QUANTIMIX EASY kit - BIOTOOLS/ 50µl	Gene Amp PCR System 9700 Applied Biosystems
L20	CTAB DNeasy® mericon™ Food – Qiagen QuickPick™ Plant DNA Bio-Nobile + magnet pipet	BioLine Bio-X-Act Short Mix / 25µl	Eppendorf
L21	CTAB, DNeasy® mericon™ Food- Qiagen	Solis Biodyne Hot Fite Polymerase/ 25µl	Eppendorf Mastercycler
L22	DNeasy® mericon™ Food- Qiagen DNeasy® Plant Mini kit-Qiagen	CMB (CULTEK MOLECULAR BIOLINE)/ 25µl	TECHGENE-FTGENE2D
L23	CTAB DNeasy® mericon™ Food- Qiagen	Bioline ImmoMix/20µl	Corbett Research RG-3000
L26	CTAB, DNeasy® mericon™ Food- Qiagen	Invitrogen/ 25µl	Eppendorf
L27	CTAB	Dream Taq Green DNA Polymerase (Thermo Scientific)/ 25µl	Biometra T3000 thermocycler (Biometra)
L28	CTAB DNeasy® mericon™ Food- Qiagen	Dominion MBL, Cordoba Spain/ 25µl	Biorad, S1000 Thermocycler
L29	CTAB DNeasy® mericon™ Food- Qiagen QuickPick™ Plant DNA Bio-Nobile + BioSprint15-Qiagen	2X GoTaq Green Master Mix- Promega / 25µl	SimpliAmp Thermal Cycle/ Applied BioSystems/ThermoFIsher
L30	CTAB, DNeasy® mericon™ Food- Qiagen DNeasy® Plant Mini kit-Qiagen QuickPick™ Plant DNA Bio-Nobile + magnet pipet	DreamTaq Green PCR Master Mix (2X) -Termo scientific / 25µl	Bio Rad myCycler™

L31	DNeasy® mericon™ Food-Qiagen DNeasy® Plant Mini kit-Qiagen	Thermo Scientific/ 25µl	Applied Biosystems (Thermal Cycle 2720)
L32	CTAB DNeasy® mericon™ Food-Qiagen	DreamTaq Green PCR Master Mix (2X) -Termo scientific / 25µl	Applied Biosystems
L34	QuickPick™ Plant DNA Bio-Nobile + magnet rack	Platinum®Taq DNA Polymerase, Invitrogen/ 25µl	Applied Biosystems

Table 3. Laboratories performing ELISA tests. Specifications are provided for the kit, the type of materials and plate reader.

Laboratory	Kit ELISA	ELISA plate/ volume loaded	Microplate reader
L02	Agritest	Nunc plates/200µl	Bio-Rad 680
L03	Agritest Loewe	Nunc MaxiSorp plates/ 200 µl	MultiSkan GO
L06	Agritest Loewe	Nunc plates/200µl	iMark Microplate Absorbance Reader (BIORAD)
L07	Agritest Loewe	Costar plates/ 100µl	Multiskan Ex
L10	Agritest Loewe	Costar plates/ 100µl	MULTISKAN FC- THERMO SCIENTIFIC
L19	Loewe	NUNC POLYSORP plates/200µl	Multiskan Ascent
L21	Loewe	NUNC Immunoplates Maxisorp F Boden 400 ml/200µl	Tecan Magellan
L22	Agritest Loewe	Nunc plates/200µl and BIOREBA commercial buffers (coating, conjugate and substrate b.)	MULTISKAN EX (THERMO ELECTRON CORPORATION)
L25	Agritest	Nunc plates/200µl	Microplate reader Biorad mod. 680
L28	Agritest	Costar plates/ 100µl	Multiskan EX Thermo Scientific
L30	Agritest Loewe	NUNC Maxi Sorp plates/200µl	TECAN
L32	Agritest Loewe	Costar plates	HumaReader HS
L33	Agritest	Thermofisher nunc - 200µl	Perkin elmer 2030 victor x5

4. METHODOLOGY FOR DATA ANALYSIS

4.1 Analysis of the results

Results were primarily analyzed based on the qualitative data received by the laboratories. In each laboratory the samples were assigned as negative, positive or undetermined, according to the interpretation criteria specific for each diagnostic method, i.e. quantitative cycle (Cq) value; OD405 values; presence/absence of the PCR amplification product.

The Organizers after decrypting the sample codes, for each laboratory assessed the number of positive agreements (PA), negative agreements (NA), positives deviations (PD) and negatives deviations (ND) according to the parameters described in Table 4. The values obtained were then used to calculate the different performance criteria (Table 5), and from these values the proficiency of each laboratory was assessed.

Beside the qualitative results, quantitative data were also recorded for qPCR assays (quantitation cycles, Cq) and for the ELISA tests (Absorbance OD_{405} values).

The proficiency was expressed as percentage, with 100% being the highest performance level (see Chabirand et al., 2014 for more detailed information).

Table 4. Definition of the parameters adapted from ISO 16140

Laboratory Results	Assigned value	
	Positive	**Negative**
Positive	PA= positive agreement	PD= positive deviation
Negative	ND= negative deviation	NA= negative agreement
Undetermined	ND= negative deviation	PD=positive deviation

Table 5. Details on the performance criteria

Performance criteria	**Definition**	**Calculation**
Accuracy (AC)	Closeness of agreement between the laboratory result and the assigned value	$AC= (N_{PA}+N_{NA})/N$
Sensitivity (SE)	Closeness of agreement between the laboratory result and the assigned value for samples for which the assigned value is positive	$SE= N_{PA}/N+$
Specificity (SP)	Closeness of agreement between the laboratory result and the assigned value	$SP=N_{NA}/N-$

Performance criteria	Definition	Calculation
	for samples for which the assigned value is negative	
Repeatibility (DA)	Closeness of agreement between independent test results obtained under conditions of repeatability, i.e. conditions under which independent test results are obtained by the same method, on identical test samples in the same laboratory, by the same operator, using the same equipment, within a short period of time	DA denotes the percentage chance of obtaining the same result (positive, negative or indeterminate) from two identical samples analyzed in the same laboratory

4.2 Categorization of the laboratories based on their performance

With regard to the different methods tested, using the values recovered for the "accuracy" the laboratories were categorized as:

(i) **"highly proficient"** when the level of accuracy corresponded to the highest value (i.e. 100%); this was the case of the laboratories in which all the samples produced the expected positive and negative results, without any positive deviation (false positive) or negative deviation (false negative). In addition, the three replicates of each sample produced identical results.

(ii) **"proficient"** when the level of accuracy was in the range of 90-100%. This category included the laboratories that obtained either one positive deviation or one negative deviation.

(iii) **"non-proficient"** when the level of accuracy was lower than 90%. This category included the laboratories that obtained more than one positive or negative deviation.

The declaration of conformity was then assigned only to the laboratories categorized as "highly proficient" and "proficient". The results obtained by the laboratories included in these two categories were also used for conducting a test performance study for the molecular methods (see paragraph 7).

5. RESULTS

The results recovered in each laboratory are available at the following link https://www.ponteproject.eu/wp-content/uploads/2017/07/EU-XF-PT-2017-02-results.zip

5.1 Homogeneity and stability

The homogeneity and the stability were assessed for all the diagnostic methods included in the PT, and were performed on three replicates for each artificially contaminated sample and three replicates of the *Xylella*-free sample. The data presented in the following 2 paragraphs refers only to the qPCR and ELISA tests. However, homogeneity and stability was also assessed for the samples tested by conventional PCR, whose results confirmed that the samples were sufficiently homogeneous and stable.

5.1.1 Homogeneity

As expected, the Cq values recovered in qPCR tests, and the OD_{405} values obtained in ELISA, showed a positive correlation between the bacterial concentrations and the resultant diagnostic values.

For qPCR, the values of the standard deviation (SD) and ΔCq (Table 6), confirm they were in the acceptable range of variation. Based on the calculation of the homogeneity, the samples are all considered to be sufficiently homogenous (Table 7 and 8).

Similarly, the analysis of the quantitative and qualitative results recovered for the ELISA tests showed that all samples were sufficiently homogeneous (Table 9).

5.1.2 Stability

Stability tests were conducted once all laboratories had completed their tests. For the qPCR, differences between the mean Cq obtained during the proficiency test by all laboratories, the stability and the homogeneity tests are reported in Table 6. Based on the overall results of the homogeneity and stability tests the samples were sufficiently homogeneous and stable and thus suitable to evaluate the laboratories' performance.

Positive results (i.e. samples sufficiently stable) were also obtained from the ELISA tests (Table 9).

Based on the analysis of the quantitative (Cq values) and qualitative (positive/negative) results the samples can be considered stable (Table 7-8). However, when qPCR was performed on the CTAB-extracts, the resultant Cq were surprisingly lower than those obtained during the homogeneity test.

This could be explained with the lower concentration of plant PCR-inhibitors that may have been precipitated and removed during the freezing/thawing steps.

Table 6. Comparative analysis of the quantitative cycle (Cq) obtained for each extraction method in qPCR assays. Values include the average of the overall Cq obtained by the participating laboratories and of the Cq values recovered for the homogeneity and stability tests. SD indicates the standard deviation among the samples considered in each tests.

Samples	10^6 cells/ml	10^5 cells/ml	10^4 cells/ml
CTAB			
Average all laboratories	20,73	24,03	27,35
SD	1,87	1,99	1,85
Homogeneity	21,47	24,50	26,51
SD	0,48	0,26	0,44
Stability	19,50	21,89	25,93
SD	1,22	0,97	1,68
Mericon Food kit, Qiagen			
Average all laboratories	22,04	25,58	28,72
SD	1,86	1,88	1,96
Homogeneity	22,95	26,13	29,66
SD	0,25	0,26	0,17
Stability	22,05	25,43	28,69
SD	0,57	0,36	0,37
QuickPick kit, Bionobile			
Average all laboratories	25,31	28,42	31,51
SD	2,12	1,45	1,47
Homogeneity	25,28	28,60	31,68
SD	0,34	0,31	0,61
Stability	24,98	28,11	31,96
SD	0,78	0,52	0,79
Dneasy plant mini kit (Qiagen)			
Average all laboratories	26,03	29,95	32,55
SD	2,42	2,31	2,41
Homogeneity	25,89	29,61	33,16
SD	0,12	0,31	0,22
Stability	24,96	28,58	31,57
SD	0,25	0,35	0,42

Table 7. Results of the homogeneity tests for the real time PCR assays performed on the samples prepared using four different extraction methods. Values reported in the table represent the quantitative cycle (Cq). The conformity (yes/no) of the quantitative and qualitative homogeneity is reported for each set of replicates.

