Evaluation of Serological and Molecular Method for Detection of *Xylella Fastidiosa* Associated with Olive Quick Decline

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Abstract

Sampling was carried out monthly for five months (March to July) on different host plants known to be infected by *X. fastidiosa* in previous surveys in order to evaluate the efficiency of a patented "sap extraction method" for favoring the detection of *X. fastidiosa*. Regarding the bacterium isolation, "sap extraction" showed to be more performing than conventional ones for olive, *N. oleander* and *P. myrtifolia*. The study confirmed that the best period for the bacterium isolation was from the end of spring to early summer. Regarding the use of Sap extraction for diagnostic purposes, slight differences were related to the type of explants used in ELISA and RT-LAMP. Likely, the surface sterilization of plant material may decrease the concentration of the bacterium in Sap extraction. In ELISA, Sap extraction looks less sensitive and has a slower reaction kinetics compared to conventional extraction method, while RT-LAMP applied with Sap Extraction looks better than conventional ones. Concerning RT-LAMP, the numbers of comparative tests should be increased. From the preliminary results of this study, it would appear that the "sap extraction method" is more effective than the conventional one for the isolation of different fungal species.

Key Words: Xylella Fastidiosa, Olive Quick Decline, Cultivated Olive.

DOI Number: 10.14704/nq.2022.20.1.NQ22261 **NeuroQuantology 2022; 20(1):229-237**

Introduction

Cultivated olive worldwide due to its fruit that can consume or for oil production that can be obtained from fruits, therefore it has high economic value, according to FAO organization about 10648187 ha of areas cultivated with olive globally with production quantity 19270115 tons, in Italy the areas for cultured olive are 1165562 ha and production 2092175 tons in 2016 (FAO, 2018b), (FAO, 2018a). Olive tree and its fruits are such great suitable hosts to many pests like insects, mites, pathogens. Among these pathogenic microorganisms include bacterium which invade xylem systems (vessels, treachery elements and parenchyma cells) and colonize lumen of bronchial system then cause clog them by presence of pathogens like aggregation of cells inside lumen of vessels and biofilm formation in case of *Xylella fastidiosa* bacterium. The presence of this bacterium was associated with a disaster disease on olive called as "Olive Quick Decline Syndrome" (OQDS). In Apulia, *X. fastidiosa* subsp. *pauca* ST53 De Donno (Saponari et al., 2017; Saponari, Boscia, Nigro, & Martelli, 2013) associated with OQDS. Apulia, i.e. *Prunus dulcis, Nerium oleander, Acacia saligna, Catharanthus roseus, Spartium junceum, Rhamnus alaternus, Rosmarinus officinalis , Myrtus communis, Vinca minor, Euphorbia terracina, Asparagus acutifolus, Grevillea juniperina, Westringia glabra, Cistus creticus, Laurus nobilis, Dodonaea viscosa purpurea, Lavandula angustifolia, Myoporum insulare* (EFSA, 2016b; European-commission, 2018; Giampetruzzi et al., 2017; M Saponari et al., 2014).

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Received: 20 December 2021 **Accepted:** 10 January 2022

More than 31 different plant species are till now reported to be hosts of *X. fastidiosa* De Donno in Successful detection of the pathogen will enhance the search of the interactivity between *X.f* and host plants. The tissue preparation by the conventional extraction of endophytic pathogen methods from plant tissue usually come with several plant components (e.g. polyphenols, plant metabolites) which can be inhibitors for (Bextine & Miller, 2004; Schrader et al., 2012) (DAS – ELISA, PCR) tests or by placed small pieces and placed on the agar media.

