Técnicas de detección y diagnóstico de *Xylella fastidiosa*

Ester Marco Noales
Colaboradora Científica Adjunta / Bacteriología
E-mail: **marco_est@gva.es**
http://www.ivia.gva.es/----

Instituto Valenciano de Investigaciones Agrarias (IVIA)

SIMPOSIO XYLELLA

SITUACIÓN ACTUAL Y PERSPECTIVAS DE LAS ENFERMEDADES CAUSADAS POR *Xylella fastidiosa* EN LA CUENCA MEDITERRÁNEA

Jueves 28 de septiembre de 2017
Ciutat Administrativa 9 D'Octubre - C/ Castán Tobeñas 77, 46018 Valencia

ORGANIZA: Conselleria d'Agricultura, Medi Ambient, Canvi Climàtic i Desenvolupament Rural
Instituto Valenciano de Investigaciones Agrarias (IVIA)

COLABORA: Ministerio de Agricultura, Pesca, Alimentación y Medio Ambiente, Consejo Superior de Investigaciones Científicas, Govern de les Illes Balears, Consiglio Nazionale delle Ricerche, University of Berkeley, proyectos europeos POnTE y XFACTORS

LABORATORIO DE BACTERIOLOGÍA IVIA

Líneas de investigación

Laboratorio Nacional de Referencia para Bacterias Fitopatógenas (MAPAMA)

GOBIERNO DE ESPAÑA
MINISTERIO DE AGRICULTURA, ALIMENTACIÓN Y MEDIO AMBIENTE

Laboratorio Nacional de Referencia de Bacterias - IVIA

Laboratorio:	Comunidad Valenciana. Moncada. Laboratorio Nacional de Referencia de Bacteriología (IVIA)
Institución:	Instituto Valenciano de Investigaciones Agrarias
Dirección Postal:	CV-315 km 10,7 - 46113 MONCADA (Valencia)
Teléfono:	96 3424000 ext. 24225
Fax:	96 3424001
Responsable:	Ester Marco Noales
Email:	emarco@ivia.es
Ténicos:	Adela Monterde, Javier Peñalver Navarro
Tipo de Trabajo:	Bacteriología
Página web:	http://www.ivia.es

GENERALITAT VALENCIANA ivia

Laboratorio Nacional de Referencia (LNR) para la identificación y el diagnóstico de bacterias fitopatógenas

- **Desde 1993**

BOLETÍN OFICIAL DEL ESTADO

Núm. 195 Jueves 13 de agosto de 2009 Sec. III. Pág. 69203

III. OTRAS DISPOSICIONES

MINISTERIO DE MEDIO AMBIENTE, Y MEDIO RURAL Y MARINO

13458 *Orden ARM/2238/2009, de 29 de julio, por la que se designan los laboratorios nacionales de referencia para la identificación y el diagnóstico de plagas y enfermedades de los vegetales.*

4. Laboratorio de bacteriología del Centro de Protección Vegetal y Biotecnología del Instituto Valenciano de Investigaciones Agrarias para la identificación y diagnóstico de bacterias fitopatógenas.

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Funciones del LNR

BOLETÍN OFICIAL DEL ESTADO

Jueves 13 de agosto de 2009 Sec. III. Pág. 69203

III. OTRAS DISPOSICIONES

MINISTERIO DE MEDIO AMBIENTE, Y MEDIO RURAL Y MARINO

13458 *Orden ARM/2238/2009, de 29 de julio, por la que se designan los laboratorios nacionales de referencia para la identificación y el diagnóstico de plagas y enfermedades de los vegetales.*

La Ley 43/2002, de 20 de noviembre, de Sanidad Vegetal, establece en el apartado 4 del artículo 47 que el Ministerio de Medio Ambiente, y Medio Rural y Marino dispondrá de laboratorios de referencia designados entre los laboratorios de identificación y diagnóstico de las comunidades autónomas u otros de reconocido prestigio, con las funciones de armonizar los métodos y técnicas que hayan de utilizarse, participar en los programas nacionales de prospección de plagas, emitir preceptivamente informe previo a la declaración de existencia de una plaga de cuarentena aparecida por primera vez en el territorio nacional, realizar las actividades relacionadas con el cumplimiento de los programas de aplicación de buenas prácticas de laboratorio que sean de su competencia, participar en los programas coordinados y efectuar los análisis y ensayos que, a efectos arbitrales o con otros fines, le sean solicitados.

