TEST PERFORMANCE STUDY

Molecular detection of *Xylella fastidiosa* through quantitative real time assays

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

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CONTENT

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1. General information

1.1 Objective

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 qPCR assays to detect *Xylella fastidiosa* in host plants. In the framework of this test performance study (TPS), five different qPCR assays were compared using a common panel of DNA templates. DNA extracts 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). More specifically, the DNA extracts used in this TPS were recovered from artificially contaminated olive sap, processed using either the DNeasy® mericon™ Food kit (Qiagen) or the CTAB-based extraction protocol.

1.2 Performance Criteria

The following performance criteria were evaluated (definition: OEPP/EPPO PM 7/76 (2), 2010; OEPP/EPPO PM 7/122 (1), 2014):

Performance criteria	Dataset used in this TPS
▪ **Diagnostic sensitivity:** Proportion of infected/infested samples testing positive compared to results from an alternative test (or combination of tests). Sensitivity = true positives/(true positives + false negatives)	Results obtained for the 9 Xf-contaminated samples tested with each protocol
▪ **Specificity:** Proportion of uninfected/uninfested samples (true negatives) testing negative compared to results from an alternative test (or combination of tests). Specificity = true negative results/(true negatives + false positives)	Results obtained for the 3 Xf-free samples tested with each protocol
▪ **Repeatability:** Level of agreement between replicates of a sample tested under the same conditions	Results obtained in the same laboratory on the 3 replicates for each set of Xf-contaminated and Xf-free samples
▪ **Accuracy:** closeness of agreement between a test result and the accepted reference value	Determined based on the results of 3 aforementioned performance criteria
▪ **Reproducibility:** Ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions (time, persons, equipment, laboratory, etc)	Identities among the results yielded for the 3 replicates of each set of Xf-contaminated/Xf-free samples, in the different laboratories (i.e. under different conditions).

1.3 Organization

This TPS was 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), as an implementation of the PT EU-XF- PT-2017-02, and in 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)".

1.4 Laboratories

The laboratories participating in the TPS are listed in Table 1, each laboratory was identified with the same anonymous alphanumeric code assigned for the PT EU-XF-PT-2017-02.

Table 1. List of the participating laboratories

INSTITUTION	COUNTRY	INSTITUTION	COUNTRY
AGES - Austrian Agency for Health and Food Safety	AUSTRIA	**CSIC** - Institute for Sustainable Agriculture	SPAIN
JKI - Julius Kuehn Institute	GERMANY	**LOSVIB -** Laboratorio Oficial de Sanidad Vegetal de las Islas Baleares	SPAIN
LOEWE - Loewe Biochemica GmbH	GERMANY	**Fera**	UK
SASA - Science and Advice for Scottish Agriculture	UK	**Di3A** - Dipartimento di Agricoltura, Alimentazione e Ambiente, Università degli Studi di Catania	ITALY
CREA-PAV - Consiglio per la ricerca e la sperimentazione in agricoltura, Centro di ricerca per la Patologia Vegetale	ITALY	**SELGE-** Institute for Sustainable Plant Protection, CNR and Department of Soil, Plant and Food Science, University of Bari	ITALY
HCPHS - Croatian Centre for Agriculture, Food and Rural Affairs	CROATIA	**CRSFA-** Centro di ricerca, Sperimentazione e Formazione "Basile Caramia"	ITALY
L27 RIH - Research Institute of Horticulture	POLAND	**Unisalento** - Dipartimento di Scienze e Tecnologie	ITALY

2. DNA TEMPLATES

This TPS was carried out using the DNA extracts recovered by the same participating laboratories during the PT EU-XF-PT-2017-02, and stored at -20°C. More specifically, the panel of DNA extracts used in this TPS included the extracts recovered either using the DNeasy® mericon™ Food kit (Qiagen) or the CTAB-based extraction protocol. Table 2 reports the list of the experimental samples tested.

Table 2. List of samples used to assess the performance of the different qPCR assays

Samples	Assigned Value	Used to calculate the performance criteria
3 replicates of DNA extracts from non-infected olives	negative	X
3 replicates of DNA extracts (from olive) containing 10^6 cells/ml	positive	X
3 replicates of DNA extracts (from olive) containing 10^5 cells/ml	positive	X
3 replicates of DNA extracts (from olive) containing 10^4 cells/ml	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	

For each protocol, qPCR reactions were set up by running two replicates per sample. All the raw data, both qualitative and quantitative results, received from the different participating laboratories were collected in separate excel files with the corresponding decrypted sample codes.

