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TRAINING WORKSHOP

DETECTION OF *XYLELLA FASTIDIOSA*

16-19 JANUARY, 2018

ANSES -PLANT HEALTH LABORATORY

ANGERS, France

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French agency for food, environmental and occupational health & safety
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SAMPLING OF PLANT MATERIAL AND SAMPLE PREPARATION IN THE LABORATORY

1 SAMPLING OF PLANT MATERIAL

1 1 SAMPLING PERIOD FOR SYMPTOMATIC OR ASYMPTOMATIC PLANTS

The concentration of the bacterium in a plant depends upon environmental factors, strains and the host plant species. To maximize the likelihood of detection, sampling should be performed during the period of active growth of the plants (Hopkins, 1981). For tropical plant species grown indoors, such as coffee plants, sampling may be performed all year round.

For outdoor plants in Europe this active growth period is usually from late spring to autumn.

Details based on specific observations during current outbreaks in Europe are presented below (EU, 2015).

(a) For *Polygala* spp., sampling can be performed from late spring to early autumn;
(b) For *O. europaea* and *N. oleander*, observations conducted in Italy (Apulia region) indicated that:
  – withering, desiccation and leaf scorching symptoms associated with *X. fastidiosa* infections are more strongly expressed in summer, although persistent during the entire year
  – in some cases, symptoms were also observed during winter at the start of the new vegetative growth.
(c) For deciduous plant species (e.g. *Prunus* spp.) in Italy (Apulia region) symptoms were consistently recorded, together with a detectable bacterium concentration, in leaves collected during summer. Asymptomatic leaves collected earlier in the vegetative period from the same trees tested negative.
(d) If necessary, dormant plants can be sampled by taking mature branches (e.g. woody cuttings), from which the xylem tissue is recovered and processed for detection of *X. fastidiosa*.

Experience in temperate areas in other parts of the world shows that in vines or deciduous trees, e.g. cherry and almond, that have been infected for some time, the bacteria do not move into the new season's growth until the middle of summer, when symptoms may also become visible. For example, the most suitable time for searching for symptoms in grapevine is late summer to early autumn when weather conditions are predominately hot and dry or when grape plants are exposed to drought stress (Galvez *et al.*, 2010).

1.2 SAMPLE COLLECTION

This section applies to sampling in places of production and in consignments. After taking samples they should be sent to the laboratory as soon as possible.

As *X. fastidiosa* is confined to the xylem tissue of its hosts, the petiole and midrib recovered from leaf samples are the best source for diagnosis as they contain a higher number of xylem vessels (Hopkins, 1981).

However, other sources of tissue include small twigs and roots of peach (Aldrich *et al.*, 1992), blueberry stem and roots (Holland *et al.*, 2014) and citrus fruit peduncles (Rossetti *et al.*, 1990).

Samples for the laboratory should be composed of branches/cuttings with attached leaves. The sample should include mature leaves. Young growing shoots should be avoided.

For small plants the entire plant can be sent to the laboratory.

For sclerotic leaves (e.g. *Coffea*) individual leaves and petioles can be sampled.

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1.3 SYMPTOMATIC PLANTS

The sample should consist of branches/cuttings representative of the symptoms seen on the plant(s) and containing at least 10 to 25 leaves depending on leaf size. Symptomatic plant material should preferably be collected from a single plant; however, a pooled sample may also be collected from several plants showing similar symptoms.

1.4 ASYMPTOMATIC PLANTS

For asymptomatic plants, the sample should be representative of the entire aerial part of the plant. Recent experimental data on the detection of *X. fastidiosa* in monumental and ancient olive trees showed that detection was more reliable when sampling the mid to upper part of the canopy. For testing individual asymptomatic plants, the number of branches to be collected is at least 4 to 10, depending on the host and plant size. There is limited experience of testing samples comprising leaves (including their petioles) collected from several asymptomatic plants. However, *X. fastidiosa* has been detected from samples of 100 to 200 leaves (including their petioles) collected from consignments of asymptomatic coffee plants (NRC, NL unpublished data).

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2 SAMPLE PREPARATION IN THE LABORATORY FOR PLANT MATERIAL

Samples should be processed as soon as possible after arrival.

If the plant samples originate from areas where infected vectors may occur, it is recommended to check whether insects are present in the sample before opening the bags. If any insects are present, samples should be stored in the refrigerator for approximately 12 h.

