

Diagnostic training workshop
Sampling and diagnostic tools for
Xylella fastidiosa
19-22 September, 2017 - Locorotondo, Italy

Laboratory training on the detection of
***Xylella fastidiosa* in plant samples**
DIAGNOSTIC PROTOCOLS

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1. PLANT SAMPLE PREPARATION

Samples for testing include cuttings with leaves of olives, cherry and *Polygala myrtifolia*. Samples were collected one week before the training and kept at 4°C.

Tissues were prepared as follow:

- DNA extraction for PCR and qPCR assays: (i) olive: a mixed sample of leaf petioles and small pieces of twigs; (ii) cherry: one sample consisting of leaf petioles; one sample consisting of xylem tissues scraped from twigs; (iii) *Polygala myrtifolia*: leaf petioles.
- ELISA and real-time LAMP: (i) olive: leaf petioles; (ii) cherry: one sample consisting of leaf petioles; one sample consisting of xylem tissues scraped from twigs; (iii) *Polygala myrtifolia*: leaf petioles.
- DTBIA: (i) olive: mature cuttings.
- Isolation: (i) olive: mature cuttings; (ii) cherry: mature cuttings and leaves; (iii) *Polygala myrtifolia* mature cuttings and leaves.

The samples to be processed for DNA extraction, ELISA and real-time LAMP assay consist of 0,5-0,8 g of tissue (leaf petioles/midribs or xylem tissue) recovered from at least 4 different cuttings representative of the whole sample, giving priority to the symptomatic portion, if any.

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2. ISOLATION PROCEDURES

2A. ISOLATION FROM LEAVES

1. Select 10-20 leaves (depending on the size).
2. Wash the leaves under tap water using a dish detergent.
3. Remove the excess of water and dry the leaves.
4. Soak sequentially the leaf midribs/petioles in 2% of bleach for 2 min and then in 70% ethanol for 2 min.
5. Rinse three times in sterile water and dry on sterile adsorbent paper.
6. Recover 0.5 g of leaf petioles or the leaf midribs;
7. Transfer the tissue in a Bioreba bag and add 5ml of PBS 1X (seal the bag under the hood)
8. Homogenize/grind the tissue.
9. Recover 100µl of the sap and add to 900µl of PBS 1X
10. Prepare a 10-fold serial dilution
11. Plate the different dilutions on PWG
12. Incubate the plates at 28°C, and monitor the colony development over 6 weeks. Plates are sealed to prevent desiccation.

2B. ISOLATION FROM TWIGS AND BRANCHES

1. Select 10-12 pieces (6-8 cm in length) and wash deeply under tap water using a dish detergent;
2. Remove the excess of water and dry the leaves.
3. Soak sequentially the cuttings in 2% of bleach for 2 min and then in 70% ethanol for 2 min.
4. Rinse three times in sterile water and dry on sterile adsorbent paper.
5. Cut each twig in half
6. Squeeze the cuttings using pliers and make 2-3 spots on the agar plates with the internal cut ends
7. Plates are then incubated as described above.

Buffer and media

All buffers and media are sterilized by autoclaving at 121°C for 20 min.

PBS 1X

20 mM NaH₂PO₄

0.5 % NaCl

Adjust pH to 6.8 by KOH before autoclaving.

PWG (Hill and Purcell, Phytopathology, 1995)

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Components	1L of substrate
Phytone peptone (BBL 4311906)	4.0 g
Trypticase peptone (BBL 4311921)	1.0 g
Hemin chloride (0.1 %)	10 ml
Phenol red (0.2 %)	10 ml
K ₂ HPO ₄	1.2 g
KH ₂ PO ₄	1.0 g
MgSO ₄ ·7SO ₄	0.4 g
Gelrite gellan gum (Gelzam TM CM; Sigma G 1910)	9.0 g
H ₂ O	820 ml

Autoclave 20 min at 120°C

After autoclaving, allow the temperature to cool down to 50°C prior to add:

Components	1L of substrate
Glutamine 4%*	100 ml
BSA 10%*	60 ml

* To be filtered

Glutamine 4%

- 4 g glutamine (Sigma G-5763)
- 100 ml H₂O
- Filter on 0.22 µm

BSA 10%

- 6 g BSA fraction V (Sigma A-9418)
- 60 ml di H₂O
- Filter on 0.22 µm

Phenol red 0.2 % (Store for a maximum of 1 month at 5-4°C.)

