Isolation And molecular characterization of *xylella fastidiosa* from different host plant species in Italy (apulia region)

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Abstract

Pure culture isolation of the *Xylella fastidiosa* strain related to the olive quick decline syndrome (OQDS), lately noticed in Apulia (southern Italy) was attempted from naturally infected symptomatic plants mainly *Olea europaea L.*, *Polygala myrtifolia* and *Rosmarinus officinalis* using printing and sap extraction methods. Prior to isolation, RT-LAMP and qPCR were used to determine the presence of *Xylella fastidiosa* in all hosts. Pure bacterial cultures were obtained from both *Olea europaea L.* and *Polygala myrtifolia* extracts plated in buffered cysteine-yeast extract (BCYE) media. Two olive isolates were subsequently typed using the Multilocus Sequencing System (MLST). Findings indicate that beside *Olea europaea*, *Polygala myrtifolia* seems to have a good potential for isolation, and printing method has presented significantly better results. MLST shows genetic commonality with De Donno (ST53) strain and ascertain that Apulia region still have the same sequence type ST53 in the region.

Keywords : *Xylella fastidiosa*; OQDS; RT-LAMP; qPCR; Pure culture isolation; BCYE; Printing method; sap extraction method; MLST.

Isolement et caractérisation moléculaire de *xylella fastidiosa* à partir de différentes espèces de plantes hôtes en Italie (région des pouilles)

Résumé

L'isolement en culture pure de la souche *Xylella fastidiosa* liée au syndrome de déclin rapide de l'olivier (OQDS), récemment observé dans les Pouilles (sud de l'Italie) a été tenté à partir des plantes symptomatiques naturellement infectée, principalement *Olea europaea L., Polygala myrtifolia* et *Rosmarinus officinalis*, en utilisant les méthodes d'impression et d'extraction de la sève. Avant l'isolement, RT-LAMP et qPCR ont été utilisés pour déterminer la présence de *X. fastidiosa* chez tous les hôtes. Des cultures bactériennes pures ont été obtenues à partir d'extraits *d'Olea europaea L.* et de *Polygala myrtifolia* étalés dans un milieu tamponné d'extrait de cystéine-levure (BCYE). Deux isolats d'olive ont ensuite été typés à l'aide du système de séquençage multilocus (MLST). Les résultats indiquent qu*'Olea europaea* a un grand potentiel d'isolement, et la méthode d'impression a présenté des résultats nettement meilleurs. MLST montre des points communs génétiques avec la souche De Donno (ST53) et confirme que nous avons toujours le même type de séquence ST53 dans la région.

Mots-clés : *Xylella fastidiosa*; Isolement de culture pure; BCYE; LAMPE RT; qPCR; MLST

العزل والتوصيف الجزيئي لبكتيريا *Xylella fastidiosa* من أنواع نباتية مضيفة مختلفة في إيطاليا (منطقة بوليا)

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ملخص

تمت محاولة عزل سلالة *Xylella fastidiosa* المرتبطة بمتلازمة التدهور السريع للزيتون (OQDS)، التي لوحظت مؤخرًا في بوليا (جنوب إيطاليا)، من نباتات مصابة بشكل طبيعي وأهمها *Olea europaea L*. و*Polygala myrtifolia* و*Rosmarinus officinalis* باستخدام طُرق الطباعة واستخراج النسغ. قبل العزل، تم استخدام RT-LAMP وqPCR لتحديد وجود *Xylella fastidiosa* في جميع العوائل. تم الحصول على مزارع بكتيرية نقية من مستخلصات *Olea europaea L*. و*Polygala myrtifolia* المطلية في وسط مستخلص (BCYE). تم بعد ذلك كتابة اثنين من عزلات الزيتون باستخدام نظام تسلسل متعدد البؤرة (MLST). تشير النتائج إلى أنه بجانب *Olea europaea*، يبدو أن *Polygala myrtifolia* تتمتع بإمكانية جيدة للعزل، وقد قدمت طريقة الطباعة نتائج أفضل بشكل ملحوظ. يُظهر MLST القواسم الجينية المشتركة مع سلالة De Donno (ST53) وتم التأكد من أن منطقة Apulia لا تزال لديها نفس نوع التسلسل ST53 في المنطقة.

الكلمات المفتاحية: *Xylella fastidiosa*؛ RT-LAMP. OQDS ؛ qPCR ؛ عزل سلالة نقية ؛ BCYE؛طريقة الطباعة؛ طريقة استخراج النسغ MLST.

