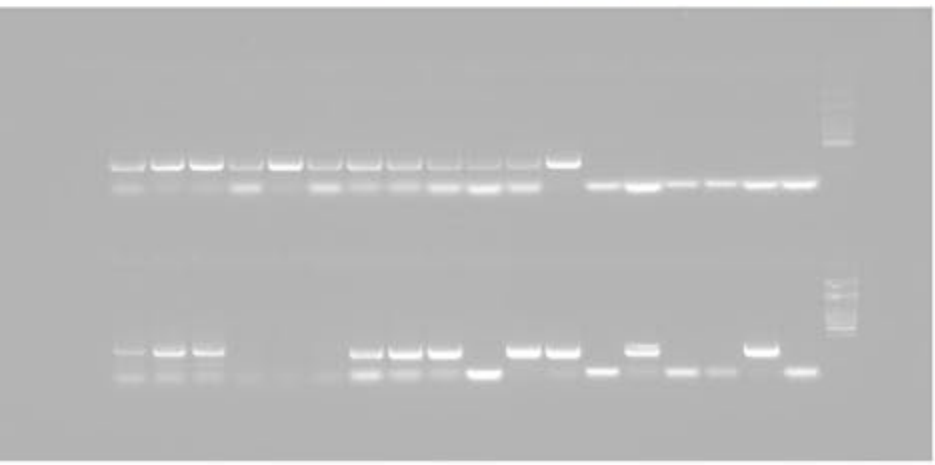
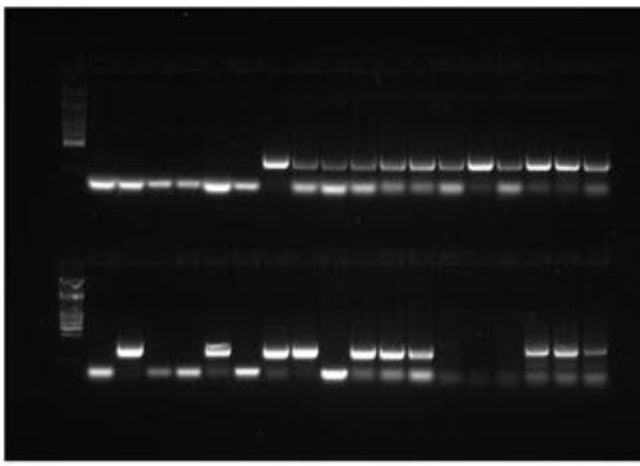
# Current protocols for the detection of *Xylella fastidiosa* in host plants and vectors







# Workshop Manual

**WORKSHOP**

# "CURRENT TOOLS FOR THE DETECTION OF *XYLELLA FASTIDIOSA* IN HOST PLANTS AND VECTORS"

October 23-24, 2014

CRSFA Centro di Ricerca, Sperimentazione e Formazione in Agricoltura "Basile Caramia"

Locorotondo (Bari), Italy

## DIAGNOSTIC PROTOCOLS

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## INDEX OF CONTENTS

## 1. CURRENT METHODS FOR THE DETECTION OF *XYLELLA FASTIDIOSA*

*Xylella fastidiosa* is a regulated plant pathogen in many parts of the world. The bacterium may be detected in host plants by traditional symptom, culture-based techniques, and more recently serological and molecular assays. Inspections of crops suspected to be infected by *X. fastidiosa* are fundamental to point out early symptoms of infection.

*Disease symptoms*. Development of symptoms induced by *X. fastidiosa* vary according to the susceptible hosts, with leaf scorching (LS) being the most common syndrome associate to the bacterial infections in almond, coffee, elm, oak, oleander pear, and sycamore. Indeed, strains of this bacterium are the causal agent of Pierce's disease (PD) of grapes, citrus variegated chlorosis (CVC), phony peach disease, plum leaf scald. Leaf scorch symptoms appear in late summer to early fall and can be distinguished from scorch-like symptoms caused by other factors (drought, salt injury, wilt diseases, etc.) by the presence of a yellow halo between the area of marginal leaf necrosis and green leaf tissue. Infected, symptomatic trees will drop leaves prematurely, and can develop dieback and irreversible decline. PD in grapevine, consists in a sudden drying of large parts of the green leaves, evolving in brown and necrotic and the surrounding tissues become yellow to red. The necrosis is often present at the leaf margins. Scorched (burnt-like) leaves usually drop from the distal and not from the usual basal end of the petiole, leaving bare petioles attached to canes, often well after normal leaf fall. PD can be confused with other disorders such as salt toxicity, boron, copper or phosphorus deficiency.