- 0.2 g phenol red
- 10 drops NaOH 20% (NaOH 5N)
- 100 ml H₂O

Hemin Cl 0.1 % (Store for a maximum of 1 month at 5-4°C.)

- 0.1 g hemin Cl (Sigma H-1652)
- 0.2 g NaOH
- 100 ml H₂O

BCYE medium (Wells et al., Appl. Env. Microbiol., 42:357-363, 1981)

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	P/1000 ml
Yeast extract	10.0 g
Activated charcoal (Vetec)	2.0 g
L-cysteine HCl (Sigma)*	0.4 g
Ferric pyrophosphate (Sigma)**	0.25 g
ACES buffer (Sigma)	10.0 g
Difco Bacto Agar	17.0 g
Demineralized water	940 ml
KOH solution 1M	40 ml

1. Warm ($\approx 50^{\circ}\text{C}$) the Aces buffer in 500 ml of distilled water;
2. Add 40 ml KOH in 1N in 440 ml distilled water;
3. Add the active charcoal in step-2 solution ;
4. Pool the step 1 and 3 solutions;
5. Add the yeast extract to pooled solution;
6. Adjust the pH (6.85);
7. Add agar and autoclave
8. Cool down the medium at 50°C and add the pre-filtered L-cystein and ferric pyrophosphate.

*L-cystein diluted in 10 ml sterile water;

**Ferric pyrophosphate needs to be diluted in 10 ml sterile water

3. DNA EXTRACTION FOR PLANT MATERIAL

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3A. CTAB-BASED EXTRACTION

1. Recover 0.5-1 g of fresh small pieces of midribs, petioles, leaf basal part or twigs (1/4 of the indicated amount, if lyophilized), transfer the tissue into the extraction bags or into suitable tubes with 5 mL of CTAB buffer/Food lysis buffer (see Mericon extraction) and homogenized using an homogenizer (e.g. Homex, Polytron, etc.).
2. Transfer 1ml of sap into 2ml microcentrifuge tubes.
3. Heat the samples at 65°C for 30 minutes.
4. Centrifuge samples at 12,000 g for 5 minutes and transfer 1ml to a new 2ml microcentrifuge tube, being careful not to transfer any of the plant tissue debris. Add 1ml of Chloroform and mix well by shaking.
5. Centrifuge sample at 16,000 g for 10 minutes. Transfer 700 µl to a 1.5ml 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.
6. Centrifuge the samples at 16,000 g for 20 minutes and decant the supernatant.
7. Wash pellet with 1ml of 70% ethanol.
8. Centrifuge sample at 16,000 g for 10 minutes and decant 70% ethanol.
9. Air dry the samples or use the vacuum.
10. Re-suspend the pellet in 100-150 µl of TE or RNase- and DNase-free water.
11. Extracts of total nucleic acid can be stored at 4°C for immediate use or at -20°C for use in the future.
12. Determine the concentration at the spectrophotometer (Nanodrop 1000 or similar). Read the absorption (A) at 260nm and at 280 nm. Optimal A₂₆₀/A₂₈₀ ratio should be close to 2 for high quality nucleic acid.
13. Use 1 µl (in a final volume of 11 µl) to set up the conventional and real time PCR assays for *X. fastidiosa* detection.