The plant Sap extraction method (CIHEAM/MAIB Patent number WO2017017555A1) is a promising technique for detecting pathogens in vascular plant Sap (xylem, phloem) fluid without large amounts of plant material. The extraction method, compared to conventional extraction techniques, gave better results in terms of sensitivity, accuracy (no false negatives), costs, time of execution and skills for the diagnosis of *X. fastidiosa* from olive trees using different detection methods: isolation, ELISA, DTBIA, PCR, Real-Time PCR, and Real-Time LAMP. The use of different plant host species and matrices (branch, leaf peduncle, etc.) is still under evaluation (Yaseen et al., 2017). Results obtained by using DTBIA prove that is a reliable technique for detecting *X. fastidiosa* in olive trees, showing characteristics of sensitivity and reliability similar to ELISA (Djelouah, Frasheri, Valentini, D'Onghia, & Digiaro, 2014).

Molecular diagnosis methods including Polymerase chain reaction PCR (Minsavage, Thompson, Hopkins, Leite, & Stall, 1994), Loop-Mediated Isothermal Amplification (LAMP) (Nagamine, Hase, & Notomi, 2002) are fine occurate methodes for this pathogen (Tomlinson & Boonham, 2008).

The aim of this study, therefor, was to evaluate patented method and the traditional sap extraction methods for the diagnosis of *Xylella fastidiosa* (De Donno strain) in olive and other host species. *X. f* has potential role of endophytic pathogen associated with OQDS (Olive Quick Decline Syndrome). To this aim comparative detection trials were conducted using different plant host species and matrices, with main detection methods being: isolation, ELISA, DTBIA, PCR and Real-time LAMP (Yaseen et al., 2017).

Material and Methods

Sampling

Infected plant samples were collected from different sites within Salento distract in Apulia region south of Italy. Plant materials, which were collected from orchards and public gardens, contained: (branches/cuttings bearing the symptoms observed on the plant(s) and containing at least 10 to 25 leaves depending on the leaf size). From symptomatic plant(s), the plant material was collected from the area close to the symptoms excluding the necrotic tissues. The plant samples were shacked very well and put in plastic bags, labeled and put in cool box EPPO Bulletin PM 7/24 (2) (2016) and transferred to the laboratory. The period of sampling it had been performed from March until July. Host plant species which were sampled belong to different botanical families (herbaceous, woody). *Olea europaea*, *Myrtus communis*, *Prunus dulcis*, *Rosmarinus officinalis*, *Acacia saligna*, *Nerium oleander, Rhamnus alaternus*, *Polygala myrtifolia*, *Asparagus* spp., *Lavandula* spp., *Cistus* spp., *Westringia fruticosa*, *Erigeron* spp. and *Spartium junceum.*

Preparation by Conventional Extraction Techniques

Midveins, petioles were recovered from mature leaves with minced stem tissues were used. Samples included 4-8 twigs per tree, 5-6 internode long, from which 2-3 basal leaves were excised and pooled to recover a total of 8-10 midveins. Petioles, midveins and minced stem tissues weighting approximately 0.5 g in total were then transferred to extraction bags with a plastic intermediate net layer and homogenized using the semi-automated Homex device. Tissue extracts obtained after homogenization of leaf tissues in extraction buffer (1:10) were transferred into micro-centrifuge tubes (1.5 ml) prior to loading ELISA plates and Real-Time LAMP tubes (Loconsole et al., 2014). As for sap extraction, twigs of appropriated size (around 10 cm) long from each sample were cut and surface sterilized by using sodium hypochlorite 5% (1min), ethanol 70% (2min) and rinsed in sterilized water (3x per 3min). The extraction method is based on a low-pressure application of the extraction liquid through the plant vessels at one terminal of the twig, for this reason, a syringe with a filter and a sterilized rubber tube for the section size of each twig were attached with parafilm to. then push gently the extraction liquid through the plant vessels, containing 5ml of 1x PBS buffer [NaCl 8 g, KCl 0.2 g, Na_2HPO_4•12 H_2O 2.9g, KH_2PO_4 0.2 g in distilled water up to one litter, pH 7.2 before autoclaving] ("EPPO Bulletin PM 7/24 (2)", 2016). Or sterile distilled water .The collected sap in (2ml eppendorf) from the other terminal of

the twigs was used for diagnosis purposes (isolation, DAS – ELISA, PCR, RT-LAMP) (Yaseen et al., 2017).