3. PROTOCOLS

The protocols with the specific indications on the reagents (brand and catalog number) and on the amplification conditions used in this TPS were provided by the Organizers (Annex I). Reagents were supplied by each laboratory and reactions were performed on different thermocyclers according to the equipment own by each laboratory. The thermocycler, the DNA templates and the protocols selected by each participant laboratories are reported in table 3. Each protocol was identified with a code as indicated in red and in parenthesis in table 3.

Table 3. Protocols, thermocyclers and DNA extracts used in this TPS by the participating laboratories

qPCR protocol / Lab Thermocycler	DNA PURIFIED USING CTAB PROTOCOL					DNeasy® mericon™ Food kit (Qiagen)				
	Harper et al., 2010 (HP)	Li et al., 2013, using standard TaqMan probe (Li-SP)	Li et al., 2013, using MGB-TaqMan probe (Li-MGB)	Francis et al., 2006 (FR-SP)	Francis et al., 2006, modified using Sybr green (FR-SG)	Harper et al., 2010 (HP)	Li et al., 2013, using standard TaqMan probe (Li-SP)	Li et al., 2013, using MGB-TaqMan probe (Li-MGB)	Francis et al., 2006 (FR-SP)	Francis et al., 2006, modified using Sybr green (FR-SG)
L01 Eppendorf Realplex 4 Mastercycler S	X	X	X	X*	X*	X	X	X	X*	X*
L09 BioRad CFX96		X	X		X	X	X	X	X	X
L10 BioRad CFX96	X	X	X	X	X	X	X	X	X	X
L11 ROCHE LightCycler 480 I	X	X	X	X	X	X	X	X	X	X
L12 IQ Cycler Biorad	X	X	X	X	X	X	X	X	X	X

qPCR protocol / Lab Thermocycler	DNA PURIFIED USING CTAB PROTOCOL					DNeasy® mericon™ Food kit (Qiagen)				
	Harper et al., 2010 (HP)	Li et al., 2013, using standard TaqMan probe (Li-SP)	Li et al., 2013, using MGB-TaqMan probe (Li-MGB)	Francis et al., 2006 (FR-SP)	Francis et al., 2006, modified using Sybr green (FR-SG)	Harper et al., 2010 (HP)	Li et al., 2013, using standard TaqMan probe (Li-SP)	Li et al., 2013, using MGB-TaqMan probe (Li-MGB)	Francis et al., 2006 (FR-SP)	Francis et al., 2006, modified using Sybr green (FR-SG)
L13 Applied biosystems 7900HT	X	X	X	X		X	X	X	X	
L16 Stratagene Mx3005P	X	X	X	X						
L20 StepOnePlus Applied Biosystems	X	X	X			X	X	X		
L21 Quant Studio 3	X	X	X	X	X	X	X	X	X	X
L22 BioRad CFX96						X	X	X	X	X
L27 BioRad CFX96	X	X	X	X	X					
L29 Applied Biosystems 7900	X	X	X	X		X	X	X	X	
L30 BioRad CFX96	X	X	X	X	X	X	X	X	X	X
L33 Applied Biosystems 7900	X	X	X	X	X					

qPCR protocol / Lab Thermocycler	DNA PURIFIED USING CTAB PROTOCOL					DNeasy® mericon™ Food kit (Qiagen)				
	Harper et al., 2010 (HP)	Li et al., 2013, using standard TaqMan probe (Li-SP)	Li et al., 2013, using MGB-TaqMan probe (Li-MGB)	Francis et al., 2006 (FR-SP)	Francis et al., 2006, modified using Sybr green (FR-SG)	Harper et al., 2010 (HP)	Li et al., 2013, using standard TaqMan probe (Li-SP)	Li et al., 2013, using MGB-TaqMan probe (Li-MGB)	Francis et al., 2006 (FR-SP)	Francis et al., 2006, modified using Sybr green (FR-SG)
HT Fast Real-time PCR System										
TOTAL LABORATORIES	12	13	13	10	8	11	11	11	9	7

*= the results from these tests were not included in the analysis due to technical problems which invalidate the results

4. ANALYSIS OF THE RESULTS

Results were analyzed based on the qualitative and quantitative data (Cq values). In each laboratory, samples were assigned as negative, positive or undetermined, according to the resultant quantitative cycle (Cq) values. For each laboratory, the Organizers determined the number of positive agreements (PA), negative agreements (NA), positives deviations (PD) and negatives deviations (ND) according to the parameters described in Table 4. These values were used to calculate the different performance criteria (Table 5) for each protocol. In order to use common threshold and cut-off values, the Organizers revised and harmonized the categorization made by each laboratory, following the rules reported in table 6.

