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WP PR Point

Diagnostic protocols for regulated pests
Protocoles de diagnostic pour les organismes réglementés

XYLELLA FASTIDIOSA

Introduction

Symptoms caused by the bacterium currently named *Xylella fastidiosa* were first observed in 1892 in the grape growing region of southern California and the syndrome was called "Pierce's disease". Subsequently, the pathogen was reported on many fruit tree and ornamental species such as peach (phony peach), plum (plum leaf scald), almond (almond leaf scorch), citrus (citrus variegated chlorosis), pear (pear leaf scorch), alfalfa (alfalfa dwarf), periwinkle (periwinkle wilt), elm (elm leaf scorch), mulberry (mulberry leaf scorch), sycamore (sycamore leaf scorch), maple (maple leaf scald), oak (oak leaf scorch), oleander (oleander leaf scorch), coffee (coffee leaf scorch) especially in northern and southern America. In Europe it has been found once on grapevine in the Kosovo region (ex-Yugoslavia). On the most of the host plants the pathogen induces leaf wilting, twig dieback and the decline of the plant. However, in the "phony peach" disease it is evident a reduction of the plant growth that shows shorter internodes. *X. fastidiosa* proliferates in the xylem vessels and natural transmission occurs via insects having a suctorial xylem sap-feeding. Transmission efficiency varies widely among vectors species. The bacterium overwinters in the xylem of the host plant as well as in weeds. From the latter it is transmitted to the host plant during spring.
This protocol currently gives methods only for strains from grapevine and citrus.

Identity

Name: *Xylella fastidiosa* Wells *et al.* 1987

Taxonomic position: Bacteria, Gracilicutes
Gram-negative aerobic rods, Category I, Group 4, Subgroup 4A
(Bergey's Manual of Determinative Bacteriology, Holt et al., 1994).

Bayer computer code: XYLEFA

Phytosanitary categorization: EPPO A1 list No. 137 & 166; EU Annex designation I/A1 – as *Xylella fastidiosa* on grapevine, peach phony rickettsia on peach and variegated chlorosis on citrus

Detection

Disease symptoms

Inspections of crops suspected to be infected by *Xylella fastidiosa* are fundamental to point out early symptoms of infection. The ELISA test and PCR are currently utilized for a rapid and reliable detection of the pathogen. There is a seasonal fluctuation in the detection of infected specimens. For Citrus Variegated Chlorosis, concerning the recovery of the pathogen from roots and stems, there are two yearly periods of detection: midsummer (June-August) and midwinter (December-February)**.** For "Pierce's disease" of grapevine, the best period for detection is late summer and early autumn.

"Pierce's disease" of grapevine

Main symptom of primary infection is the leaf scorching. An early sign of the disease is the sudden drying of a part of the leaf lamina which turns brown and very often is surrounded by a yellowish or a reddish halo. Subsequently, the wilting spreads and the whole leaf shrivels and drops; remnants of the leaf remain attached to the petiole. The wilting of the twig usually starts from the tip. Infected stems mature irregularly showing

patches of green tissues. Infected plants sprout after the healthy ones. Heavily infected plants may die in one or two years. Symptoms on one-year-old plants can be rarely pointed out and in winter a reduction of the population of the pathogen has been frequently recorded. Symptoms along the twigs can be confused with those incited by phytopathogenic fungi such as *Eutypa lata* and *Phomopsis viticola*.

b) Citrus Variegated Chlorosis (CVC) of *Citrus* spp.

The disease is locally named "amarelinho" in Brasil and "pecosita" in Argentina. Symptoms are more evident on 3-6 years-old trees and mainly on sweet orange cultivars. Affected trees show the leaves with chlorotic yellow spots resembling zinc deficiency; the lower surface show brownish necrotic spots slightly raised. Fruits are much smaller than the normal and very firm. Blossom and fruit set occur at the same time on healthy and affected trees but fruits remain small and ripe earlier. The growth rate of affected trees is greatly reduced and twigs and branches may wilt but, usually, the plant do not die. The roots do not show any apparent symptoms.