Unlike the majority of the *X. fastidiosa* diseases, CVC symptoms do not include scorched leaves, but typical irregular chlorosis in mature leaves recognized by interveinal yellowing on the upper side of the leaf and corresponding brownish gumlike material over the side.

Indeed, there are several hosts that may carry the pathogen with, but more often without showing symptoms, such as grasses, sedges and trees.

*Isolation.* Isolation by pathogen cultivation *in vitro* is the most definitive and direct for pathogen detection and identification because whole bacterial cells and their biochemical and physiological properties are observed. However, cultivation of *X. fastidiosa* in the laboratory is time consuming, ranging from 3 to 20 days, and labor intensive, particularly when a large number of samples are involved.

*Serology*. Serological methods target the unique properties of bacterial cell surface. Among them, enzyme-linked immunosorbent assay (ELISA) is commonly used and has a high throughput capacity because of the simplicity in sample preparation and the use of the 96-well plate format.

*Molecular tests*. Molecular techniques include PCR and PCR derivatives including RFLP and RAPD analysis, as well are real-time PCR. Extraction of *X. fastidiosa* DNA from culture and host species for PCR and related molecular analyses has been achieved from tissue by both standard commercial column kits and by basic CTAB or, in the case of cultures, Tris-EDTA-Sarkoysl techniques. The available whole genome sequences of *X. fastidiosa* strains make it feasible to design PCR primers at various levels of specificity. Several specific PCR primer sets are currently available for *X. fastidiosa* detection including the most thoroughly tested RST31/33 primer set, derived from the RNA polymerase genomic locus, those derived from16S rRNA gene and gyrase genes (*gyr*B), and those genomic-specific targeting the conserved hypothetical HL protein.

## 2. OBJECTIVE

The purpose of this work instruction is to describe current procedures for detection of *X. fastidiosa* in plant samples and insect vectors, by Enzyme-Linked ImmunoSorbent Assay (ELISA) using a commercial kit (Loewe, Germany), conventional PCR using 16s rDNA, housekeeping genes (Minsavage et al., 1994; Rodriguez et. al., 2003), and quantitative (q) PCR (Harper *et al*., 2010).
It is important to remark that these procedures need to be periodically revalidated in the light of new technical advances and findings.

## 3. SAMPLE PREPARATION

### 3.1 PLANT SAMPLES

Samples must be received by the diagnostic labs in good conditions, with the bags properly sealed, clearly labeled and accompanied by the proper documentation.
During the registration of the samples, the bag (especially those harboring weed samples) must be inspected for the presence of any winged insects and bags contaminated with bugs should be annotated, kept separately from the other sample bags and managed carefully (i.e. exposed to low temperature -4°C- for at least 3h prior processing the plant tissues).
Leaf peduncles and midribs or basal portion excised from mature leaves are the most suitable tissues for *X. fastidiosa* detection in perennial crops. For annual herbaceous plants stem, leaf peduncles and basal part from basal leaves can be used (Annex I).
For each sample, at least 0,5-0,8 g of tissue should be recovered from 5-10 leaves (according to the leaf size and consistency) and used for ELISA sap preparation or DNA extraction. Leaves selected for the extraction should be representative of the whole sample, giving priority to the symptomatic leaves, if any.

### 3.2 INSECT SAMPLES

Detection can be performed on insects recovered from sticky traps, or stored in ethanol or collected by sweeping net and properly stored prior to be tested. Insects from the traps should be removed using the proper solvent and then washed in 95% ethanol and then in deionized water to remove any residual oil. After field collection, insects can be tested immediately or stored in ethanol or at -20°C. For DNA isolation, insect heads are removed and used for extraction, using CTAB-based protocol or commercial kit.

## 4. DIAGNOSTIC PROCEDURES

### 4.1 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The procedure hereafter described refers to the use of the ELISA kit from LOEWE (Biochemica GmbH, Germany). Preliminary interlaboratory tests had showed its suitability for the detection of *X. fastidiosa* strain CoDiRO in olive tissues.
Each test should include the positive and the negative controls included in the kit, as well as the buffer. If avalailble healthy and positive controls for each of the plant species included in the test should be used.