The performance was expressed as percentage, with 100% being the highest performance level (see for more information Chabirand et al., 2014; OEPP/EPPO PM 7/122 (1), 2014).

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 (Chabirand et al., 2014; OEPP/EPPO PM 7/122 (1), 2014)

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 for samples for which the assigned value is negative	$SP = N_{NA}/N-$
Repeatability (DA)	Closeness of agreement between independent test results obtained under conditions of repeatability, i.e. conditions	DA denotes the percentage chance of obtaining the same result (positive, negative or

Performance criteria	Definition	Calculation
	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	indeterminate) from two identical samples analyzed in the same laboratory
Reproducibility	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)	based on the number of interlaboratory pairs of same results/total number of interlaboratory pairs

Table 6. Rules adopted for the harmonization of the interpretation of the results

Result replicate 1	Result replicate 2	Final assessment
Positive (Cq ≤ 35)	Positive (Cq ≤ 35)	Sample assigned as positive
Positive	Undetermined (35≤Cq<40)	Sample assigned as negative
Undetermined (35≤Cq<40)	Undetermined (35≤Cq <40)	Sample assigned as undetermined
Negative (N/A)	Undetermined (35≤Cq <40)	Sample assigned as negative
Negative (Cq= N/A)	Negative (Cq= N/A)	Sample assigned as negative

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 Qualitative results

Tables 7 and 8 report the values of the performance criteria yielded for each qPCR protocol performed on the DNA templates prepared using the DNeasy® mericon™ Food kit (Qiagen) or the CTAB-based extraction protocol, respectively.

As shown in the tables, the highest values (for all performance criteria) were obtained when qPCR reactions were performed on the DNA templates recovered using the DNeasy® mericon™ Food kit (Qiagen). In this case, values of 100% were consistently yielded for the qPCR protocols HP, FR-SP and FR-SG. Whereas, as shown in table 7, the qPCR Li-SP and Li-MGB, yielded values slightly lower than those obtained with the other qPCR protocols. More specifically, this was the determined by the results gained in one laboratory where one expected negative sample was categorized as "undertermined" (i.e. positive deviation), yielding Cq values of 36.66 (Li-SP) and 37.80 (Li-MGB).

When DNA templates recovered using CTAB-based extraction protocol were subjected to qPCR, consistent values of 100% where obtained using the qPCR HP and FR-SG; whereas, the remaining protocols generated values lower than 100% for the sensitivity, accuracy, repeatability and reproducibility. These lower values were determined by 7 and 13 presumptive negative samples which, instead, yielded undetermined results (positive deviations), i.e. Cq values close to or >35.00, with Li-SP and Li-MGB, respectively. These positive deviations affected the accuracy (96%), the reproducibility (92%) and the repeatability (97%). Furthermore, the protocol FR-SP produced, in one laboratory, three negative deviations corresponding to three undetermined results (average Cq values of 36.49, 35.81 and 37.09) for 3 replicates contaminated with the lowest bacterial concentration (10^4 cells/ml) which determined values of sensitivity of 97%, accuracy 98% and reproducibility 98%.

Besides the occurrence of samples producing undetermined results, none of the qPCR assays produced false positive or false negative results.

Table 7. Values recovered for the different performance criteria using five different qPCR protocols on the DNA purified using the DNeasy mericon Food Kit (QIAGEN).