		CTAB			Mericon Food kit (Qiagen)		
		10^6 cells/ml	**10^5 cells/ml**	**10^4cells/ml**	**10^6 cells/ml**	**10^5 cells/ml**	**10^4cells/ml**
Replicate 1	repetition 1	21.96	24.85	25.82	22.72	26.34	29.79
Replicate 2		21.01	24.26	26.98	22.68	26.4	29.85
Replicate 3		21.45	24.33	26.23	22.85	25.93	29.53
Replicate 1	repetition 2	20.86	24.57	26.44	23.06	26.3	29.72
Replicate 2		22.03	24.26	26.94	23.35	25.75	29.4
Replicate 3		21.53	24.75	26.65	23.04	26.03	29.64
Quantitative homogeneity		yes	yes	yes	yes	yes	yes
Qualitative homogeneity		yes	yes	yes	yes	yes	yes
		QuickPick			Dneasy plant mini kit (Qiagen)		
		10^6 cells/ml	**10^5 cells/ml**	**10^4cells/ml**	**10^6 cells/ml**	**10^5 cells/ml**	**10^4cells/ml**
Replicate 1	repetition 1	25.57	28.74	31.22	25.79	29.7	32.88
Replicate 2		25.11	28.2	32.42	26.06	29.67	33.32
Replicate 3		25.45	28.9	31.84	25.92	29.32	32.97
Replicate 1	repetition 2	25.39	28.84	31.07	25.78	30.02	33.06
Replicate 2		24.65	28.22	32.36	25.8	29.17	33.47
Replicate 3		25.48	28.68	31.19	25.98	29.75	33.23
Quantitative homogeneity		yes	yes	yes	yes	yes	yes
Qualitative homogeneity		yes	yes	yes	yes	yes	yes

Table 8. Results of the stability tests for the real time PCR assays performed on the samples prepared using the 4 different extraction methods. Values reported in the table represents the quantitative cycle (Cq). The conformity (yes/no) of the quantitative and qualitative stability is reported for each set of replicates.

		CTAB			Mericon Food kit (Qiagen)		
		10^6 cells/ml	**10^5 cells/ml**	**10^4cells/ml**	**10^6 cells/ml**	**10^5 cells/ml**	**10^4cells/ml**
Replicate 1	repetition 1	17.93	23.24	24.14	22.1	25.62	28.65
Replicate 2		18.46	20.98	24.47	22.39	25.82	28.98
Replicate 3		19.72	21.42	27.93	21.41	24.96	28.65
Replicate 1	repetition 2	19.55	21.47	25.34	22.63	25.35	29.12
Replicate 2		21.41	22.98	25.7	22.47	25.76	28.7
Replicate 3		19.95	21.24	28.02	21.27	25.07	28.05
Quantitative stability		no	no	yes	yes	yes	yes
Qualitative stability		yes	yes	yes	yes	yes	yes
		QuickPick			Dneasy plant mini kit (Qiagen)		
		10^6 cells/ml	**10^5 cells/ml**	**10^4cells/ml**	**10^6 cells/ml**	**10^5 cells/ml**	**10^4cells/ml**
Replicate 1	repetition 1	24.38	28.44	31.37	25.02	28.2	31.08
Replicate 2		24.97	28.69	33.01	25.27	28.7	32.09
Replicate 3		25.66	27.44	31.99	24.65	28.46	32.04
Replicate 1	repetition 2	24.06	28.1	30.87	25.1	29.2	31.47
Replicate 2		24.67	28.45	31.89	25.07	28.6	31.18
Replicate 3		26.11	27.53	32.63	24.67	28.34	31.55
Quantitative stability		yes	yes	yes	yes	yes	yes
Qualitative stability		yes	yes	yes	yes	yes	yes

Table 9. Results of the homogeneity and stability tests for the ELISA tests. OD_{405} values are indicated for each replicate.

HOMOGENEITY TEST									
		AGRITEST				LOEWE			
		5X 10^6 cells/ml	**5X 10^5 cells/ml**	**5X 10^4cells/ml**	**healthy**	**5X 10^6 cells/ml**	**5X 10^5 cells/ml**	**5X 10^4cells/ml**	**healthy**
Replicate 1	repetition 1	2,525	1,722	0,197	0,037	2,510	1,449	0,148	0,040
Replicate 2		2,522	1,830	0,170	0,037	2,555	1,443	0,174	0,037
Replicate 3		2,649	1,777	0,168	0,013	2,488	1,391	0,157	0,010
Replicate 1	repetition 2	2,491	1,745	0,197	0,040	2,471	1,345	0,141	0,033
Replicate 2		2,485	1,835	0,169	0,028	2,533	1,447	0,174	0,019
Replicate 3		2,524	1,903	0,160	0,017	2,476	1,411	0,153	0,011
Quantitative homogeneity		yes	yes	no	yes	yes	yes	no	yes
Qualitative homogeneity		yes	yes	yes	yes	yes	yes	yes	yes
STABILITY TEST									
		AGRITEST				LOEWE			
		5X 10^6 cells/ml	**5X 10^5 cells/ml**	**5X 10^4cells/ml**	**healthy**	**5X 10^6 cells/ml**	**5X 10^5 cells/ml**	**5X 10^4cells/ml**	**healthy**
Replicate 1	repetition 1	2,733	1,557	0,111	0,045	2,730	1,602	0,130	0,045
Replicate 2		2,762	1,415	0,105	0,040	2,720	1,450	0,101	0,037
Replicate 3		2,796	1,643	0,099	0,020	2,747	1,629	0,123	0,025
Replicate 1	repetition 2	2,728	1,552	0,108	0,047	2,740	1,603	0,127	0,042
Replicate 2		2,768	1,423	0,105	0,036	2,715	1,445	0,103	0,030
Replicate 3		2,805	1,637	0,104	0,023	2,735	1,637	0,128	0,021
Quantitative stability		no	no	no	yes	no	no	no	yes
Qualitative stability		yes	yes	yes	yes	yes	yes	yes	yes

5.2 Results of the molecular tests

5.2.1 Qualitative results

A total of 28 laboratories out 35, performed at least one molecular test, either conventional and/or real time PCR assays.

Below are reported the details about the number of laboratories testing one or more extraction procedure(s) and those performing PCR and/or qPCR.

Table 10. Details on the number of laboratories performing the different molecular protocols.

DNA Extraction Procedures	N. of Laboratories performing molecular tests	
	qPCR	PCR
CTAB	20	25
Dneasy Mericon Food kit (Qiagen)	17	22
Dneasy Plant mini kit (Qiagen)	4	6
Quick Pick (Bionobile)	12	9

5.2.1.1 Quantitative PCR assays

As shown in Figures 1-4, all laboratories that performed qPCR assays using the DNA extracts recovered from CTAB, Mericon food kit and DNeasy plant mini kit produced results with an accuracy of 100%. Thus, for the diagnosis of *X. fastidiosa* through qPCR for all these methods **all** the participating laboratories were "**highly proficient**" (Table 11).

With regard to the forth method used for DNA extraction, the Quickpick kit (Bionobile), 10 laboratories out of 12 reached an accuracy level of 100%, whereas in the remaining two laboratories the accuracy was 92% (L19) and 67% (L14), respectively. Specifically, L19 obtained one positive deviation, L14 obtained four negative deviations (false negative) for the three replicates containing 10^4 cells/ml and for one replicate containing 10^5 cells/ml. Based on these results, for the qPCR assays performed on the DNA extracts recovered with the Quickpick kit, 10 laboratories where categorized as "highly proficient", L19 was categorized as "proficient" and L14 was categorized as "non-proficient", i.e. non-conform to the PT (Table 11). It is important to note that for the Quickpick kit, the best performance is obtained using the available

automatized platform. The two laboratories that yielded the lowest accuracy values did not used such platforms but the extraction was performed manually, using a magnetic pipet.

In all laboratories, the positive amplification control (PAC) (provided by the Organizers) produced clear amplification curves with positive Cq values. Only in one laboratory, it was necessary to dilute the PAC 1:20 in order to get the proper amplification curve.

Table 11. Summary of the performance criteria recovered in the different laboratories for real time PCR (qPCR) (Harper et al., 2010) performed using DNA extracts prepared using four different methods of extraction. The table reports the number of samples with positive agreement (PA), negative agreement (NA), positive deviation (PD) and negative deviation (ND), and the resultant values for the different performance criteria.

Performance parameters and criteria	DNA extraction methods					
	CTAB	**MERICON Food kit**	**Quick pick**			**DNeasy plant minikit**
	Laboratories					
	L01,L04,L09,L10, L11,L12,L13,L16, L17,L18,L19,L20, L21,L23,L26,L27 L28,L29,L30,L33	**L01,L04,L09,L10, L11,L12,L13,L18, L19,L20,L21, L22,L23,L26, L28,L29,L30**	**L01,L03,L11,L13, L20,L24,L29,L30,L34,L35**	**L14**	**L19**	**L04, L18, L22, L30**
N. of PA	9	9	9	5	9	9
N. of NA	3	3	3	3	2	3
N. of ND	0	0	0	4	0	0
N. of PD	0	0	0	0	1	0
Sensitivity	100%	100%	100%	56%	100%	100%
Specificity	100%	100%	100%	100%	67%	100%
Repeatability	100%	100%	100%	89%	89%	100%
Accuracy	100%	100%	100%	67%	92%	100%
CATEGORY	**Highly proficient**	**Highly proficient**	**Highly proficient**	**Non-proficient**	**Proficient**	**Highly proficient**
Conformity	**YES**	**YES**	**YES**	**NO**	**YES**	**YES**

5.2.1.2 PCR assays

As shown in Figures 1-4, based on the PCR tests the values of the "accuracy" recovered in the majority of the laboratories corresponded to 100%, thus the majority of the laboratories were categorized as "highly proficient" for the PCR detection of *X. fastidiosa*. However, in some laboratories negative deviations (false negative) were obtained for the samples containing the lowest bacterial concentration (10^4 cells/ml). In fact, some of these samples did not produced any DNA amplicon (no band visualized after the electrophoresis) or only faint DNA bands visualized which determined problems unclear interpretation of the results (see Table 12). Indeed, two laboratories reported that the amplicons (DNA bands visualized after electrophoresis) yielded on the DNA extracts obtained using the DNeasy plant mini kit (Qiagen) were less intense than those obtained on the extracts prepared using the Mericon food kit or the CTAB procedure. In all laboratories, the positive amplification control (provided by the organizer) yielded the expected DNA amplicon. Only in one laboratory, it was reported that a dilution of 1:20 was necessary in order to get the proper amplification band.

With regard to the laboratories categorized as "proficient" or "non-proficient", hereafter are described the main deviations encountered in the results:

1) L32 failed to detect the bacterium in one replicate containing 10^4 cells/ml, after the extraction with CTAB. The laboratory indicated that the volume of the sap received for this sample was lower that the volume necessary to perform the extraction, this may have affected the result. The value of the accuracy for this test was 92%, thus the laboratory was categorized as "proficient" and conform to the PT.