Isolation of X. fastidiosa

The bacterium *X. fastidiosa* is very difficult to isolate and grow in axenic culture even from symptomatic plants. The specific media used for culturing and growth of *X. fastidiosa* (PWG, BCYE, PD2) are described in Annex 2, 3 and 4. Moreover, two different methods were used for the bacterium isolation as further described.

Printing Method

Stems or petioles were directly printed on the surface of the plate. This way of isolation has been fully described by CNR – IPSP, and UNIBA – DISSPA laboratories in Bari. At first, several centimeters were cut out from both sides of the twig in order to make suitable size section for isolation and remove ethanol from the printing surface. Twigs were taken with pliers and clenched until cut zone moisten. Then prints were made. Then plates were closed with parafilm and put at 28±1°C for incubation.

Patent Plant Sap Extraction

About 100 µl of plant Sap extracted by the patented method was pipetted from the eppendorf onto plates containing BCYE, PWG, and PD2 media. Then the cover of petridish was closed. The plates were then tightened with parafilm and incubated at 28 ±1°C.

Purification of Primary Colonies

The plates were observed daily until the appearance of colonies similar to those of *X. fastidiosa*. The plates containing other microorganisms were discarded after transfer of *Xylella*-like colonies. These colonies were transferred to a new fresh growth medium to obtain pure culture .Purified colonies were taken and suspended in glycerol for preservation at -80˚C.

Detection Methods

Serological Tests

I. Antibody Sandwich Enzyme-linked Immunosorbent Assay

Antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was performed according to the protocol described by (Loconsole et al., 2014) using Agritest commercial kit. Elisa plate was coated with capture antibody (IgG) diluted at a ratio 1:500 in coating buffer. After incubation at 37˚C for 4 hours, wells were washed 3 times with washing buffer. Sap obtained by both extraction methods was used as antigen, and the plate was incubated overnight at 4˚C. After washing the conjugated antibody specific for *X. fastidiosa* was added and the plate was incubated at 37˚C for 4 hours. A solution of P–Nitrophenyl Phosphate (0.01mg/ml) in substrate buffer was added, and the colorimetric reaction was recorded after two hours at 405nm using a plate reader (Bio-Tek KC4, v.3.1). Samples with absorbance values above three times the average absorbance values of the known negative samples were considered positive for *X. fastidiosa*

II. Direct Tissue Blot Immuno Assay (DTBIA)

Fresh cross-sections of leaf petioles and/or young twigs (2 mm in diameter) apical shoots excluded, were printed onto Protran (Sigma-Aldrich) nitrocellulose membrane of 0.45µm pore size. Each type of sample was printed twice and the membranes were left to dry for 20-30min at room temperature. After that, they were covered with a blocking solution of 1% BSA on a shaker for one hour and washed with PBS containing 0.05% tween 20 (3x per 3min). Then membranes were exposed for two hours at room temperature to alkaline phosphatase-conjugated polyclonal antibodies to *X. fastidiosa* (Agritest commercial kit) at a dilution of 1:500 in conjugate buffer followed by tree washing as described above. Membranes were then stained by immersion in a solution of Stigma Fast™ BCIP-NBT in distilled water and incubated at room temperature until a purple-violet color appeared within the spotted sections. The reaction was stopped by washing with tap water. After drying at room temperature, the membranes were observed under a low power magnification lens (x10-x20) (Djelouah et al., 2014).