- Armonizar métodos y técnicas
- Participar en los programas nacionales de prospección de plagas
- Emitir informe previo a la declaración de existencia de una plaga de cuarentena aparecida por primera vez en el territorio nacional
- Efectuar los análisis y ensayos que le sean solicitados

- **Analizar muestras enviadas por los Servicios de Sanidad Vegetal de las Comunidades Autónomas.**

- **Analizar muestras enviadas por los Servicios de Sanidad Vegetal de las Comunidades Autónomas.**

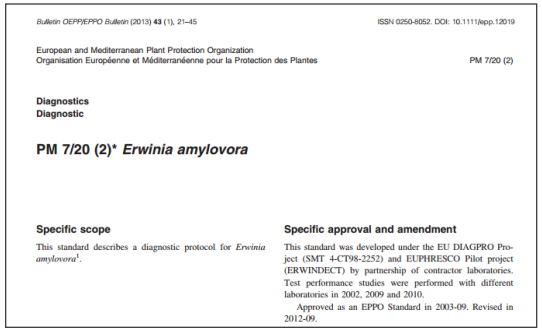
- **Analizar muestras de material vegetal importado, enviado desde los Puntos de Inspección Fronteriza (PIF).**

GENERALITAT VALENCIANA ivia

- **Analizar muestras enviadas por los Servicios de Sanidad Vegetal de las Comunidades Autónomas.**

- **Analizar muestras de material vegetal importado, enviado desde los Puntos de Inspección Fronteriza (PIF).**

- **Transferir protocolos y metodología disponible.**



Bulletin OEPP/EPPO Bulletin (2013) **43** (1), 21–45 ISSN 0250-8052. DOI: 10.1111/epp.12019

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/20 (2)

Diagnostics
Diagnostic

PM 7/20 (2)* *Erwinia amylovora*

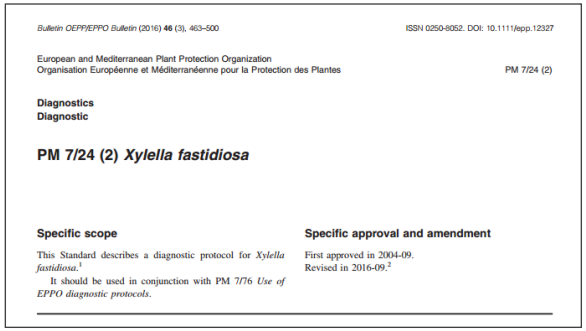
Specific scope

This standard describes a diagnostic protocol for *Erwinia amylovora*[1].

Specific approval and amendment

This standard was developed under the EU DIAGPRO Project (SMT 4-CT98-2252) and EUPHRESCO Pilot project (ERWINDECT) by partnership of contractor laboratories. Test performance studies were performed with different laboratories in 2002, 2009 and 2010.

Approved as an EPPO Standard in 2003-09. Revised in 2012-09.



Bulletin OEPP/EPPO Bulletin (2016) **46** (3), 463–500 ISSN 0250-8052. DOI: 10.1111/epp.12327

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/24 (2)

Diagnostics
Diagnostic

PM 7/24 (2) *Xylella fastidiosa*

Specific scope

This Standard describes a diagnostic protocol for *Xylella fastidiosa*.[1]

It should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Specific approval and amendment

First approved in 2004-09.
Revised in 2016-09.[2]

- **Analizar muestras enviadas por los Servicios de Sanidad Vegetal de las Comunidades Autónomas.**

- **Analizar muestras de material vegetal importado enviado desde los Puntos de Inspección Fronteriza (PIF).**

- **Transferir protocolos y metodología disponible.**

- **Impartir cursos o atender consultas de los Laboratorios de Diagnóstico de las Comunidades Autónomas sobre técnicas y métodos.**

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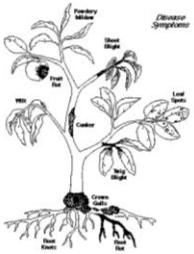
DIAGNÓSTICO Y DETECCIÓN

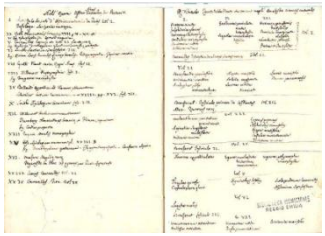
Diagnóstico

Identificar la naturaleza y la causa de la enfermedad

Detección

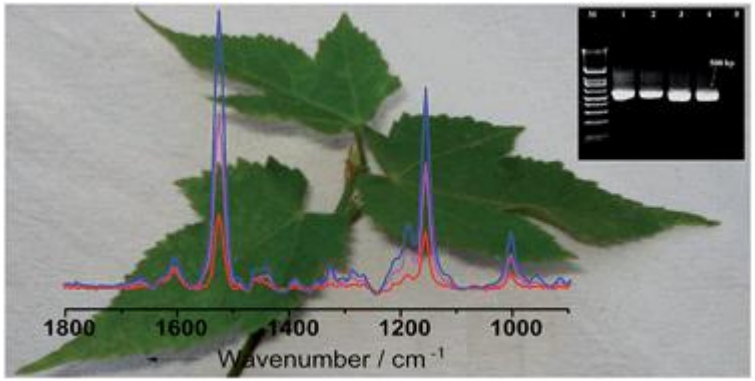
Determinar la presencia del organismo diana en una muestra





