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RESEARCH PAPERS

On-site detection of *Xylella fastidiosa* in host plants and in "spy insects" using the real-time loop-mediated isothermal amplification method

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Summary. A recent severe outbreak of *Xylella fastidiosa* associated with 'olive quick decline syndrome' (OQDS) was reported in Apulia (Southern Italy). In this study an on-site real-time loop-mediated isothermal amplification (real-time LAMP) was developed for detecting *X. fastidiosa* in host plants and insects. A marked simplification of the DNA extraction procedure was obtained by heating the samples in a portable Smart-Dart device and using an optimized enhancer reaction buffer. The connection to a tablet or Smartphone allowed to visualize the results of the reaction in real time. Compared to PCR and ELISA, with which it showed comparable results in terms of sensitivity and reliability in the *X. fastidiosa* detection, this simplified real-time LAMP procedure proved to be "user friendly", displaying the advantages to be an on-site detection method of easy handling, rapid execution and low cost.

Key words: real-time LAMP, *Philaenus spumarius*, olive quick decline syndrome (OQDS).

Introduction

Xylella fastidiosa (Xf), a xylem-limited fastidious bacterium, is the recognized agent of a large number of diseases including Pierce's disease of grapevine, citrus variegated chlorosis (CVC), plum leaf scald, phony peach, pear leaf scald, alfalfa dwarf and coffee, almond, and oleander leaf scorch (Purcell and Finlay, 1979; Purcell, 1997; Chatterjee *et al.*, 2008).

Some natural Auchenorrhyncha insect vectors (mainly sharpshooter leafhoppers and froghoppers or spittlebugs) contribute to its rapid spread, and adult insects remain infectious throughout their life (Purcell and Finlay, 1979; Redak *et al.*, 2004).

Until few years ago, the presence of this bacterium was confined to the American continent, except for few sporadic reports of interception on commodities in some Asian and European countries (EFSA, 2013). As first report in the European and Mediterranean region, *X. fastidiosa* was associated to the severe olive quick decline syndrome (OQDS) in Lecce province (Apulia, southern Italy), where it is rapidly spreading (Saponari *et al.*, 2013). The Apulian *X. fastidiosa* isolate was identified as a strain of the subspecies *pauca*, to which the name Codiro was assigned (Cariddi *et al.*, 2014; Elbeaino *et al.*, 2014). Besides olive (*Olea europaea*), *Xf*-Codiro strain can infect several other plant species, i.e. *Prunus dulcis*, *P. avium*, *Nerium oleander*, *Vinca minor*, *Polygala myrtifolia*, *Westringia fruticosa*, *Acacia saligna*, *Spartium junceum*, *Rosmarinus officinalis*, *Myrtus communis*, etc. (Saponari *et al.*, 2014; D. Boscia, personal communication). Insects of *Aphrophoridae* (*Philaenus spumarius* L. and *Neophilaenus campestris* Fallén) and *Cicadellidae* (*Euscelis lineolatus* Brullé) proved to harbour *X. fastidiosa*(Elbeaino *et al.*, 2014), but only *P. spumarius* has shown, to date, to be a true vector of it (Locon-

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sole *et al*., 2014). Moreover, for the monitoring of the pathogen in areas that are apparently *Xf*-free, the detection of *X. fastidiosa* in the above insects should be useful since it can reveal the presence of the inoculum before symptom development in plants. For this reason, to these insects we conventionally assigned the name of 'spy insects'. In such a situation, the rapid and reliable detection of the pathogen on a large scale is essential for the promptness application of efficient control measures.

To date, the approaches for the diagnosis of *X. fastidiosa* (EPPO, 2004) include the traditional isolation on culture media, serological [i.e. indirect immuno-fluorescence microscopy, IIF, double antibody sandwich enzyme-linked immunosorbent assay, DAS-ELISA (Janse and Kokoskova, 2009) and direct tissue blot immunoassay, DTBIA (Djelouah *et al*., 2014)], and molecular techniques [i.e. polymerase chain reaction, PCR (Pooler and Hartung, 1995; Rodrigues *et al*., 2003; Huang *et al*., 2006; Huang, 2009), real-time PCR (Schaad *et al*., 2002; Francis *et al*., 2006) and loop-mediated isothermal amplification, LAMP (Harper *et al*., 2010)].