CTAB BUFFER

2% CTAB (Hexadecyl trimethyl-ammonium bromide)
Autoclaved 0.1M TrisHCl PH 8
Autoclaved 20mM EDTA
Autoclaved 1.4M NaCl
Adjust PH to 8.0

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3B. DNEASY® MERICON™ FOOD STANDARD PROTOCOL (QIAGEN) (MODIFIED) FOR MANUAL EXTRACTION

1. Recover 0.5-1 g of fresh small pieces of midribs, petioles, leaf basal part or twigs (1/4 of the indicated amount, if lyophilized), transfer the tissue into the extraction bags or into suitable tubes with 5 mL of Food lysis buffer and homogenized using an homogenizer (e.g. Homex, Polytron, etc.).
2. Transfer 1ml of sap into 1.5ML microcentrifuge tubes.
3. Incubated for 30 min at 60°C. To enhance inhibitor precipitation, cool the sample to room temperature (15–25°C) on ice after incubation.
4. Centrifuge for 5 min at 2500 x g.
5. Pipet 500 µl chloroform into a 2 ml microcentrifuge tube.
6. Carefully transfer 700 µl of the clear supernatant from step 4 to the microcentrifuge tube containing the chloroform. Be sure not to carry over material from the bottom phase, which contains precipitated food debris.
7. Vortex the microcentrifuge tube from step 6 vigorously for 15 s and centrifuge at 14,000 x g for 15 min.
8. Pipet 350 µl of Buffer PB into a fresh 2 ml microcentrifuge tube. From this step, it is alternatively possible to use the automated platform QIACUBE (see below).
9. Add 350 µl of the upper, aqueous phase from step 7 and mix thoroughly by vortexing.
10. Pipet 600 µl of the mixture from step 9 into the QIAquick spin column placed in a 2 ml collection tube. Centrifuge at 17,900 x g for 1 min and discard the flow-through. Reuse the collection tube in step 11.
11. Add 500 µl Buffer AW2 to the QIAquick spin column, centrifuge at 17,900 x g for 1 min and the discard flow-through. Reuse the collection tube and centrifuge again at 17,900 x g for 1 min to dry the membrane.
12. Transfer the QIAquick spin column to a 1.5 ml or 2 ml microcentrifuge tube (not supplied), and pipet 100 µl Buffer EB directly onto the QIAquick membrane. Incubate for 1 min at room temperature (15–25°C), and then centrifuge at 17,900 x g for 1 min to elute.
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. Use 1 µl (in a final volume of 11 µl) to set up the conventional and real time PCR assays for *X. fastidiosa* detection

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Method with Qiacube

Material	Transfer 350 µl of the aqueous phase from step 7 to a 2 ml safe-lock tube, and place it onto the QIACube shaker
Elution volume	Set up 100 µl
Vessel	2 ml safe-lock microcentrifuge tube*
Adapter	Shaker adapter for 2 ml microcentrifuge tubes (marked with "2")

* Sample Tubes RB, 2 ml (cat. no. 990381); see www.qiagen.com/MyQIACube.

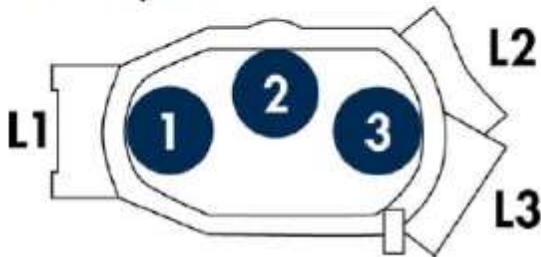
Disposable Tips

Disposable Filter-Tips, 1000 µl

Reagent Bottle Rack

Rack labeling strip	Unlabeled
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Rotor Adapter



Position	Reagent
1	Buffer PB
2	–
3	–
4	–
5	Buffer AW2
6	Buffer EB

Position	Labware	Lid position
1	QIAquick® spin column	L1
2	–	–
3	Elution Tube (1.5)	L3



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3C. QUICKPICK™ SML PLANT DNA KIT-BASED EXTRACTION (BIO-NOBILE)