2) L28 failed to correctly detect the bacterium in the three replicates containing 10^4 cells/ml regardless the method used for the DNA extraction (see Table 12). This condition may underline that the PCR conditions (i.e. reagents, master mix) used in this laboratory were not as efficient as those used in other laboratories. The accuracy calculated for this laboratory, on the base of the PCR results, was lower than 90%, thus the laboratory was categorized as "non-proficient" and not conformed to the PT.

3) The laboratories L01, L19, L30 and L34 failed to detect the bacterium by PCR in some replicates containing 10^5 and/or 10^4 cells/ml processed using the Quickpick kit (see Table 12), resulting in several negative deviations, which determined accuracy values lower than 100%. Thus, these laboratories were either "non-proficient" (i.e. L01, L19 and L34) or "proficient" (i.e. L30). A justification for the lower performance of these

laboratories may be found in the type of experimental sample provided in this PT (not optimal for this kit), or in the lack in these laboratories of an automated platform that negatively impacted the quality of the extracts (i.e. the perfect removal of the beads), or to the lack of experience of these laboratories in using this specific kit.

4) Three laboratories (L04, L06, L18) failed to detect the bacterium in the samples containing 10^4 cells/ml extracted using the DNeasy Plant Mini kit, which determined accuracy values lower than 90%. Thus, all these laboratories were categorized as not-proficient and not conformed for the detection of *X. fastidiosa* by using the DNeasy Plant Mini kit (see Table 12).

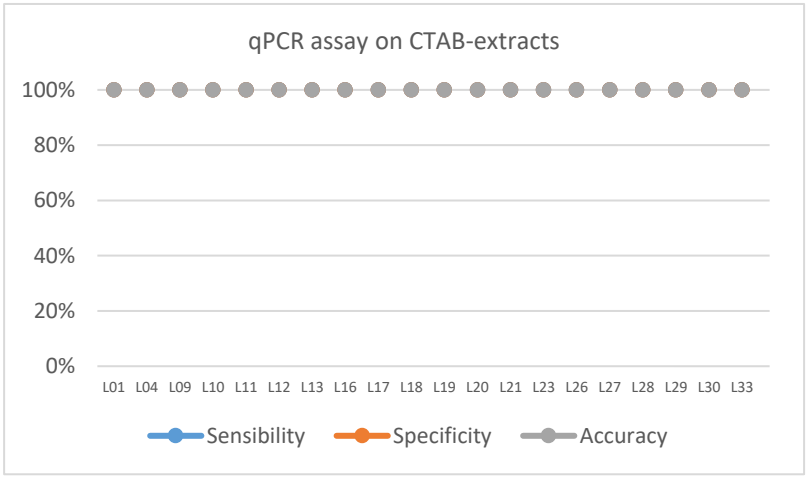
Table 12. Summary of the performance criteria recovered in the different laboratories for PCR (Minsavage et al., 1994) performed using the DNA extracts prepared using four different methods of extraction. The table reports the **n**umber of samples with positive agreement (PA), negative agreement (NA), positive deviation (PD) and negative deviation (ND), and the resultant values for the different performance criteria.

Performance parameters and criteria	DNA extraction methods													
	CTAB			MERICON Food kit		Quick pick					DNeasy plant minikit			
	Laboratories													
	L01,L04,L05,L06 L07,L08,L09,L10 L11,L12,L13,L15 L16,L17,L18,L19 L20,L21,L23,L26 L27,L29,L30,	L28	L32	L01,L04,L06,L07 L08,L09,L10 L11,L12,L13, L18,L19,L20, L21,L22,L23, L26,L29,L30, L31,L32	L28	L03,L11, L13,L20, L29	L01	L19	L30	L34	L22,L30, L31	L04	L06	L18
N. of PA	9	5	8	9	6	9	3	6	8	5	9	5	6	6
N. of NA	3	3	3	3	3	3	3	3	3	3	3	3	3	3
N. of ND	0	4	1	0	3	0	6	3	1	4	0	4	3	3
N. of PD	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sensitivity	100%	56%	89%	100%	67%	100%	33%	67%	89%	56%	100%	56%	67%	67%
Specificity	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Repeatability	100%	89%	89%	100%	100%	100%	100%	100%	89%	89%	100%	89%	100%	78%
Accuracy	**100%**	**67%**	**92%**	**100%**	**75%**	**100%**	**50%**	**75%**	**92%**	**67%**	**100%**	**67%**	**75%**	**75%**
Category	**Highly proficient**	**Non-Proficient**	**proficient**	**Highly proficient**	**Non-Proficient**	**Highly Proficient**	**Non-Proficient**	**Non-Proficient**	**Proficient**	**Non-Proficient**	**Highly Proficient**	**Non-Proficient**	**Non-Proficient**	**Non-Proficient**
Conformity	**YES**	**NO**	**YES**	**YES**	**NO**	**YES**	**NO**	**NO**	**YES**	**NO**	**YES**	**NO**	**NO**	**NO**

The following graphs show the values for the different performance criteria (sensibility, sensitivity and accuracy) obtained in each laboratory using the four different extraction methods, in relation to conventional and real time PCR assays.

Figure 1. Performance values recovered in each laboratories for qPCR (A) and PCR (B) tests performed on CTAB-extracts.

(A)



(B)

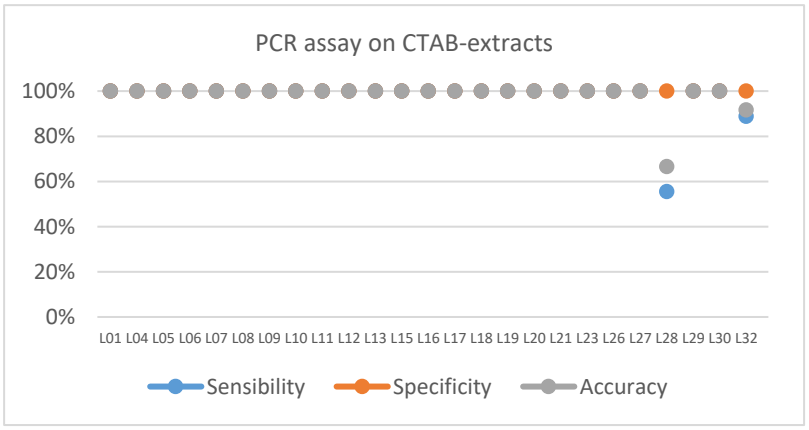
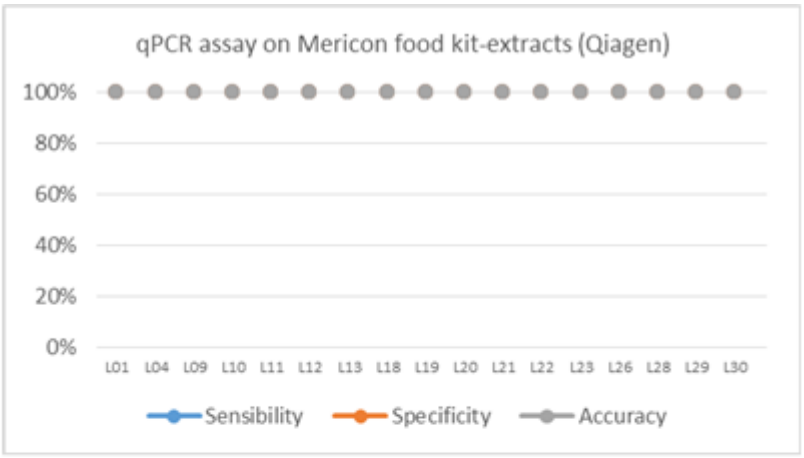


Figure 2. Performance values recovered in each laboratories for qPCR (A) and PCR (B) tests performed on extracts prepared using the Mericon food kit (Qiagen).

(A)



(B)

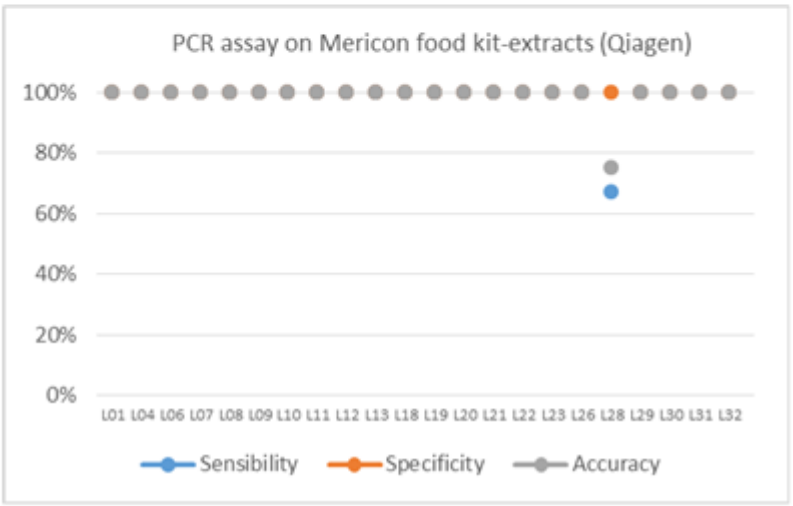
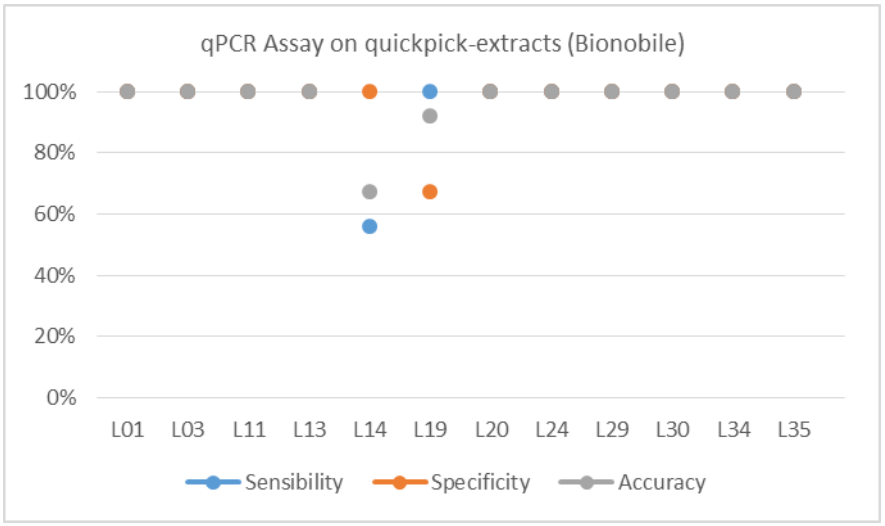


Figure 3. Performance values recovered in each laboratories for qPCR (A) and PCR (B) tests performed on extracts prepared using the QuickPick kit (Bionobile).