For isolation, samples may be kept refrigerated for up to 3 days. For other, tests samples may be stored refrigerated for up to 1 week.

Samples should be inspected for symptoms and, if present, symptomatic leaves (including their petioles) should be selected and processed (removing the necrotic and dead tissue). If no symptoms are noted, leaves should be representative of the entire sample received in the laboratory.

Dirty samples should be cleaned.

For isolation, samples should be surface disinfected.

From the sample received, indications on the minimum number of leaves (including their petioles) to be used and approximate weight of the laboratory sample are given in Table 1.

Table 1. Number of leaves (including their petioles) to be used and approximate weight of the laboratory sample

Type of sample	Host plants/type of tissue	Minimum number of leaves per laboratory sample	Approximate weight of the laboratory sample
Samples from individual plants with leaves	Petioles and/or midribs or leaves of large size such as *Coffea* sp., *Ficus* sp., *Vitis* sp., *Nerium oleander*	5	0.5–1 g
	Petioles and/or midribs of leaves of small size such as *Polygala myrtifolia* and *Olea* sp.	25	0.5–1 g
	Plant species without petioles or with small petiole and midrib	25	0.5–1 g
Dormant plants or cuttings	Xylem tissue	N.A.	0.5–1 g
Composite sample from several coffee plants from a single lot with leaves (NRC, NL, procedure)	Samples of asymptomatic plants collected from, e.g., imported consignments or nursery monitoring	100–200	10–50 g

Tissue (preferably petioles and midribs or stem portions) should be recovered from leaves and used directly for the preparation of the plant extract. The sample is processed according to the test to be used as described in this protocol.

For isolation see the specific protocol below

For DNA extraction (Anses LSV protocol)

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Weight 0,5 to 1 g of petioles, midribs or stems

Material is cut in small section (about 2 mm max)

Material is put into a plastic bag (with filter)

- For QuickPick™ extraction: Sterile demineralized water is added into the bag (1 mL / g)
- For CTAB extraction: CTAB buffer is added into the bag (1 mL / g) (preferably 1.5 g / 7.5 mL)

Then, the material is roughly crushed

After 15 min of maceration under shaking:

- For QuickPick™ extraction: 250 µL are transferred
- For CTAB extraction: 1 mL is transferred into a 2 mL plastic microtube (2 tubes per sample, plus 1 tube for the positive isolation control (PIC)). Macerate is ready for DNA extraction.

References: Bulletin OEPP/EPPO Bulletin (2016) 0 (0), 1–38, Diagnostic, PM 7/24 (2) *Xylella fastidiosa*

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SAMPLING OF VECTORS AND SAMPLE PREPARATION IN THE LABORATORY

Field-collected insects can be analyzed to detect *X. fastidiosa* by molecular tests The enzyme-linked immunosorbent assay (ELISA) test is not sensitive enough, as the bacterium only colonizes the insect foregut where, in spite of its multiplication, it is generally present at low levels (Purcell *et al.*, 2014).

1 SAMPLE COLLECTION

Adult vectors should preferably be collected with sweeping nets (adults) or aspirators. Sticky traps are usually not as effective as active sampling for xylem feeders, but insects may be trapped accidentally and specimens collected from sticky traps can be used for testing. Identification keys with pictures are available online (Purcell *et al.* 2014).

Vectors can be removed from the traps using small forceps/pincers and a suitable solvent. After removal from the traps, insects should be rinsed in ethanol/acetone. Traps should be serviced on a weekly basis.

Sampling for insects should preferably be done from late spring until early autumn to maximize the likelihood of detection of the bacterium.

If insects cannot be processed immediately, they should be stored in 95–99% ethanol or at −20°C or −80°C. Sticky traps can also be stored at −20°C.

2 SAMPLE PREPARATION OF VECTORS IN THE LABORATORY

Since *X. fastidiosa* only colonizes the foregut and does not systemically spread into the body, only the head of the insect should be used for DNA extraction, thus avoiding the extraction of several contaminants that may inhibit the enzymatic reactions (Purcell *et al*., 2014). Experience in Italy on *Philaenus spumarius* shows that up to 5 insects can be pooled to perform one test. Inhibitors may be present in the eyes and could affect PCR sensitivity Removing the eyes is recommended (B. Legendre pers. comm., 2014).