1. For each sample, weight 0.5-1 g (according to the plant species) of fresh small pieces of midribs, petioles, basal leaf part, or xylem. Put in a plastic bag with gaze. (It is possible to stop the test here by storing the bags at <-20°C). Crush the plant tissues in sterile water (5 mL/g).
2. Soak for at least 15 min, under gentle shaking. If not used immediately for the step 3, macerates have to be stored at 5°C before testing during the day or the bags stored at <-20°C. (NB: store water as negative extraction control). Take about 1 ml in 1.5 microtubes.
3. Take 1 X 250 µL of each plant extract and put in a new 1.5mL microtube. Centrifuge for 20 min at 20,000 g. Discard the supernatant. It is possible to stop the test here by storing the pellets at <-20°C.
4. Resuspend the pellet in 75 µL of lysis buffer with 5 µL of proteinase K (reagents from Bio-Nobile kit).
5. Mix thoroughly and lyse the sample for 20 minutes at 65°C with regular shaking (at minimum 1 time each 5 min).

Method with magnet pipet

1. During the lysis step pipette QuickPick™ SML Plant DNA reagents into tubes as follows:
2. Tube 2: 5µL Plant DNA Magnetic Particles and 125µL Plant DNA Binding Buffer
*Important: never vortex the magnetic particles. But gently suspend and homogenize the particles before pipetting.
3. Tube 3: Plant DNA Wash Buffer
4. Tube 4: Plant DNA Wash Buffer
5. Tube 5: Plant DNA Wash Buffer
6. Tube 6: Plant DNA Elution Buffer
7. Remove tube 1 from 65°C. Centrifuge the tube for 5 minutes at 18,000 g. Gently transfer the supernatant into tube 2. Mix tube 2 gently and incubate at room temperature for 10 min. Mix the suspension continuously during this step.
8. Pick up the QuicPick tip with the QuicPik 1. Collect the magnetic particles from tube 2 and release them into tube 3 (washing buffer). Wash the magnetic particles by missing the

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suspension gently for 20 seconds using the QuicPick tip. Repeat the washing steps in tubes 4 and 5 (Wash Buffer).

9. Collect the Magnetic Particles from tube 5 with the QuicPick 1 and release them into tube 6 (Elution Buffer). Mix tube 6 continuously and incubate at room temperature for 10 minutes (use a tube rotator or mix manually). During elution Magnetic Particles should disperse.
10. Collect the magnetic particles from tube 6 and discard them and the tip. The eluate in tube 6 containing the purified DNA is ready to be used on the same day, or to be store at -20C until use.

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4. REAL-TIME PCR

Harper *et al.*, 2010; erratum 2013

XF-F (forward) 5'-CAC GGC TGG TAA CGG AAG A-3'

XF-R (reverse) 5'-GGG TTG CGT GGT GAA ATC AAG-3'

XF-P (probe) 5' 6FAM -TCG CAT CCC GTG GCT CAG TCC-BHQ-1- 3'

PCR conditions: pre-incubation at 50°C for 2 minutes followed by 95°C for 10 minutes, followed by 40 cycles of (94°C for 10 seconds and 62°C for 40 seconds).

Reagents	[Concentrated Sol.]		[Final Sol.]		Vol. for one tube	
Ultra pure water					6.60	μL
TaqMan™ Fast Advanced Master Mix (AB) (Cod. 4444557)	2	x	1	x	10	μL
XF-F	10	μM	0.30	μM	0.6	μL
XF-R	10	μM	0.30	μM	0.6	μL
XF-P	10	μM	0.10	μM	0.2	μL
	PCR Mix Volume				18	μL
	DNA Sample Volume				2	μL
	Total Volume total per reaction				20	μL

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6. CONVENTIONAL PCR (MINSAVAGE ET AL., 1994)

Primers RST31 and RST33, which generate a PCR product of 733 base pairs

RST31 (forward): 5'-GCGTTAATTTTCGAAGTGATTTCGATTGC-3

RST33 (reverse): 5'-CACCATTCGTATCCCGGTG-3'

Reagents	[Concentrated Sol.]		[Final Sol.]		Vol. for one tube	
Ultra pure water					9.5	μL
2x Master Mix DreamTaq (COD. 4472942)	2	x	1	x	12.5	μL
RST 31	10	μM	0.2	μM	0.5	μL
RST 33	10	μM	0.2	μM	0.5	μL
	PCR Mix Volume				23	μL
	DNA Sample Volume				2	μL
	Total Volume total per reaction				25	μL

PCR amplification conditions

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

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).