DNA Extraction and Polymerase Chain Reaction (PCR)

DNA material from plant samples was extracted using the CTAB method, initially described by (Murray & Thompson, 1980). Leaves and petioles were taken from samples and homogenized in CTAB extraction buffer (Hexadecyl trimethyl-ammonium bromide) (Annex 1) (2ml CTAB for Homex and add 3ml to homogenize with fingers afterward). After homogenization, 1 ml of

suspension was transferred to Eppendorf and incubated for 30 min on 65˚C. The solution was centrifuged for 5 min at 10 000 rpm and supernatant (500 µl) was transferred into a new tube. Samples were extracted with chloroform-isoamyl alcohol (24:1, 500µl) and centrifuged 5 min at 10 000 rpm. Again, the supernatant was transferred to a new tube (400 µl) and diluted with cold isopropanol (400 µl). This dilution was gently mixed and stored at - 20˚ C. Experiment can be continued either 2 hours after storing or the next day. Next step requires centrifuging for 20 min at 13 000 rpm. The supernatant was discarded, whereas residue washed off with 1 ml of ethanol 70%. One more centrifuge was needed, 10 min at 13 000 rpm. After removing ethanol, eppendorf were air – dried. Eventually, remained pellets were suspended adding 100 ml of sterile water per tube and mixing with the vortex. DNA material prepared in this way can be used immediately, or stored at -20˚ C.

Amplification of obtained DNA was done based on a protocol designed by (Minsavage et al., 1994). The test was performed by PCR using RST31 and RST33 primers. These primers are specific, targeting RNA polymerase sigma – 70-factor genomic region, composed of 733 bp. RST31 and RST33, forward and reverse primers are unreliable for detection of genetically various strains of *X. fastidiosa,* however high accuracy for subspecies pauca has been confirmed (Harper, Ward, & Clover, 2010). All the reactions were visualized after electrophoresis in 1.2 % TAE agarose gel.

Real-time LAMP

The protocol was based on the kit and device designed by Enbiotech Srl (Italy). Real-time LAMP assay was performed employing primers designed by (Harper et al., 2010). 5µl of crude plant Sap obtained by homogenization of small pieces of midribs and petioles in ELISA-Extraction buffer (1:10 w: v) and 5µl by CIHEAM patent plant Sap extraction method were transferred in the tube containing the LAMP extraction buffer, gently vortexed, and incubated for 10min at 65°C. The samples were then heated for 10 minutes at 65°C. Aliquots of the LAMP MIX were prepared by adding in each tube labeled “primer MIX”, 22.5 µl of LAMP MIX, 30 µl of mineral oil and finally 2.5 µl of denatured sample (Yaseen et al., 2015) followed by gently vortex and briefly centrifugation. The amplification program used on the device included one step at 65°C for 25min. Amplification curves were observed in case of a positive sample. No amplification curve indicated a negative sample. The fluorescence units were shown on the Y-axis and the time to amplification on the X-axis of the amplification plot. The device is equipped with software that based on the amplification curves assigns positive/negative reactions to wells with unknown samples.

Results and Discussion

Result of March

The first sampling was carried out in March. 27 samples from host plants were selected in the infected area of Gallipoli, of which 10 were known to be infected by *X. fastidiosa* since 2017. The isolation was carried only on six of them using the two methods of isolation described in material and methods (2.3.1 - 2.3.2). The six plants were still positive in PCR, and they were used for isolation. All 27 samples were also tested by ELISA using only the conventional extraction method (Table 1).