Before DNA extraction, it is imperative to remove the solvent (ethanol/acetone). To achieve this, the insects can be transferred for a few minutes to a dry filter paper and may be further dried in a SpeedVac centrifuge, to facilitate evaporation of the solvent. Total DNA can be extracted from single (or pooled) insect heads following different procedures.

2 1 HEAD SAMPLING

Insect is laid on a paper in order to absorb ethanol

Transferred the insect on a new paper and place it under the binoculars microscope,

The insect body is maintained with forceps/pincers

Then the scalpel blade is inserted between head and thorax

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A light pressure is applied in order to separate the head form the thorax

The head is then transferred into a 2 mL microtube.

Fig. 1. Sampling of the spittlebug head with scalpel for analysis

2 2 CRUSHING (ANSES LSV PROTOCOL)

200 µL are added into the tube containing the head

10 stainless steel beads (3 mm diameter) are added into the tube

Tubes are disposed into the specific rack of the bead beating mixer mill (RETSCHMM400).

Crushing in running during 2 minutes at 30 Hertz

Then microtubes are put down into a magnetic rackl (for example DynaMagtm-2)

The whole macerate is transferred into a new 2 mL microtube and then centrifuged 20 min at 20 000 g.

The sample (pellet) is ready for the DNA extraction (for example with the QuickPick™ plant DNA kit (Bio-Nobile))

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If using the QuickPick™ plant DNA kit, the following volumes will be used:

Reagent/buffer	Volume (µL/sample)
Lysis buffer	37,5
Proteinase K	2,5
Binding buffer	62,5
Beads suspension	2,5
Washing buffer (step 1) Washing buffer (step 2) Washing buffer (step 3)	125 125 125
Elution buffer	25

Note: If the beads will be used anew, a decontamination procedure is applied: the beads are soaking into sodium hypochlorite (2.6%) then rinsed twice into demineralized water

References: Bulletin OEPP/EPPO Bulletin (2016) 0 (0), 1–38, Diagnostic, PM 7/24 (2) *Xylella fastidiosa*

DNA EXTRACTION FOR PLANT MATERIAL

1 QUICKPICK™ 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).

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For information: Method with a magnet rack

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.

For information: 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 QuickPick tip with the QuickPick 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 QuickPick tip. Repeat the washing steps in tubes 4 and 5 (Wash Buffer).

21. Collect the Magnetic Particles from tube 5 with the QuickPick 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.

2 OTHER PLANT DNA EXTRACTIONS

2.1 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 700ml to a 1.5 ml microcentrifuge tube and add 450 ml (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. 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 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.

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

2.2 DNEASY® MERICON™ FOOD STANDARD PROTOCOL (QIAGEN) (MODIFIED)

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REAL-TIME PCR

Prepare amplification positive and negative controls

1 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'

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	0.3	µM	0.6	µL
XF-R	10	µM	0.3	µM	0.6	µL
XF-P	10	µM	0.1	µM	0.2	µL
BSA (Optionnal)	50	µg/µL	0.3	µg/µL	0.12	µL
	PCR Mix Volume				18	µL
	DNA Sample Volume				2	µL
	Total Volume total per reaction				20	µL

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).

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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'

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/

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ISOLATION

Xylella fastidiosa is very difficult to isolate and grow in axenic culture, even from symptomatic plants. The bacterium does not grow on most common culture media, and requires specific media. PD2 (Davis *et al.* 1980), BCYE (Wells *et al.*, 1981) or PWG (modified after Hill & Purcell, 1995) are widely used for the isolation from different host species.

The use of at least two different media is recommended, in particular when isolation is attempted for new hosts or in the case of a first detection.

It is very important to surface disinfect the sample to avoid growth of saprophytes because *X. fastidiosa* grows very slowly (the colonies can take up to 28 days to be visible) and can be readily overgrown by other microorganisms in the plates.

As a control, whenever possible a suspension of a *X. fastidiosa* strain at a concentration of about 10^6–10^7 cfu mL−1 should be plated onto the same medium. Colonies are small, and depending on the strain the colony size is 1–1.5 mm in diameter after 1–3 weeks of incubation at approximately 28°C.

Plates should to be sealed or kept in plastic bags to prevent desiccation during incubation.

- Colony morphology

The colony morphology of *X. fastidiosa* is variable (Davis *et al.*, 1981; Chen *et al.*, 2005). Colonies on the media recommended in this protocol are as follows.