The following steps must be followed:

1.Coat the plate

Dilute the IgG (anti -*Xf*.-IgG) 1:200 in coating buffer (i.e. 50µl in 10ml buffer, or at equal ratio for other volumes) and load 100 or 200 µl to each well of the microtiter plate. Cover the plate tightly and place it in a humid box. Incubate the plate at 37°C for 4 h.

2.Washing step

Remove the sap from the wells and wash 4 times the plates using the washing buffer, remove any liquid by blotting the plate on paper towels.

3.Plant sap preparation and antigen incubation

Homogenize the samples in extraction buffer 1:10 (w/V): weigh at least 0,5 g of leaf petioles and basal portion of the leaves, cut in small pieces using a razor blade (while processing the samples, sterilize the blade between samples). Transfer the plant tissue into the extraction bags and add 5 ml of extraction buffer; crush with a hammer and grind by a semi-automated homogenizer (i.e. Homex). Transfer 1 ml of sap into a microcentrifuge tube that store at 4°C until use, allowing plant debris precipitation. Load 100 or 200 µl of plant extract to each well of the microtiter plate. Cover the plate and incubate at 4°C overnight in a humid box.

4.Washing step

Repeat step 2.

5.Add the detection antibody

Dilute enzyme-conjugated antibodies (anti-*Xf*.-APconjugate) diluted 1:200 in conjugate buffer. Add 100 or 200 µl to each well of the microtiter plate. Cover the plate and incubate at 37°C for 4h in a humid box.

6.Washing step

Repeat step 2.

7.Add Substrate

Dissolve the p-nitrophenylphosphate (0.6-1 mg/ml) in substrate buffer and add 100 or 200 µl per well. Incubate at room temperature (18-25°C) till the yellow color reaction start to develop and read the plate at 60-120-180 min (if necessary, prolong the reaction over-night) using a plate reader at $\lambda$ =405 nm. The enzymatic reactions can be stopped by adding 25 µl 3 M NaOH (Sodium Hydroxide) to each well.

## BUFFERS REQUIRED FOR ELISA

**PBS** ( PH 7,4)

NaCl 8 g
$KH_2$ PO4 anhydrous 0,2 g
$Na_2HPO_4$ anhydrous 1,15 g
KCl 0,2
$NaN_3$ 0,2 g
Bring final volume to 1 L with distilled water

**WASHING BUFFER (PBST)**

PBS 1 L
Tween-20 0,5 ml
Store at room temperature

**COATING BUFFER (1 L; PH 9,6)**

$Na_2CO_3$ anhydrous 1,59 g
$NaHCO_3$ 2,93 g
$NaN_3$ 0,2 g
Store at 4°C

**EXTRACTION BUFFER** (1L; PH 7,4)

PBST 1 L
Polyvinylpyrrolidone (PVP-25) 20 g
Bovin serum albumin (BSA) 2g
Store at 4°C

**CONJUGATE BUFFER** ( 1 L; PH 7,4)

PBST 1 L
PVP-25 20 g
BSA 2 g
Store at 4°C

**SUBSTRATE BUFFER** (1 L; PH 9,8)

Diethanolamine 97 ml
$NaN_3$ 0,2 g
$MgCl_2$ x $6H_2O$ 0,2 g

Adjust pH with HCl and bring to final volume of 1 liter with distilled water
Store at 4°C

## TEMPLATE FOR ELISA PLATE

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | | | | | | | | | | | | |
| **B** | | | | | | | | | | | | |
| **C** | | | | | | | | | | | | |
| **D** | | | | | | | | | | | | |
| **E** | | | | | | | | | | | | |
| **F** | | | | | | | | | | | | |
| **G** | | | | | | | | | | | | |
| **H** | | | | | | | | | | | | |

## FORMAT FOR RESULT REPORTING

| SAMPLES | OD405 60 min | OD405 120 min | OD405 180 min | OD405 ON | STATUS Positive/negative* |
|---|---|---|---|---|---|
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| Positive Control | | | | | |
| Negative Control | | | | | |
| Buffer | | | | | |

## 4.2 DNA ISOLATION AND PCR ASSAYS

Plant tissues can be either processed by using a semi authomated homogenizer (i.e. Homex, Bioreba) or similar equipment; or by grinding the tissue in liquid nitrogen.
Insects can be homogenized in microcentrifuge tubes containing tungsten carbide beads and DNA isolation from plant tissue and insects can be made using a commercial kit or CTAB-based extraction. Each extraction must include the positive and negative controls.
Details on both protocols are provided below.