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7. Real-time fluorescence LAMP assay*

7A. USING PLANT TISSUES

1. For each sample cut 4 small slices (1–2 mm) from 4 different olive petioles (1- year old) and place them in the tube containing 200 µL of extraction buffer.
2. Vortex gently.
3. Incubate the samples for 10 min at 65°C.
4. Prepare the aliquots of the LAMP MIX by adding in each tube labelled “primer MIX”, 22.5 µl of LAMP MIX, 30 µl of mineral oil and finally 2.5 µl of denatured sample.
5. Vortex gently and briefly centrifuge.
6. Set the following amplification program on the device: one step at 65°C for a minimum of 25min. If you use real-time PCR device, you have to use FAM as fluorophore and to set the detecting camera each min.
7. Amplification curves will be observed in case of a positive sample. No amplification curves will indicate a negative sample. The fluorescence units are shown on the Y-axis and the time to amplification on the x-axis. If you use ICGE device the device is equipped with software that based on the amplification curves assigns positive/negative reactions to wells with unknown samples. If you use a real time PCR device, positive results will be associated for samples showing Real-Time Exponential Curve followed with Plateau phase.

7B. USING PLANT SAP

1. Prepare 0.5-0.1 g of fresh small pieces of midribs, petioles, leaf basal part or twigs, xylem tissue (1/4 of the indicate amount, if lyophilized) and homogenize in ELISA-Extraction buffer (1:10 w:v).
2. Transfer 5 µl of crude sap in the tubes containing the LAMP extraction buffer
3. Vortex gently.
4. Incubate the samples for 10 min at 65°C.

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5. Prepare the aliquots of the LAMP MIX by adding in each tube labelled “primer MIX”, 22.5 µl of LAMP MIX, 30 µl of mineral oil and finally 2.5 µl of denatured sample.
6. Vortex gently and briefly centrifuge.
7. Set the following amplification program on the device: one step at 65°C for 25min.

If you use real-time PCR device, you have to use FAM as fluorophore and to set the detecting camera each min.

8. Amplification curves will be observed in case of a positive sample. No amplification curves will indicate a negative sample. The fluorescence units are shown on the Y-axis and the time to amplification on the x-axis. If you use ICGE device the device is equipped with software that based on the amplification curves assigns positive/negative reactions to wells with unknown samples. If you use a real time PCR device, positive results will be associated for samples showing Real-Time Exponential Curve followed with Plateau phase.

Extraction buffer (1L; pH 7.4)

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

* The protocol is based on the kit and device designed by Enbiotech srl (Italy). The original protocol is described in: Yaseen T, Drago S, Valentini F, Elbeaino T, Stampone G, Digiario M and D’Onghia AM (2015). On-site detection of *Xylella fastidiosa* in host plants and in “spy insects” using the real-time loop-mediated isothermal amplification method. *Phytopathologia Mediterranea* 54, 488–496.

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8. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

PLEASE REFER TO THE TECHNICAL SHEET FOR ADDITIONAL INFORMATION

The general procedure includes the following steps:

1. Coat the plate

Dilute the IgG (anti -*Xf*.-IgG) in coating buffer following the manufacturer's instructions of the kit, and load 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 for 3 min the plates using the washing buffer, remove any liquid by blotting the plate on paper towels.

3. Plant sap preparation and Antigen incubation

Load 200 µl of plant sap 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) in conjugate buffer according to the manufacturer's instructions. Add 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 200 µl per well. Incubate at room temperature (18-25°C) till the yellow color reaction starts to develop and read the plate at 60-120-180 min 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.