Faced with the extraordinariness of the *Xf*-problem in Italy, actions to isolate in purity the bacterial agent (Cariddi *et al*., 2014; Elbeaino *et al*., 2014) and to identify appropriate diagnostic methods for its detection in plant hosts, particularly in olive, were immediately taken. Diagnostic protocols based on ELISA and conventional PCR, already used in the past for the detection of *X. fastidiosa* from other plant species, were applied to olive samples and compared and validated via an interlaboratory ring-test, during which both procedures proved to be equally effective (Loconsole *et al*., 2014).

Although ELISA and DTBIA are the preferred assays for large scale monitoring of *X. fastidiosa* in olive trees (Djelouah *et al*., 2014; Loconsole *et al*., 2014), PCR remains the best method for the detection of all *X. fastidiosa* subspecies in plant material (Loconsole *et al*., 2014) and insects (Elbeaino *et al*., 2014) due to its high sensitivity and specificity. By contrast, PCR is time-consuming and requires technical skills as well as expensive laboratory's equipment and reagents (Kogovšek *et al*., 2014).

A promising alternative to PCR is the loop-mediated isothermal amplification (LAMP) technique (Notomi *et al*., 2000) for its easy handling and high reliability. The use of six primers provides a greater level of specificity compared to that obtainable with the two primers normally used in PCR. The LAMP reaction, occurring at isothermal conditions, can be performed in a simple heat block or water bath without any sophisticated equipment. In addition, amplification can be directly observed by colorimetric or fluorescent dyes and does not require gel running (Notomi *et al*., 2000). This technique was already successfully applied for the detection of 20 isolates of four different *X. fastidiosa* subspecies (*fastidiosa, multiplex, sandyi* and *pauca*) in different plant species (*V. vinifera, V. rotundifolia* and *Quercus rubra*) and insects (the blue-green sharpshooters *Graphocephala atropunctata*), using standardized extraction protocols (Harper *et al*., 2010).

In consideration of the above mentioned advantages, in this study a new, simpler, rapid and on-site version of real-time LAMP has been applied for the detection of *X. fastidiosa* from olive trees and other plant species and from 'spy insects', in comparison with ELISA and PCR. The reaction was performed in a Smart-Dart device and the results visualized at an electronic device tablet or Smartphone, in form of quantitative curves.

Materials and methods

Sampling of plant material

The setting up of the real-time LAMP assay was carried out using samples coming from the infected area of Lecce, seven of which were olive trees showing OQDS symptoms, and ten were infected plants of the following species (two samples/each): oleander (*Nerium oleander*), cherry (*Prunus avium*), almond (*P. dulcis*), myrtle leaf milkwort (*Polygala myrtifolia*) and golden wreath wattle (*Acacia saligna*). The *Xf*-infection of all selected sources had been previously confirmed by ELISA and PCR (data not shown). Negative controls of the same host species were from plants maintained under screen house at the CIHEAM- Mediterranean Agronomic Institute of Bari.

As for field evaluation, a total of 33 olive samples, four of which from apparently symptomless trees, were collected from four different olive orchards located in the infected area of Lecce province, where symptoms of OQDS were prevalent. Three olive samples collected from the *Xf*-free area in Bari province were used as negative controls. From six olive trees showing few symptoms, two distinct samples

were separately collected from symptomatic and asymptomatic branches.

According to quarantine recommendations, samples were stored at 4°C in hermetically sealed plastic bags until laboratory processing.

Capture of insects

Two olive orchards with high incidence of OQDS symptoms were repeatedly visited during winter and spring in Lecce area for insect captures. Insects were also collected from one olive orchard located in the *Xf*-free area of Bari province. Two different capture methods were adopted: (i) three-five yellow sticky traps per orchard were placed outside the olive canopies; (ii) a sweeping net was used to manually trap the insects in the olive orchards from both olive canopy and ground vegetation. After 20 exposure days, the sticky traps were placed into plastic bags and stored at 4°C. Once in laboratory, all Auchenorrhyncha species were removed from the traps by applying a solvent oil around the insect, and then washed in 95% ethanol and in de-ionized water to remove any oil residue. The insects captured with sweeping net were carefully collected by aspiration directly *in loco*, put in small tubes containing 95% ethanol and brought to the laboratory for identification and testing by both real-time LAMP and PCR assays. The identification of insect species was done according to the procedure described by Elbeaino *et al.* (2014).