### 4.2.1 DNA EXTRACTION USING COMMERCIAL KIT

DNeasy Plant Mini Kit, Cat. No. 69104 – Qiagen, Valencia, CA

1. Weigh out 200 mg fresh tissue (1/4 if lyophilized) and homogenize with mortar and pestle in liquid nitrogen and transfer powered tissue into 2ml microcentrifuge tubes. Remaining tissues can be stored at -20ºC for future use. If processing insects, Homogenize the excised insect tissue in a 2 ml tube with 1-2 tungsten carbide beads for max 15-20 sec at 24/sec frequency, in a Mill300 mixer.
2. Add 800 µl of the Qiagen DNeasy Plant Mini extraction kit AP1 buffer and 8 µl of RNase A stock solution (100 mg/ml) into a sample tube.
3. Incubate cellular lysate at 65ºC for 10 min.
4. Add 260 µl of Buffer AP2 to the lysate, vortex briefly and incubate on ice for 5 min.
5. Centrifuge at 20,000 x g (14,000 rpm) for 10 min.
6. Pipet lysate into a QIAshredder Mini Spin Column (lilac colored column) in a 2 ml collection tube and centrifuge for 2 min at 20,000 x g (14,000 rpm), then, discard the column (typically about 500 µl of lysate can be recovered).
7. Measure the volume and add 1.5 volumes of Buffer AP3/E to the lysate and mix by pipetting.
8. Transfer 650 µl of the mixture including any precipitate to the DNeasy Mini Spin Column sitting in a 2 ml collection tube. Centrifuge at 6000 x g (8000rpm) for 1 min. (Discard flow through).
9. Repeat Step 12 with the remaining portion of the mixture. Discard flow-through and collection tube.
10. Place the spin column in a new 2 ml collection tube. Add 500 µl of Buffer AW to the column and centrifuge at 8000 rpm for 1 min. Discard flow-through.
11. Add another 500 ml of AW and centrifuge for 2 min at 20,000 x g (14,000 rpm) to dry the membrane.
12. Transfer the spin column to a 1.5 ml microcentrifuge tube and pipet 200 µl of Buffer AE (room temperature) onto the column membrane. Incubate for 5 min at room temperature and then centrifuge for 1 min at 6,000 x g (8000rpm) to collect DNA elution (do not allow the column to dry).
13. Extracts of total genomic DNA can be stored at 4º C for immediate use or at -20ºC for use in the future.

14. Determine the concentration at the spectrophotometer (Nanodrop or similar). Read the absorption (A) at 260nm and at 280 nm. Optimal A260/280 ratio should be close to 2 for high quality nucleic acid.
15. Adjust the concentration to 50-100ng/µl, and use 2µl (in a final volume of 25µl) to set up the PCR reactions.

NOTES: Prepare all buffers according to manufacturer's instructions supplied with the kit.

#### 4.2.2 CTAB-BASED TOTAL NUCLEIC ACID EXTRACTION FROM TISSUE PLANT

1. Weigh out 0,5-0,8 g of fresh small pieces of midribs and petioles (1/4 if lyophilized), transfer the tissue into the extraction bags and add 2ml of CTAB. Crush with a hammer and homogenize.
2. In each extraction bag add 3ml of CTAB.
3. Transfer 1ml of sap into a 2ml microcentrifuge tube.
4. Heat samples at 65°C for 30 minutes.
5. Centrifuge samples at 10,000 rpm for 5 minutes and transfer 1ml to a new 2ml micro-centrifuge tube, being careful not to transfer any of the plant tissue debris. Add 1ml of Chloroform:Isoamyl Alcohol 24:1 and mix well by shaking or vortex.
6. Centrifuge sample at 13,000 rpm for 10 minutes. Transfer 750 ml to a 1.5 ml microcentrifuge tube and add 450 µl (approximately 0.6V) of cold 2-Propanol. Mix by inverting 2 times. Incubate at 4°C or -20°C for 20 minutes.
7. Centrifuge the samples at 13.000 rpm for 20 minutes and decant the supernatant.
8. Wash pellet with 1ml of 70% ethanol.
9. Centrifuge sample at 13,000 rpm for 10 minutes and decant 70% ethanol.
10. Air dry the samples or use the vacuum.
11. Re-suspend the pellet in 100µl of TE or RNAse- and DNase-free water.
12. Extracts of total nucleic acid can be stored at 4º C for immediate use or at -20ºC for use in the future.
13. Determine the concentration at the spectrophotometer (Nanodrop 1000 or similar). Read the absorption (A) at 260nm and at 280 nm. Optimal A260/280 ratio should be close to 2 for high quality nucleic acid.
14. Adjust the concentration to 50-100ng/µl, and use 2 µl (in a final volume of 20-25µl) to set up the conventional and real time PCR reactions.