Diferentes agentes causales pueden producir síntomas similares

Detección temprana: prevención de la extensión de la enfermedad



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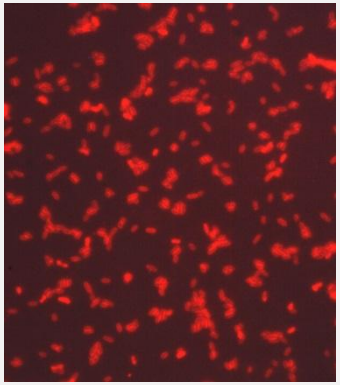
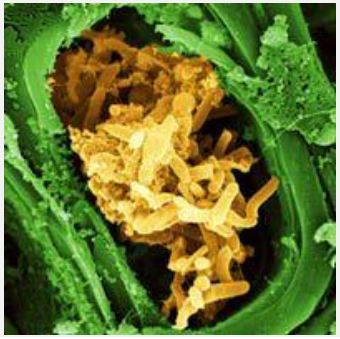
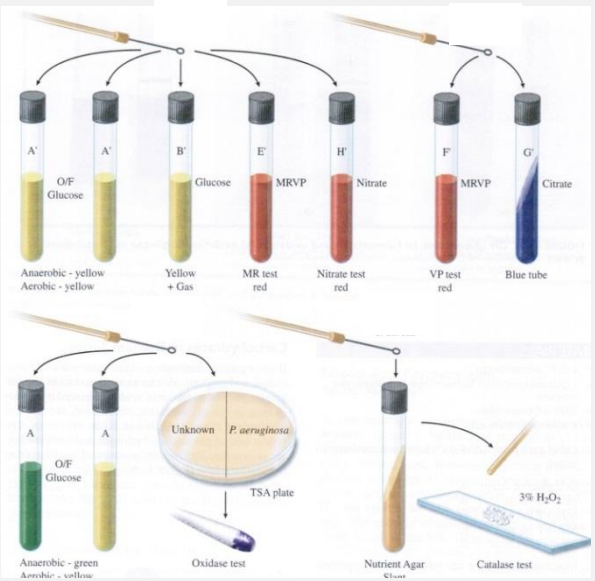
Observación visual de síntomas

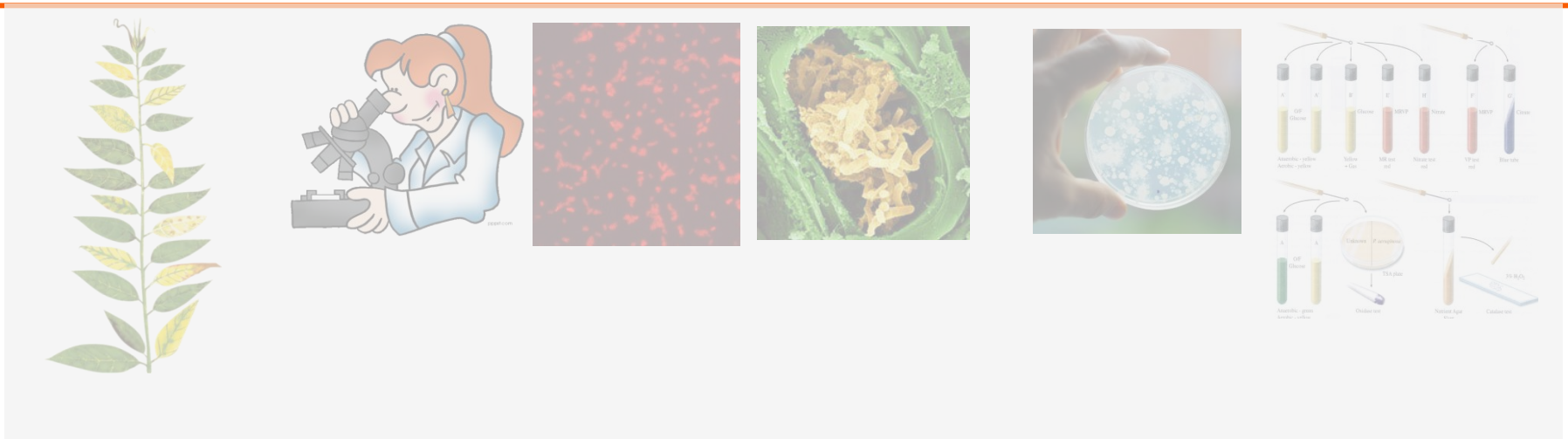




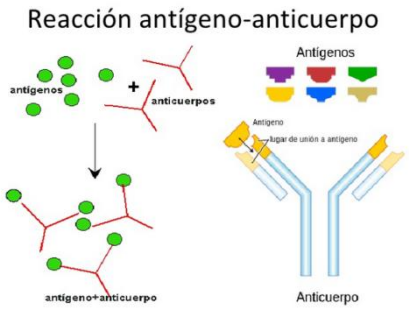
Observación microscópica de los patógenos