(A)



(B)

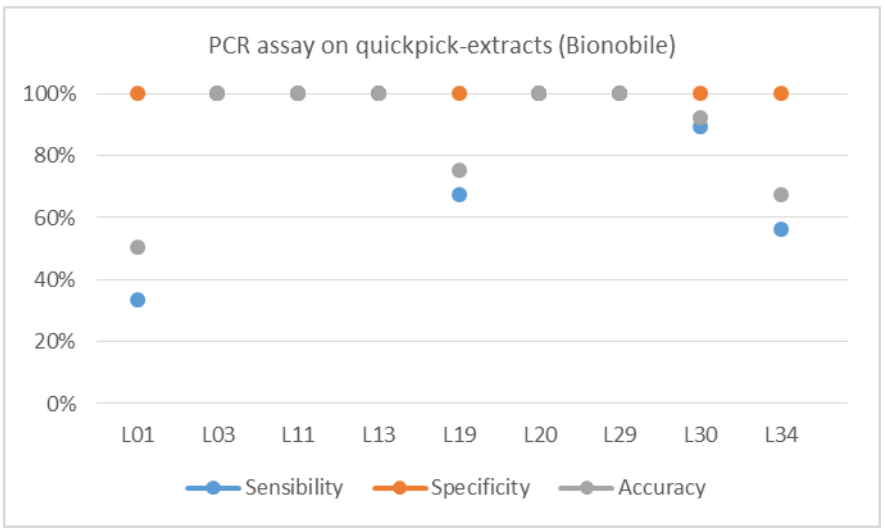
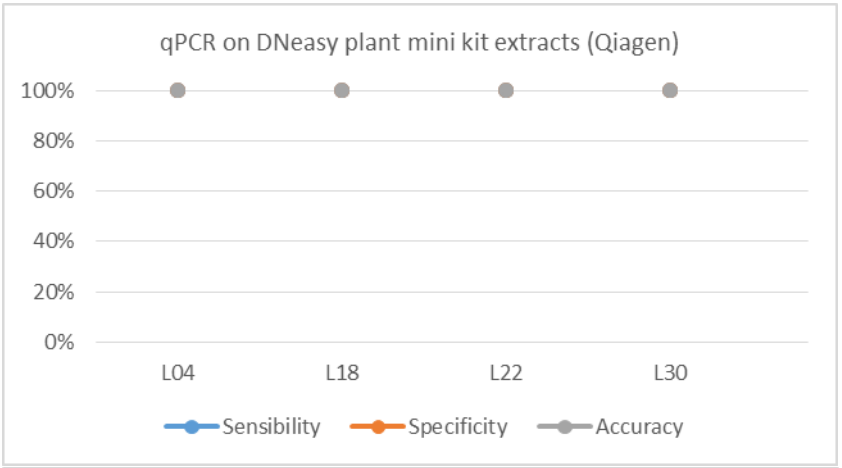
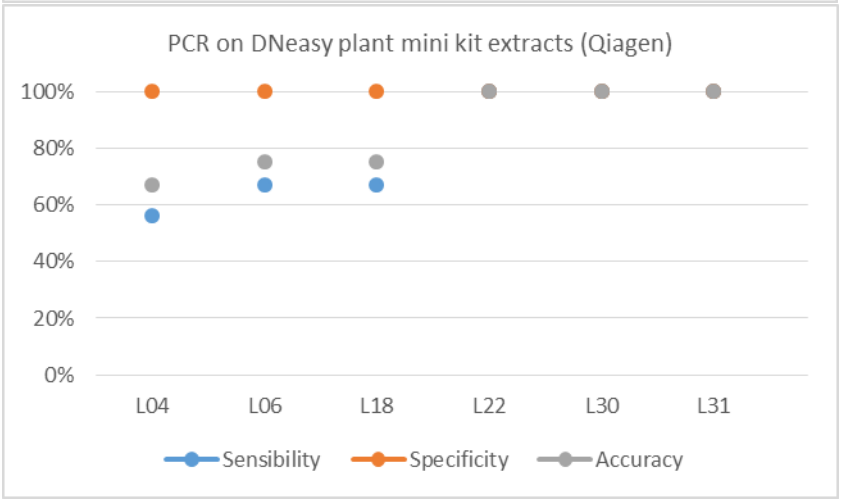


Figure 4. Performance values recovered in each laboratories for qPCR (A) and PCR (B) tests performed on extracts prepared using the DNeasy Plant mini Kit (Qiagen).

(A)



(B)



5.2.2 Quantitative results

This section includes the data on the values of the quantitation cycles (Cq) recovered in each laboratory for the qPCR assays performed on the DNA extracts recovered with four different extraction methods.

In figures 5-8 are graphically shown using boxplots, the Cq obtained in each laboratory for the different DNA extracts. As shown, the range of Cq values recovered for the *Xylella*-contaminated samples, varied in the different laboratories, as a consequence of the different reagents and master mixes used, and to the difference in the quantity/quality of the DNA extracts recovered using the four extraction protocols. In some laboratories, the Cq values were significantly above the median of the overall Cq values, regardless the type of DNA extracts (i.e. L04), indicating that the qPCR master mix and amplification condition in this laboratory were less efficient than those used in the other laboratories.

Indeed, within each laboratory differences in Cq values were observed for the samples containing the same bacterial concentration, but processed through different DNA extractions protocols, indicating that the each extraction protocol yielded DNA extracts of different quantity and quality.

The Cq values obtained in the laboratory L27 were affected by the use of diluted DNA extracts, which determined higher Cq values than those obtained in the other laboratories.

Figure 5. Range of the values of the quantitation cycle (Cq) recovered in each laboratory on the DNA extracts prepared from the samples containing 10^6, 10^5 and 10^4 CFU/ml, using CTAB extraction procedure.

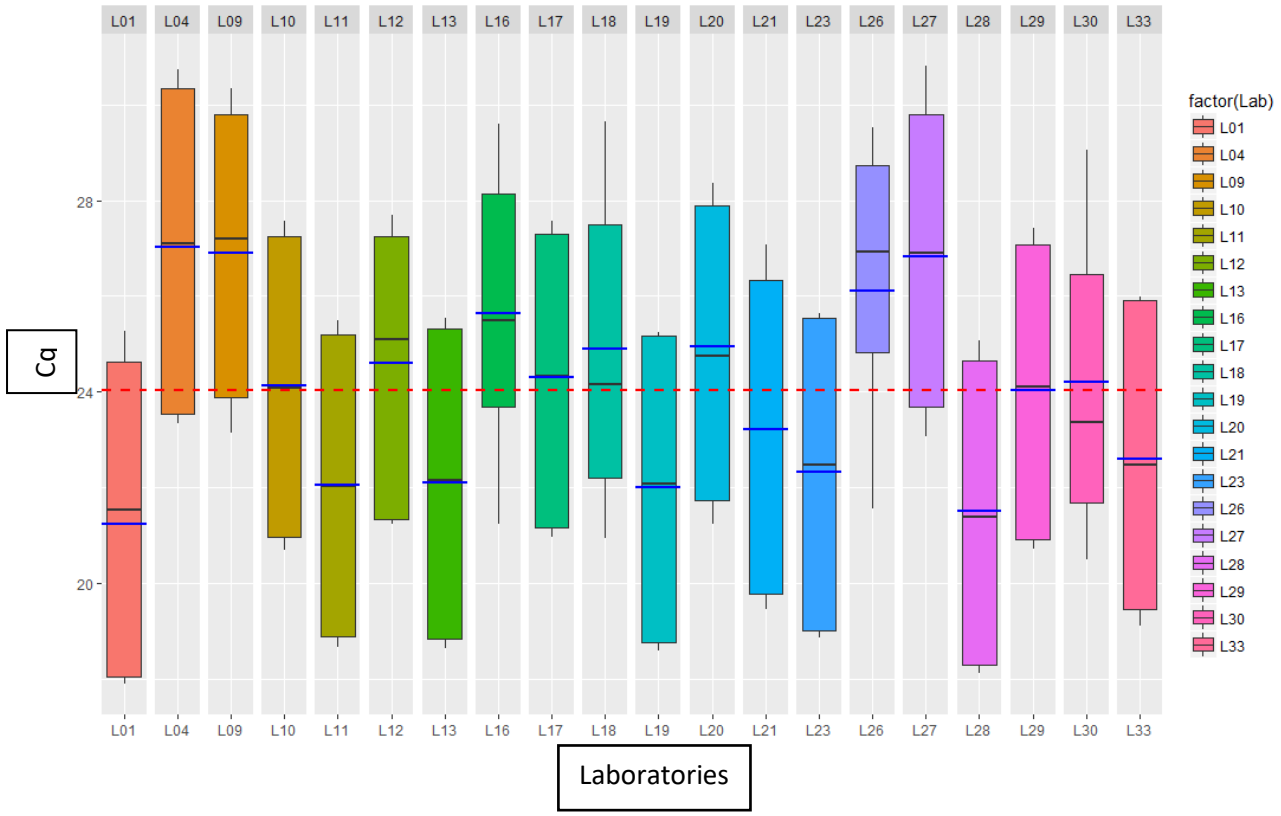


Figure 6. Range of the values of the quantitation cycle (Cq) recovered in each laboratory on the DNA extracts prepared from the samples containing 10^6, 10^5 and 10^4 CFU/ml, using the Mericon food kit (Qiagen).

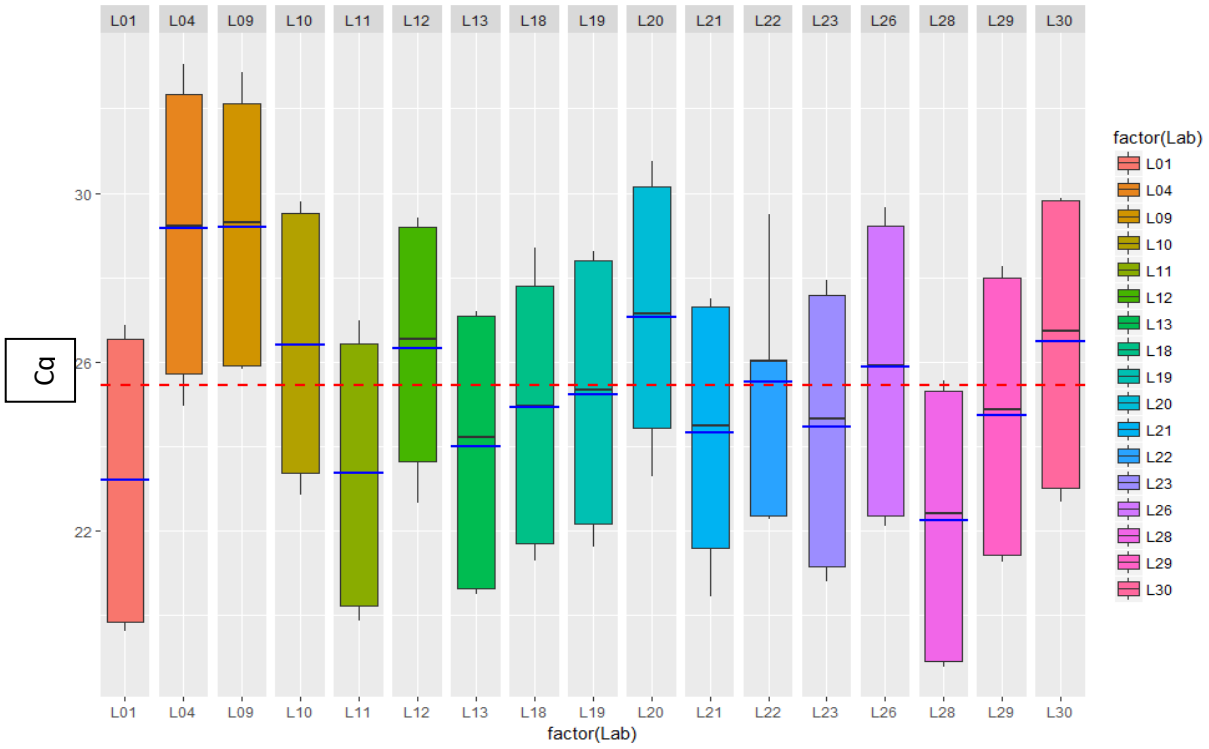


Figure 7. Range of the values of the quantitation cycle (Cq) recovered in each laboratory on the DNA extracts prepared from the samples containing 10^6, 10^5 and 10^4 CFU/ml, using Quick pick kit (Bionobile).