DATA	qPCR PROTOCOLS				
	HP	Li-SP	Li-MGB	FR-SP	FR-SG
N. lab	11	11	11	9	7
N+	99	99	99	81	63
PA	99	99	99	81	63
ND	0	0	0	0	0
- Undetermined (suspicious) of N+	0	0	0	0	0
N-	33	33	33	33	21
NA	33	32	32	33	21
PD	0	1	1	0	0
- Undetermined (suspicious) of N-	0	1	1	0	0
% Accuracy	**100**	**99**	**99**	**100**	**100**
% Sensitivity	100	100	100	100	100
% Specificity	100	97	97	100	100
% Repeatability	**100**	**99**	**99**	**100**	**100**
% Reproducibility	**100**	**99**	**99**	**100**	**100**

PA positive agreements; NA negative agreements; PD positive deviations; ND negative deviations; N+ number of samples with positive assigned value (Σ PA+ Σ ND); N- number of samples with negative assigned value (Σ NA+ Σ PD); N total number of samples (N+ + N-).

Table 8. Values recovered for the different performance criteria using five different qPCR protocols on DNA purified using CTAB-based extraction protocol.

DATA	qPCR PROTOCOLS				
	HP	Li-SP	Li-MGB	FR-SP	FR-SG
N. of laboratories	12	13	13	10	8
N. of results obtained	144	156	156	120	96
N+	108	117	117	90	72
PA	108	117	117	87	72
ND	0	0	0	3	0
- Undetermined (suspicious) of N+	0	0	0	3	0
N-	36	39	39	30	24
NA	36	32	26	30	24
PD	0	7	13	0	0
- Undetermined (suspicious) of N-	0	7	13	0	0
% Accuracy	**100**	**96**	**92**	**98**	**100**
% Sensitivity	**100**	**100**	**100**	**97**	**100**
% Specificity	**100**	**82**	**67**	**100**	**100**
% Repeatability	**100**	**97**	**97**	**100**	**100**
% Reproducibility	**100**	**96**	**92**	**98**	**100**

PA positive agreements; NA negative agreements; PD positive deviations; ND negative deviations; N+ number of samples with positive assigned value (Σ PA+ Σ ND); N- number of samples with negative assigned value (Σ NA+ Σ PD); N total number of samples (N+ + N-).

5.2 Quantitative results

This section includes comparative analyses of the values of the quantitation cycles (Cq) recovered for the different qPCR assays. The Cq values obtained from each qPCR protocols for each series of samples processed either using the DNeasy® mericon™ Food kit (Qiagen) or the CTAB-based protocol are graphically shown with boxplots in figure 1.

The qPCR efficiencies for the five tested qPCR protocols was determined using the average Cq recovered for the three series of Xf-contaminated samples (10-fold dilutions). The slopes of the linear regression, that measure the assay's efficiency, were between 3.065 and 3.91, corresponding to the optimal qPCR efficiency values, ranging from 90% to 110% (Table 9, Figure 1). Similarly, the R^2 values that measure the performance of the assay were greater than 0.99, regardless the procedures used to prepare the DNA templates.

For a given sample, the Cq values varied according to the method used for the purification of the DNA and in relation to the qPCR protocol used for the amplification (Fig. 2 A-C).

In general, higher values (ΔCq= ~ 1) were obtained, regardless the qPCR protocol used, when the DNA purified using the DNeasy® mericon™ Food kit (Qiagen) was used.

Whereas with regard to the qPCR protocols, the assays based on Li et al (2013) consistently produced the lowest Cq values for all sets of samples. For both qPCR protocols Li-SP and Li-MGB, the standard deviation (SD) values were in the range of +1.07-1.71 and +0.97-1.13 when using CTAB-extracts, and in the range of +1.28-1.57 and +1.78-1.93 when using DNA recovered with the DNeasy® mericon™ Food kit (Qiagen), respectively. On the other hand, as reported in the previous paragraph, Li-SP and Li-MGB protocols generated the highest number of "undetermined" results, which impacted the specificity and the accuracy of the assays.

The values of Cq generated using the HP protocol were slightly higher (ΔCq ~ 1) than those recovered Li-SP and Li-MGB, with SD values comprised in the range of +1.14-1.17 and +1.20-1.31 for CTAB and DNeasy® mericon™ Food kit (Qiagen) extracts, respectively.

The values of Cq generated using the protocols FR-SP and FR-SG were the highest among the tested protocols, i.e. ΔCq= ~ 3-4. Similarly, the SD of the Cq values recovered from these two protocols were higher than those recovered with the other protocols, being comprised in the range of +2.27-2.68 and +1.98-2.73 for CTAB and DNeasy® mericon™ Food kit (Qiagen) extracts, respectively.