Introduction

2013 is registered as the horribilis year for olive trees in south-eastern Italy (Almeida *et al.* 2016). Olive cultivation in Apulia has a more significant spread compared to other Italian regions and, this explains the strong economic impact caused in Apulian region. For almost ten years in the province of Lecce (Almeida *et al.* 2008), olives have been threatened and affected by an alien disease that commences with foliage desiccation, swiftly ending up with the death of the full tree. This condition was unknown and called "olive quick decline syndrome" (OQDS) (Cariddi *et al.* 2014). OQDS is a devastating disease distinguished by yellow leaves and twigs desiccation, ending by tree death. In the primary stages, symptoms seem on the apical parts of trees, and by and by, they spread to the rest of the crown, which gained a burned-like aspect in the final stages of disease (Chatterjee *et al.* 2008). The outset of this economically destructive disease has been correlated with *X. fastidiosa* infection

Xylella fastidiosa is a Gram-negative pathogen that has previously caused huge damage in the South America and United States (Chen *et al.* 2014). These alarming bacteria build biofilms inner xylem vessels, causing their occlusion, which in the end impairs mineral salt uptake and water (Cornara *et al.* 2017). *X. fastidiosa* originates in the Americas where it has caused several diseases to economically important crops (Davis *et al.* 1983). Outside the Americas, the species has been stated on both grapevine and almond in Iran (Della *et al.* 2001). The geographical distribution and progression of *X. fastidiosa* subspecies in the Americas designate that diverse subspecies have appeared in geographical isolation: subsp. *multiplex* in North America, subsp*. pauca* in South America, subsp. *fastidiosa* in Central America, and subsp*. sandyi* detected only in southern regions of the United States (Denancé *et al.* 2017). In spite of the fact that *X. fastidiosa* populations appeared to have historically remained largely isolated because of geographical barriers, the new human-mediated invasions are responsible for important plant diseases driven by bacterial introductions in new areas. Examples include the identification in Taiwan of isolates on Nashi pears, causing pear leaf scorch, genetically related to the North American strains of *X. fastidiosa* subsp. *fastidiosa* (Djelouah *et al.* 2014) but recently identified as new species (EFSA, 2015),the genotype responsible for disease in grapevines in the US, which originated from Central America (EFSA, 2016), the finding in Brazil of plum trees harboring isolates belonging to the North American subsp. *multiplex* (EFSA,2018), finally, the report of subsp*. pauca* in Central America (López *et al.* 2017). and of subsp. *fastidiosa* in Israel (EPPO*,* 2018).

The assessment confirms the absence of affective way of eradicating the bacterium from a diseased plant in field conditions (Giampetruzzi *et al.* 2015). The effectiveness of biological and chemical control measures has been assessed in recent experiments (Giampetruzzi *et al.* 2017). Researches indicate that they could temporarily decrease disease severity in some situations, however there is no certitude that they could

eliminate *X. fastidiosa* over a long duration of time or in field conditions. The ongoing agronomic practices are mostly based on uprooting the infected olive trees and their surrounding ones, with later planting of more resistant olive cultivars like Leccino and 'FS17 (D'Attoma *et al.* 2019). Unfortunately, these two practices are having an unfortunate impact on the economy (costs of the new installments, the loss of production for some years and, the last but not less important the oil quality produced by more resistant cultivars may not match the high standards of the previous ones) and on the environment (the majority of these trees were several years old) (Cardone *et al.* 2021, Bozzo *et al.* 2022, Frem *et al.* 2021). *X. fastidiosa* has a broad host range worldwide and it is expected that the list of host plants will be extended in the future. Therefore, understanding the strain diversity and the evolutionary dynamic of adaptation of this pathogen to new hosts has become a key point for the development of innovative disease management strategies. Actually, the bacterial population structure and genetic diversity, as well as the genetic evolutionary relationships among *X. fastidiosa* strains within the agricultural population is unclear. Hence, advances in the understanding of *X. fastidiosa* population structure and genetic diversity will highly help the development of effective disease control (Vanhove *et al.* 2019). Since more than one subspecies have been found associated with ornamental plants, and knowing that the pathogen continues to spread to new areas in Apulia, continuous isolation from known host plants and characterization of the bacterium is required.

The objective of this paper is to isolate the bacterium from known hosts present in the Apulia region using printing and sap extraction approaches, identify which host plant is easy for pure culture isolation of *X. fastidiosa* and molecular characterization of the isolates by Multilocus Sequence Typing (MLST).

Material and methods

Sampling site and protocol

Sampling from different hosts of the bacterium was carried out in the first (2022) in the demarcated area in Apulia region, situated in the south of Italy (fig.1). Samples were collected and host species are scattered all over the region showing different growth stage. Plant material was taken from orchards within the area, depending on the availability of symptomatic material. Collection of plant parts from symptomatic hosts was done using a special tool that was disinfected with ethanol 70% when we moved from one sample to another. For the different hosts, we followed simple criteria, such as cutting few twigs per tree from the area close to the symptoms but not from the necrotic parts and from different sides of the canopy. The number and sampled plants were: 8 samples of *Olea europaea L.,* 8 samples of *Polygala myrtifolia* and 8 samples of *Rosmarinus officinalis L.* Samples were put in labelled bags and placed in a cool box in the car. Direct exposure to sunlight was avoided and the isolation step started no later than one day after sampling.