Identification

Isolation

Xylella fastidiosa is difficult to isolate and grow in axenic culture. It does not grow on most common bacterial media.

a) Grapevine

To isolate *X. fastidiosa* directly on the bacterial culture media, the procedure described for the preparation of plant extract for ELISA can be followed (see DAS-ELISA test). Isolation can be performed from leaf veins, petiole, small twigs or roots. In any case, it is critical to properly surface sterilize the sample and to dilute the plant extract in sample buffer. The xylem sap obtained either from the crushing of leaf vein, petiole or small twig or from extraction with vacuum infiltration of small twigs and roots can be streaked on to CS20, PD2, PD3 or B.CYE media (Appendix I). Alternatively, the twigs can be sliced tangentially with a sterile scalpel and the slices can be directly placed on the agar medium in Petri dishes. The plates have to be incubated at 28°C for 8-10 days. The plates are kept in plastic bags to prevent desiccation.

b) Citrus spp.

Symptomatic leaves are surface-sterilized with 10% bleach for 5 min, followed by two rinses in sterile distilled water. Midribs and petioles are aseptically excised and placed on to sterile Petri dishes containing 1-2 ml of PBS. Sections of 2-3 mm are obtained with a sterile scalpel. The sections are grinded and the sap is streaked on to PW or SPW medium. The plates are incubated at 28°C for 21 days. For isolation from roots and stem, after their surface-disinfection to be performed as described above, segments of 4-12 mm in diameter and 2-3 cm long are vacuum infiltrated with succinate-citrate-phosphate buffer (1.0 g/l disodium succinate, 1.0 g/l trisodiumcitrate, 1,5 g/l K_2HPO_4, 1.0 g/l K_2HPO_4, pH 7.0) as described for grapevine. Then, the vacuum extract (3-4 ml per sample) is centrifuged at 4.500 *g* for 15 min and resuspended in 0.8 ml of buffer. One drop (5 µl) is, subsequently, placed on to PW, SPW, CVC1 or CVC2 media (Appendix I). The plates are incubated at 27-30°Cand they are kept in plastic bags to prevent desiccation. The plates are observed for colony development at weekly intervals for a month with a binocular microscope.

Colonies morphology and microscopical observation

a) Grapevine

After 10 days of incubation at 28°C on to PD2, PD3, CS20 or B.CYE media, *X. fastidiosa* yields colonies (Figure.1) of 0.5-2.0 mm in diameter, circular, with entire margins and convex elevation. Sometimes colonies are produced that are also circular with undulate margins with an umbonate or flat elevation.

b) *Citrus* spp.

On SPW medium, isolated colonies are visible within 7 days after the streaking. After 21 days of incubation at 27°C, their diameter is 0.35 mm. On PW medium, appearance of colonies occurs after 10-14 days after the isolation. On CVC1 and CVC2 media, colony development occurs after 25-30 days from the isolation. Because of their elongated shape, the microscopic observation is very useful for detection. Under dark field microscopy the bacterium has a rod shaped appearance with the following dimensions: 0.2-0.35 µm x 1-4 µm. Under electron microscopy *X. fastidiosa* shows a characteristic rippled wall.

Pathogenicity test

Inoculation techniques must deliver the inoculum directly into the xylem vessels.

a) Grapevine

For the pathogenicity tests use young grapevine plants grown in pots and colonies of the bacteria grown for 8-10 days on PD2, PD3, CS20 or B.CYE at 28-30°C. Before inoculation, plants are removed from the pots and roots are washed with water to remove soil particles. Suspensions of the bacteria to be tested should have a density of c. 10^7-10^8 cfu.ml^{-1} in PBS. Test plants are cut off 15 cm above the collar and the roots are trimmed and immediately immersed into the bacterial suspension. The stem is fitted to a Tygon tube connected to a vacuum and negative pressure is applied for 90 min. Control plants have to be treated in the same way except that PBS is used instead of the bacterial suspension. Then, the plants are repotted and observed for symptom development. Alternatively, only one root per plant is trimmed and connected with a Tygon tube to a 10-ml pipet containing 10 ml of the bacterial suspension. The plants are repotted with the pipet reservoir connected to the root. The inoculum should be absorbed in 3-4 days. Symptoms appear usually after 60-80 days from the inoculation. Initially, small necrotic spots appear along the major veins. Afterwards such spots enlarge and coalesce. Subsequently, scorch symptom are apparent along the edge of the leaf that can also wither. Reisolation has to be performed as described for the primary isolation.

b) *Citrus* spp.