On all media, colonies are circular, smooth-edged and slightly convex.

On PD2 and BCYE they are opaque and whitish (Figs 2 and 3 respectively). On BCYE they contrast with the black (charcoal) medium (Fig. 4).

On modified PWG colonies are shiny and translucent. They take the color of the medium (light caramel) (Figs 5 and 6).

- Cell morphology

Under dark field microscopy, the bacterium has a rod-shaped appearance with the following dimensions: 0.2–0.35 µm by 1–4 µm. Under the electron microscope, *X. fastidiosa* shows a characteristic rippled wall (Newman *et al.*, 2003; Alves *et al.*, 2009).

- Interpretation of isolation results

The isolation is negative if no bacterial colonies with growth characteristics and morphology similar to *X. fastidiosa* are observed. Depending on the different sub-species, colonies can be visible after 2–3 weeks but the plates should be observed for up 6 weeks.

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The isolation is positive if bacterial colonies with growth characteristics and morphology similar to *X. fastidiosa* are observed within the above-mentioned period on at least one medium. The reference culture should also have grown on the media used. The presumptive identification of *X. fastidiosa* colonies should be confirmed by serological or molecular tests.

Isolations procedures as currently implemented in different laboratories are presented below. No comparison of these procedures has been performed. Consequently, no recommendation can be made so far regarding the advantages and disadvantages of different procedures.

The conditions for surface disinfection can vary according to the plant tissues, the most commonly used procedures are reported below.

After disinfection of the leaf or stem with 70% (v/v) ethanol, a part of petiole, midrib or stem approximately 1 cm long is collected with a sterile scalpel. Symptomatic leaves should be used preferably if available. The midrib or petiole is briefly soaked in ethanol at 96% (v/v) and flamed very quickly to achieve surface disinfection without causing a significant temperature rise in the tissues which could kill the bacteria. The sample is immediately placed in a sterile Petri dish with 1–2 mL of sterile saline solution or sterile demineralized water, comminuted and left to soak for at least 15 min, under gentle shaking. 100 µL of the macerate is plated without dilutions. Plates should be sealed or kept in plastic bags to prevent desiccation.

The plates are incubated at 28°C.

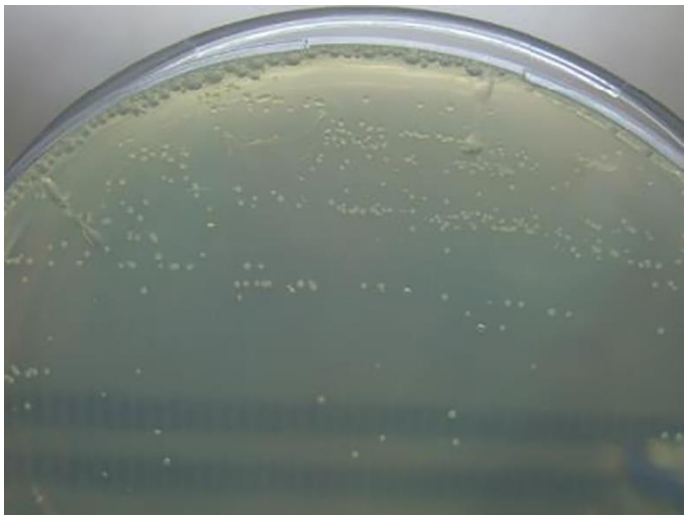

Fig. 2 Colonies of *Xylella fastidiosa* subsp. *fastidiosa* on PD2 (size < 2 mm after 3 weeks).

Fig. 3. Collection strain of *Xylella fastidiosa* subsp. *fastidiosa* ATCC 35879 on BCYE (size < 2 mm after 3 weeks).

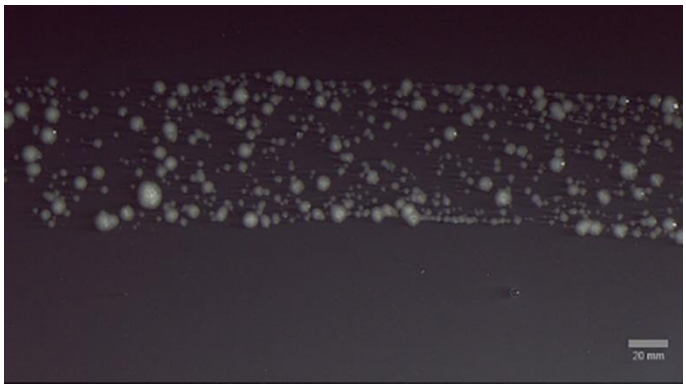

Fig. 4 Colonies of *Xylella fastidiosa* subsp. *pauca* strain CoDiRO (ST53) on BCYE after 2 weeks. Courtesy M. Saponari, Institute for Sustainable Plant Protection (CNR). (Other pictures of colonies are available in the EPPO Global database.)

