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DIAGNOSTIC TRAINING WORKSHOP

DETECTION OF *XYLELLA FASTIDIOSA*

PROTOCOLS

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Euphresco

DNA extraction for plant material

A. 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 and homogenized using a homogenizer (e.g. Homex, Polytron, etc.).
2. Transfer 1 ml of sap into 2 ml microcentrifuge tubes.
3. Heat the samples at 65°C for 30 minutes.
4. Centrifuge samples at 12,000 g for 5 minutes and transfer 1 ml to a new 2 ml micro-centrifuge tube, being careful not to transfer any of the plant tissue debris. Add 1 ml of Chloroform:Isoamyl Alcohol 24:1 and mix well by shaking.
5. Centrifuge sample at 16,000 g for 10 minutes. Transfer 700µl 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.
6. Centrifuge the samples at 16,000 g for 20 minutes and decant the supernatant.
7. Wash pellet with 1 ml of 70% ethanol.
8. Centrifuge sample at 16,000 g for 10 minutes and decant 70% ethanol.
9. Air dry the samples or use a vacuum.
10. Re-suspend the pellet in 100-150 µl of TE or RNAse- and DNase-free water.
11. Determine the concentration using a spectrophotometer (Nanodrop 1000 or similar). Read the absorption (A) at 260nm and at 280nm. Optimal A260/280 ratio should be close to 2 for high quality nucleic acid.
12. Extracts of total nucleic acid can be stored at 4°C for immediate use or at -20°C for use in the future.

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

B. DNeasy® mericon™ Food Standard Protocol (Qiagen) (Modified)

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 and homogenized using an homogenizer (e.g. Homex, Polytron, etc.).
2. Transfer 1 ml of sap into 1.5 ml microcentrifuge tubes.
3. Incubated for 30 minutes 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. Pipette 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 plant debris.
7. Vortex the microcentrifuge tube from step 6 vigorously for 15 seconds and centrifuge at 14,000 x g for 15 minutes.
8. Pipette 350 µl of Buffer PB into a fresh 2 ml microcentrifuge tube, add 350 µl of the upper, aqueous phase from step 7 and mix thoroughly by vortexing.
9. Pipette the solution from step 8 into the QIAquick spin column placed in a 2 ml collection tube. Centrifuge at 17,900 x g for 1 minute and discard the flow-through. Reuse the collection tube in step 10.
10. 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 minute to dry the membrane.
11. Transfer the QIAquick spin column to a 1.5 ml or 2 ml microcentrifuge tube (not supplied), and pipette 100 µl Buffer EB directly onto the QIAquick membrane. Incubate for 1 minute at room temperature (15–25°C), and then centrifuge at 17,900 x g for 1 minute to elute.
12. Determine the concentration using a spectrophotometer (Nanodrop 1000 or similar). Read the absorption (A) at 260nm and at 280nm. Optimal A260/280 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

C. QuickPickTM SML Plant DNA Kit-based extraction (Bio-Nobile)

1. For each sample, weigh 0.5-1 g (according to the plant species) of fresh small pieces of midribs, petioles, basal leaf part or twigs. Put in a plastic bag with gauze. (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 minutes, 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).
3. Take 2 X 250 µL of each plant extract and put in 2 X 2 ml (or 1.5ml) microtubes. Prepare positive extraction control(s) (one per plant genus): 250 µL of plant extract + 5 µL of Xf lysis (concentration about 10^7/mL) in a separate room.
   Centrifuge for 20 minutes at 20,000 g. Discard the supernatant. It is possible to stop the test here by storing the pellets at <-20°C.
4. Re-suspend 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).

<u>Method with the robot</u> (BioSprint 15 Qiagen or KingFisher mL or Flex Thermo Scientific)

6. During the lysis step pipette QuickPick™ SML Plant DNA reagents into plastic tubes for the robot according this following scheme:

	A		B	C	D	E
Buffers	Plant DNA Binding Buffer	Plant DNA Magnetic Particles*	Plant DNA Wash Buffer	Plant DNA Wash Buffer	Plant DNA Wash Buffer	Plant DNA Elution Buffer
Volume (µL)	125	5	250	250	250	50

*Important: never vortex the magnetic particles. But gently suspend and homogenise the particles before pipetting.