For inoculation, a suspension of 10^8 to 10^9 CFU/ml in PBS is prepared from colonies grown on to PW or SPW at 28°C. Inoculation can be performed by following three techniques. A) 20 µl of the suspension are placed on the surface of the citrus stem. Then, the stem is pierced through the inoculum droplets with a syringe. Multiple inoculation sites on the same stem raise the possibility of successful inoculation. B) Alternatively, by means of a razor blade a flap of a citrus branch is raised by cutting tangentially upward. Then, the inoculum is applied to the sliced branch by placing 10.30 µl of the suspension underneath the flap of tissue. C) The flap of tissues is placed into a microcentrifuge tube containing 500 µ of the bacterial suspension for 2 h. Afterwards, the wound is wrapped with grafting tape. Their inoculations can be repeated after 2 months to increase the possibility of reproducing the disease.

Symptoms appear 10 months after the inoculation on leaves that show chlorotic spots. Re-isolation have to be carried out by following the procedure described for the primary isolation.

DAS-ELISA test

Kits for the serological detection of *Xylella fastidiosa* are supplied by AGDIA Inc., Elkhart, IN, USA.

a) Grapevine

1 - *Plant extract*

A critical step for successfully performing the ELISA test is the obtaining of the xylem sap where the pathogen survives. For recovering the bacterium from the leaf, 1.0 g of petiole or leaf vein is first surface sterilized in 0.5% of sodium hypochloride for 10 min and then rinsed in four changes of sterile distilled water and dried in a laminar flow cabinet. Subsequently, it is ground in a sterile mortar containing 5 ml of sample buffer (SB) (disodiumsuccinate 1.0 g/l; trisodium citrate 1.0 g/l; K_2HPO_4 1.5 g/l; KH_2PO_4 1.0 g/l; 0.02 M of sodiumascorbate; 5% of acid-washed insoluble polyvinylpyrrolidone; pH 7.0) or grape sample extraction buffer provided by Agdia (Tris (hydroxymethyl) aminomethane 60.5g/l ; sodium chloride 8.0g/l ; polyvinylpyrrolidone (PVP), MW 24-40 20.0g/l ; polyethylene glycol 10.0g/l ;sodium azide 0.2g/l ; tween-20 0.5g/l ; pH 8.2). In case of twig or root, they have to be cut into 1-2 cm sections, stripped of bark, surface sterilized for 5 min in 0.5% of sodium hypochloride with 3% of ethyl alcohol added. Then the pieces have to be rinsed in four changes of sterile distilled water and crushed in a sterile mortar containing 5 ml of SB. Alternatively, twigs or roots of grapevine have to be cut to 5-6 cm lengths, stripped of bark and surface sterilized as described above. Then, both ends of the twig or root have to be connected to two pieces of sterilized vacuum tube (e.g. Tygon®). One piece of tube is, in turn, connected, through a glass tube, to a vacuum flask that inside has a sterile microcentrifuge tube for collecting the liquid. The other end of the Tygon tube is filled with 15-20 ml of SB and then sealed with parafilm or a sterile plastic tip. A negative vacuum pressure is applied to the flask to allow the buffer to pass through the xylem tissue and to be collected in the microcentrifuge tube. The collected liquid is, then, centrifuged for 3 min at 12.000 *g* and the pellet resuspended in 0.6 ml of SB.