Crecimiento en medios de cultivo y realización de pruebas



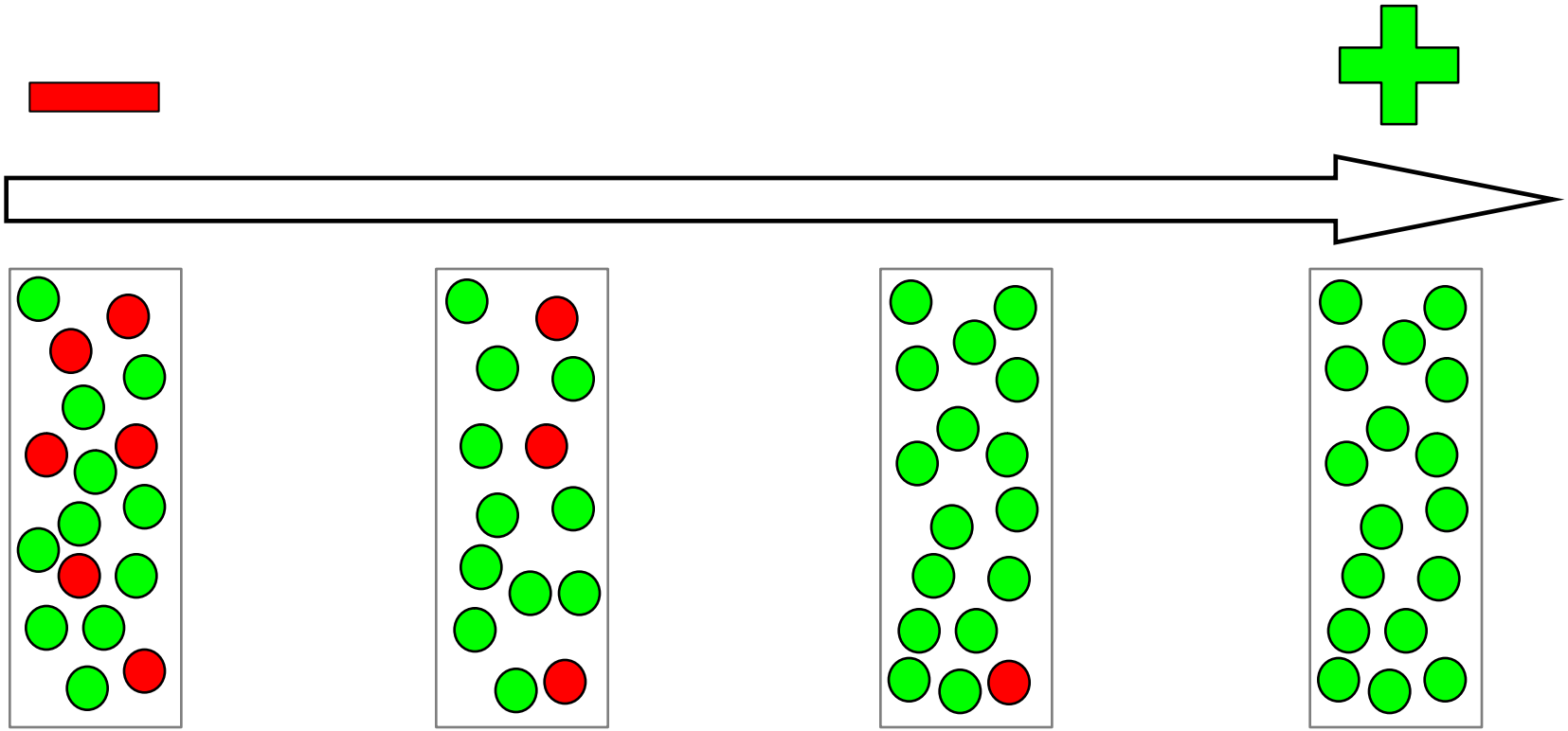
Técnicas serológicas



Peter Perlmann y
Eva Engvall, 1971

ELISA
(*Enzyme Linked ImmunoSorbent Assay*)