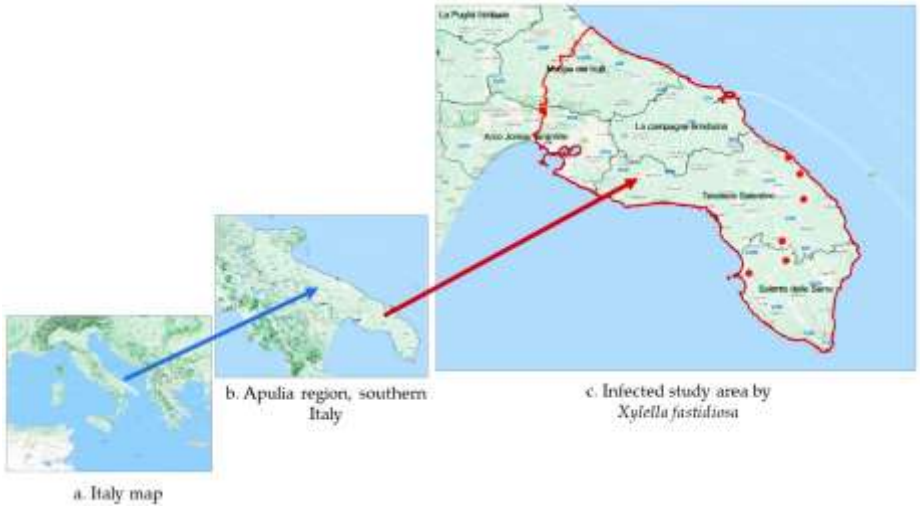

Figure 1. Sampling sites (Red dots), Apulia Region, extremely damaged by *Xylella fastidiosa*

Molecular analyses

RT-LAMP and qPCR were applied as diagnostic methods for the detection of *X. fastidiosa*. Negative samples were discarded, while positive ones were kept for next steps of the experiment.

Real-Time Loop-Mediated Isothermal Amplification method (Real-Time LAMP)

Real-Time LAMP has been developed for detecting *X. fastidiosa* in host plants and insect (Janse *et al.* 2010). It has been successfully used so far and showed sensitivity and reliable results. The DNA extraction procedure was simplified and obtained by heating the samples in a portable smart-Dart device using an optimized reaction buffer. The connection to a tablet or smartphone allowed visualization of the results of the reaction in real time. The RT-LAMP protocol started by cutting small slices of midribs and petioles (0.5g) from leaf samples, transferring them into extraction bags, adding 5 ml of extraction buffer (1:10 v: w), then crushing them with a hammer and homogenizing them. After homogenization, 1ml of suspension was transferred in an Eppendorf tube (1.5ml). In the tubes containing 200 µl of extraction buffer (supplied in the kit, Enbiotech, Italy), 5 µl of the homogenate were added, vortexed gently and incubated for 10min at 65°C. Next step requires the preparation of the aliquots of the RT-LAMP Mix by adding in each tube labeled “primer Mix” 22.5 µl of rt-LAMP Mix, 30 µl of mineral oil, 2.5 µl of denatured sample. The whole reaction was finally mixed by vortexing. Eventually, the amplification program is set on the device: one step at 65°C for 25 min. In case of positive samples, amplification curves are observed, while no amplification curve is obtained from negative samples.

DNA extraction

DNA material was extracted from plant samples using the standard CTAB based procedure (Killiny *et al.* 2014). Fresh small pieces of leaf midribs, petioles and twigs were taken from samples and homogenized in 5 ml of CTAB extraction buffer. After homogenization, 1 ml of suspension was transferred in 2 ml Eppendorf tubes and incubated for 30 min at 70°C. DNA was extracted by adding 1 ml of chloroform and centrifuging for 10 min at 13000 rpm. Then, 750 µl of supernatant were transferred to a new tube and diluted with cold isopropanol (450 µl). This dilution was gently mixed and stored at - 20°C for 20 min. Next step requires centrifugation for 20 min at 13000 rpm. The supernatant was discarded, whereas the pellet was washed off with 1 ml of ethanol 70%. One more centrifugation was needed for 10 min at 13000 rpm. After removing ethanol, Eppendorf tubes were air-dried. Eventually, remained pellets were suspended by adding 100 µl of sterile water in each tube and mixing with vortex. DNA material prepared in this way can be used immediately, or stored at -20°C.

Quantitative real-time PCR (qPCR)

Positive samples were detected also by the real-time PCR (qPCR). The DNA was extracted using the CTAB protocol previously described. Real-time Polymerase Chain Reaction method has been confirmed to be reliable and showed high accuracy for the detection of various strains of *X. fastidiosa* (Li *et al.* 2007). All compounds and volumes used for PCR mixture (11 µl) are shown in the following: 3.8 µl of molecular grade water, 5.5 µl of 2X Taq Man Universal PCR Master Mix (AB)* (cod.4364338), 0.3 µl of 10 µM Forward primer RST31, 0.3 µl of 10 µM Reverse primer RST33, and 0.1 µl of 10 µM Taq Man probe. Final master mix contained 10 µl. In order to reach final volume of 11 µl, 1 µl DNA material was added. Tubes were put in Thermocycler (BioRad) for PCR execution. Finally, the following program was applied: pre-incubation at 50°C for 2 min followed by 95°C for 10 min, followed by 40 cycles of 94°C for 10 seconds and 62°C for 40 seconds. FAM Cq values higher than 34,99 determined negative samples, FAM Cq values in the range of 32 to 34,99 suggested the samples needed to be tested again and FAM Cq values less than 32 determined positive samples.