Table 1. Detection of X. fastidiosa infection at 14 March, 2018

ID	Species	PCR	Isolation		ELISA CONV
			SAP	print	
A ID1	*Lavandula* spp	no test	no test	no test	+
A ID2	*Cistus* spp	no test	no test	no test	+
A ID3	*Rosmarinus officinalis*	no test	no test	no test	+
A ID4	*Rosmarinus officinalis*	no test	no test	no test	+
A ID5	*Rosmarinus officinalis*	no test	no test	no test	+
A ID6	*Rosmarinus officinalis*	no test	no test	no test	-
A ID7	*Olea europaea*	+	+	+	+
A ID8	*Myrtus communis*	+	-	-	-
A ID9	*Myrtus communis*	no test	no test	no test	+
A ID10	*Asparagus* spp	no test	no test	no test	+
A ID11	*Asparagus* spp	no test	no test	no test	-
A ID12	*Asparagus* spp	no test	no test	no test	-
A ID13	*Prunus dulcis*	no test	no test	no test	-
A ID14	*Rhamnus alaternus*	+	-	-	+
A ID15	*Rhamnus alaternus*	no test	no test	no test	+
A ID16	*Myrtus communis*	no test	no test	no test	-
A ID17	*Prunus dulcis*	+	-	-	-
A ID18	*Prunus dulcis*	no test	no test	no test	-
A ID19	*Nerium oleander*	+	+	+	+
A ID20	*Erigeron* spp	no test	no test	no test	-
A ID21	*Nerium oleander*	no test	no test	no test	+
A ID22	*Acacia saligna*	no test	no test	no test	+
A ID23	*Acacia saligna*	no test	no test	no test	-
A ID24	*Myrtus communis*	no test	no test	no test	-
A ID25	*Acacia saligna*	no test	no test	no test	-
A ID26	*Polygala myrtifolia*	no test	no test	no test	+
A ID27	*Polygala myrtifolia*	+	+	+	+
	Total positive	**6**	**3**	**3**	**15**

The data show that the isolation of *X. fastidiosa* occurred only from three plants out of 6 (AID7, AID~~8~~19, and AID27) by both methods of isolation, and that these three plants tested positive by both ELISA and PCR, while the other three were positive only by PCR. ELISA test detected 15 positive plants out of the 27 tested. Regarding fungal isolation, no colonies were obtained from any sample plated on MEA and PDA media by both conventional and Sap extraction methods.

Result of April

During April the sampling was carried out twice: on 5 and 18 April, respectively. A total of 49 samples from different host plants were collected. The first sampling was carried out, only on olive trees for a total of 20 plants. All samples were assayed by PCR, DTBIA, and ELISA using both extraction methods without surface sterilization of plant material (Table2).

The results show (Table2) that 18/20 plants were tested positive by PCR, whereas 15/20 were positive by ELISA conventional extraction. The ELISA SAP extraction detected 16 samples as positives out of the 20 ones tested, of which, two more positive samples compared to conventional ELISA, while 2 positives samples were negative with ELISA SAP extraction. The PCR detected two more positive samples then ELISA performed with both extraction techniques. However, all the samples tested positive by DTBIA method. No isolation of any pathogen, including *X. fastidiosa* was carried on these samples.

During the second sampling of April, 13 plants known to be *X. fastidiosa* positive from the previous survey were collected and tested by serological and molecular assays.

All plants were tested positive to *X. fastidiosa* by PCR, while colonies of *X. fastidiosa* were obtained on BCYE media only from four plants of different species (*Nerium oleander Rhamnus alaternus, Myrtus communis, and Olea europaea*) using both methods of isolation. Positive reactions were obtained for 9 out of 13 samples by ELISA conventional extraction and DTBIA, while only two of them tested positive by ELISA-SAP extraction.

No fungal colonies were obtained in any sample inoculated on MEA and PDA media by conventional methods, whereas colonies of *Alternaria* spp. were obtained by Sap extraction.