Although, using the panel of samples prepared for this TPS, the values of sensitivity recovered from the protocols FR-SP and FR-SG were 100%, the analysis of the Cq values put in evidence that this assay has lower sensitivity, as confirmed by the highest Cq values, which would have been more evident testing samples containing bacterial concentrations lower than 10^4 CFU/ml (as used in this TPS) and closer to the detection limit of the qPCR assays.

Table 9. Results of the linear regression analysis

| DNA extracts recovered using CTAB-based protocol	
qPCR protocols	**Linear regression and R^2 values**
HP	y = 3,335x + 17,36 R^2 = 0,9995
Li-SP	y = 3,16x + 17,117 R^2 = 0,9993
Li-MGB	y = 3,365x + 15,727 R^2 = 0,9997
FR-SP	y = 3,065x + 20,65 R^2 = 0,9965
FR-SG	y = 3,75x + 19,61 R^2 = 0,9995
DNA extracts recovered using DNeasy® mericon™ Food kit (Qiagen)	
qPCR protocols	**Linear regression and R^2 values**
HP	y = 3,465x + 18,62 R^2 = 0,9973
Li-SP	y = 3,365x + 18,6 R^2 = 0,9971
Li-MGB	y = 3,445x + 17,813 R^2 = 0,998
FR-SP	y = 3,525x + 20,77 R^2 = 0,9999
FR-SG	y = 3,91x + 20,93 R^2 = 0,9934