2 - *Procedure*

Flat-bottom microtiter plates are coated with 0.2 ml of gamma globulin in coating buffer (Na_2CO_3: 1.59 g; $NaHCO_3$: 2.93 g; $NaNO_3$: 0.2 g, distilled water 1 liter; pH 9.6) in each well. The plates are incubated at 4°C overnight. Then, the plates are washed with PBS-Tween (NaCl: 8.0 g; KH_2PO_4: 0.2 g; $Na_2HPO_4.12\ H_2O$: 2,9 g; KCl: 0.2 g; $NaNO_3$ 0.2 g; distilled water 1 liter; pH 7.4). 0.2 ml of plant extract in SB are added to the wells and incubated overnight at 4°C. The plates are washed with PBS-Tween and 0.2 ml of alkaline phosphatase conjugated antibody in conjugated buffer (Polyvinylpyrrolidone: 20 g; Ovo albumin: 2 g; PBS-Tween 1 liter) are added. The plates are incubated at 37°C for 4 h. After washing with PBS-Tween, 0.2 ml of substrate solution (*p*-nitrophenyl phosphate: 1mg/ml) in substrate buffer (diethanolamine: 98 ml; NaN_3 0.2 g; distilled water 1 liter). The plates are incubated in the dark, at room temperature for 30-60 min. The reaction is terminated by adding 50 µl of 3M NaOH per well. Plates are read at 405-410 nm in a microplate auto reader. A suspension of 10^5 colony forming units/ml of a *Xylella fastidiosa* type-strain in SB has to be used as positive control, whereas SB only is used as negative control. A mean absorbance greater than the mean of the negative control wells plus four times the standard deviation indicates out a positive reaction.

b) *Citrus* spp.

1 - *Plant extract*

Leaves are taken from symptomatic trees. The leaf midribs and the petioles are aseptically excised. Subsequently, they are surface-disinfected for 5 min with 10% bleach and, then, rinsed two times with sterile distilled water. Midribs and petiole are homogenized or grinded in sterile mortars (1 g of midribs or petiole in 3 ml of Phosphate Buffer Saline (PBS, 0.01 M KPO_4, 0.15 M NaCl, 0.02% sodium azide) plus 2% polyvinylpyrrolidone. 0.2 ml of the plant extract are added to the wells in the ELISA microplate.

2 - *Procedure*

Similar to that of the grapevine test.

Polymerase Chain Reaction (PCR)

a) Grapevine

1 - *DNA extraction*

A sample of 1.0 g of leaf veins of the plant is ground to powder in liquid nitrogen and transferred directly into 10 ml of 65°C preheated extraction buffer (100 mM Tris-HCl, 20 mM EDTA, 500 mM NaCl, 1,25 % sodium-dodecylsulphate, pH 8.0) containing 0.1 g of polyvinylpyrrolidone. After mixing the sample is incubated at 65°C in a water bath for 20 min. Then, 4 ml of 5M potassium acetate are added and placed in ice for at least 20 min. Subsequently, the mixture is centrifuged at 12.000 *g* for 10 min at 4°C. To the supernatant 1 ml of 5% hexadecyltrimethylammonium bromide (CTAB) and 10 ml of chloroform are added. The mixture is then centrifuged at 12.000 *g* for 10 min at 4°C and 2/3 volume of isopropanol is added to precipitate DNA (Berisha *et al*., 1998).

2 - *Primers*

Primers RST31 and RST33 which generate a PCR product of 733 base pairs are used for detection of *X. fastidiosa* (Minsavage *et al*., 1994). RST31: 5'-GCGTTAATTTTCGAAGTGATTCGATTGC-3'; RST33: 5'-CACCATTCGTATCCCGGTG-3'.

3 - *PCR amplification*

Individual PCR samples (50 µl) contain 1X amplification buffer, 100 µM of each dNTP, 50 µM of each primer, 1,25 U of *Taq* DNA polymerase, 4 µl of plant sample extract (see ELISA test for extraction protocol) or 100 ng of purified *X. fastidiosa* genomic DNA in 4 µl of TAE buffer (40mM Tris acetate, 1mM EDTA, pH 8.0) for positive control. Each reaction is covered with 50 µl of mineral oil before running of the PCR reactions. Sterile 500-µl microcentrifuge tubes are used. PCR is carried out with an initial denaturation at 95°C for 1 min followed by 40 cycles of denaturation step at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and DNA extension at 72°C for 45 sec. A final cycle consists of the denaturation and annealing step followed by an extension cycle at 72 °C for 5 min before cooling at 4°C. After the amplification, the products (15 µl) have to be analyzed by electrophoresis in a 1 % agarose gel in TAE buffer. The gel has to run at 5 V/cm for 1,5 h and then stained with ethidium bromide (10 mg/ml) for 20-30 min. PCR products can

be photographed. A PCR product of 733 bp indicates the presence of a *X. fastidiosa* DNA fragment in the sample.

b) *Citrus* spp.