DAS-ELISA

Plant samples were tested by DAS-ELISA (Loconsole *et al*., 2014) using specific antibodies to *X. fastidiosa* (LOWE Biochemica GmbH), according to the manufacturer's instructions. Extracts were obtained from petiole and midvein tissues as bulk samples grounded with a semi-automated extractor in the presence of phosphate-buffered saline. Absorbance values were recorded at 405 nm using an automatic microplate reader (Multiskan Ascent, Labsystems). Samples with absorbance readings exceeding three times that of the healthy control were considered positive.

Total nucleic acids extraction

For PCR, total nucleic acids were extracted from plant (mixture of leaf petiole and midvein tissues) and 'spy insect' samples using a CTAB-based extraction buffer (Lin and Walker, 1997). Samples were surface sterilized and homogenized in a 2 mL tube using a semi-automated homogenizer (Omex, Bioreba) in presence of 1.5 mL extraction buffer (20 mM EDTA, 350 mM Sorbitol in 100 mM Tris-HCl, pH 7.5 plus 2.5% PVP and 0.2% β-mercaptoethanol). Tubes were centrifuged at 16,000 g for 20 min and the pellets resuspended in 300 µL of buffer containing 20 mM EDTA, 350 mM Sorbitol in 100 mM Tris–HCl, pH7.0, plus 300 µL of DNA lysis buffer (50 mM EDTA, 2 M NaCl, 2% (w/v) CTAB in 200 mM Tris–HCl, pH 7.5, and 200 µL of 5% sarcosyl), mixed well and incubated at 65°C for 45 min. Extracted DNA was purified using chloroform-isoamyl alcohol (24:1) and precipitated with isopropanol after a 30 min incubation at -20°C. The total DNA preparation was resuspended in 200 µL of 0.5x TE.

In the case of real-time LAMP, a simple nucleic acid extraction procedure was adopted. Plant tissues (thin slices of one-year-old twigs, 1–2 mm of thickness) or single captured 'spy insects' (adults not grinded) were immersed in 1 ml extraction buffer (1% Triton x-100, 20 mM Tris-HCl, 20 mM EDTA) and denaturised at 95°C for 10 min. Due to the non-destructive procedure adopted for nucleic acids extraction from insects, the same insect samples were successively undergone to another, destructive, extraction for PCR test, in order to compare the results of the two assays.

The optimal kind and amount of plant tissue to be used by real-time LAMP was previously determined by comparative trials. Main leaf veins, petioles or twig slices were separately collected from seven infected olive trees and tested. Once established that twig slices gave the best results in terms of speed of reaction, series from 1 to 5 slices per plant were immersed in 200 µL of extraction buffer (four replicates per trial, on a total of 7 olive trees). After denaturation of the extracted TNAs at 95°C for 10 min, different aliquots of the extract (5 µL, 2.5 µL and 1 µL) were tested by real-time LAMP.

PCR assay

For PCR, RST31/RST33 primers (Minsavage *et al.*, 1994), which target the 16S rDNA gene, were used. Reactions were conducted in a final volume of 25 µL with the following final concentrations: 1x FlexiGo Taq Buffer (Promega, Madison, WI, USA), 1 mM $MgCl_2$, 0.2 µM each of forward and reverse primers,

0.2 mM dNTPs, 1U Taq polymerase enzyme and 50 ng of total DNA template. Thermocycling conditions were as follows: 94°C for 4 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, then a final extension of 7 min at 72°C.

Real-time LAMP assay

Real-time LAMP assay was carried out employing primers designed by Harper *et al.* (2010) at *rimM* open reading frame of *X. fastidiosa* 16S rRNA. Differently from the procedure adopted by Harper *et al*. (2010), in this study the DNA extraction was extremely simplified by merely dipping a small slice (1–2 mm) of olive shoot (one year old) in 200 µL of extraction buffer and heating at 95°C for 10 min. The same simple procedure was adopted for testing the insects, for which it was not necessary to grind the whole body in liquid nitrogen and to purify the DNA trough the DNeasy Plant Mini Kit (Qiagen). Another difference with the above paper consisted in the way used to visualize the results, that in this experiment was through a portable electronic device and tablet or Smartphone that produced quantitative curves, whereas in the above mentioned work they were shown by a colorimetric change in the presence of hydroxylnapthal blue dye.