### 4.2.3 CTAB-BASED TOTAL NUCLEIC ACID EXTRACTION FROM INSECTS

1. Homogenize the excised insect tissue in a 2 ml tube with 1-2 tungsten carbide beads (for max 15-20 sec at 24/sec frequency, in Mill300 mixer (Qiagen).
2. Add in each tube 500 µl of CTAB and mix well by shaking or vortexing.
3. Heat the samples at 65°C for 20 minutes.
4. Add 500 µl of Chloroform:Isoamyl Alcohol 24:1 and mix well by shaking or vortexing.
5. Centrifuge sample at 13,000 rpm for 10 minutes. Transfer 400 µl to a 1.5 ml microcentrifuge tube and add 240 µl (approximately 0.6 V) of cold 2-Propanol. Mix by inverting 2 times. Incubate at 4°C or -20°C for 20 minutes.
6. Centrifuge the samples at 13.000 rpm for 20 minutes and decant the supernatant.
7. Wash pellet with 1ml of 70% ethanol.
8. Centrifuge sample at 13,000 rpm for 10 minutes and decant 70% ethanol.
9. Air dry the samples or use the vacuum.
10. Re-suspend the pellet in 70 µl of TE or RNAse- and DNase-free water.
11. Continue as described in the previous paragraph 4.2.3.

**BUFFER REQUIRED FOR THE EXTRACTION:**

**CTAB BUFFER**

2% CTAB (Hexadecyl trimethyl-ammonium bromide) (any vendor)
Autoclaved 0.1M TrisHCl PH 8 (any vendor)
Autoclaved 20mM EDTA (any vendor)
Autoclaved 1.4M NaCl (any vendor)
1% PVP-40

## 4.3 PCR ASSAYS

### 4.3.1 PRIMERS (DESALT PURIFICATION)

The primer sets hereafter reported have been previously tested and proved to be suitable for the detection of *X. fastidiosa* CoDiRO strain in olive tissues.

1) Primers RST31 and RST33, which generate a PCR product of 733 base pairs, (Minsavage et al ., 1994).

| |
|---|
| RST31 (forward): 5′-GCGTTAATTTTCGAAGTGATTCGATTGC-3′<br>RST33 (reverse): 5’-CACCATTCGTATCCCGGTG-3 |

2) Primers FXYgyrR499 and RXYgyr907, which generate a PCR product of 428 base pairs (Rodrigues et al., 2003).

| |
|---|
| FXYgyr499 (forward): 5’-CAGTTAGGGGTGTCAGCG-3’<br>RXYgyr907 (reverse): 5’-CTCAATGTAATTACCCAAGGT-3’ |

3) Primer HL5 and HL6, which generate a PCR product of 221 base pairs (Francis et al., 2006).

| |
|---|
| HL5 (forward): 5’-AAGGCAATAAACGCGCACTA-3’<br>HL6 (reverse): 5’-GGTTTTGCTGACTGGCAACA-3’ |

4) Primers and probe for quantitative real-time PCR (Harper et al., 2010).

| |
|---|
| XF-F(forward) 5’-CAC GGC TGG TAA CGG AAG A-3’<br>XF-R (reverse) 5’-GGG TTG CGT GGT GAA ATC AAG-3’<br>XF-P (probe) 5’ 6FAM -TCG CAT CCC GTG GCT CAG TCC-BHQ-1- 3’ |

### 4.3.2 PCR REACTIONS AND CONDITIONS

Two different PCR mix (A and B) can be used. Preliminary tests have demonstrated that the GoTaq DNA polymerase (Promega) was the most efficient for the amplification of the targets in different plant extracts. The use of a layer of mineral oil over the PCR reactions proved to be useful to effectively prevent carryover contamination, especially when a large number of samples have to be processed routinely. Each reaction should include the positive, the negative and the non-template controls. For qPCR, samples should be run in duplicate wells.