Isolation of *X. fastidiosa* from positive plant samples

When the presence of bacterial DNA was ascertained by one of the detection methods previously described, the next step of isolation started. First of all, preparing of the appropriate growth medium was necessary. Considering the rigorous conditions needed for cultivation of *X. fastidiosa*, we selected different media proposed as the most efficient for *pauca* subspecies and the CoDiRO strain.

Preparation of growth media

BCYE or buffered charcoal – yeast extract medium, is complemented growth substrate (Lin *et al.* 2005). Ingredients and their quantity for preparation of this medium are presented in table 1.

Table 1. Material for BCYE medium (Wells et al., 1981)

Ingredients	Grams per 1 L
Yeast extract (Sigma)	10.0
Activated charcoal (Vetec)	2.0
L-cysteine HCL (Sigma)	0.4
Ferric pyrophosphate (Sigma)	0.25
Aces buffer (Sigma)	10.0
Difco Bacto Agar (Sigma)	17.0

Aces buffer was suspended with 500 ml of distilled water in 1l bottle and hydrated at 50°C. Solution of 40 ml 1N KOH and 440 ml of distilled water was made in another bottle in which we suspended the active charcoal. We pooled the two bottles together and we added the yeast extract to the mixture. Before putting agar in the bottle, pH was measured (optimum value is 6.9-7) and then the medium was autoclaved for 20 min at 120°C. Two solutions of ferric pyrophosphate and L-cysteine HCl were prepared. Each of these products needed to be suspended separately in 10 ml of distilled water. After the medium cooled down at 50°C, the two solutions were added after being sterilized with a 0.2 µm size filter membrane. Finally, the medium was gently shaken and poured into plates (Lindstedt *et al.* 2005).

PD3 medium has been used as the primary substrate for Pierce's disease strains of *X. fastidiosa* subsp. *fastidiosa*. For 1 l of medium, ingredients were added in the following order: tryptone (4g), soytone (2g), trisodium citrate (1g), sodium succinate (1g), hemin chloride (0.01g), soluble potato starch (2g), MgSO4-7H2O (1g), K2HPO4 (1.5g), KH2PO4 (1g), agar (15g), phenol red 0.2% solution (10ml) (Loconsole *et al.* 2014a).

Isolation methodology

Printing method

Isolation of colonies has been carried out through direct printing of twigs on the substrate. Twigs of 8-10cm long were washed under tap water, surface sterilized using sodium hypochlorite 2% for 2 min and ethanol 70% for 2 min, and rinsed three times in sterilized water. Each twig was cut in half and squeezed at one end with a plier, while the other end was gently pressed on plates with BCYE medium to make imprints (Fig.2). Plates then were closed with parafilm, incubated at 28°C (Loconsole *et al.* 2014b) and continuously checked for the appearance of Xylella-like colonies.

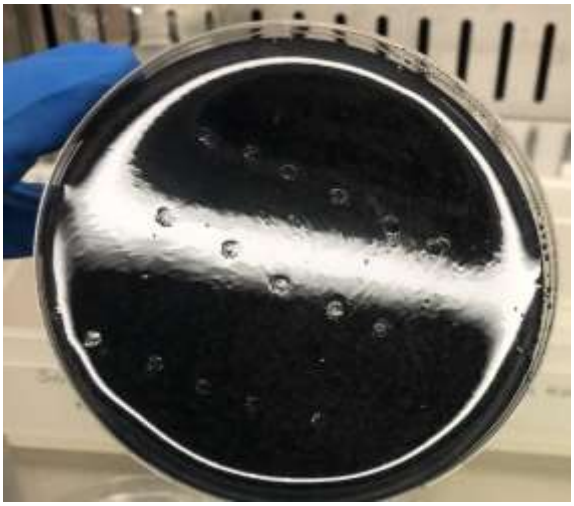

Figure 2. Direct printing of twigs on BCYE medium

Isolation with Sap Extract Method

In this technique, sterilized rubber tubes and twigs were attached with parafilm to syringe containing 2ml of sterile water or PBS buffer. The syringe was gently and slowly pressed in order to push the plant sap out of the twig which was collected in an Eppendorf tube. After that, the sap was pipetted (2-3 droplets) into a Petri plate. In order to spread the drops from one brim to another, the plate was turned vertically. Under the hood, the plate was dried, then sealed with parafilm and stored at 28°C in the incubator.

After the appearance of the first colonies, purification was needed. This was based on making dilution of colonies in water and pipetting to fresh growth medium. This step called subculture and consist on transferring of one or more colonies to the fresh growth medium to obtain pure colonies. In this way the amount of cultured *X. fastidiosa* inoculum was increased and its preservation was prolonged.