RESULTS INTERPRETATION:

The manufacturer's instruction must be followed.

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BUFFERS REQUIRED for ELISA

PBS (pH 7,4)

NaCl 8 g

KH₂ PO₄ anhydrous 0.2 g

Na₂HPO₄ anhydrous 1.15 g

KCl 0.2

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)

Na₂CO₃ anhydrous 1.59 g

NaHCO₃ 2.93 g

Store at 4°C

Extraction buffer/Conjugate buffer (1L; pH 7.4)

PBST 1 L

Polyvinylpyrrolidone (PVP-25) 20 g

Bovin serum albumin (BSA) 2g

Store at 4°C

Substrate buffer (1 L; pH 9.8)

Diethanolamine 97 ml

MgCl₂ x 6H₂O 0.2 g

Adjust pH with HCl and bring to final volume of 1 L with distilled water.

Store at 4°C

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9. DIRECT TISSUE BLOT IMMUNOASSAY (DTBIA)*

1. Preparation of the membrane

Prepare the nitrocellulose membrane (0.45µm) by marking with a pencil the grid for positioning the individual sample (premarked membrane could be also supplied with the diagnostic kit)
Select at least four olive mature twigs from each sample; make a fresh clean cut, squeeze and press gently the surface on the membrane. Prepare at least 2 spots for each twig for a total of eight spots.

Sterilize the shears between samples using a 10% bleach solution.

Dry the membrane for at least 30 min at room temperature.

2. Blocking

Prepare the blocking solution: 1% (w/v) of non-fat dry milk in 1x PBS.

Place the membrane in a box of the appropriate size and pour the blocking solution.

Incubate for 2 hours with shaking at room temperature or overnight at 4°C.

3. Washing

Discard the blocking solution

Wash the membrane with an appropriate volume of washing buffer, for 3 minutes at room temperature, on a shaker (optional)

Repeat the washing step three times

4. Conjugated antibody incubation

Dilute the enzyme-conjugated Xf-specific antibodies following the manufacturer's instruction** in conjugate buffer and incubate the membrane with an appropriate volume (covering the membrane) for 2 hours at room temperature with shaking.

5. Washing

Repeat step 3.

6. Substrate development

Dissolve 1 tablet of BCIP-NBT (Sigma Fast) in 10ml of distilled water.

Cover the membrane with this solution and incubate at room temperature till the appearance of purple-violet color in the positive samples (about 5 to 10 min.)

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Stop the reaction by washing the membrane with water

7. Results recording

Dry the membrane and observe the imprints using a low power magnification (x10-x20)
The presence of purple-violet precipitates in the imprints indicates positive reactions.

BUFFERS REQUIRED for DTBIA

PBS (pH 7,4)

NaCl 8 g
KH₂ PO₄ anhydrous 0,2 g
Na₂HPO₄ anhydrous 1,15 g
KCl 0,2
Bring final volume to 1 L with distilled water

Washing buffer (PBST)

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

Conjugate buffer (1 L; pH 7,4)

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

* The original protocol is described in: Djelouah, K., Frasher, D., Valentini, F., D'Onghia, A. M. & Digiario, M. (2014). Direct tissue blot immunoassay for detection of *Xylella fastidiosa* in olive trees. *Phytopathologia Mediterranea*, 53. doi: 10.14601/Phytopathol_Mediterr-14603.

**see the technical fact sheet for additional information.

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ANNEX I



(A) Olive twigs selected for the test. Leaves showing leaf scorching and symptomless leaves selected for the sample preparation are shown along with the petioles and midribs excised for the extraction.

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(B) Preparation of the tissues from samples of cherry. In the upper panel: leaves of cherry collected in late summer and showing leaf scorch symptoms, and the leaf petioles used for the test. In the lower panel are shown the cuttings used to recover the xylem tissue after removing the bark.

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(C) *Polygala myrtifolia* showing symptoms of leaf scorching and necrosis. Tissues recovered for the tests are also shown.