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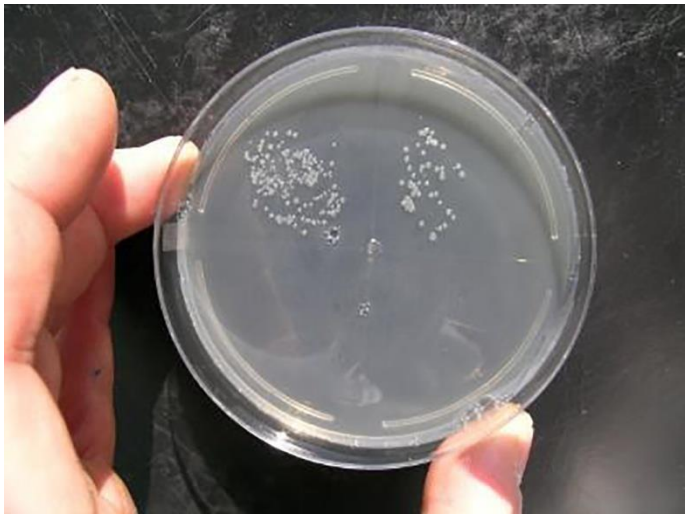

Fig. 5 *Xylella fastidiosa.* subsp. *fastidiosa* isolated from *Coffea canephora* on modified PWG (size < 2 mm after 3 weeks). Courtesy Anses.

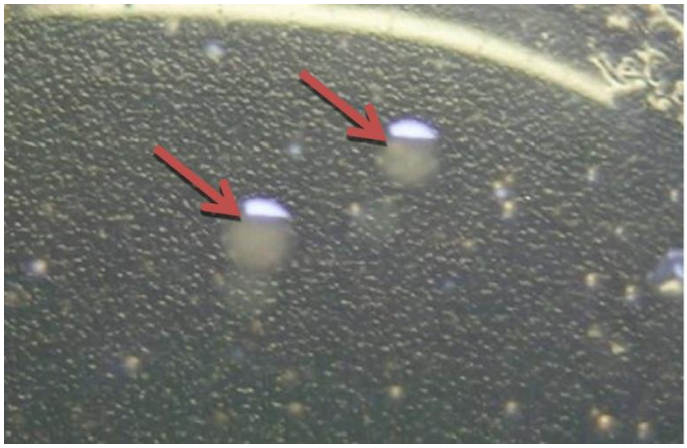

Fig. 6 *Xylella fastidiosa* subsp. *pauca* isolated from *Coffea arabica* on modified PWG (size <2 mm after 3 weeks) Courtesy Anses (the background is a sheet of black paper below the plate).

References: Bulletin OEPP/EPPO Bulletin (2016) 0 (0), 1–38, Diagnostic, PM 7/24 (2) *Xylella fastidiosa*

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MEDIA

Note: Ingredients should be dissolved in the order given.

1 PWG MEDIUM MODIFIED

[Anses based on Hill & Purcell (1995) and information provided at the COST Workshop – Bari 2010 (Rodrigo Almeida pers. comm.)

Gelrite gellan gum (Gelzam™ CM; Sigma G 1910)	**9.0 g**
Phytone peptone (BD/211906)	4.0 g
Bacto tryptone (Fisher Scientific 11778143 = BD Difco™ 211705)	1.0 g
$MgSO_4 \bullet 7H_2O$	0.4 g
K_2HPO_4	1.2 g
KH_2PO_4	1.0 g
Stock solution of red phenol (0.2% aqueous solution) see below	10 mL
Stock solution of Hemin chloride (0.1% solution NaOH 0.05 N)	10 mL
Sterile distilled or deionized water	830 mL
BSA (Sigma Aldrich, A7030)	3 g
L-glutamine (Sigma Aldrich, G3126)	4 g

1. Use a 2 L bottle and autoclave at approximately 121°C for 20 min. Ingredients except BSA and L-glutamine are added mixed and dissolved in the order given.