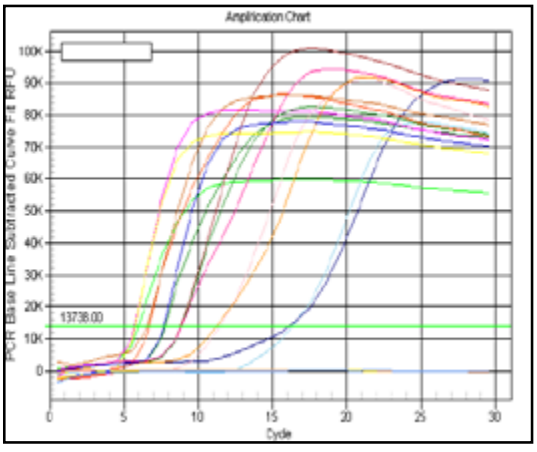
The real-time LAMP assay was performed with a Bio-Rad IQ™ 5 thermocycler (Bio-Rad Laboratories), employing a reaction volume of 25 µL containing 1x Isothermal Master-Mix (OptiGene Ltd. Horsham) with a ds-DNA binding dye, 1 µM of each internal primer (FIP and BIP), 0.1 µM of each external primer (F3 and B3), 0.5 µM of each loop primer (LF and LB), and 5 µL template DNA. The best amplification conditions were established at 65°C (temperature suggested by the company for the best activity of the enzyme) for 20 min, as it was assessed by testing serial dilutions of the *Xf*-DNA (Figure 1). The final emission reaction was measured after a 5 min enzyme inactivation at 80°C, even if this phase can be eliminated since it does not influence the results. For each reaction series, a negative control was included.

The same protocol (20 min amplification at 65°C) was applied in Smart-DART™ (Diagenetix), a portable device equipped with D&A software that can process eight samples each time on-site (Figure 2). In this case a new optimized enhancer reaction buffer (EnbioTaq buffer, from Enbiotech, Palermo, Italy) was used following manufacturer's instructions.

Results and discussion

Real-time LAMP in comparison with ELISA and PCR in plant material

Results by real-time LAMP assay confirmed the detection of *X. fastidiosa* in all infected samples of olive and other host species that had been positive by



Fluor	WellType	Ident	Rep	Ct	Log SQ	SQ	SQ Mean	SQ SD	Ct Mean	Ct SD	Set Point
FAM	A04 NTC	-	1	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	B04 NTC	-	1	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	C04 NTC	-	1	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	A01 Std	10 ng	1	5.97	1.000	1.00E+01	1.00E+01	0.00E+00	5.82	0.134	N/A
FAM	B01 Std	10 ng	1	5.72	1.000	1.00E+01	1.00E+01	0.00E+00	5.82	0.134	N/A
FAM	C01 Std	10 ng	1	5.76	1.000	1.00E+01	1.00E+01	0.00E+00	5.82	0.134	N/A
FAM	D01 Std	1ng	2	6.70	0.000	1.00E+00	1.00E+00	0.00E+00	6.64	0.086	N/A
FAM	F01 Std	1ng	2	6.58	0.000	1.00E+00	1.00E+00	0.00E+00	6.64	0.086	N/A
FAM	A02 Std	0.1ng	3	7.77	-1.000	1.00E-01	1.00E-01	0.00E+00	7.73	0.061	N/A
FAM	H01 Std	0.1ng	3	7.69	-1.000	1.00E-01	1.00E-01	0.00E+00	7.73	0.061	N/A
FAM	B02 Std	0.01ng	4	8.81	-2.000	1.00E-02	1.00E-02	0.00E+00	8.85	0.029	N/A
FAM	C02 Std	0.01ng	4	8.86	-2.000	1.00E-02	1.00E-02	0.00E+00	8.85	0.029	N/A
FAM	D02 Std	0.01ng	4	8.87	-2.000	1.00E-02	1.00E-02	0.00E+00	8.85	0.029	N/A
FAM	E02 Std	1pg	5	11.41	-3.000	1.00E-03	1.00E-03	0.00E+00	11.30	0.165	N/A
FAM	F02 Std	1pg	5	11.18	-3.000	1.00E-03	1.00E-03	0.00E+00	11.30	0.165	N/A
FAM	A03 Std	0.1pg	6	16.31	-4.000	1.00E-04	1.00E-04	0.00E+00	16.18	0.191	N/A
FAM	B03 Std	0.1pg	6	16.04	-4.000	1.00E-04	1.00E-04	0.00E+00	16.18	0.191	N/A
FAM	C03 Std	0.01pg	7	N/A	-5.000	1.00E-05	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	D03 Std	0.01pg	7	N/A	-5.000	1.00E-05	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	E03 Std	0.01pg	7	N/A	-5.000	1.00E-05	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	F03 Std	1fg	8	N/A	-6.000	1.00E-06	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	G03 Std	1fg	8	N/A	-6.000	1.00E-06	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	H03 Std	1fg	8	N/A	-6.000	1.00E-06	0.00E+00	0.00E+00	.00	0.000	N/A