Table 2. Detection of X. fastidiosa infection at 5 April, 2018

ID	Species	PCR	ELISA		DTBIA
			CONV	SAP	
C2ID1	*Olea europaea*	+	+	+	+
C2ID5	*Olea europaea*	+	+	+	+
C2ID16	*Olea europaea*	+	+	+	+
C2ID14	*Olea europaea*	+	+	+	+
C2ID30	*Olea europaea*	+	+	-	+
C2ID43	*Olea europaea*	+	+	+	+
C2ID56	*Olea europaea*	+	+	+	+
C2ID63	*Olea europaea*	+	+	+	+
C2ID77	*Olea europaea*	+	-	+	+
C2ID98	*Olea europaea*	+	+	-	+
A6ID170	*Olea europaea*	+	+	+	+
A6ID158	*Olea europaea*	-	-	-	+
A6ID138	*Olea europaea*	+	+	+	+
A6ID113	*Olea europaea*	+	+	+	+
A6ID100	*Olea europaea*	+	+	+	+
A6ID69	*Olea europaea*	+	+	+	+
A6ID26	*Olea europaea*	-	-	-	+
A6ID13	*Olea europaea*	+	-	+	+
A6ID9	*Olea europaea*	+	+	+	+
A6ID10	*Olea europaea*	+	-	-	+
	Total positive	**18**	**15**	**16**	**20**

Table 3. Detection of X. fastidiosa infection at 18 April, 2018

ID	Species	PCR	Isolation		DTBIA	ELISA	
			SAP	print		CONV	SAP
BID1	*Spartium junceum*	+	-	-	-	-	-
BID3	*Prunus dulcis*	+	-	-	-	+	-
BID6	*Nerium oleander*	+	+	+	+	+	-
BID7	*Rhamnus alaternus*	+	+	+	+	+	-
BID8	*Rhamnus alaternus*	+	-	-	+	+	-
BID9	*Prunus dulcis*	+	-	-	-	-	-
BID12	*Acacia saligna*	+	-	-	+	-	-
BID14	*Polygala myrtifolia*	+	-	-	+	+	+
BID19	*Nerium oleander*	+	-	-	+	+	-
BID20	*Myrtus communis*	+	+	+	+	+	-
BID27	*Rosmarinus officinalis*	+	-	-	-	-	-
BID28	*Olea europaea*	+	+	+	+	+	+
BID29	*Olea europaea*	+	-	-	+	+	-
	Total positive	**13**	**4**	**4**	**9**	**9**	**2**

Result of May

Sampling was carried out on 8th of May. A total of 20 olive plants were collected and plant Sap was obtained extracted by both extraction methods. Only one sample was negative out of 20 tested by PCR, RT-LAMP, DAS- ELISA (Table 4).

Table 4. Detection of X. fastidiosa infection at 8 May, 2018

ID	Species	PCR	Isolation		ELISA		RT-LAMP	
			SAP	Print	CONV	SAP	CONV	SAP
CID1	*Olea europaea*	+	-	-	-	-	-	-
CID2	*Olea europaea*	-	-	-	-	-	-	-
CID3	*Olea europaea*	+	-	+	+	+	+	+
CID4	*Olea europaea*	+	-	-	+	+	+	+
CID5	*Olea europaea*	+	-	+	+	+	+	+
CID6	*Olea europaea*	+	-	-	+	-	+	-
CID7	*Olea europaea*	+	-	-	+	-	+	+
CID8	*Olea europaea*	+	+	-	+	-	+	+
CID9	*Olea europaea*	+	+	+	+	-	+	+
CID10	*Olea europaea*	+	+	-	+	+	+	+
CID11	*Olea europaea*	+	-	-	+	-	+	+
CID12	*Olea europaea*	+	-	-	+	-	+	+
CID13	*Olea europaea*	+	-	-	+	-	+	+
CID14	*Olea europaea*	+	-	-	+	-	+	+
CID15	*Olea europaea*	+	-	-	+	-	+	+
CID16	*Olea europaea*	+	-	-	+	-	+	+
CID17	*Olea europaea*	+	+	+	+	+	+	+
CID18	*Olea europaea*	+	-	+	+	-	+	+
CID19	*Olea europaea*	+	+	+	+	-	+	-
CID20	*Olea europaea*	+	-	+	+	-	+	+
	Total positive	**19**	**5**	**7**	**18**	**5**	**18**	**16**

Result of June

Sampling was carried out on 7th, 20 and 27th of June. A total of 49 samples were collected from different host plants, including 22 olive trees. On the 7th of June 17 samples from olive trees and 1 sample from an almond tree were collected and tested by ELISA, DTBIA, and PCR. The plant sap was extracted by both extraction methods without surface sterilization of plant material. *X. fastidiosa* colonies were obtained from 4 samples by both isolation methods and another one only by the conventional method of isolation (Table5).