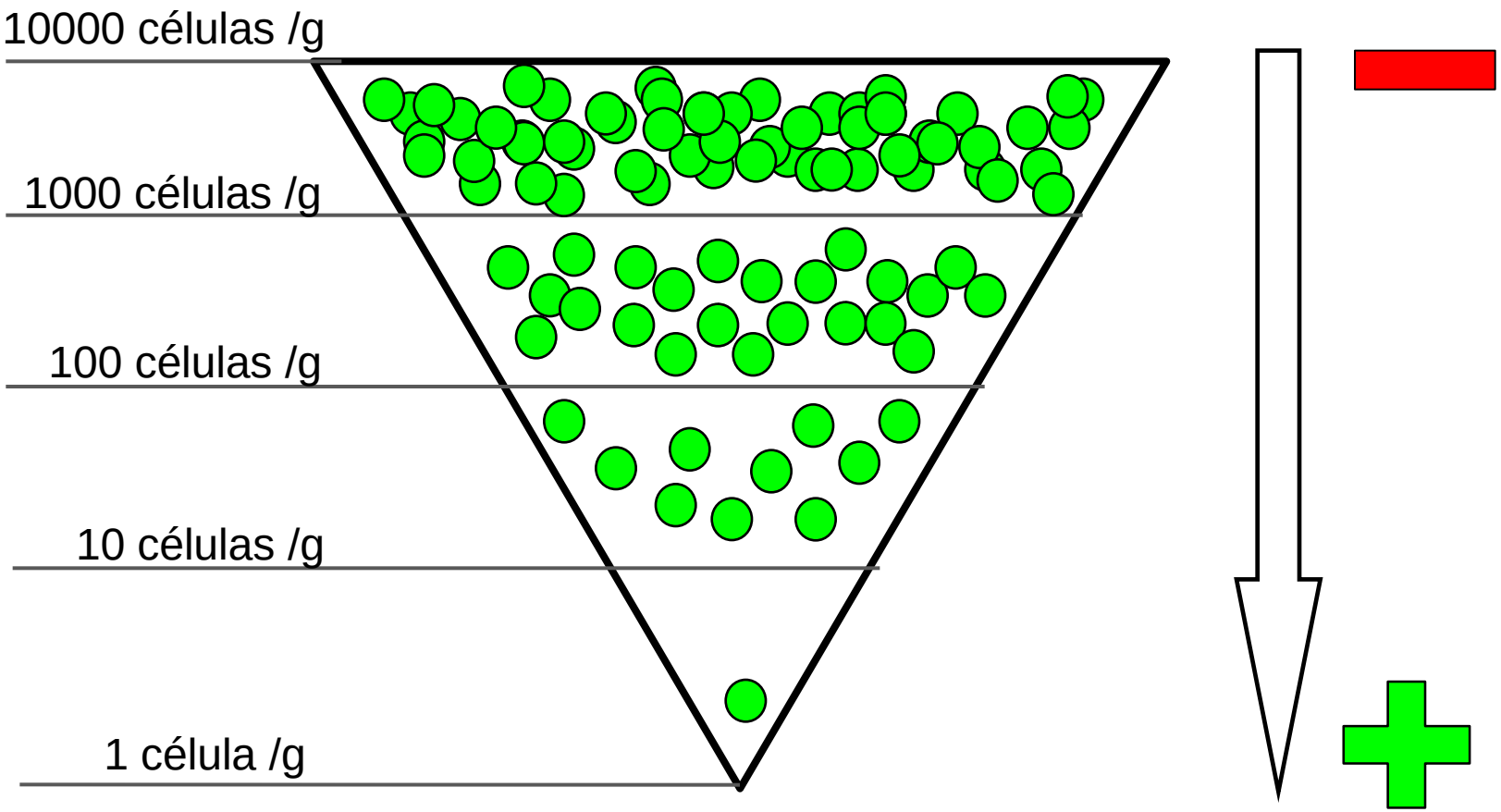
Especificidad

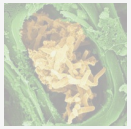


Falso positivo

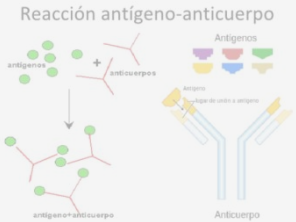
Verdadero positivo

Sensibilidad

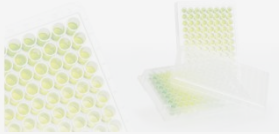