Figure 1. Real-time LAMP detection limit for DNA extracted from *X. fastidiosa*-DNA. A suspension of 10ng/µL DNA was serially diluted in distilled water. All dilution higher than 0.01pg react positively in a maximum of 16.30 min. Xf- dilution less than 0.1pg and negative samples showed linear curves. Dilutions were tested in duplicate on a real-time IQ5 Bio-Rad device.

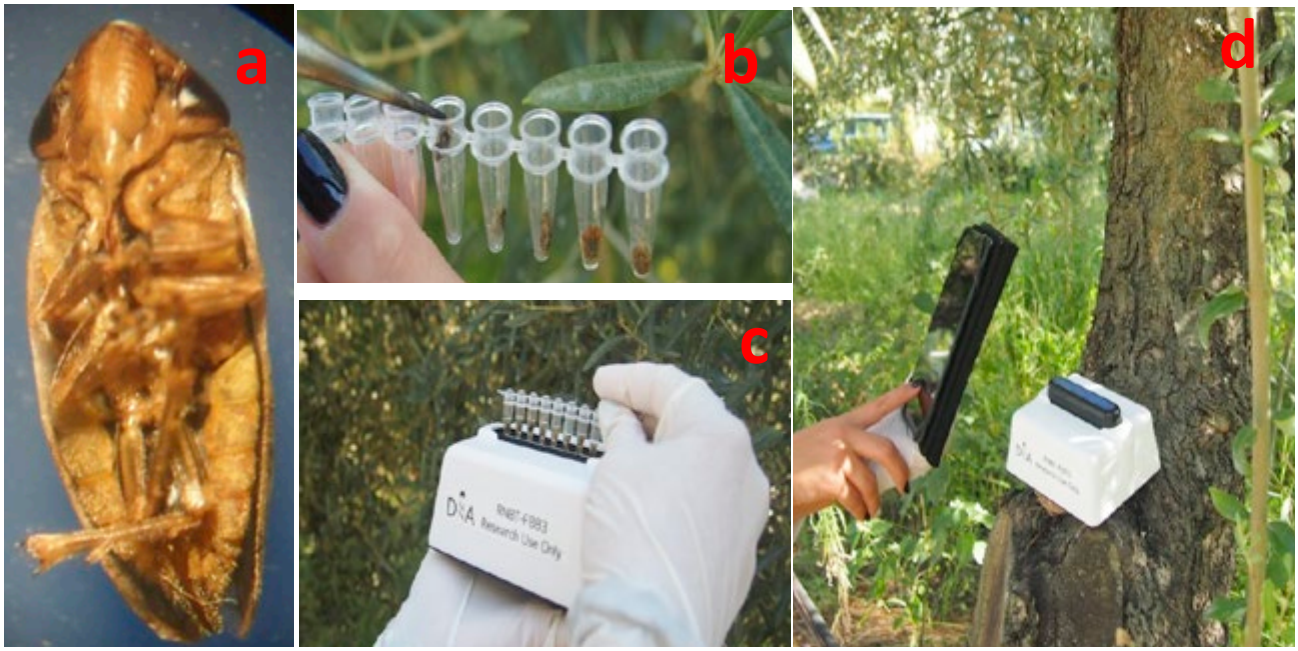


Figure 2. Overview of the on-site detection of *X. fastidiosa* in 'spy insects' using real-time LAMP in SMART DART device directly in the field: a) adult of *P. spumarius*; b) collected insects in 0.2 mL extraction buffer; c) DNA extraction; d) real-time LAMP results acquisition by the tablet.