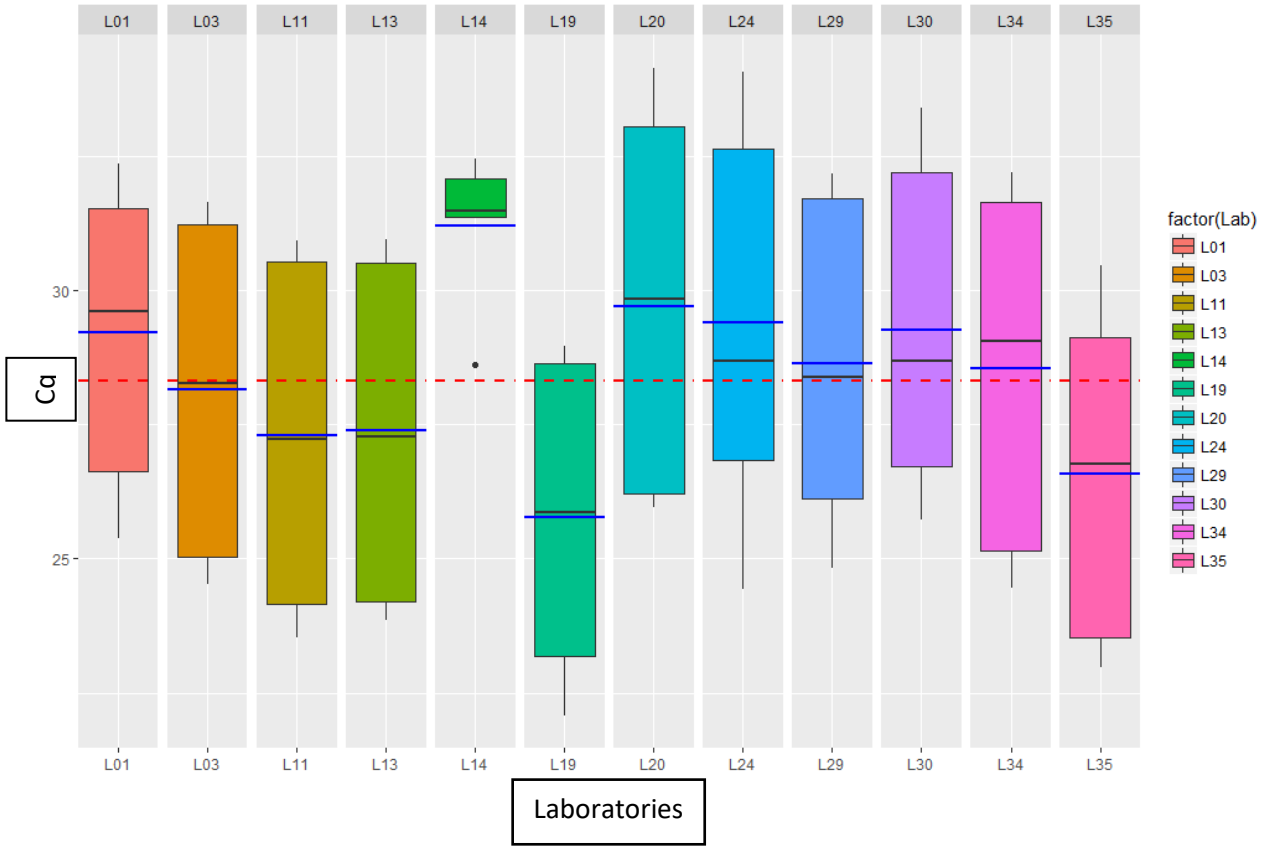
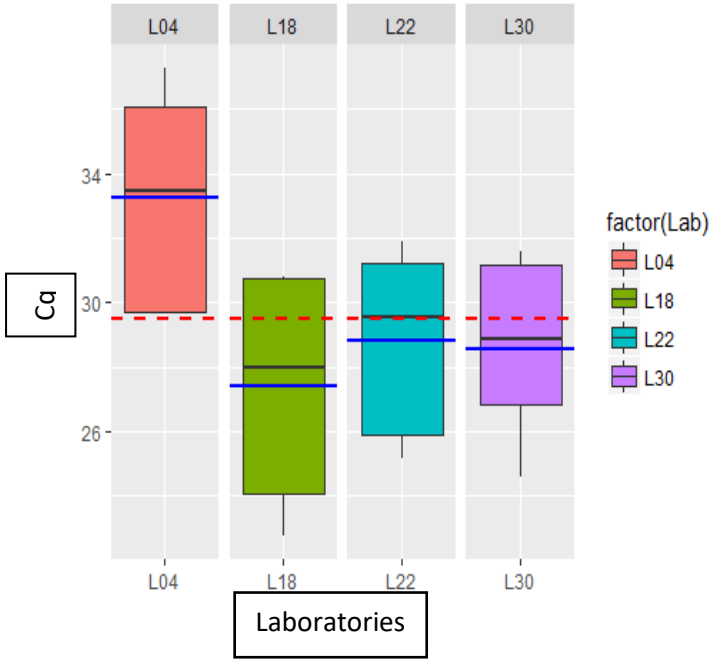


Figure 8. Range of the values of the quantitation cycle (Cq) recovered in each laboratory on the DNA extracts prepared prepared from the samples containing 10^6, 10^5 and 10^4 CFU/ml, using the DNeasy plant mini kit (Qiagen).



5.3 Results of the ELISA tests

5.3.1 Qualitative results

The results herein discussed, refer to the dataset obtained in 13 laboratories which performed ELISA tests using one or two different commercial kits.

As for the molecular tests, the samples were categorized in each laboratory as positive, negative or undetermined, based on the criteria provided by the manufacturers and/or based on the interpretation criteria routinely used in each laboratory; i.e. a sample is assessed as "positive" when the OD_{405} value is at least three times higher than the OD_{405} value of the negative control, conversely it is categorized as negative when the value is below this threshold.

In general, the accuracy of the results obtained in each laboratory for the ELISA tests was lower than the accuracy values obtained using the molecular tests. The majority of the deviations were recorded for the samples containing the lowest bacterial concentration (5x10^4 CFU/ml), with several replicates testing negative, i.e. producing negative deviations.

However, excluding from the analysis these replicates and considering the results obtained for the *Xylella*-contaminated samples containing 5x10^6 CFU/ml and 5x10^5 CFU/ml and the *Xylella*-free samples, all laboratories were proficient with an accuracy of 100%.

The graphs reported in Figure 9 show the diagnostic sensibility, specificity and accuracy values recovered in each laboratory using the two different ELISA kits on the full panel of samples.

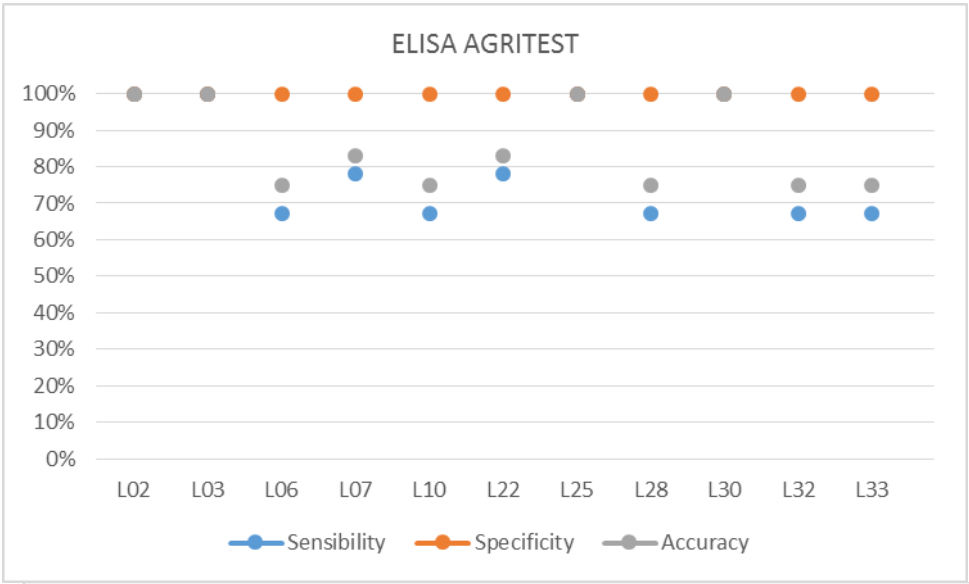
More details are provided in Table 13, with the values of sensitivity, specificity and accuracy recovered in the different laboratories and the subsequent laboratory categorization and assessment of the conformity.