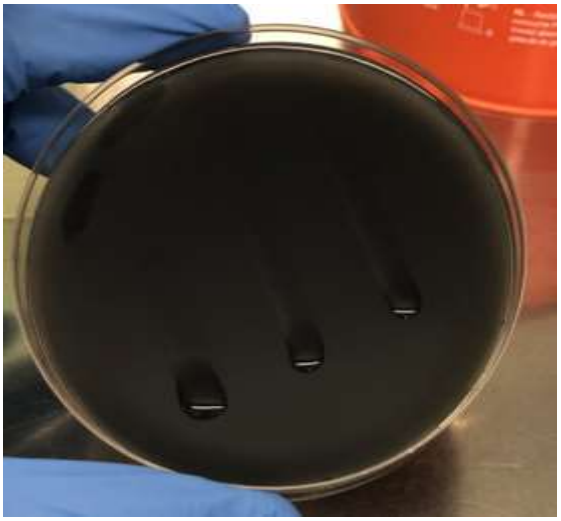

Figure 3. Sap Extract Methodon BCYE medium

Molecular characterization of isolates: Multilocus Sequence Typing (MLST)

After the appearance of *X. fastidiosa* colonies on agar plates, DNA material was extracted following these steps: Many colonies were transferred from the plate and diluted in tube containing 200µl of PBS buffer, and incubated for 15 minutes at 70°C. Then 200 µl of chloroform were added, and the suspension was centrifuged for 10 minutes at 13000 rpm. The supernatant was transferred to a new tube. Seven different PCR primers were used to amplify housekeeping genes with different biological function (*holC, nuoL, gltT, cysG, petC, leuA, lacF*) in *X. fastidiosa* genome, as shown in table 2. All compounds and volumes used for PCR mixture are shown in the following: 50µl of 2X master Mix PCR (Qiagen), 41µl of distilled water, 2µl from each primer forward and reverse (10mM) and 5µl of DNA to reach a final volume of 100µl. For these primers the following program was applied: Denaturation at 94°C for 3 min, then 35 cycles of 30 seconds at 94°C, 30 seconds at 55 °C, 1 min at 72°C and final extension at 72°C for 5 min. All amplified seven housekeeping genes were sequenced as described by Scally *et al* (2005).

Sequence similarities were examined using BLAST at the National Center for Biotechnology Information website (https://www.ncbi.nlm.nih.gov/data-hub/genome/GCF_000811965.1/). Alignment of all subspecies were used as input data to create the MLST tree and sequence identities were checked using pubmlst website available online.

Table 2. List of primers used for amplifying *X. fastidiosa* housekeeping genes

Gene	Function	Biochemical function	Primer sequences (forward/reverse)	length (bp)
holC	DNA polymerase III holoenzyme, chi subunit	Replication	*5'-GATTTCCAAACCGCGCTTTC-3'* *5'-TCATGTGCAGGCCGCGTCTCT-3'*	***379***
nuoL	NADH-ubiquinone oxidoreductase, NQO12 subunit	Aerobic respiration	*5'-CATTATTGCCGGATTGTTAGG-3'* *5'-GCGGGAAACATTACCAAGC-3'*	***1821***
gltT	Glutamate symport protein	Transport of amino acids	*5'-TTGGGTGTGGGTACGTTGCTG-3'* *5'-CGCTGCCTCGTAAACCGTTGT-3'*	***951***
cysG	Siroheme synthase	Biosynthesis of heme, porphyrin	*5'-GGCGGCGGTAAGGTTG-3' 5'-GCGTATGTCTGTGCGGTGTGC-3*	***1170***
petC	Ubiquinol cytochro. *c* oxidoreductase, cytochrome *c*1 subunit	Electron transport	*5'-CTGCCATTCGTTGAAGTACCT-3'* *5'-CGTCCTCCCAATAAGCCT-3'*	***533***
leuA	2-Isopropylmalate synthase	Amino acid biosynthesis	*5'-GGGCGTAGACATTATCGAGAC-3'* *5'-GTATCGTTGTGGCGTACACTG-3'*	***1218***
lacF	ABC transporter sugar permeas	Transport of carbohydrates	*5'-TTGCTGGTCCTGCGGTGTTG-3'* *5'-CCTCGGGTCATCACATAAGGC-3'*	***730***

Results

RT-LAMP Quantitative real-time (q) PCR results of plant samples

X. fastidiosa was detected by RT-LAMP and qPCR in all symptomatic plants sampled. 12 plants from different species have been confirmed positive. For quantitative PCR, reactions yielded quantitative cycles (Cq). In table 3 and Figure 4 results of qPCR tests and RT-LAMP for two sets of samples are presented.