1 - *DNA extraction*

The technique described for the obtaining of the plant extract to be used in the DAS-ELISA test can be followed also for the PCR. Plant extract from twigs, are obtained by vacuum-extraction as described for grapevine.

2 - *Primers*

Primers CVC-1 and 272-2-int that generate a PCR product of 500 base pairs are used for the detection of *X. fastidiosa* in this case. DNA fragment are used (Pooler and Hartung, 1995): CVC-1:5'-AGATGAAAACAATCATGCAAA-3'; 272-1-int: 5'-GCCGCTTCGGAGAGCATTCCT-3'.

Primers 272-1-int and 272-2-int that generate a PCR product of 600 bp may also be used for citrus as well as for grapevine.

3 - *PCR amplification*

Individual PCR samples (25 µl) containing: PCR buffer (20 mM NaCl, 50 mM Tris pH 9.0 1% Triton X-100, 0.1% gelatin, 3mM $MgCl_2$), 200 µl dNTP, 0.4 µM primer, 1.0 unit of *Taq* DNA polymerase, 1-5 µl of plant sample extract or 5 x 10^3 cfu/ml of *X. fastidiosa* (CVC strain) cells as positive control. PCR is carried out with an initial lysis of bacteria at 94°C for 4 min, followed by an amplification profile of 94°C for 1 min, primer annealing at 62°C for 1 min, a DNA extension at 72°C for 1 min for 30 cycles, followed by a final extension at 72°C for 10 min. PCR products are analyzed by agarose gel electrophoresis and visualized with ethidium bromide.

Membrane Entrapment Immunofluorescence of plant extracts (MEIF)

This technique, developed for *Xanthomonas axonopodis* pv. *citri* (Brlansky *et al*., 1990), proved useful also for the preliminary detection of CVC (Hartung *et al*., 1994). It is not conclusive for the completion of the diagnostic process.

The plant extract is prepared as described for the ELISA test. Subsequently, it is centrifuged for 5 min at 1.000 *g* and the supernatant is transferred to a sterile syringe and pushed to a multiple holder adapter containing a 5.0 µm membrane for trapping plant cellular debris plus a 0.2 µm polycarbonate membrane for trapping bacterial cells. After having pushed the extract, the 0.2 µm membrane is removed and incubated for 1 h in *X. fastidiosa*-specific immunoglobulin in Tris-Bovine Serum Albumin-Gelatin Buffer (20 mM Tris, 0.9% NaCl, pH 8,2 plus 0.1% Bovine Serum Albumin and 1% gelatin). Then, the membrane is washed with PBS containing 2% polyvinylpyrrolidone (PVP-40) and incubated for 1 h in tetramethyl-rhodamine isothiocyanate (TRITC)-labeled goat anti-rabbit IgG. After a brief washing with PBS, the membrane is mounted in a epifluorescence microscope for the observation. Cells of *X. fastidiosa* appear as fluorescent rods.

Dot Immunoblotting Assay (DIBA)

This technique (Lee *et al*., 1992) is useful for the preliminary detction of CVC. It is not conclusive for the completion of the diagnostic process.