Figure 1. Standard curves represented as linear regression of the quantitation cycle (Cq) values (Y axis) versus the concentration of the spiked samples (X axis). Different colors indicate the Cq generated using different qPCR protocols. A) qPCR reactions set up using DNA extracts prepared using the CTAB-based protocol. B) qPCR reactions set up using DNA extracts purified using the DNeasy® mericon™ Food kit (Qiagen).
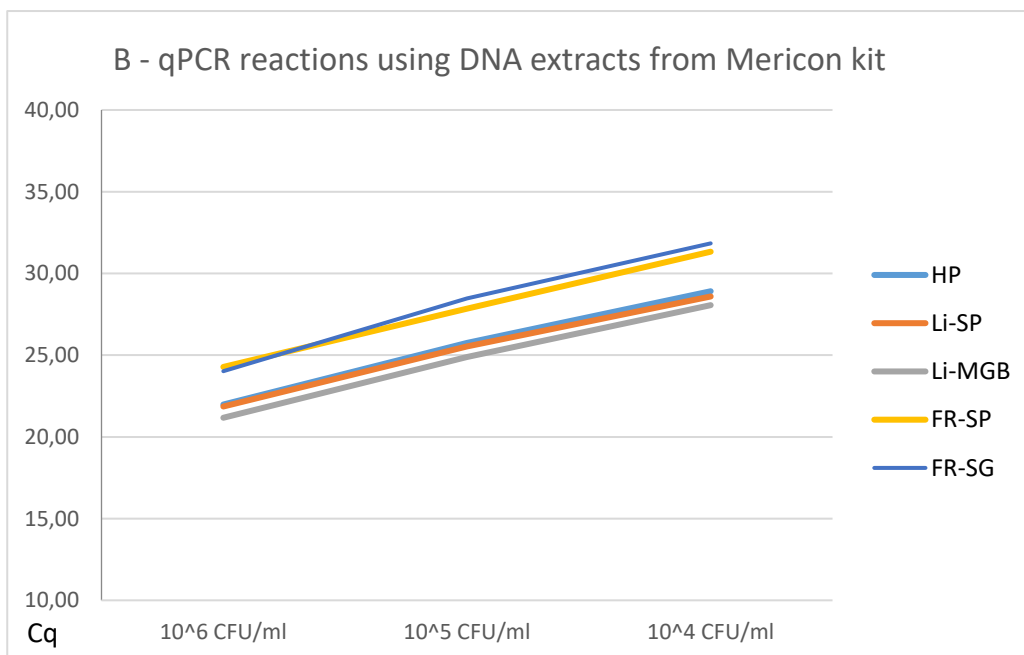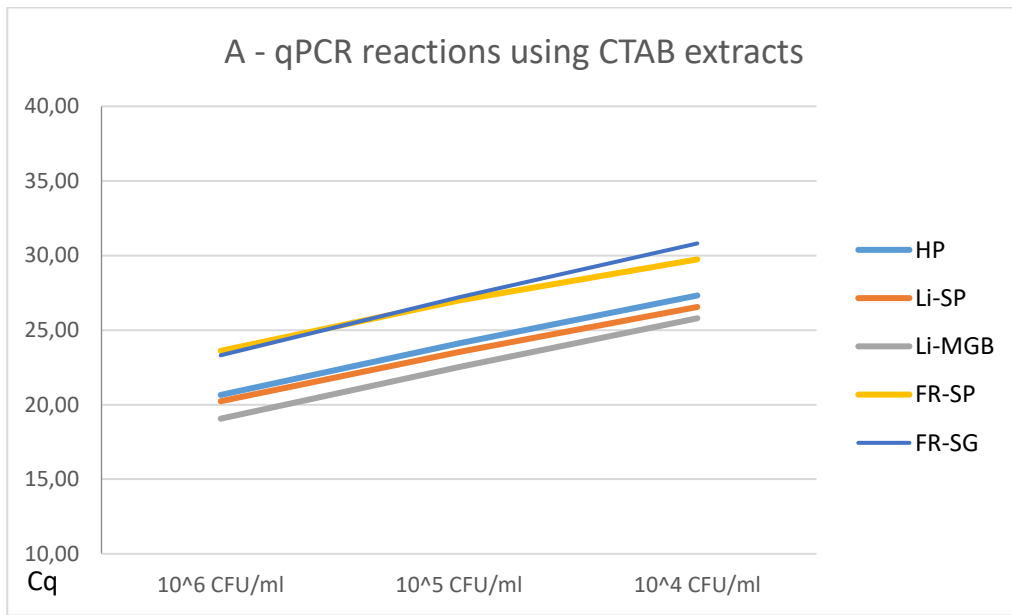