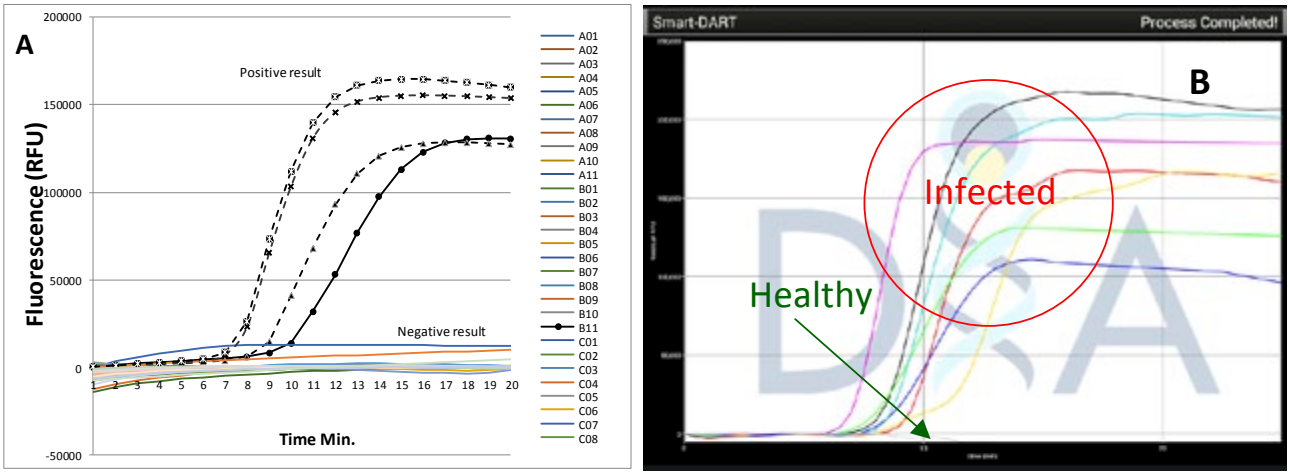


Figure 3. *Xf*-infected olive samples showing positive results in real-time LAMP with 20 min reaction time in both real-time IQ5 Bio-Rad device (A), and in Smart-Dart device equipped with D&A software (B). *Xf*-negative samples showed linear curves.

ELISA and PCR in previous tests, and the absence of the infection in the negative controls. Stem section samples gave a faster reaction than leaf petiole and midvein tissues (data not shown).

Results 100% matching to those from laboratory were obtained using Smart-Dart device directly in the field, but with the undoubted advantages of being on-site, simple and rapid testing (Figure 3).

As for the field evaluation, results of Table 1 show that *X. fastidiosa* was not detected in all olive samples from OQDS symptomatic trees. In particular, it was detected only in 20 out of 26 symptomatic olive trees when tested by DAS-ELISA and in 22 samples when tested by PCR and real-time LAMP (Table 1). Conversely, all samples collected from asymptomatic olive trees were negative in ELISA, PCR and real-time LAMP. According to these results, real-time LAMP proved the same efficacy of PCR in the detection of *X. fastidiosa* in olive trees.

The negative results from the four symptomatic samples obtained by all the three diagnostic techniques could be attributed to other pathogens associated to OQDS (Nigro *et al.*, 2014) or to the uneven distribution of *X. fastidiosa* in the olive tree (Djelouah *et al.*, 2014).

Regarding to the six olive trees showing a sectorial distribution of OQDS symptoms on the canopy, the absence of *X. fastidiosa* in the asymptomatic branches was assessed in half of the sampled trees using the three diagnostic techniques. Conversely, in the symptomatic branches the infection was revealed in the six tested trees by PCR and real-time LAMP and only in five trees by ELISA (Table 1).

Real-time LAMP was able to detect the bacterium also in the other plant species tested: oleander (*Nerium oleander*), cherry (*Prunus avium*), almond (*P. dulcis*), myrtle leaf milkwort (*Polygala myrtifolia*) and golden wreath wattle (*Acacia saligna*).

In real time LAMP, the best results for the DNA extraction were obtained when only one olive slice of one-year-old twig (1–2 mm of thickness) was dipped in 200 µL of extraction buffer. Using 2 or 3 olive slices the amplification time suffered some delays, while with 4-5 olive slices there were also some false negatives (Table 3). Five µL of the extraction template represented the optimal volume to be used in real-time LAMP reaction with an amplification time of about 16 min. By reducing the volume of the template the reaction developed more slowly or gave some false negative results (Table 3).

A consistent improvement in the speed and reliability of *Xf*-detection has been obtained by using the new enhancer reaction buffer (EnbioTaq buffer, Enbiotech, Italy) (Figure 4).

Real-time LAMP in comparison with PCR in *X. fastidiosa* detection from 'spy insects'

During a four-month period in the winter and spring 2014, a total of 236 adult specimens of *Auche-*

Table 1. Detection of *X. fastidiosa* from symptomatic and 'healthy' olive plants, using real-time LAMP, PCR and ELISA.