7. Remove tubes from 65°C. Centrifuge each tube for 5 minutes at 18,000 g.

8. Gently transfer the supernatant into tube A with binding buffer and magnetic beads. Put the plate with tubes in the robot.
9. Verify the position of plastics and magnets inside the robot before starting. Start the robot with the specific program (duration: around 31 min).
10. At the end of this program, transfer the DNA (tube E) in a new tube (NB: before transferring well verify the absence of magnetic particles by putting the tube E on a magnet for 10 seconds).

<u>Method with a magnet rack</u>

11. During the lysis step pipette QuickPick™ SML Plant DNA reagents into one new tube per extract as follows :Tube 2: 5µL Plant DNA Magnetic Particles and 125µL Plant DNA Binding Buffer.
    *Important: never vortex the magnetic particles, but gently suspend and homogenise the particles before pipetting.
12. 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 minutes. Mix the suspension continuously during this step.
13. Centrifuge tube 2 for 5 seconds at 250 g for pelleting the particles. Then put the tube 2 on the magnet for a minimum of 5 minutes. Pipette and discard the liquid without the particles.
14. Take the tube 2 off the magnet. Add 250 µL of Plant DNA Wash Buffer. Shake the suspension gently for 1 minute (washing of the magnetic particles). Put the tube 2 on the magnet for pelleting the magnetic beads for 5 minutes at a minimum. Then pipette and discard the liquid without the particles.
15. Repeat the washing steps 2 times (Wash Buffer).
16. Take tube 2 off the magnet. Add 50µL of Plant DNA Elution Buffer. Incubate for 10 minutes at room temperature by shaking gently and continuously. Centrifuge for 5 seconds at 250 g for pelleting the magnetic beads. Place the tube on the magnet for a minimum of 5 minutes. Transfer the supernatant (eluate) in a new tube 3 for storing DNA.

17. The eluate containing the purified DNA is ready to be used on the same day, or to be stored at -20°C until use.

Method with magnet pipette

18. During the lysis step pipette QuickPick™ SML Plant DNA reagents into tubes as follows:
    - 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.

    - Tube 3: Plant DNA Wash Buffer
    - Tube 4: Plant DNA Wash Buffer
    - Tube 5: Plant DNA Wash Buffer
    - Tube 6: Plant DNA Elution Buffer

19. 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.
20. Pick up the QuicPick tip with the QuicPick 1. Collect the magnetic particles from tube 2 and release them into tube 3 (washing buffer). Wash the magnetic particles by mixing the suspension gently for 20 seconds using the QuicPick tip. Repeat the washing steps in tubes 4 and 5 (Wash Buffer).
21. 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.
22. 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 stored at -20°C until use.

Reagents	[Concentrated Sol.]		[Final Sol.]		Vol. for one tube	
Ultra pure water					6.48	µL
TaqMan™ Universal PCR Master Mix (AB)* (Cod. 4364338)	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
BSA*	50	µg/µL	0,30	µg/µL	0.12	µL
*optional	PCR Mix Volume				18	µL
	DNA Sample Volume				2	µL
	Total Volume total per reaction				20	µL

Real-time PCR

Prepare amplification positive and negative controls

A) 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).

B) Francis et al., 2006

HL5 5'-AAGGCAATAAACGCGCACTA-3'
HL6 5'-GGTTTTGCTGACTGGCAACA-3'

Reagents	[Concentrated Sol.]		[Final Sol.]		Vol.for one tube	
Ultra pure water					6.8	µL
Master Mix SYBR SELECT MASTER MIX (COD. 4472942)	2	x	1	x	10	µL
HL5	10	µM	0.3	µM	0.6	µL
HL6	10	µM	0.3	µM	0.6	µL
	PCR Mix Volume				18	µL
	DNA Sample Volume				2	µL
	Total Volume total per reaction				20	µL

Quantitative real-time PCR amplification conditions

Denaturation	95	°C		5	min
Number of cycles : 40					
Denaturation	95	°C		20	s

Hybridation/					
Elongation	60	°C		40	s
Melt	65	°C	to	95° C	Increment 0.5°C 0:05

PCR for MLST (Yuan *et al*., 2010)