**REACTION MIX (OPTION A):**

| **Reagent** | **Volume** |
|---|---|
| Total genomic DNA | 2 µl |
| 5X Green GoTaq Buffer (Promega) | 5 µl |
| 10 µM Forward Primer | 0.5 µl |
| 10 µM Reverse Primer | 0.5 µl |
| 10 mM dNTPs (any vendor) | 0.4 µl |
| GoTaq G2 DNA Polymerase (Promega, cod. M7845) | 0.2 µl |
| Molecular grade water | 16.4 µl |
| **Total** | **25 µl** |

**REACTION MIX (OPTION B):**

| **Reagent** | **Volume** |
|---|---|
| Total genomic DNA | 2 µl |
| 2X GoTaq Green Master Mix (Promega, cod. M7122) | 12.5 µl |
| 10 µM Forward Primer | 0.5 µl |
| 10 µM Reverse Primer | 0.5 µl |
| Molecular grade water | 9.5 µl |
| **Total** | **25 µl** |

**REACTION MIX FOR QUANTITATIVE (q)PCR**

| **Reagent** | **Volume** |
|---|---|
| Total genomic DNA | 1 µl |
| 2X master mix for probes | 5.5 µl |
| 10 µM Forward Primer | 0.3 µl |
| 10 µM Reverse Primer | 0.3 µl |
| 10 µM TaqMan probe | 0.1 µl |
| Molecular grade water | 5.5 µl |
| **Total** | **11 µl** |

**PCR CONDITIONS**

| **Primers RST31/RST33** | |
|---|---|
| 94°C 5 min | 1 cycle |
| 94°C 30 sec | 35 cycles |
| 55°C 30 sec | |
| 72°C 45 sec | |
| 72°C 7 min | 1 cycle |

| **Primers XF1-F/XF6-R, FXYgyrR499/RXYgyr907** | |
|---|---|
| 94°C 5 min | 1 cycle |
| 94°C 30 sec | 35 cycles |
| 50°C 30 sec | |
| 72°C 40 sec | |
| 72°C 7 min | 1 cycle |

| **Primers Harper *et al.*, 2010 for qPCR** | |
|---|---|
| 50°C 2 min | 1 cycle |
| 95°C 10 min | 1 cycle |
| 94°C 10 sec | 39 cycles |
| 62°C 40 sec | |

### 4.3.3 GEL ELECTROPHORESIS

Load 8-10 µl of PCR products on 1.2% Agarose gel in TAE 1X (STOCK 1lt 50X: Tris 242g, Acetic Acid 57 ml, EDTA 0,5 M-ph8 100ml) previously added of "GelRed Nucleic Acid Stain" (1µl/100ml of gel) (BIOTIUM, cod. 410003-0.5ml).

**TEMPLATE FOR CONVENTIONAL PCR AND REAL TIME QPCR REACTIONS**

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | | | | | | | | | | | | |
| **B** | | | | | | | | | | | | |
| **C** | | | | | | | | | | | | |
| **D** | | | | | | | | | | | | |
| **E** | | | | | | | | | | | | |
| **F** | | | | | | | | | | | | |
| **G** | | | | | | | | | | | | |
| **H** | | | | | | | | | | | | |

**FORMAT FOR PCR RESULT REPORTING**

| SAMPLES | Positive/negative* |
|---|---|
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| | |
| | |
| Negative control | |
| Positive control | |
| Non-template control | |

*Positive= presence of DNA band of expected size; Negative=absence of DNA band of expected size.

**FORMAT FOR QUANTITATIVE PCR RESULT REPORTING**

| SAMPLES | Cq Values | STATUS Positive/negative* |
|---|---|---|
| 1 | | |
| 2 | | |
| 3 | | |
| 4 | | |
| 5 | | |
| 6 | | |
| 7 | | |
| 8 | | |
| 9 | | |
| 10 | | |
| 11 | | |
| 12 | | |
| 13 | | |
| Negative control | | |
| Positive control | | |
| Non-template control | | |

*Negative samples: If a sample produces a FAM Cq =0.00 or >35.00 then it is determined to be negative for *Xylella fastidiosa*.
Positive samples: If a sample produces a FAM Cq value in the range of 0.00< FAM Cq <35.00 the sample is determined to be positive for *Xylella fastidiosa.*
Samples which produce a Fam Cq value in the range of 32.01>FAM Cq >34.99 need to be tested again in real-time PCR to confirm the first run results.