	Site	ELISA	PCR	Symptoms	Real-time LAMP
1	Bari	-	-	absent	-
2	Bari	-	-	absent	-
3	Bari	-	-	absent	-
4	Lecce 1	-	-	absent	-
5	Lecce 1	-	-	absent	-
6	Lecce 1	-	-	absent	-
7	Lecce 1	-	-	absent	-
8	Lecce 1	+/+	+/+	present/absent	+/+
9	Lecce 1	-/-	+/-	present/absent	+/-
10	Lecce 1	+/+	+/+	present/absent	+/+
11	Lecce 1	+/-	+/-	present/absent	+/-
12	Lecce 1	+/+	+/+	present/absent	+/+
13	Lecce 1	+/-	+/-	present/absent	+/-
14	Lecce 1	+	+	present	+
15	Lecce 1	+	+	present	+
16	Lecce 2	+	+	present	+
17	Lecce 2	+	+	present	+
18	Lecce 2	-	-	present	-
19	Lecce 2	+	+	present	+
20	Lecce 2	+	+	present	+
21	Lecce 2	+	+	present	+
22	Lecce 2	+	+	present	+
23	Lecce 2	-	+	present	+
24	Lecce 3	+	+	present	+
25	Lecce 3	+	+	present	+
26	Lecce 3	-	-	present	-
27	Lecce 3	+	+	present	+
28	Lecce 3	+	+	present	+
29	Lecce 4	+	+	present	+
30	Lecce 4	-	-	present	-
31	Lecce 4	+	+	present	+
32	Lecce 4	+	+	present	+
33	Lecce 4	-	-	present	-
	No. Total infected	20	22		22

Table 2. Number of Auchenorrhyncha insects (males and females) captured from two *Xf*-infected olive orchards and one non-infected olive orchard.

Area	Olive grove	*P. spumarius*		*N. campestris*		*E. lineolatus*		Infected/ captured
		♂	♀	♂	♀	♂	♀	
Xf-contaminated	1	3	8	5	9	33	13	17/71
	2	24	26	13	11	2	1	11/77
	Infected/ captured	4/27	5/34	4/18	5/20	5/35	3/14	28/148
Xf-free	3	11	15	7	6	25	22	0/86
	Infected/ captured	0/11	0/15	0/7	0/6	0/25	0/22	0/86

Table 3. Detection of *X. fastidiosa* from olive plants by real-time LAMP. Infected tissues for DNA extraction were thin slices of one-year-old olive twigs, 1-2 mm of thickness, from a number of slices ranging from 1 to 5 for each extraction.

Sample	Number of cuttings used for DNA extraction	RT-LAMP time of amplification min	
		Replicate 1	Replicate 2
Olive XF Positive	1	18.67	18.38
Olive XF Positive	2	20.19	20.74
Olive XF Positive	3	23.21	23.86
Olive XF Positive	4	N/A	N/A
Olive XF Positive	5	N/A	N/A
Olive XF Negative	1	N/A	N/A
Olive XF Negative	2	N/A	N/A
Olive XF Negative	3	N/A	N/A
Olive XF Negative	4	N/A	N/A
Olive XF Negative	5	N/A	N/A
Buffer Control	0	N/A	N/A
Buffer Control	0	N/A	N/A
Positive Control XF 10ng/μl	/	12.44	12.27
Water Control	/	N/A	N/A

norrhyncha were captured and comparatively tested by PCR and real-time LAMP. In particular, 148 individuals were captured from two olive orchards severely affected by OQDS, whereas the remaining 86 insects were from an asymptomatic olive orchard located in the *Xf*-free area. Results showed no difference between the two diagnostic techniques in detecting *X. fastidiosa* in the 28 insects that were from the *Xf*-contaminated groves (18.9% infected insects); none of the 86 insects collected from *Xf*-free area was found positive.

Looking at the different insect species analysed in this study, the highest infection levels were found within *Neophilaenus campestris* (9/38; 23.7%), followed by *Euscelis lineolatus* (8/49; 16.3%) and *Philaenus spumarius* (9/61; 14.7%). No significant difference in the infection rate was found correlated to the sex of insects (Table 2).

In the detection of *X. fastidiosa*, a slight improvement in terms of speed of reaction was obtained by using the insect heads as sample for testing (data not shown).