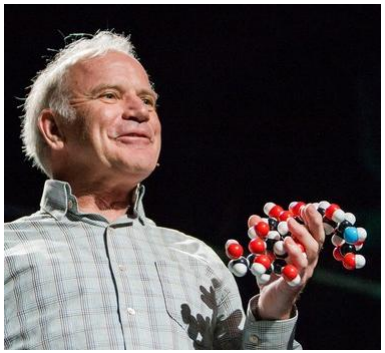
Técnicas serológicas



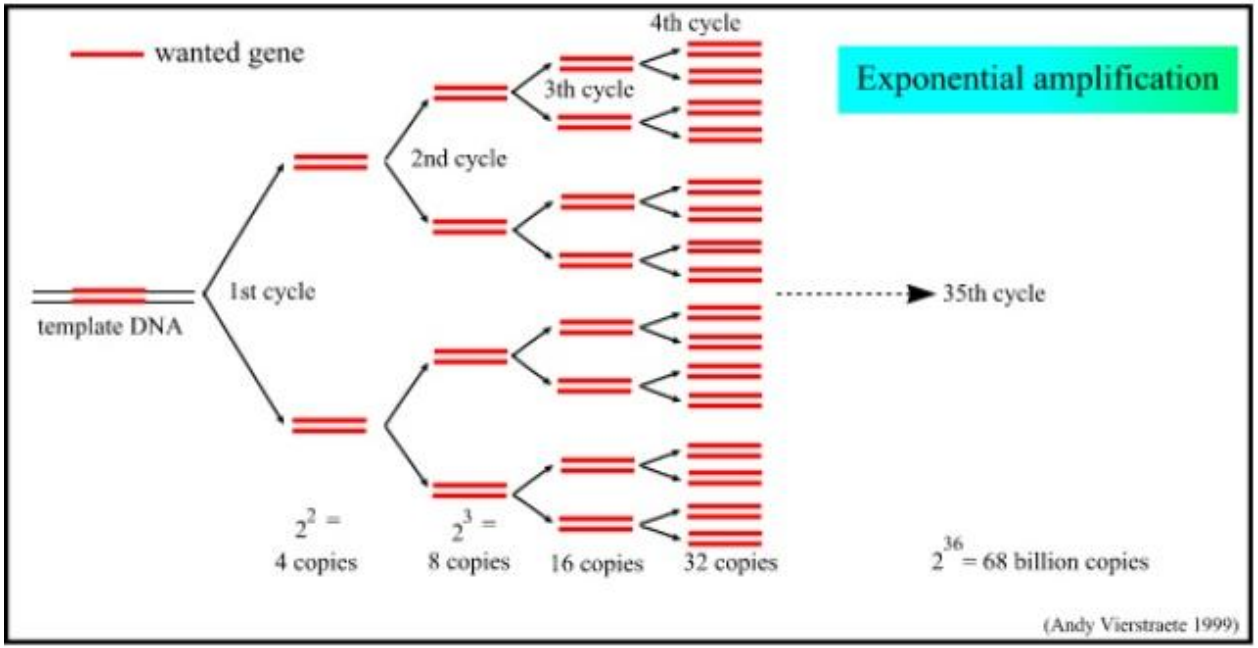
Peter Perlmann y
Eva Engvall, 1971

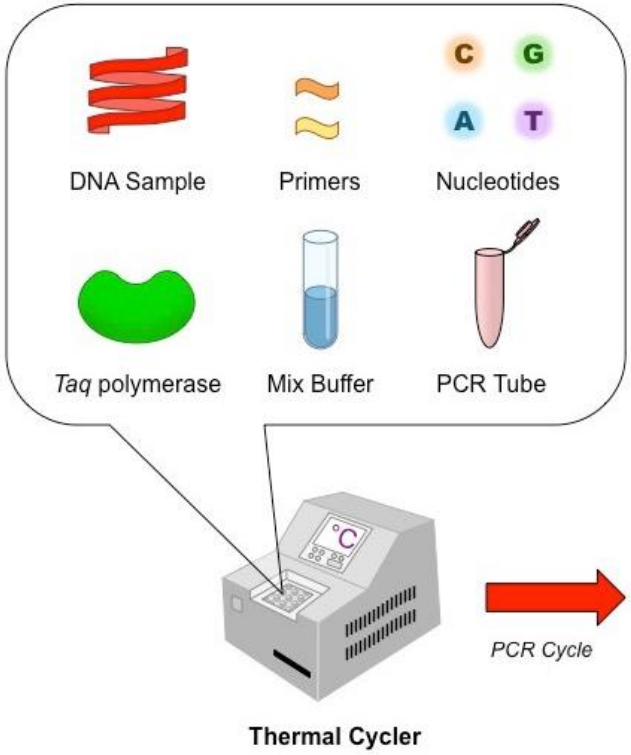


PCR (*Polymerase Chain Reaction*)