## 4.4 RECOMMENDATION FOR MOLECULAR TESTS

**A) To avoid risks of contamination:**

1. While processing the samples wear always gloves and change them frequently. Do not use the same gloves for tissue preparation and for PCR/qPCR set up.

2. Keep the lab bench top clean, surface sterilize frequently the bench with 10% bleach solution and 70% ethanol.

3. Use filter tips for all steps of the DNA isolation and PCR/qPCR set up;

4. Perform the different procedures in separated areas; tissue preparation, DNA isolation and PCR/qPCR reactions should be performed in distinct area of the laboratory.

**B) To avoid false negative:**

1. The quality of the extracts should be always checked at the spectrophotometer or by agarose gel visualization.

2. If performing qPCR assays, a plant DNA internal control can be used (i.e. assays targeting the cytochrome oxidase gene).

## 4.5 INTERPRETATION OF THE RESULTS

1. Test results will be reported as falling in one of three categories:

a. *X. fastidiosa* positive: results indicate that *X. fastidiosa* was detected in the sample.

b. No *X. fastidiosa* found: test results did not indicate that *X. fastidiosa* was present in the sample.

c. *X. fastidiosa* questionable: test results were inconclusive, therefore re-testing should be considered.

2. No testing procedure is completely accurate. Therefore if a sample is designated as "No *X. fastidiosa* Found", this does not mean that the tree/plant from which the sample was taken is disease-free. A "No *X. fastidiosa* Found" designation means only that no *X. fastidiosa* was detected in the sample. This could be due to several reasons including but not limited to:

a. No *X. fastidiosa* was present.

b. *X. fastidiosa* was present but below the limit of detection.

c. *X. fastidiosa* was present but the sample was inadequate for testing (sample in poor condition, wrong type of tissue sampled, operator errors, etc.).

d. The test failed.

# ANNEX 1: PLANT SAMPLES AND TISSUE PREPARATION

(**A**) Olive twigs collected from a sampled tree. Leaves showing leaf scorching and symptomless leaves selected for the sample preparation are shown along with the petioles and midribs excised for the extraction.

(**B**) Leaves of almond (**up**) and cherry (**down**) collected in late summer and showing leaf scorch symptoms. The petioles and the basal parts of the almond (up) and cherry (down) leaves used for the extraction are shown.

(**C**) Oleander (**up**) and polygala (*Polygala myrtifolia*) (**down**) showing symptoms of leaf scorching and necrosis. Tissues used for the extraction are also shown.

(**D**) Samples and tissues selected for grape (**upper left side**), citrus (**upper right side**) and bermuda grass (*Cynodon dactylon*, L.) (**bottom**).

(**E**) Samples and tissues selected for *Calendula arvensis* (**left side**) and *Malva sylvestris* (**right side**).