Table 5. Detection of X. fastidiosa infection at 7 June, 2018

ID	Species	PCR	isolation		DTBIA	ELISA	
			SAP	print		CONV	SAP
DID1	*Olea europaea*	+	-	-	+	+	+
DID2	*Olea europaea*	+	-	-	+	+	+
DID3	*Olea europaea*	+	+	+	+	+	+
DID4	*Olea europaea*	+	-	+	+	+	+
DID5	*Olea europaea*	+	+	+	+	+	+
DID6	*Olea europaea*	+	+	+	+	+	+
DID7	*Olea europaea*	-	-	-	-	-	-
DID8	*Olea europaea*	-	-	-	-	-	-
DID9	*Olea europaea*	-	-	-	-	-	-
DID10	*Olea europaea*	+	-	-	+	+	-
DID11	*Olea europaea*	-	-	-	+	+	+
DID12	*Prunus dulcis*	-	-	-	-	-	-
DID13	*Olea europaea*	-	-	-	-	-	-
DID14	*Olea europaea*	-	-	-	-	-	-
DID15	*Olea europaea*	-	-	-	-	-	-
DID16	*Olea europaea*	-	-	-	-	-	-
DID17	*Olea europaea*	+	+	+	+	+	+
DID18	*Olea europaea*	-	-	-	-	-	-
	Total positive	**8**	**4**	**5**	**9**	**9**	**8**

The results presented in table (5) showed that only 8 samples tested positive out of the 18 tested by PCR, while DTBIA and conventional ELISA detected 9 positives samples out of the 17 samples. One sample tested negative by PCR was tested positive by both ELISA and DTBIA, while ELISA Sap extraction failed only in one sample that was tested positive by ELISA conventional extraction and PCR. The sample of almond was tested negative by all the tests used.

RT-LAMP, PCR, ELISA, DBTIA and isolation were performed on 12 olive samples collected on 20th of June (Table6) using both extraction methods.

Table 6. Detection of X. fastidiosa infection at 20 June, 2018

ID	Species	PCR	Isolation		DTBIA	ELISA		RT-LAMP	
			SAP	Print		CONV	SAP	CON	SAP
EID1	*Olea europaea*	-	-	-	-	-	-	-	-
EID2	*Olea europaea*	+	-	+	+	+	+	+	+
EID3	*Olea europaea*	-	-	-	-	-	-	-	-
EID4	*Olea europaea*	-	-	-	-	+	+	-	-
EID5	*Olea europaea*	+	-	+	+	+	+	-	+
EID6	*Olea europaea*	+	+	+	+	+	-	+	+
EID7	*Olea europaea*	+	-	-	+	-	-	-	-
EID8	*Olea europaea*	-	-	-	-	-	-	-	-
EID9	*Olea europaea*	-	-	-	-	-	-	-	-
EID10	*Olea europaea*	-	-	-	-	-	-	-	-
EID11	*Olea europaea*	-	-	-	-	-	-	+	+
EID12	*Olea europaea*	-	-	-	-	-	-	-	-
	Total positive	**4**	**1**	**3**	**4**	**4**	**3**	**3**	**4**

The results presented in Table 7 show that only 3 out of the 12 samples tested positive with all diagnostic methods. Regarding RT-LAMP with conventional extraction, only 3 samples tested positive out of the 4 confirmed by PCR. The slight difference in the results was related to the type of extraction used in ELISA, RT-LAMP and bacteria isolation. ELISA Sap extraction looks less sensitive (3 positive/4), while rt-LAMP applied with Sap Extraction looks better than conventional extraction (4 positive/4). Moreover, plant material from different host plants was collected on 27th June and tested by PCR and DTBIA. The results are shown below in Table (7).