Figure 2. Quantitation cycle (Cq) yielded using five qPCR protocols on DNA extracts recovered using CTAB and DNeasy® mericon™ Food kit (Qiagen) extraction protocols, on samples contaminated with three different bacterial concentrations: 10^6 CFU/ml (A), 10^5 CFU/ml (B) and 10^4 CFU/ml (C). The average Cq values and the overall standard deviations recovered in the different laboratories are indicated on the top of the boxplots.
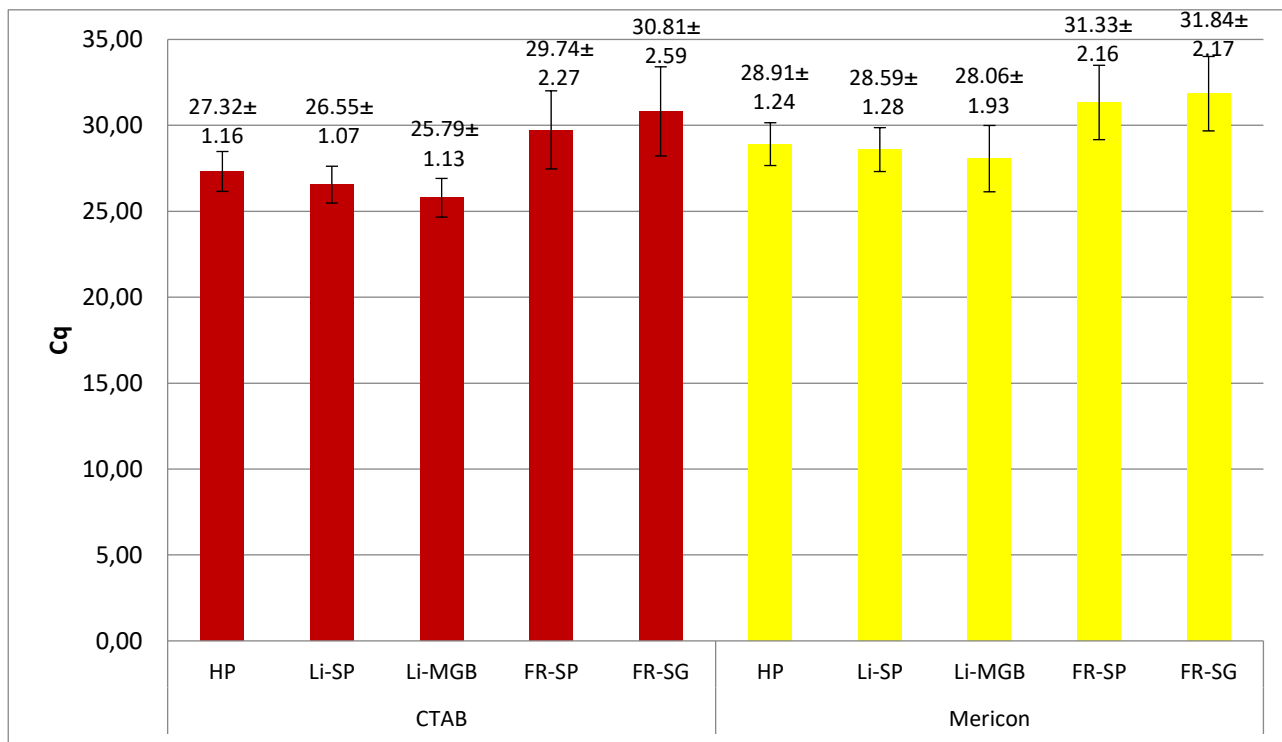
A - 10^6 CFU/ml
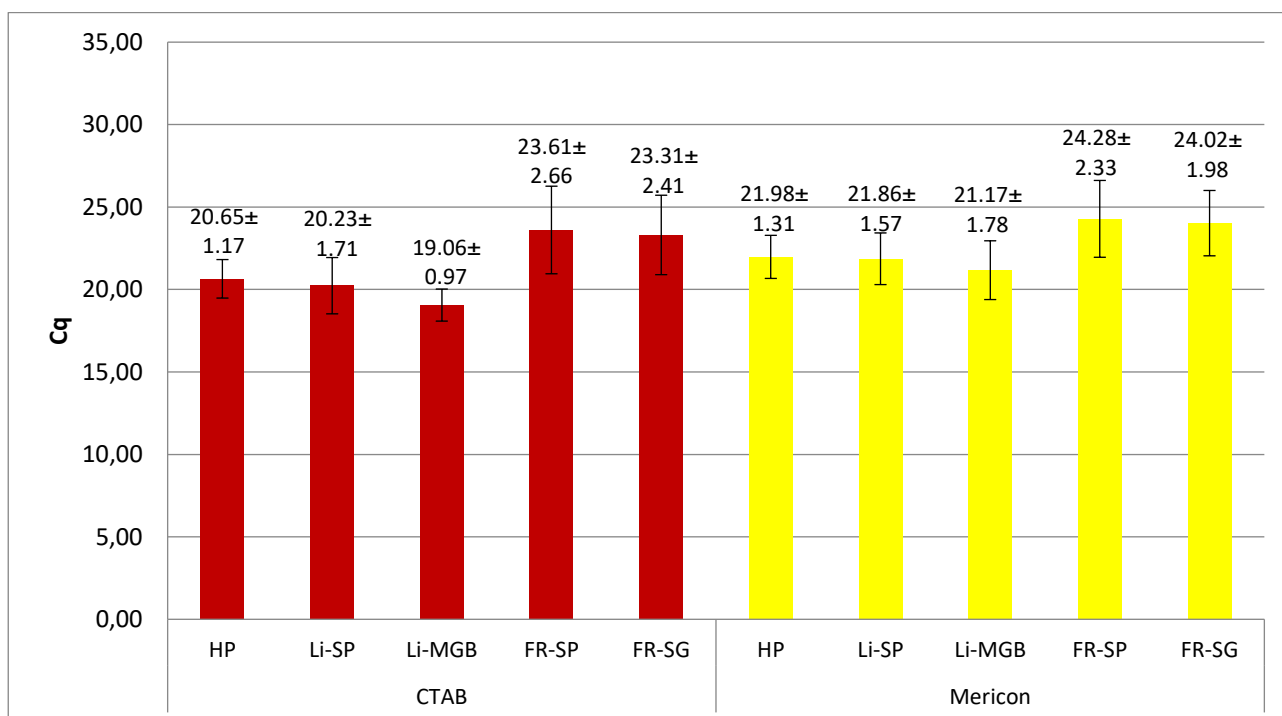
B- 10^5 CFU/ml
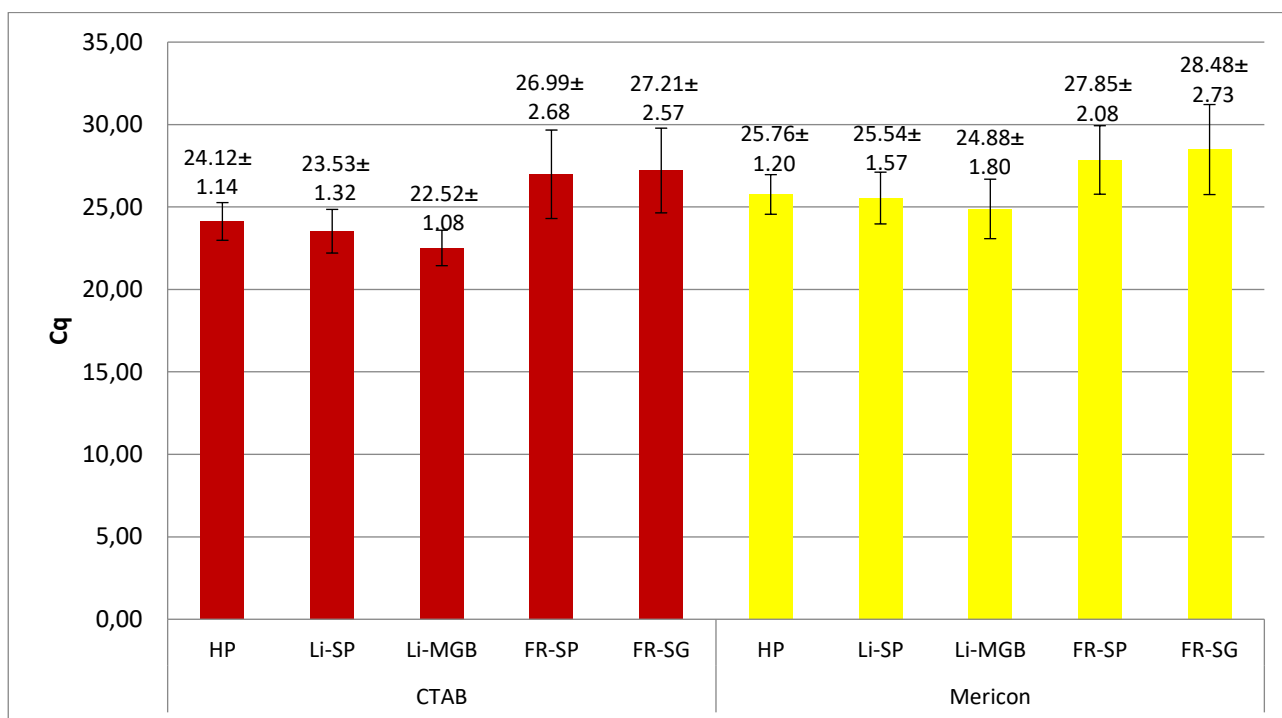
C - 10^4 CFU/ml