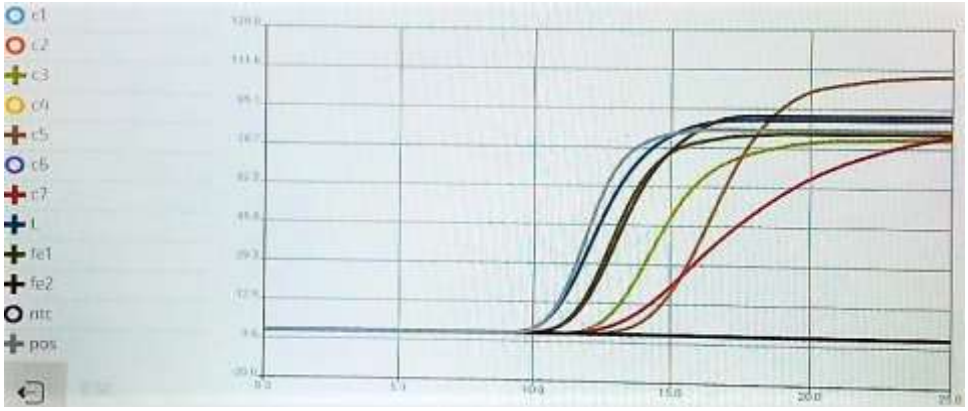

Figure 4. LAMP test for samples tested

Table 3: Results of the qPCR for samples tested (negative samples are not mentioned)

Well	Fluor	Content	Sample	Cq	Cq Mean
A01	FAM	Unkn	1	20.58	20.58
A02	FAM	Unkn	6	18,99	18,99
A03	FAM	Unkn	9	19,68	19,68
A04	FAM	NTC			0,00
B01	FAM	Pos Ctrl	DNA	19,61	19,61
B02	FAM	Unkn	16	29,40	29.40
B03	FAM	Unkn	17	18,26	18,26
B04	FAM	Unkn	18	15,20	15,20
C01	FAM	NTC			0,00
C02	FAM	Unkn	19	31,10	31,10
C03	FAM	Unkn	20	19,69	19,69
D01	FAM	Unkn	21	17,18	17,18
D02	FAM	Unkn	22	17,41	17,41
D03	FAM	Unkn	23	17,52	17,52
E01	FAM	FAM	NTC		0,00
E02	FAM	Pos Ctrl	DNA	19,61	19,61
E03	FAM	NTC			0,00
F01	FAM	Unkn		29,52	29.52
F02	FAM	NTC			0,00
F03	FAM	Pos Ctrl	DNA	19,61	19,61
G01	FAM	Pos Ctrl	EXTRACT	16,53	16,53
G02	FAM	Unkn	15	31,46	31,46
G03	FAM	Neg Ctrl	EXTRACT		0,00
H01	FAM	Neg Ctrl	DNA		0,00
H02	FAM	Pos Ctrl	EXTRACT	16,53	16,53

Isolation of *X. fastidiosa* from samples positive in qPCR or RT-LAMP

Isolation is presented in table 4, specifying the results of detection and isolation by each approach. Samples were collected one day before the isolation

Tables 4. Isolation of *X. fastidiosa* from samples positive in qPCR and RT-LAMP

Sample Name	Code	RT-LAMP	qPCR	Printing method	Sap method
1- *Olea europaea*	*1-Oe*	Pos+	Pos+	**Pure colonies**	**Pure colonies**
6- *Olea europaea*	*6-Oe*	Pos+	Pos+	**Pure colonies**	**Pure colonies**
9- *Rosmarinus officinalis*	*9-Ro*	**/**	Pos+	**/**	**/**
15- *Olea europaea*	*15-Oe*	Pos+	Pos+	**/**	**/**
16- *Olea europaea*	*16-Oe*	Pos+	Pos+	**Pure colonies**	**Pure colonies**
17- *Olea euopaea*	*17-Oe*	**/**	Pos+	**Pure colonies**	**/**
18- *Rosmarinus officinalis*	*18-Ro*	Pos+	Pos+	**/**	**/**
19- *Rosmarinus officinalis*	*19-Ro*	**/**	Pos+	**/**	**/**
20- *Polygala myrtifolia*	*20-Pm*	Pos+	Pos+	**/**	**/**
21- *Polygala myrtifolia*	*21-Pm*	/	Pos+	**Pure colonies**	**/**
22- *Polygala myrtifolia*	*22-Pm*	**/**	Pos+	**/**	**/**
23- *Polygala myrtifolia*	*23-Pm*	**/**	Pos+	**Pure colonies**	**Pure colonies**

/: This symbol means in the RT-LAMP, and qPCR columns that the corresponding method were not used in the detection. However, in the last two columns, it indicated that no colonies are obtained.

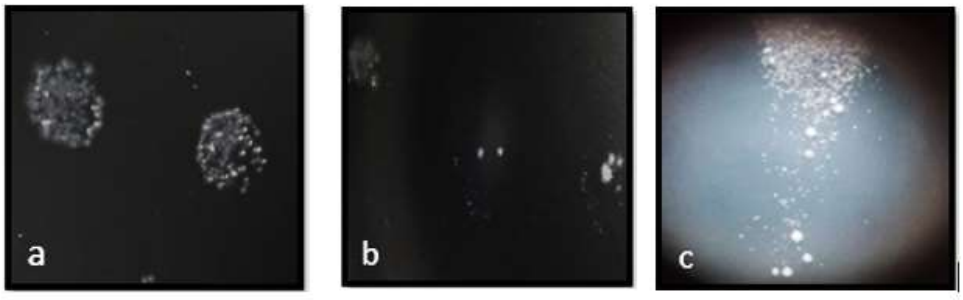

Figure 5. *Xylella fastidiosa* colonies on BCYE agar medium growing obtained from Olive samples (a) show the high number of colonies growing mostly together using printing method ;(b) shows pure colonies after sub-cultures (c) Shows colonies growing using sap extraction method.