Kary Mullis, 1983

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Nucleotides
Taq polymerase
Mix Buffer
PCR Tube
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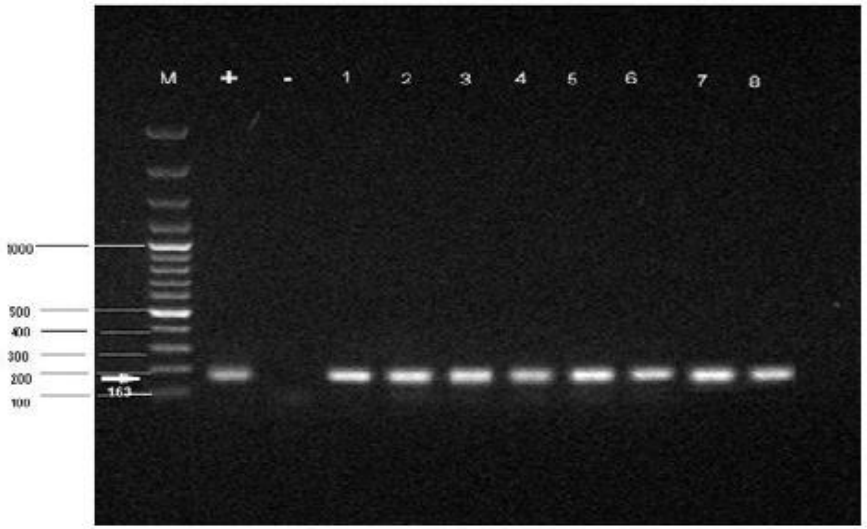
PCR Cycle
Thermal Cycler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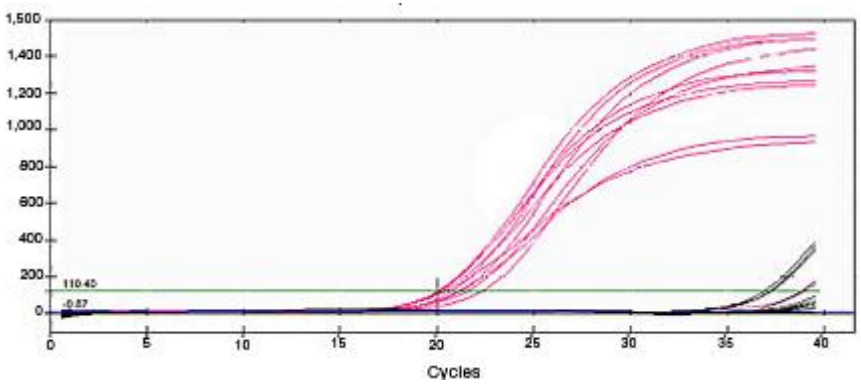
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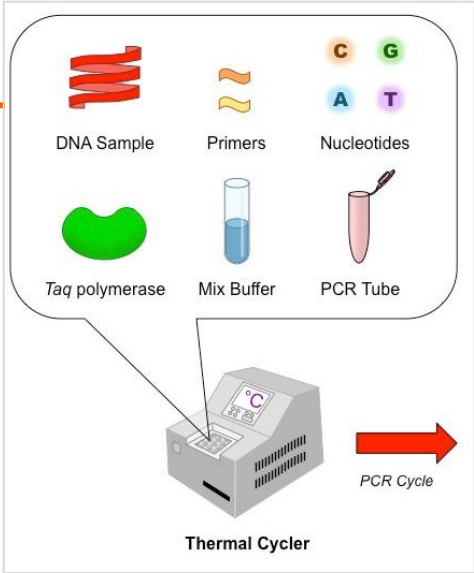
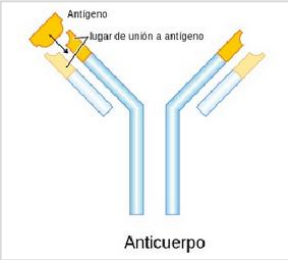
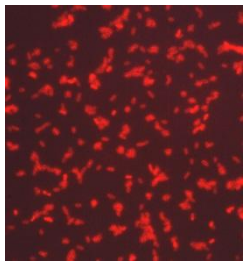
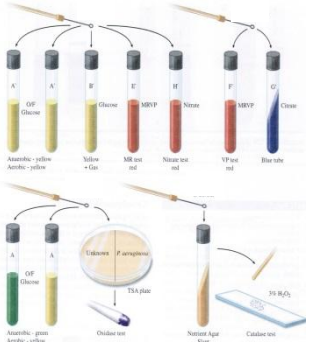




Información complementaria





PROTOCOLOS DE DIAGNÓSTICO

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

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PM 7 - Diagnostic Protocols

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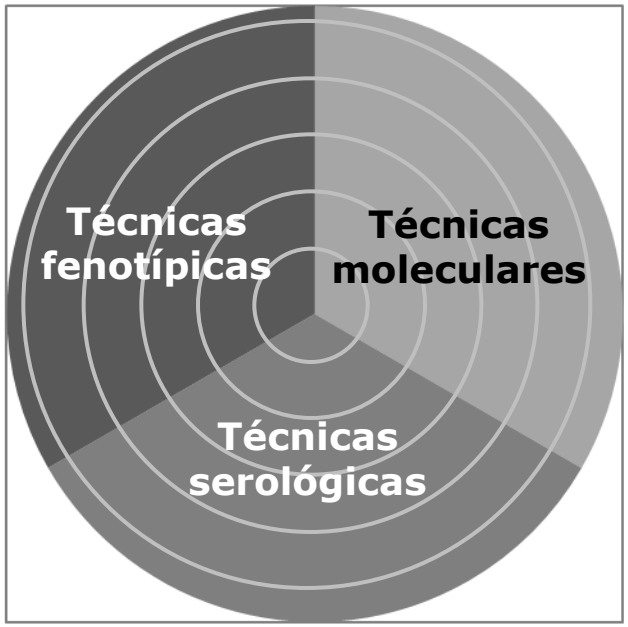