1. The target sequences are those of seven house-keeping genes:

Gene	Gene Function	Amplicon size
leuA	2-isopropylmalate synthase	708
petC	ubiquinol cytochrome C oxidoreductase, cystochrome C1 subunit	533
malF	ABC transporter sugar permease	730
cysG	siroheme synthase	600
holC	DNA polymerase III holoenzyme, chi subunit	379
nuoL	NADH-ubiquinone oxidoreductase, NQO12 subunit	557
gltT	glutamate symport protein	654

2. Primer sequences of the seven house-keeping genes:

Gene	Primer Sequences
leuA	for - 5' GGTGC ACGCC AAATC GAATG 3' rev - 5' GTATC GTTGT GGCGT ACACT G 3'
petC	for - 5' GCTGC CATTC GTTGA AGTAC CT 3' rev - 5' GCACG TCCTC CCAAT AAGCC T 3'
malF	for - 5' TTGC TGGT CCTG CGGT GTTG 3' rev - 5' GACAGCAGAAGCACGTCCCAGAT 3'
cysG	for - 5' GCCGA AGCAG TGCTG GAA G 3' rev - 5' GCCAT TTTCG ATCAG TGCAA AAG 3'
holC	for - 5' ATGGC ACGCG CCGAC TTCT 3' rev - 5' ATGTC GTGTT TGTTC ATGTG CAGG 3'
nuoL	for - 5' TAGCG ACTTA CGGTT ACTGG GC 3' rev - 5' ACCAC CGATC CACAA CGCAT 3'
gltT	for - 5' TCATG ATCCA AATCA CTCGC TT 3' rev - 5' ACTGG ACGCT GCCTC GTA AA CC 3'

PCR for MLST

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	36.2	N.A.
PCR buffer (*Invitrogen*)	10 x	5	1x
$MgCl_2$	50 mM	1.5	1.5 mM
dNTPs	20 mM	0.5	0.2 mM
Forward primers (leuA-for, petC-for, malF-for, cysG-for, holC-for, nuoL-for, gltT-for)	20 µM	0.75	0.3 µM

Reverse primers (leuA-rev, petC-rev, malF-rev, cysG-rev, holC-rev, nuoL-rev, gltT-rev)	20 µM	0.75	0.3 µM
DNA Polymerase Platinum (*Invitrogen*)	5 U/µL	0.3	0.03 U/µL
Subtotal		45	
Genomic DNA		5	
Total		50	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.

PCR conditions: 95ºC for 3 min, 35 cycles of (95ºC for 30 s, 65°C for 30 s and 72°C for 60 s) and a final step of 72°C for 10 min

If the amplicons are of good quality and at the expected size, a template will be sent for sequencing with reverse and forward primers. The results of sequencing will be compared with sequences available on http://pubmlst.org/xfastidiosa/

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 (pre-marked membrane can also be supplied with the diagnostic kit).
- Select at least four olive mature twigs from each sample; make a fresh clean cut, squeeze and press gently onto the surface of the membrane. Prepare at least 2 spots for each twig and a total of eight spots.
- Sterilize the shears between samples using a 10% bleach solution.
- Dry the membrane for at least 30 minutes 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 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. Incubation

- Dilute the 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 minutes).
- Stop the reaction by washing the membrane with distilled 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
KH_2 PO4 anhydrous	0.2 g
Na_2HPO_4 anhydrous	1.15 g
KCl	0.2 g
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

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., Frasheri, D., Valentini, F., D'Onghia, A. M. & Digiaro, 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 factsheet for additional information

Real-time fluorescence LAMP assay*

A. 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 minutes at 65°C.

4. Prepare the aliquots of the LAMP MIX 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.

5. Vortex gently and briefly centrifuge.

6. Set the following amplification program on the device: one step at 65°C for 25 minutes.

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. The device is equipped with software that based on the amplification curves assigns positive/negative reactions to wells with unknown samples.

B. Using plant sap

1. Prepare 0.5-0.1 g of fresh small pieces of midribs, petioles, leaf basal part or twigs (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 minutes at 65°C.

5. Prepare the aliquots of the LAMP MIX 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.

6. Vortex gently and briefly centrifuge.

7. Set the following amplification program on the device: one step at 65°C for 25 minutes.

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. The device is equipped with software that based on the amplification curves assigns positive/negative reactions to wells with unknown samples.

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, Digiaro 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.

Sponsors, Organisers and Participants