According to the obtained results by qPCR and LAMP, 12 samples (3 samples from *Olea europaea L*., 4 samples from *Polygala myrtifolia* and 5 samples from *Rosmarinus officinalis*) were confirmed to be negative for *X. fastidiosa*. After about 15 days, many extremely slow-growing bacterial colonies appeared in the plates of 6 samples (1-*Oe*, 6-*Oe*,16-*Oe*,17-*Oe*,21-*Pm* and, 23-*Pm*). (Fig5). The morphological features of colonies are discernible under microscope and show the following characteristics summarized in Table 5.

Table 5. Morphology 15-day-old colonies of the Apulian strain of *Xylella fastidiosa* in BCYE media

Colony morphology	**BCYE**
Elevation	Convex
Form	Circular
Margin	Entire
Size(mm)	Less than1.0
Surface appearance	Smooth
Texture	Viscous
opacity	Opalescent
pigmentation	None

Colonies were obtained with both techniques (syringe and printing) for (1-*Oe*), (2-*Oe*), (16-*Oe*) and (23-*Pm*). From the PCR-positive *Rosmarinus officinalis*, no any *X. fastidiosa* – like colonies were obtained (Table.5).

MLST of *X. fastidiosa* pure isolates and Phylogenetic tree analysis

Isolates from two chosen samples (1-*Oe* and 6-*Oe*) were used to amplify seven housekeeping genes, partially showed in Figure 6, and then MLST was conducted. The BLAST software used afterwards showed that sequences of the 7 genes from the two isolates are genetically identical with an overlapping set of alleles previously confirmed to belong to De Donno strain (ST53), with no variations. The isolates shared the same sequence type, ST53. The results obtained are consistent with those previously described by Elbeaino *et al.*2014 and proved that all isolates collected so far in Apulia belonged to ST53 strain.

Phylogenetic tree analysis using Neighbor-net reconstruction of relationships among all 85 sequence types (ST) of *Xylella fastidiosa* (by Split Tree 4.14.6) confirm that our isolates have the same sequence type of ST53 (Figure 7).

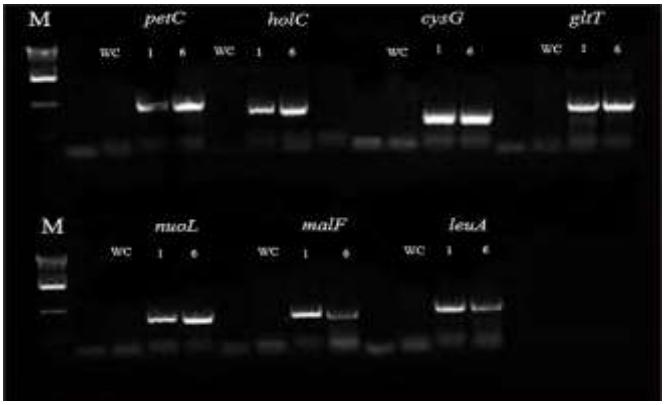

Figure 6. Results PCR to the primers of the seven housekeeping genes for samples 1 and 6 isolates (WC= water control)