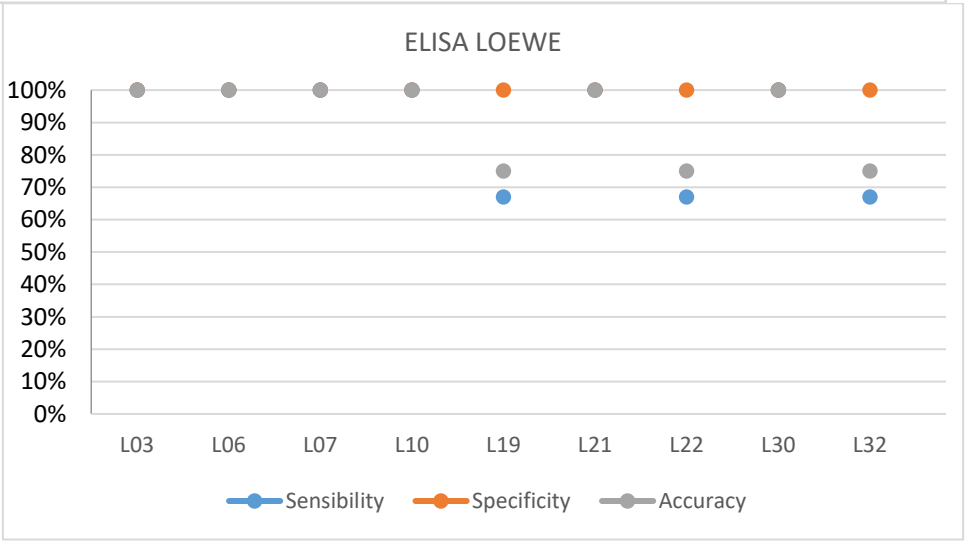
The overall results confirm the lower sensitivity of the ELISA tests compared to the molecular tests; however, in this specific PT, several parameters may have influenced the performance of the laboratories: (i) the use of different plates, (ii) different volume of the samples loaded into the plates, (iii) use of in-house prepared buffers (Table 3).

Table 13. Summary of the performance criteria recovered in the different laboratories for ELISA tests performed using two different commercial kits. The table reports the number of samples with positive agreement (PA), negative agreement (NA), positive deviation (PD) and negative deviation (ND), and the resultant values for the different performance criteria.

Performance parameters and criteria	LABORATORIES					
	L02, L03, L25,L30	**L07**	**L22**	**L06,L10,L28, L32, L33**	**L03, L06, L07,L10, L21,L30**	**L19, L22, L32**
	ELISA KIT					
	Agritest				**Loewe**	
N. of PA	9	7	7	6	9	6
N. of NA	3	3	3	3	3	3
N. of ND	0	2	2	3	0	3
N. of PD	0	0	0	0	0	0
Sensitivity	100%	78%	78%	67%	100%	67%
Specificity	100%	100%	100%	100%	100%	100%
Repeatability	100%	89%	89%	100%	100%	100%
Accuracy	**100%**	**83%**	**83%**	**75%**	**100%**	**75%**
Category	**Highly proficient**	**Non-proficient**	**Non-proficient**	**Non-proficient**	**Highly proficient**	**Non-proficient**
Conformity	**YES**	**NO**	**NO**	**NO**	**YES**	**NO**

Figure 9. Performance values recovered in each laboratories for the ELISA test performed using the kits Agritest and Loewe.





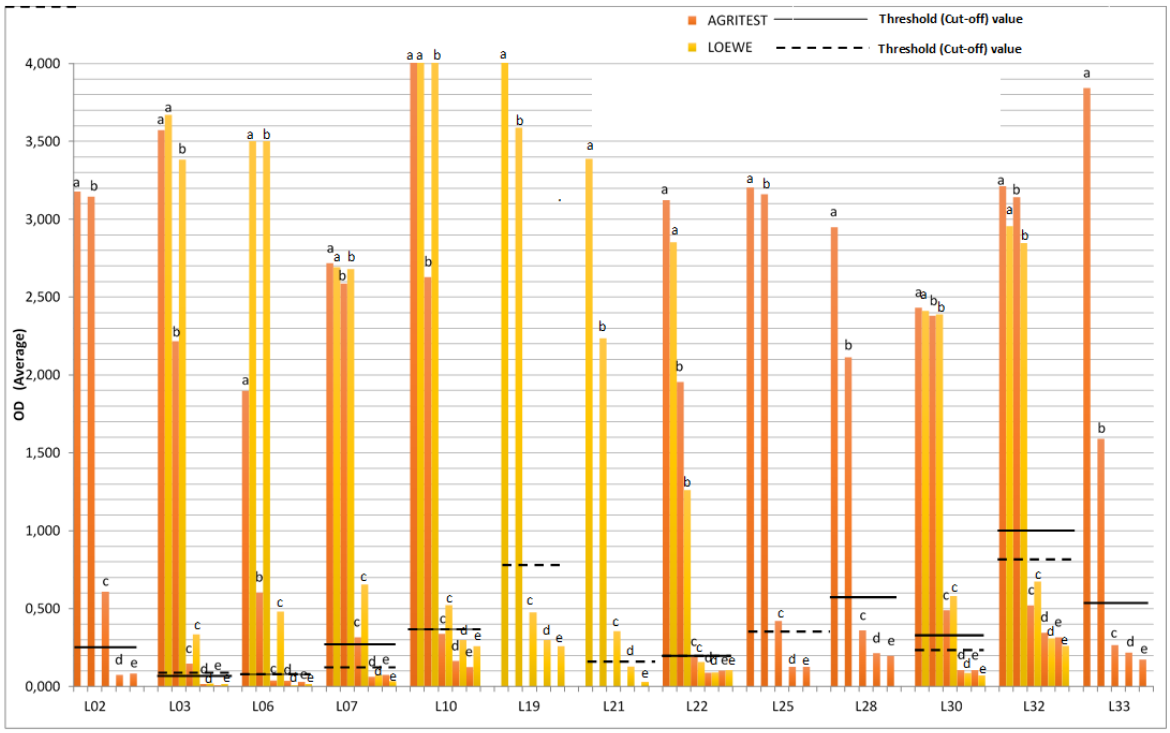
5.3.2 Quantitative results

The values of absorbance (OD_{405}), recovered in each laboratory by reading the plates three hours after adding the substrate, are reported in Figure 10. The values refer to the average of the absorbance of three replicates (loaded in double wells) for each sample.

Analysis of the OD_{405} values obtained using the three 10-fold serial diluted samples (5x10^6, 5x10^5 and 5x10^4 cells/ml) showed that the samples containing 5x10^6 and 5x10^5 cells/ml generated in the majority of the laboratories, and with both commercial kits, OD_{405} values higher than 2.5 and 1.3, respectively. With the only exception of L06 which obtained lower OD_{405} values (1.898 and 0.603).

Conversely, the OD_{405} values recovered from the samples containing 5x10^4 cells/ml were, in several laboratories, below the threshold for positives calls, thus gathering several negative deviations which reduced the accuracy of the test and the proficiency of the laboratories.

Figure 13. Graph showing the values of absorbance (OD_{405}) obtained, using either one or two kit, in each laboratory by testing the panel of samples provided for the proficiency test. The threshold values for the identification of the status of the blind samples are indicated with dashed and continous lines. (a) samples containing 5x10^6 CFU/ml; (b) samples containing 5x10^5 CFU/ml; (c) samples containing 5x10^4 CFU/ml; (d) Xf-free samples; (e) negative control.



6. OVERVIEW ON THE PERFORMANCE OF THE LABORATORIES

Despite the use different methods of extraction and different qPCR master mixes, the totality of the laboratories that performed the detection of *X. fastidiosa* by qPCR, regardless to the method of DNA extraction used, resulted proficient, with only one exception (Table 14).

The rate of proficient laboratories decreased for PCR and for ELISA tests, due to the lack of detection in samples containing the lowest bacterial concentrations, impairing the results produced by some laboratories. For PCR assays, the highest number of non-proficient laboratories occurred when using the Quick Pick kit (Bionobile) for the extraction of the DNA. As previously mentioned this may be the consequence of the use of the manual magnet pipet as alternative to an automated platform, and to the fact that some laboratories were not used and trained to use this specific kit.

In conclusion, this PT provided a good overview on the laboratory performance for the diagnostics currently used in the EU/Mediterranean countries for the detection of *X. fastidiosa* in the plant samples; the results indicated that using the most sensitive and the most widely adopted diagnostic protocol (i.e. qPCR) the laboratories's performance was very satisfactory; at the same time useful insights were obtained to achieve a better performance for the unsatisfactory laboratories, i.e. select different protocol for DNA extraction, different reagents and amplification conditions.

In table 14 are reported the number of proficient and non-proficient laboratories for each protocol.

Table 14. Number and percentage of laboratories and considered "conformed/not conformed to the PT" for each method

	Diagnostic protocols									
Status of the laboratories	CTAB		MERICON		QUICK PICK		DNeasy plant		ELISA	
	qPCR	PCR	**qPCR**	PCR	qPCR	PCR	**qPCR**	PCR	Agritest	Loewe
CONFORM (Highly proficient and proficient)	**20** **100%**	24 96%	**20** **100%**	21 95%	11 92%	6 67%	**4** **100%**	3 50%	4 36%	6 67%
NON-CONFORM (Non-proficient)	**0**	1	**0**	1	1	3	**0**	3	7	3
Total number of laboratories	**20**	25	**20**	22	12	9	**4**	6	11	9

7. TEST PERFORMANCE STUDY (TPS) FOR THE MOLECULAR ASSAYS

To assess the efficiency and accurateness of the methods used by laboratories that performed proficiently in this PT, their results were used to evaluate the performance of the molecular diagnostic methods. The protocols and the number of laboratories that fulfilled the requirement for the TPS are specified in Table 15. In the TPS, it was also evaluated the conventional PCR assay performed on the DNA extracted using the Quick Pick (Bionobile), although the total number of laboratories was lower than the minimum number of laboratories recommended in the EPPO 7/122 (i.e. 10 Laboratories).

Table 15. Number and code of the proficient laboratories used to gather the data used for the test performance study. The protocols subjected to the test performance study are also indicated.

METHODS	CTAB		MERICON		QUICK PICK	
	qPCR	PCR	qPCR	PCR	qPCR	PCR
DECLARED CONFORMITY (N. lab)	20	24	17	21	11	6
Code lab.	L01,L04,L09,L10 L11,L12,L13,L16 L17,L18,L19,L20 L21,L23,L26,L27 L28,L29,L30,L33	L01,L04,L05, L06,L07,L08, L09,L10,L11, L12,L13,L15 L16,L17,L18, L19,L20,L21, L23,L26,L27, L29,L30,L32	L01,L04,L09, L10,L11,L12, L13,L18,L19, L20,L21,L22, L23,L26,L28, L29,L30	L01,L04,L06, L07,L08,L09, L10,L11,L12, L13,L18,L19, L20,L21,L22, L23,L26,L29, L30,L31, L32	L01,L03, L11,L13, L20,L24, L29,L30, L34,L35, L19	L03,L11, L13,L20, L29,L30

7.1 Analysis of results

Analysis of the results was performed for each method using the same parameters and performance criteria reported in section 4.1 and Tables 4-5, by adding the assessment of the “Reproducibility” .

The Reproducibility is defined as the ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions (time, persons, equipment, location, etc). The reproducibility is calculated based on the number of interlaboratory pairs of same results/total number of interlaboratory pairs.

Analyses included also the quantitative results obtained for the qPCR, expressed as Cq values.