After autoclaving, allow the temperature to cool to 50°C and under a horizontal air flow add filtered sterile BSA dissolved in 50 mL of deionized water and L-glutamine dissolved in 100 mL of water at about 50°C.

Stock solution of red phenol

Red phenol (0.2% aqueous solution)	50 mg
Sterile distilled or deionized water	25 mL
Store for a maximum of 1 month at 5 ± 4°C.	

In case of solubility problems in water, dissolving in 70% ethanol is possible.

Stock solution of Hemin chloride

Hemin chloride (0.1% solution NaOH 0.05 N)	50 mg
Solution NaOH 0.05 N	50 mL
Store for a maximum of 1 month at 5 ± 4°C.	

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2 BCYE MEDIUM MODIFIED

Component (supplier/order no.)	Quantity
Demineralized water	940 mL
Aces Buffer (Sigma/A-3594)	10 g
KOH solution 1M	40 mL*
Yeast extract (BD Difco™/212750)	10 g
Activated charcoal (Sigma/C-9157)	2 g
Agar no. 1 (Oxoid/LP011 or Bacto agar BD Difco™)	17 g
*Adjust the pH to 6.9 before adding the agar. This is done by adding approximately 40 mL KOH 1M until the appropriate pH value is reached. Adjust the total volume to 980 mL with the demineralized water.	
Component (supplier/order no.)	**Quantity**
1. Agitate for at least 1 min. The final pH is approximately 6.9.	
Cysteine hydrochloride (Sigma/C-7880)	5 mL
Ferric pyrophosphate (Sigma/P-6526)	15 mL

Stock solutions (filter sterile)

Component (supplier/order no.)	Final per L	Concentration	Dissolve in
Cysteine hydrochloride (Sigma/C-7880)	400 mg	400 mg per 5 mL	Distilled water
Ferric pyrophosphate (Sigma/P-6526)	250 mg	250 mg per 15 mL	Distilled water
The compound ferric pyrophosphate needs to be heated, under agitation, at 75°C for approximately 15–20 min.			

References: Bulletin OEPP/EPPO Bulletin (2016) 0 (0), 1–38, Diagnostic, PM 7/24 (2) *Xylella fastidiosa*

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IMMUNOFLUORESCENCE (IF) TEST

1 INSTRUCTIONS TO PERFORM AN IF TEST

Plant extract used for IF could be prepared as described in the part “Isolation” or in the part “Sample preparation in the laboratory for plant material”. For the second option, it is highly recommended to perform IF on the raw extract and on a range of serial dilutions. Indeed, the concentration of vegetal debris / sediment could be very high and, in this case, the fluorescence could fade down and the test could give a false negative result.

Use multiwell microscope slides for example 8 windows of at least 8 mm diameter.

Test control material in an identical manner to the sample(s).

2 PREPARATION OF TEST SLIDES

Prepare the test slides using one of the following procedures:

Prepare decimal dilutions (1⁄10, 1⁄100) of plant extract in suspension buffer or in sterile demineralized water. Pipette a measured standard volume (40 µL is appropriate for 8 mm window diameter) of the sample extract and each dilution onto a row of windows. The second row can be used as duplicate.

3 FIXATION OF BACTERIAL CELLS

Dry the droplets at ambient temperature or by warming (maximum temperature 45°C). Fix the bacterial cells to the slide by covering the windows with ethanol (>95%) and heating (15 min at a maximum temperature of 60°C) or at ambient air.

Slides should be preferably be used as soon as possible but if necessary fixed slides may be stored frozen in a desiccated box (up to a maximum of 3 months) prior to further testing.

4 IF-PROCEDURE

Prepare a working dilution of the antibody in IF buffer.

Cover each test window completely with the antibody dilution. The volume of antibody applied on each window should be at least the volume of extract applied (40 µL)

Incubate the slides (under a cover) for 30 min at ambient temperature (18–25°C).

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Shake the droplets off each slide and rinse carefully with IF buffer. Wash by submerging for approximately 5 min in IF buffer–Tween and subsequently in IF buffer.

Avoid causing aerosols or droplet transfer that could result in cross-contamination. Carefully remove excess moisture by blotting gently.