Conclusions

Results obtained in this study have shown the remarkable potential of the real-time LAMP in the detection of *X. fastidiosa* from plant materials and 'spy insects'. Worth mentioning is the first application of this technique in the detection of *X. fastidiosa* in olive trees and in other plant hosts, i.e. cherry, myrtle

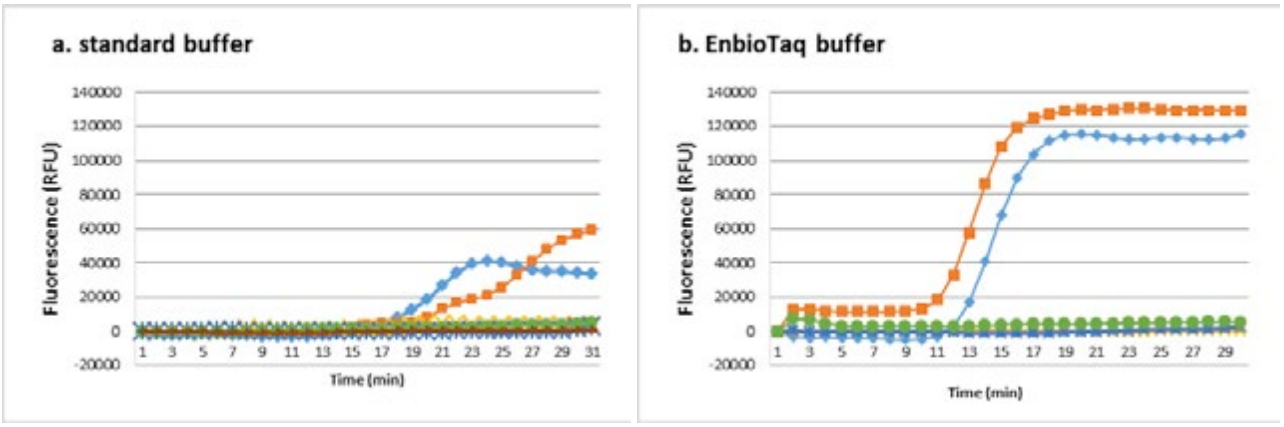


Figure 4. Comparison of real-time LAMP reaction in detecting *Xf* with the standard buffer (a) and with the EnbioTaq buffer (b). In both trials DNA was extracted using the rapid extraction method. Samples were tested in SMART-DART device.

leaf milkwort and golden wreath wattle, as well as in insect species, which may harbour the pathogen in Apulian infected areas (*P. spumarius*, *N. campestris* and *E. lineolatus*).

The technique was improved by developing a rapid DNA extraction technique from plant material using single twig slices in 200 µL of extraction buffer, and non-destructive procedures for the DNA extraction from insects. A further amelioration in the bacterium detection was obtained by using the "EnbioTaq buffer" that enhances and stabilizes the LAMP enzyme's activity, speeds up the reaction rate and eliminates the appearance of false positives. The same technique showed the additional and not negligible advantage to allow the use of the same tested insect for further identification and investigations. Real-time LAMP showed the same results of PCR in assessing the presence of the bacterium in plants and insects; interestingly, it proved to be more reliable than ELISA using olive plant material. Compared to ELISA and PCR, the real-time LAMP is easier, faster and cheaper. In fact, it requires approximately 20 min, a time even shorter than that for the detection of other pathogens (i.e. *Ca* Phytoplasma vitis) in previous works (Kogovšek *et al*., 2014). Since the test may be conducted on-site, an additional advantage of this technique is that it avoids the need to move plant material or insects samples from the infectious sites to the laboratory, thus limiting the risk of pathogen dissemination in *Xf*-free areas.

The rate of *Xf*-positive insects detected in this study is significantly lower than that found by Elbeaino *et al.* (2014) in the same infected areas one year before (23.7% *vs* 77.8% with *N. campestris*, 14.7% *vs* 40% with *P. spumarius* and 16.3% *vs* 34.8% with *E. lineolatus*). In the evaluation of these differences, it must be considered that the periods of insect captures were diverse (from October to December in the first study and from January to May in the second one). However, further investigations on population dynamics of insects harbouring *X. fastidiosa* should be carried out to better analyse this aspect.

Hence, real-time LAMP displays to be a very promising method for the large-scale detection of *X. fastidiosa* in plant material of several host species and in 'spy insects', as demonstrated in this study.

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