6. CONCLUSIONS

The accuracy, repeatability and reproducibility of the qPCR protocols tested in this TPS were in the range of 92-100%, thus the molecular diagnostic tests (including both the plant DNA extraction procedures and the qPCR protocols) showed robustness (PM 7/76) and proved to be suitable for the diagnosis of *X. fastidiosa* in plant materials.

The data summarized in table 10 show that:

- The qPCR protocols HP, FR-SP and FR-SG 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 qPCR protocols Li-SP and Li-MGB, produced the lowest Cq values, but although showed general good performances (values >92%) these were the lowest among the five protocols compared, and determined by the occurrence of PD, higher in the case DNA templates were obtained using the CTAB-based protocol.

- Modifying the TaqMan probe designed by Li et al. (2013) by including the MGB reporter, did not improve any of the performance parameters.

- HP protocol generated Cq values consistently lower than those obtained using both qPCR protocols based on the assay designed by Francis et al (2006). This is in the nature of the assays, the primers of Harper et al (2010) amplify a multi-copy gene, whereas the primers of Francis et al (2006) are designed to amplify a single-copy gene.

Table 10. Summary of the values of the performance criteria obtained for the five different qPCR protocols using extracts recovered using for CTAB-based and DNeasy® mericon™ Food kit (Qiagen) protocols. Percentage below 100% are shaded.

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

7. REFERENCES

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