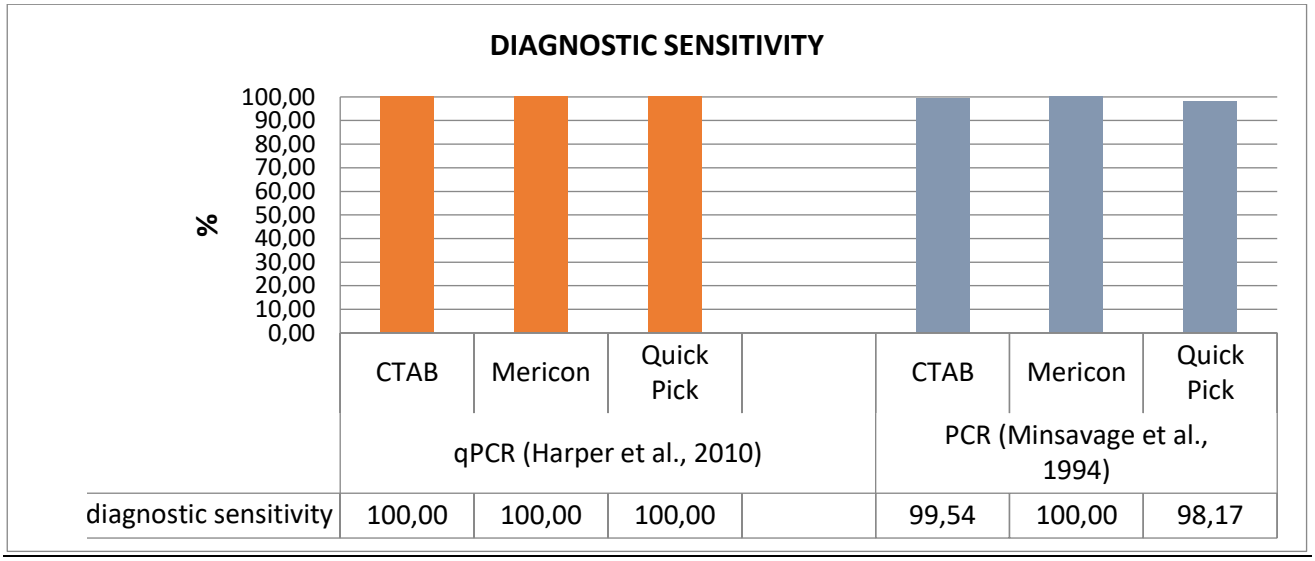
7.2 Results of qPCR assays

As shown in Figures 11-15, qPCR assays, regardless the extraction method, consistently resulted in performance values of sensitivity, specificity, accuracy, repeatability and reproducibility of 100%. The only exception occurred when using the Quick Pick kit (Bionobile) that yielded a slightly lower specificity (97%) then the other methods. This result was the consequences of a single negative deviation occurred in one of the 11 laboratories. This lower specificity determined reduced values of accuracy (99.27%), repeatability (99%) and reproducibility (99.20%).

7.3 Results of PCR assays

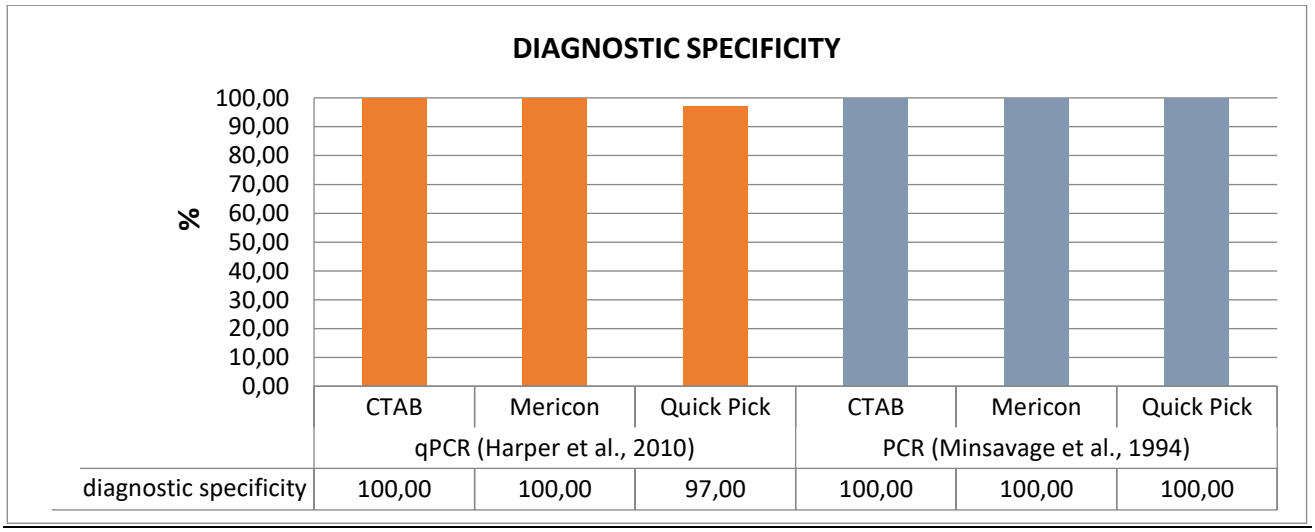
For the PCR tests, as shown in the graphs in Figure 11-15, the highest values were achieved, for all performance criteria, with the Mericon Food kit (values of 100%). The remaining two methods (CTAB and Quick Pick kit) generated values slightly lower than 100% for the sensitivity, accuracy, repeatability and reproducibility. Such results were the consequence of the presence of one undetermined result (Negative deviations) for the sample with the lowest bacterial concentration (10^4 cells/ml), yielding faint DNA band and thus unclear interpretation of the results.

Figure 11. Diagnostic sensitivity calculated using the results obtained in qPCR and PCR assays using the DNA extracts prepared following three different extraction protocols (CTAB, Mericon food kit, Quick Pick).



diagnostic sensitivity	100,00	100,00	100,00	99,54	100,00	98,17

Figure 12. Diagnostic specificity calculated using the results obtained in qPCR and PCR tests using the DNA extracts prepared following three different extraction protocols (CTAB, Mericon food kit, Quick Pick).



diagnostic specificity	100,00	100,00	97,00	100,00	100,00	100,00

Figure1 3. Accuracy calculated based on the number of samples resulting in positive and negative agreement in relation to the total number of samples.

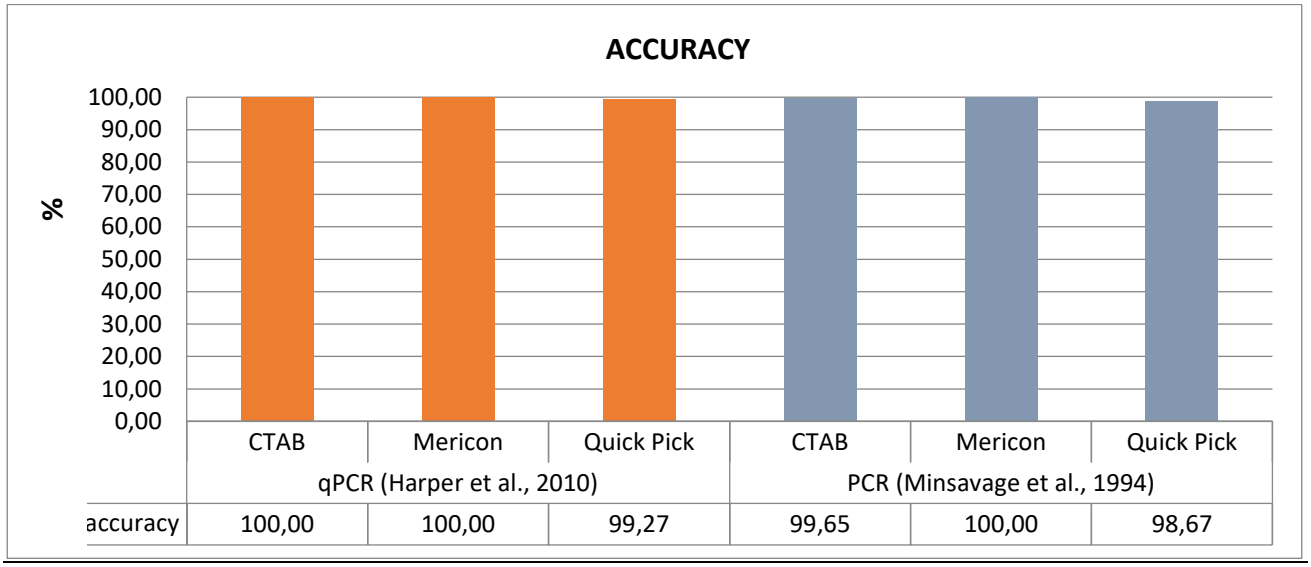


Figure 14. Repeatability calculated based on the number of replicates for each sample containing 10^6, 10^5 and 10^4 cells/ml, resulting in positive and negative agreement in relation to the total number of replicates tested under the same conditions.