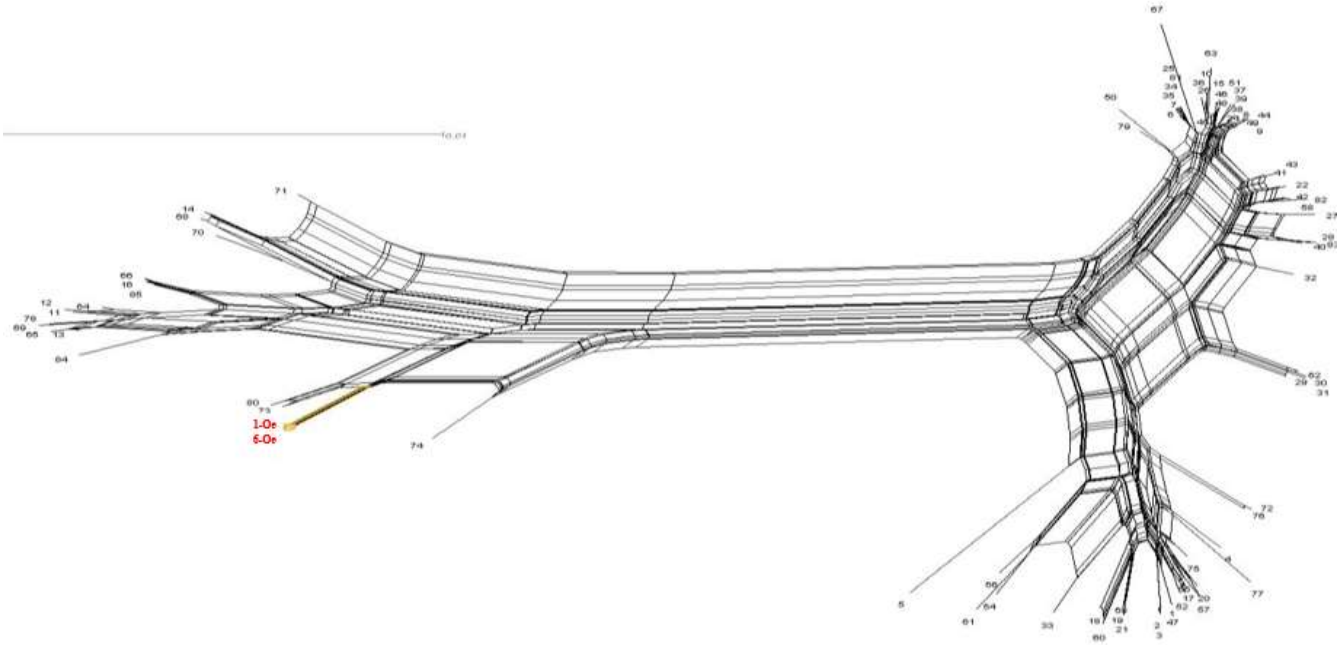

Figure 7. Phylogenetic tree by Neighbor-net reconstruction of relationships among all 85 sequence types (ST) of *Xylella fastidiosa*

Discussion

In 2013, *Xylella fastidiosa* was detected in olive trees (*Olea europaea L*.) in southern Italy (Apulia region) (Sicard *et al.* 2021). It represented the first outbreak of this quarantine pathogen under field conditions in the European Union, and it was the first documented event of widespread infections in this plant species. Infected trees exhibit a severe disease termed olive quick decline syndrome (OQDS) (Beretta *et al.* 2022). *X. fastidiosa* colonies were successfully obtained only from some plants. For some other plants, isolation plates were highly contaminated. Some samples did not develop either *X. fastidiosa* – like colonies or contaminants. This is explained by the fact that *X. fastidiosa* grows relatively slowly, colonies can take up to 15 days to be visible and can be readily overgrown by other microorganisms in the plates. Also, the heavy contamination by bacteria other than Xylella leads to the failure of the isolation which apparently inhibits occurrence and growth of the colonies. This difficulty to isolate *X. fastidiosa* on culture media from positive samples is also probably due to the fastidious nature of the bacterium, which is influenced by biotic and abiotic factors or may be at low concentrations in affected plants (Amanifar *et al.* 2014).

Multilocus sequence typing (MLST) analyses showed that olive-infecting isolates of *X. fastidiosa* were genetically related to subspecies *pauca* and all harbored the sequence type 53 (ST53) and are consistent with those previously described by Elbeaino *et al.* 2014. Prior researches using the HiSeq 4000 Illumina platform and PacBio RSII platform described the complete genome sequence of *X fastidiosa* subsp *pauca* strain De Donno (Giampetruzzi *et al.* 2017). Illumina sequencing yielded a total of 5,700,601 2 × 150-bp high-quality paired reads, of which 1% (87,950 reads) low-quality reads were discarded. In parallel, 105,585 fastq reads, with a mean length of 8,527 bp (longest read, 56,602 bp), were obtained by PacBio sequencing (Giampetruzzi *et al.* 2017). De novo hybrid genome assembly was also investigated and published using both Illumina and PacBio data set with SPAdes version 3.9.0 (5, 6) (Giampetruzzi *et al.* 2017). The final assembly resulted in a single circular 2,508,465-nucleotide (nt) chromosome with 52% C+G content. In addition, a circular plasmid of 35,273 nt, named pXF-De Donno, with a C+G content of 49.6%, was also identified. Nucleotide coverage was, on average, 1,765.5× for the plasmid (standard deviation of 216.9×) and 636.5× for the chromosome (standard deviation of 76.2×). Functional annotation by submission to the NCBI Prokaryotic Genome Automatic Annotation Pipeline resulted in the identification of 6 rRNA genes (2 operons), 49 tRNA loci, 2,381 genes, 2,322 protein-coding genes, 3 noncoding RNAs in the chromosome, and 39 protein-coding genes in the plasmid (Giampetruzzi *et al.* 2017). The complete genome description of the strain De Donno offers insights into the biology of this devastating olive disease.

Conclusion

In order to address all issues presented in the introduction and to achieve expected results, the isolation from different plant hosts, molecular characterization of isolates (MLST) have been carried out. Broadly translated our findings indicate that olives have a good potential for isolation and according to results we suggested it for an easy isolation. Concerning the isolation approach printing method for the isolation has presented satisfying results. The isolation from some positive samples have failed and some plants haven't developed either *X. fastidiosa* – like colonies or contaminants. Subsequently, some samples were highly contaminated which apparently inhibits occurrence and growth of *X. fastidiosa* colonies. For that, it is very important to exclude or at least to minimize negative effect of the contamination with other microorganisms. Molecular characterization carried out from two isolates of Olea europaea has confirmed genetic commonality with De Donno (ST53) strain.

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