The plant extract is prepared as described for the ELISA test. Subsequently, it is centrifuged for 5 min at 1.000 *g*. An aliquot of 2.0 µl of the supernatant is spotted on to a nitrocellulose membrane and allowed to dry. Then, it is blocked for 1 h in buffer (10.0 mM Tris, 0.15 M NaCl, 0.1% Tween 20, pH 8.0) (PBS-Tween) containing 1% of Bovin Serum Albumin and 2.0% Triton X-100. After rinsing with PBS-Tween, the membrane is incubated overnight in IgG (1 µg/ml) specific for *X. fastidiosa* in conjugate buffer (PBS added with 2% PVP-40 and 0.5% Bovin Serum Albumin). The membrane is rinsed with PBS-Tween and, then, incubated for 2-4 h in goat anti-rabbit IgG with alkaline phosphatase in the conjugate buffer. The membrane is rinsed with PBS-Tween, whereafter substrate (5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium) BCIP-NBT is added. A purple blue coloration of the membrane indicates the presence of the pathogen in the sample.

Reference strains

CVC strains are not maintained in ATCC. Single researchers have their own strains.

Pierce's disease ATCC 35877, 35879

Plum leaf scald ATCC 35871, 33490

Almond leaf scorch ATCC 35870

Mulberry leaf scorch ATCC 35868

Phony peach ATCC 33489

Elm leaf scorch ATCC 35873

Requirements for a positive diagnosis

- Disease symptoms, morphological and pathogenic characteristics of the pathogen should be in accordance with the descriptions.
- The detection techniques must yield a clear cut positive result.
- A reference strain of the pathogen must be included as a positive control.

Report on the diagnosis

The procedures for detection and identification described in this protocol should have been followed:

- Information about the origin of the infected material
- Description of disease symptoms
- Description of the importance of the problem
- Description of the isolation and detection techniques
- Fulfillment of Koch's postulate
- Comments on the certainty or doubts about the identification

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This protocol was originally drafted by: / Ce protocole a été initialement préparé par:

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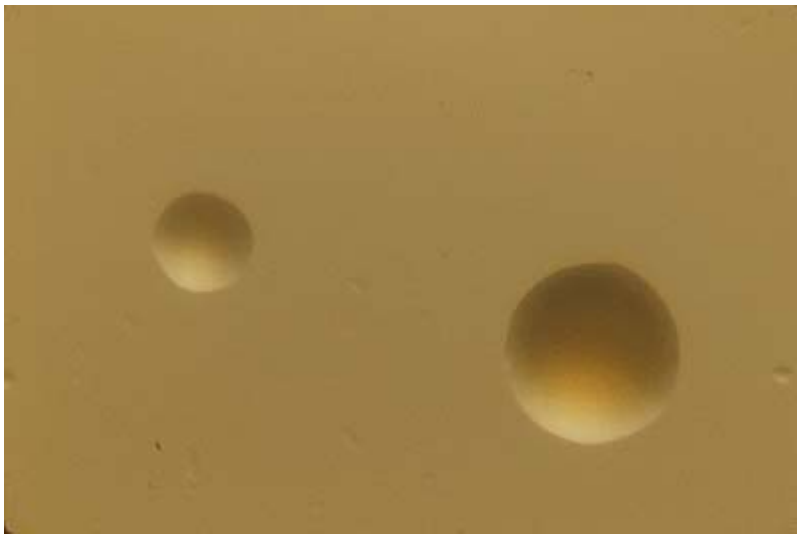
Figure 1 – Colonies of *Xylellla fastidiosa* from grapevine (x 4) on to CS 20 medium, after 10 days of incubation at 30°C. (Photo: C.J. Chang, University of Georgia, Griffin, USA).



Appendix I - Bacterial culture media

The following media are currently used for the primary isolation of *X. fastidiosa* from plant tissues.
It is important to remind that all of the ingredients have to be dissolved in the order given. It is recommended to use more than one single medium for the primary isolation.

1- PD2 (Davis *et al*., 1980)(For the isolation of *X. fastidiosa* from several host plants including grapevine)

Deionized distilled water 1.0 l
Soy peptone 2.0 g
Bacto tryptone 4.0 g
Disodium succinate 1.0 g
Trisodium citrate 1.0 g
K_2HPO_4 1,5 g
KH_2PO_4 1.0 g
Hemin chloride stock solution (0.1% in 0.05 N NaOH) 10.0 ml
Bacto agar 15.0 g
$MgSO_4.7H_20$ 1.0 g
Bovine serum albumin fraction V (20% w/v) 10.0 ml
pH 6.9

Autoclave at 121°C for 15 min.
Bovine serum albumin is filter sterilized and added to the rest of the medium at 50°C.