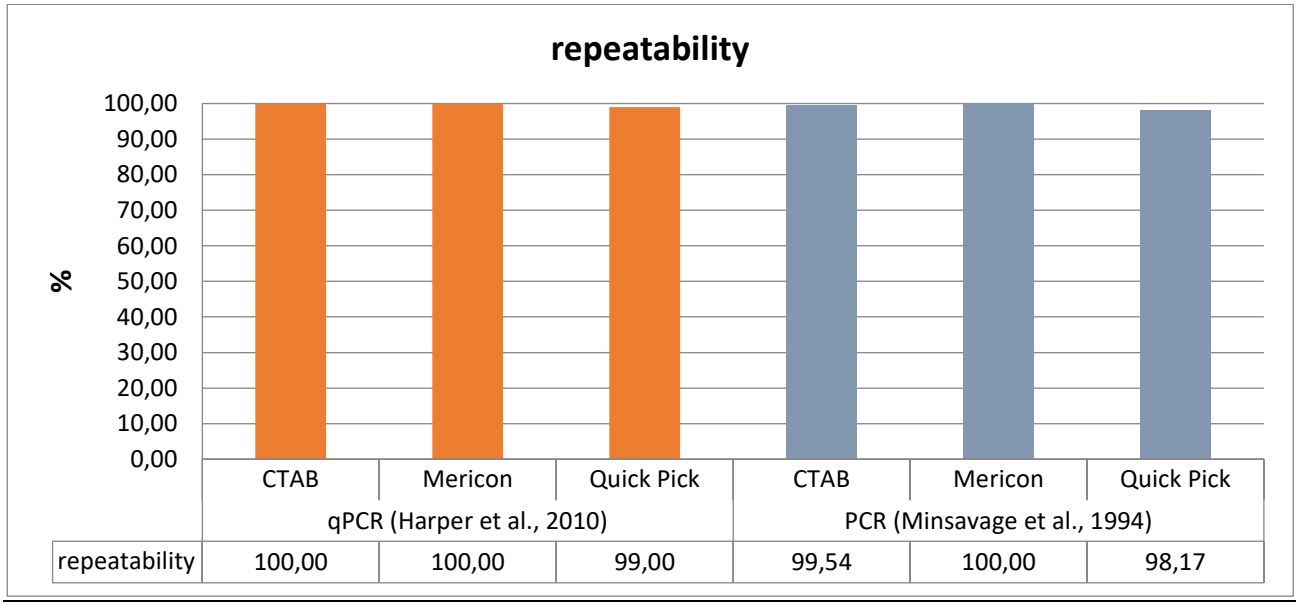
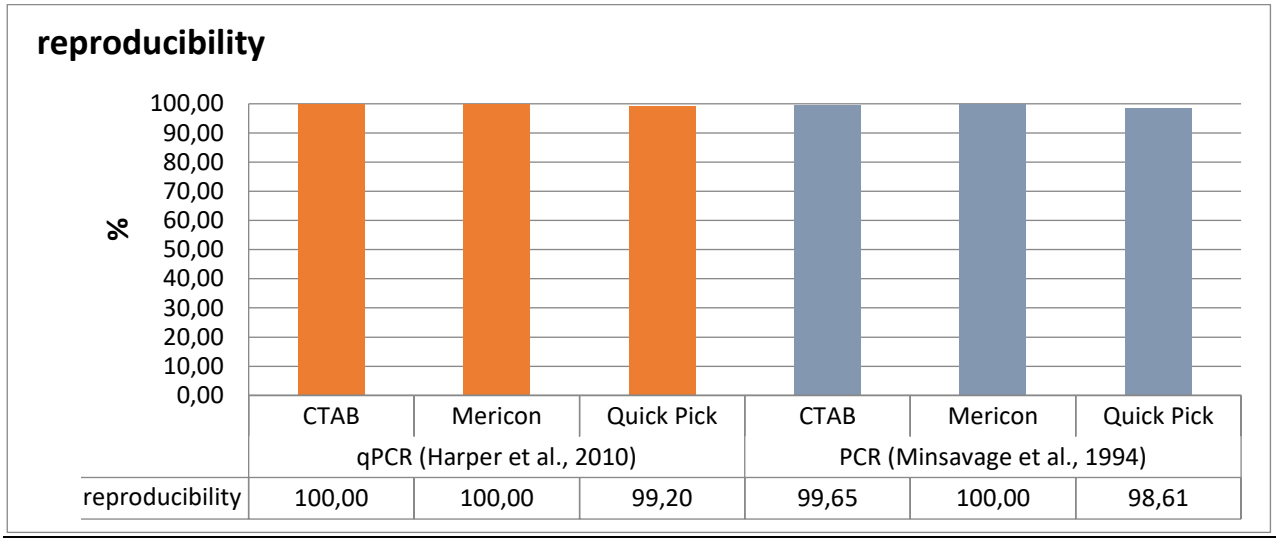


Figure 15. Reproducibility for the overall sample pairs tested in the different laboratories by qPCR and PCR.



7.4 Quantitative results

Analysis of the Cq values obtained in qPCR using the three 10-fold serial diluted samples (containing the following bacterial concentrations: 10^6, 10^5 and 10^4 cells/ml) clearly showed for all methods an optimal amplification efficiency. Despite the different methods of extraction and the different qPCR master mixes used in the different laboratories, the $\triangle$Cq among the dilutions corresponded to the expected value of "3" (approximately). In fact, when the Cq values were plotted against the concentration in each spiked sample, the slope of the line that measure the assay's efficiency were between 3.1 and 3.3, corresponding to the optimal qPCR efficiency values, ranging from 90% to 110% (Table 16, Figure 16). Similarly, the R^2 values that measure the performance of the assay were greater than 0.99 for CTAB, Qiagen Mericon Food kit and Quickpick, Bionobile.

Table 16. Results of the linear regression analysis

Extraction method	Linear regression and R^2 values
CTAB	y = 3,3022x + 17,681 R^2 = 1
Qiagen, mericon food kit	y = 3,3366x + 18,746 R^2 = 0,9988
Bionobile, Quickpick	y = 3,104x + 22,206 R^2 = 1

The standard deviation (SD) of the Cq values recovered from each method and each set of samples was comprised between 1.45 (Bionobile, Quick Pick) and 2.24 (CTAB), these values are affected by the use of different qPCR conditions (amplification master mixes, reaction volumes, etc.) in the different laboratories. In general, the lowest Cq values were obtained with the DNA recovered using CTAB protocol, followed by the Qiagen Mericon Food kit (Figure 17).

Figure 16. Standard curves represented as linear regression of the quantitation cycle (Cq) values versus the concentration of the spiked samples. Different colors indicate the Cq generated using the DNA extracted using 3 different extraction methods.

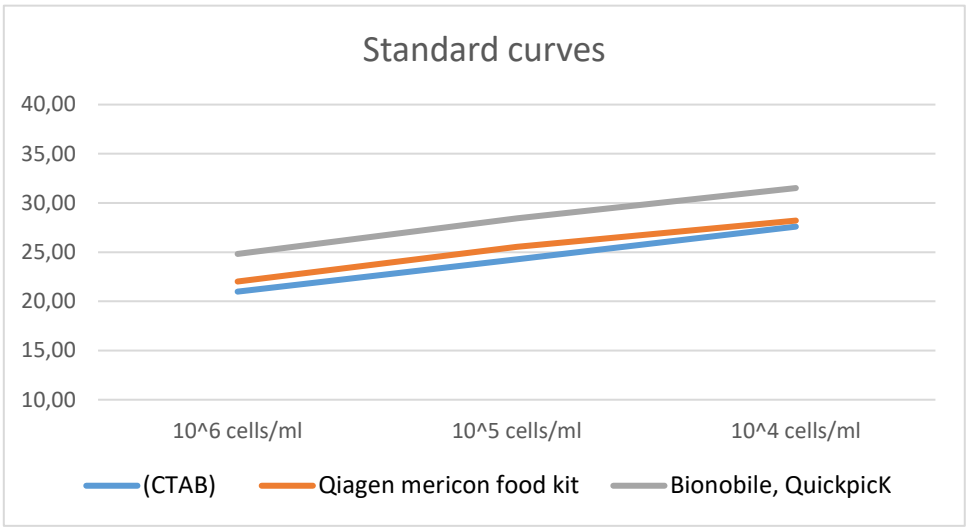
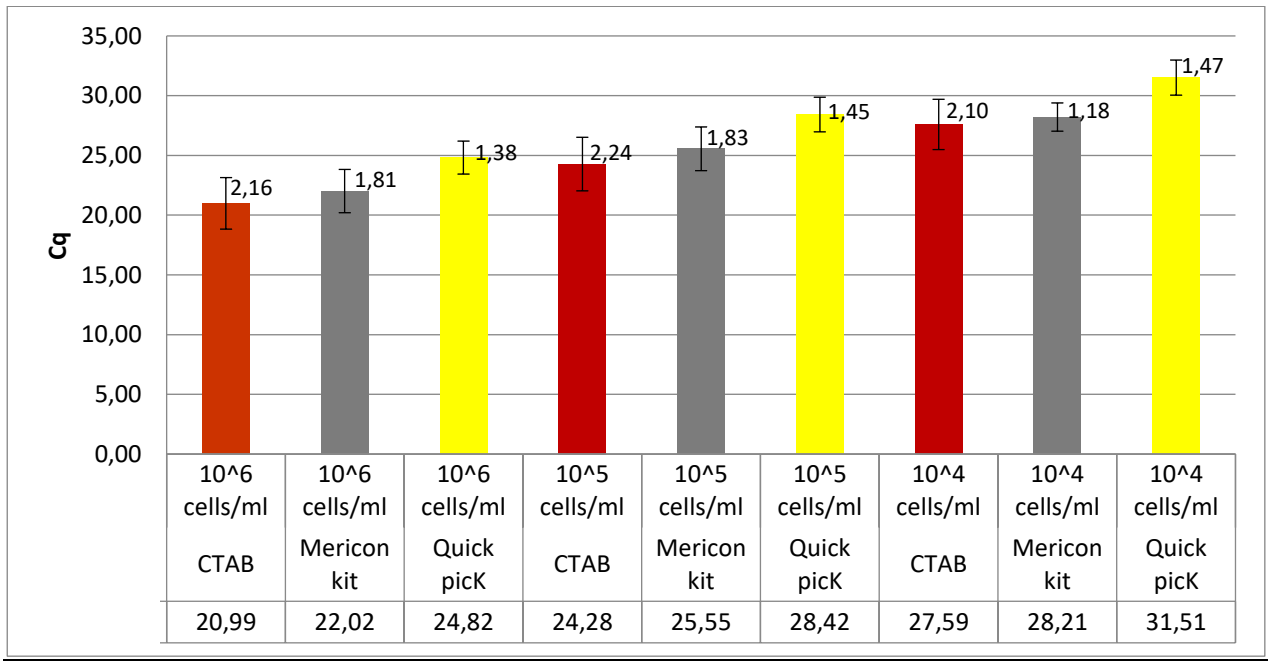


Figure 17. Average of the quantitation cycle (Cq) yielded using the DNA extracts recovered from different procedures on samples containing different bacterial concentrations. The overall standard deviations among the Cq values recovered in the different laboratories is indicated on the top of histogram.



7.5 Summary of the Test Performance Study

In Table 17 are summarized the values of the performances generated for each diagnostic method evaluated in this TPS.

The Real-Time PCR Harper *et al*., 2010 (erratum 2013) presents the best performance values, regardless to the method of extraction of the target DNA they were always higher than 97%. In fact, with the exception of the Quickpick kit (Bionobile) which produced values of 97% (for the specificity), with the remaining two protocols values of 100% were generated for all parameters.

The PCR assays (Minsavage *et al*., 1994) on the DNA extracts recovered with the Mericon Food kit, produced consistent values of 100% for all parameters. Whereas, for the PCR assays performed on CTAB and Quick pick extracts, parameters were slightly below 100%.

Despite the use of different amplification conditions and master mix, the TPS 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 PT, and currently being the most common used protocols, confirming their suitability for the diagnosis of *X. fastidiosa* in plant materials.

Table 17. Values of the performance criteria obtained for qPCR and PCR assays on CTAB, Mericon food kit an Quick pick kit extraction procedures.

Diagnotic method	Accuracy %	Sensitivity %	Specificity %	Repeatability %	Reproducibility %
CTAB/qPCR	100	100	100	100	100
CTAB/PCR	99.65	99.54	100	99.54	99.65
Qiagen mericon/qPCR	100	100	100	100	100
Qiagen mericon/PCR	100	100	100	100	100
Bionobile QuickPick/qPCR	99.65	100	97	99	99.20
Bionobile QuickPick/PCR	98.67	98.17	100.00	98.17	98.61

8. REFERENCES

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