# ANNEX 2: EQUIPMENT, MATERIALS AND REAGENTS

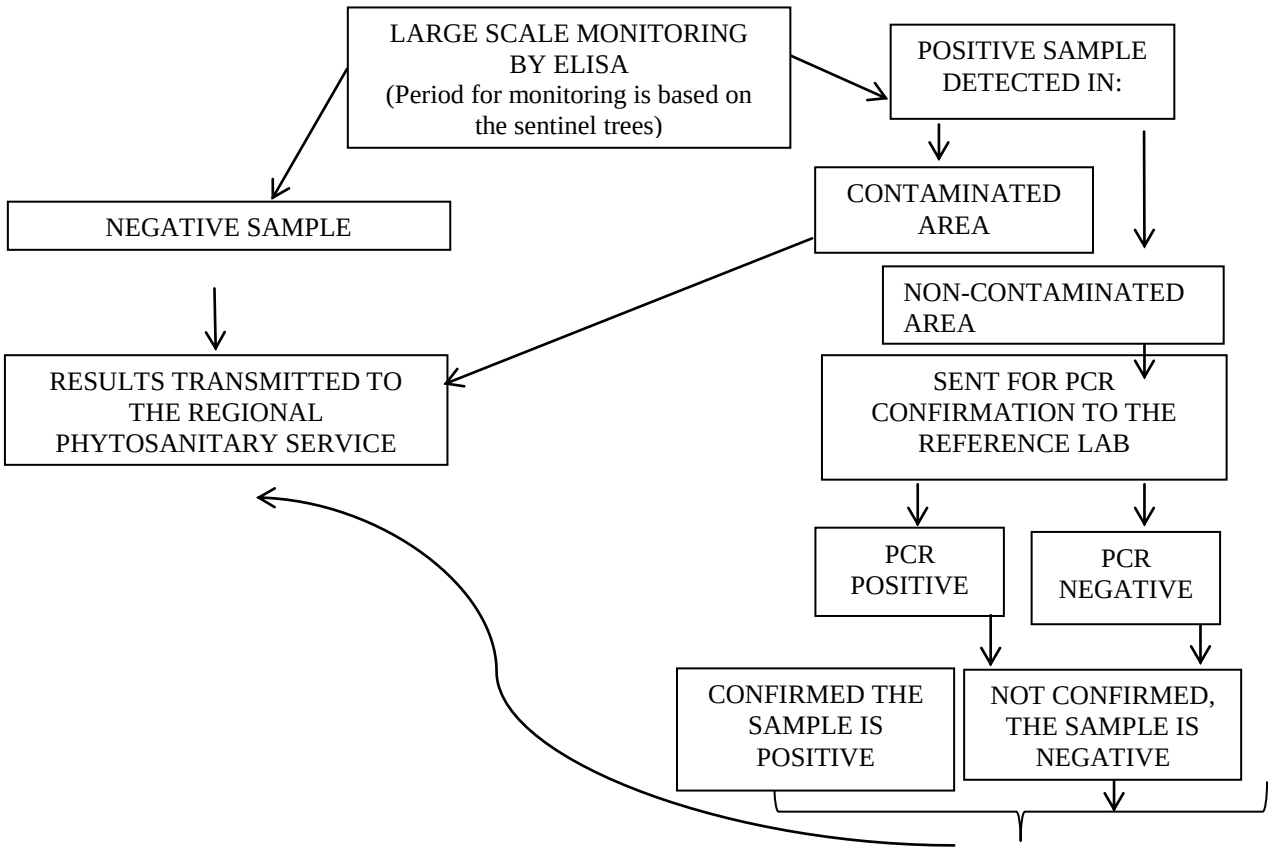
## A) ELISA TESTS

- Reagent set for the serological detection of *Xylella fastidiosa* (Loewe Biochemica GmbH-Germany, Cat. No. 07119S)
- Chemical reagents for buffers preparation
- 4-Nitrophenyl phosphate Na-salt
- ELISA plates
- (e.g. Falcon flexible plates or Nunc-Immuno Plates MaxiSorp F96)
- Bioreba bags (BB6430100B) or similar
- Microcentrifuge tubes
- Disposable pipettes (any manufacturer)
- Micropipettes (P20, P200, P1000, multichannel pipette)
- Homex (Bioreba) or similar tissue homogenizer
- Incubator at 37°C
- Microplate auto reader (405 nm)
- Analytical balance (any manufacturer)

## B) PCR AND QUANTITAVE PCR

- (Optional) PCR Workstation (any vendor)
- Homex (Bioreba) or similar tissue homogenizer
- Bench-top microcentrifuge capable of 14,000 rpm (any vendor)
- Vortex (any vendor)
- PCR unit (any vendor)
- qPCR unit (any vendor)
- Analytical balance (any vendor)
- Thermoblock capable of 65-70°C + 2°C
- NanoDrop (microvolume spectrophotometer or similar)
- Dedicated annually-calibrated pipets (P10, P20, P200, P1000).
- Freezer (-20°C, non-frost-free) (any vendor)
- Bioreba bags (BB6430100B) or similar;
- Microcentrifuge tubes 2ml, 1,5ml
  (pre-sterilized, certified DNase & RNase free, any vendor)
- Sterile filter (barrier) pipette tips (P10,P200, P1000, any vendor)
- Disposable paster pipette (any vendor)
- PCR tube 0.2ml (any vendor)
- Molecular grade water (any vendor)
- Ethanol (96-100%, any vendor)
- Isopropanol (any vendor)
- Chloroform (any vendor)

# ANNEX 3: FLOW DIAGRAM OF THE DIAGNOSTIC PROCEDURE USED IN APULIA FOR THE XYLELLA FASTIDIOSA MONITORING PROGRAMME



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