Cover the test windows with the working dilution of FITC conjugate. The volume of conjugate applied on the windows should be at least the volume of antibody applied.

Incubate the slides (under a cover) for 30 min at ambient temperature (18–25°C).

Shake the droplets of conjugate off the slide. Rinse and wash as before (3.5).

Carefully remove excess moisture by blotting gently.

Pipette 5–10 µL of 0.1 M phosphate-buffered glycerol or a commercial anti-fading mountant on each window or distribute a sufficient amount across the slide, apply a coverslip and avoid exposure of the slides to excess light.

5 READING THE IF TEST

Examine test slides on an epifluorescence microscope with filters and light source suitable for excitation of FITC, under oil, glycerol or water immersion at a magnification of 500–1000. Scan windows across two diameters and around the perimeter. For samples showing no or low numbers of cells observe at least 40 microscope fields.

Check the positive control slide first. Cells should be bright fluorescent and the cell wall completely stained. The IF test should be repeated if the staining is aberrant.

Observe for bright fluorescing cells with characteristic morphology of the target organism in the test windows of the test slides. The fluorescence intensity should be equivalent to the positive control. Cells with incomplete staining or with weak fluorescence should be disregarded.

If any contamination is suspected the test should be repeated.

This may be the case when all slides in a batch show positive cells due to the contamination of buffer or if positive cells are found (outside of the slide windows) on the slide coating.

There are several problems inherent to the specificity of the immunofluorescence test. Background populations of fluorescing cells with atypical morphology and cross reacting saprophytic bacteria with size and morphology similar to the target organism may occur in the plant or seed sample.

Consider only fluorescing cells with typical size and morphology.

6 INTERPRETATION OF THE IF READING

The IF test is positive when fluorescing morphologically typical cells are detected in the sample extract.

The detection threshold of the IF test is usually between 10^3 and 10^4 cells per mL of sample extract.

7 BUFFERS

IF-BUFFER [10 MM PHOSPHATE BUFFERED SALINE (PBS), PH 7.2]

This buffer is used for dilution of antibodies

$Na_2HPO_4.12H_2O$ 2.7 g

$NaH_2PO_4.2H_2O$ 0.4 g

NaCl 8.0 g

Distilled water 1.0 L

Dissolve ingredients, check pH 7.2 and sterilize by autoclaving

at 121°C for 15 min

IF-BUFFER–TWEEN

This buffer is used to wash slides

Add 0.1% Tween 20 to the IF buffer

PHOSPHATE BUFFERED GLYCEROL, PH 7.6

This buffer is used as a mountant fluid on the windows of IF slides to enhance fluorescence

$Na_2HPO_4.12H_2O$ 3.2 g

$NaH_2PO_4.2H_2O$ 0.15 g

Glycerol 50 mL

Distilled water 100 mL

Check pH 7.6 and sterilize by autoclaving

8 ANTISERA

A commercial polyclonal antibody is available from Loewe.

Analytical specificity on pure cultures (data is provided by the supplier; Loewe) concentrations of up to 10^4 cfu mL−1 tested on pure cell cultures).

Inclusivity: 100%

Number of *X. fastidiosa* strains tested: 5 (*X. fastidiosa, X. fastidiosa, X. fastidiosa* subsp. *multiplex, X. fastidiosa* subsp. *fastidiosa*; CoDiRO, Lecce, IT).

Exclusivity: 100%

Number of non-target strains: 9 (*Agrobacterium vitis, Clavibacter michiganensis* subsp. *michiganensis, C. m*. subsp. *sepedonicus, Dickeya chrysanthemi, Pseudomonas syringae* pv. *syringae, Rhodococcus fascians, Xylophilus ampelinus, Xanthomonas vesicatoria, Xanthomonas campestris* pv. *campestris*).

No cross-reaction observed.

A preliminary test performance study on diagnostic sensitivity was performed during a workshop in Germany involving 13 laboratories using naturally infected coffee plant samples.

Diagnostic sensitivity 100% of agreement at 10^4 cells per mL.

Repeatability: 100%

References:Bulletin OEPP/EPPO, Bulletin OEPP/EPPO (2009) Bulletin 39, 413–416 , PM 7/97 (1), Indirect immunofluorescence test for plant pathogenic bacteria and Bulletin OEPP/EPPO Bulletin (2016) 0 (0), 1–38, Diagnostic, PM 7/24 (2) *Xylella fastidiosa*