2 - PD3 (Davis *et al*., 1980)
It is the same as PD2 but bovine serum albumin is replaced with soluble potato starch (2 g/l). So, all of the ingredients can be directly sterilized in autoclave.

3 - CS20 (Chang and Walker, 1988)(For the isolation of *X. fastidiosa* from several host plants including grapevine)

Deionized distilled water: 1100 ml
Soy peptone 2.0 g
Bacto tryptone 2.0 g
Hemin chloride stock solution (0.1% in 0.05 N NaOH), 15.0 ml
$(NH_4)_2HPO_4$ 0.8 g
KH_2PO_4 1.0 g
$MgSO_4.7H_2O$ 0.4 g
Phenol red stock solution (0.2%) 5.0 ml
L-glutamine 6.0 g
Dextrose 1.0 g
L-histidine-HCl 1.0 g
Potato starch soluble 2.0 g
Bacto agar 12.0 g
pH 6.6
Autoclave at 121°C for 15 min.

To prepare Hemin chloride 0.1%, dissolve 1.2 g of NaOH in 600 ml of distilled water and add 0.6 g of HeminCl and dissolve. Keep in a dark container.

To prepare Phenol red 0.2%, dissolve 0.6 g of phenol red in 30 drops of 20% NaOH and bring the volume up to 300 ml with distilled water. Store in the refrigerator.

4- B.CYE (Wells *et al*, 1981)

Deionized distilled water	1.0 l
Yeast extract	10.0 g
Activated charcoal	2.0 g
L-cysteine HCl.H20	0.4 g
Ferric pyrophosphate(soluble)	0.25 g
ACES (N-2-acetamido-2-aminoéthane sulfonic acid)	10.0 g
Bacto agar	17.0 g

L-cysteine HCl and Ferric pyrophosphate are dissolved, filter sterilized (0.2 µm filter), and added to the autoclaved basal media, pH 6,9

5 - PW (For isolation of *X. fastidiosa* from several host plants including *Citrus* spp.)(Davis *et al*., 1983)

Deionized distilled water	1.0 l
Soytone	4.0 g
Bacto Tryptone	1.0 g
Hemin chloride stock solution (0.1% in 0.05N of NaOH)	10.0 ml
$MgSO_4.7H_2O$	0.4 g
K_2HPO_4	1,2 g
Phenol red stock solution (0.2%)	10.0 ml
L-glutamine	4.0 g
Bovine Serum Albumin fractionV (20% w/v)	30.0 ml
Bacto agar	12.0 g
pH	6,6

Bovin Serum Albumin is filter sterilized and added to the rest of the medium at 50°C.

6 - SPW (For stimulating the growth of *X. fastidiosa* to be isolated from sweet orange) (Hartung *et al.,* 1994). It is the same as PW supplemented with:

Malt extract	5.0 g
Sucrose	10.0 g
Myo-inositol	0.1 g
Thiamine chloride	0.01 g
Pyridoxine chloride	0.01 g
Nicotinic acid	0.005 g
Glycine	0.002 g

7 - CVC1 (it has been developed for the isolation of *X. fastidiosa* from *Citrus* spp.) (Chang *et al*., 1993)

Deionized water	970 ml
Bacto-peptone	4.0 g
Tryptone	1.0
K_2HPO_4	1,2 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.4 g
Phenol red stock solution (0.2%)	10.0 ml
Agar	12.0 g

After autoclaving the following filter-sterilized compounds are added :

Glutamine stock solution (8%)	50 ml
Bovine serum albumin stock solution Frac V (10%)	60 ml
pH	6.5

8 - CVC2 (Chang *et al*., 1993)

It is the same as CVC1 plus 10.0 ml of hemin chloride stock solution (0.1%) to be added before autoclaving.

Appendix II Decision scheme for testing plant samples to detect *Xylella fastidiosa*. It can be adopted either for visibly infected or for apparently healthy specimens. Both isolation and screening tests have to be performed.