Table 7. Detection of X. fastidiosa infection at 27 June, 2018

ID	Species	PCR	Isolation		DTBIA
			CONV	SAP	
GID1	*Olea europaea*	+	+	+	+
GID2	*Olea europaea*	-	-	-	-
GID3	*Nerium oleander*	+	-	+	+
GID4	*Asparagus* spp	-	-	-	-
GID5	*Myrtus communis*	+	+	+	+
GID6	*Prunus dulcis*	-	-	-	-
GID7	*Acacia saligna*	+	+	-	+
GID9	*Poligala myrtifolia*	+	+	+	+
GID10	*Lavandula* spp	-	-	-	-
GID11	*Wrestingia* spp	-	-	-	-
	Total positives	**5**	**4**	**4**	**5**

Only 5 plants from different hosts were tested positive out of the 10 tested by PCR. Colonies of *X. fastidiosa* were acquired from all the positive samples. The slight difference in the results was related to the type of extraction used for the isolation.

Result of July

The last sampling was carried out on the 11th of July, 2018. A total of 21 samples from different host plants were collected. As shown in Table (8), there were 13 samples out of 21 were tested positive for at least one diagnostic technique; 10 tested positive by conventional PCR; one sample of *Rosmarinus officinalis* was positive only in ELISA performed by both extraction methods; the bacterium was isolated.

Table 8. Detection of X. fastidiosa infection at 11 July, 2018

ID	Species	PCR	Isolation		ELISA		RT-LAMP	
			SAP	Print	CONV	SAP	CONV	SAP
LID1	*Nerium oleander*	+	+	+	-	-	+	+
LID2	*Rhamnus alaternus*	+	-	+	+	-	+	-
LID3	*Rhamnus alaternus*	-	-	+	-	-	-	-
LID4	*Myrtus communis*	-	-	-	-	-	-	-
LID5	*Prunus dulcis*	+	-	+	-	-	-	-
LID6	*Prunus dulcis*	-	-	-	-	-	-	-
LID7	*Myrtus communis*	-	-	-	-	-	-	-
LID8	*Myrtus communis*	-	-	-	-	-	-	-
LID9	*Myrtus communis*	+	-	-	-	-	+	+
LID10	*Myrtus communis*	-	-	-	-	-	-	-
LID11	*Myrtus communis*	-	-	-	-	-	-	-
LID12	*Acacia saligna*	+	+	+	+	-	+	+
LID13	*Acacia saligna*	+	-	-	+	+	+	+
LID14	*Westringia fruticosa*		-	-	-	-	-	-
LID15	*Lavandula* spp.	-	-	-	-	-	-	-
LID16	*Rosmarinus officinalis*	-	-	-	+	+	-	-
LID17	*Polygala myrtifolia*	-	+	+	-	-	+	+
LID18	*Cistus* spp.	+	-	-	+	+	+	+
LID19	*Rosmarinus officinalis*	+	-	-	+	+	+	+
LID20	*Rosmarinus officinalis*	+	-	-	+	+	+	+
LID21	*Olea europaea*	+	-	-	-	-	+	+
	Total positives	**10**	**3**	**6**	**7**	**5**	**10**	**9**

With conventional print in one sample of *Rhamnus alaternus* tested negative by all the diagnostic methods. A 7 samples were tested positive by ELISA conventional extraction compared with 5 by ELISA Sap extraction. The positive sample of *Rhamnus alaternus* tested negative by PCR, ELISA, and RT-LAMP indicating that the detection failure may have been due to the absence of the bacteria in the material examined. The presence of the bacterium was detected by RT-LAMP in ten samples with conventional extraction but one of them was not detected with Sap extraction. Sap extraction method seems again slightly successful for *X. fastidiosa* diagnostic purpose.

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