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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

Protocolo EPPO de diagnóstico para *Xylella fastidiosa*

Bulletin OEPP/EPPO Bulletin (2016) **0** (0), 1–38 ISSN 0250-8052. DOI: 10.1111/epp.12327

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/24 (2)

Diagnostics
Diagnostic

PM 7/24 (2) *Xylella fastidiosa*

Specific scope

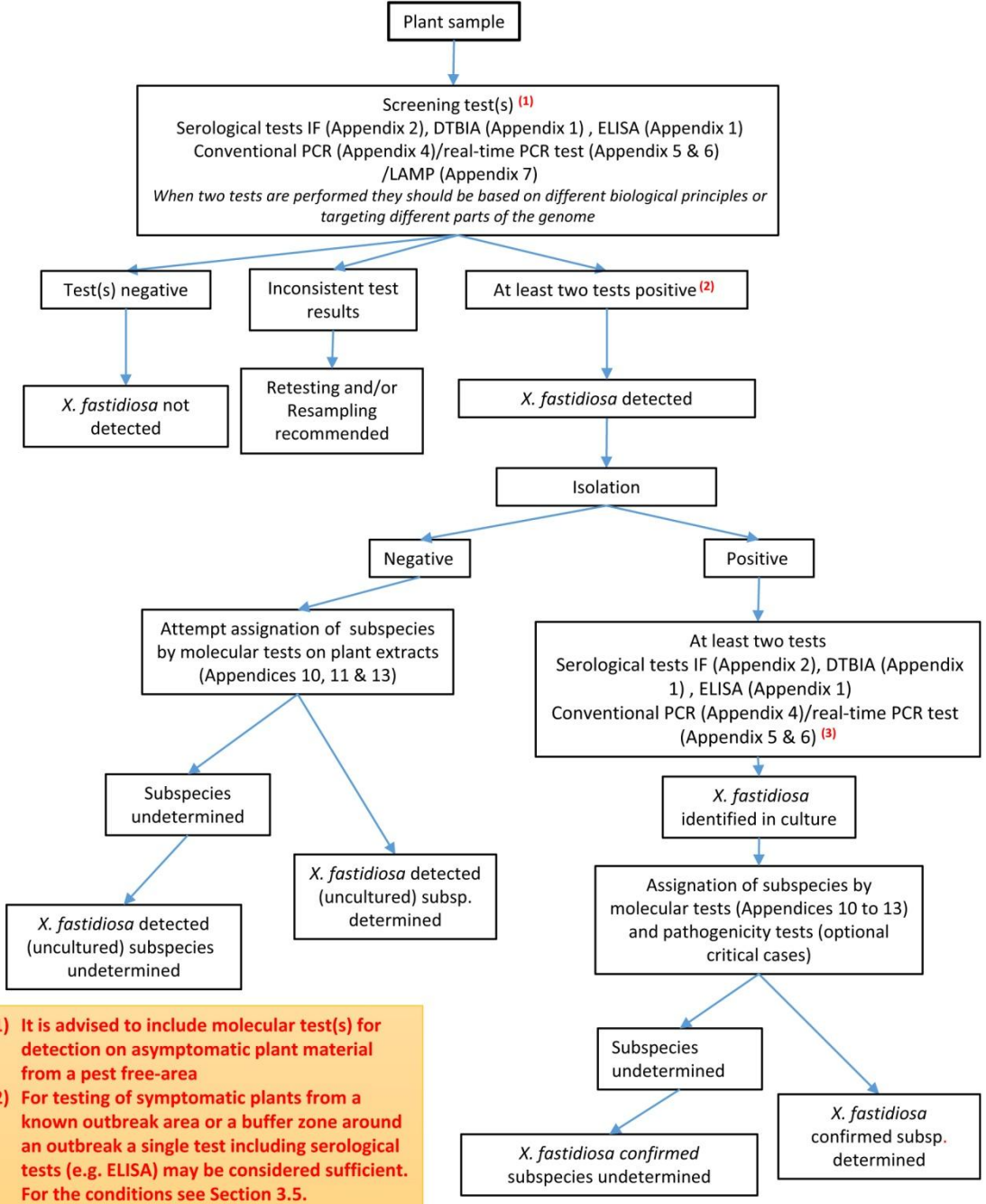
This Standard describes a diagnostic protocol for *Xylella fastidiosa*.[1]

It should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Specific approval and amendment

First approved in 2004-09.
Revised in 2016-09.[2]

Protocolo de diagnóstico de *X. fastidiosa* en material vegetal

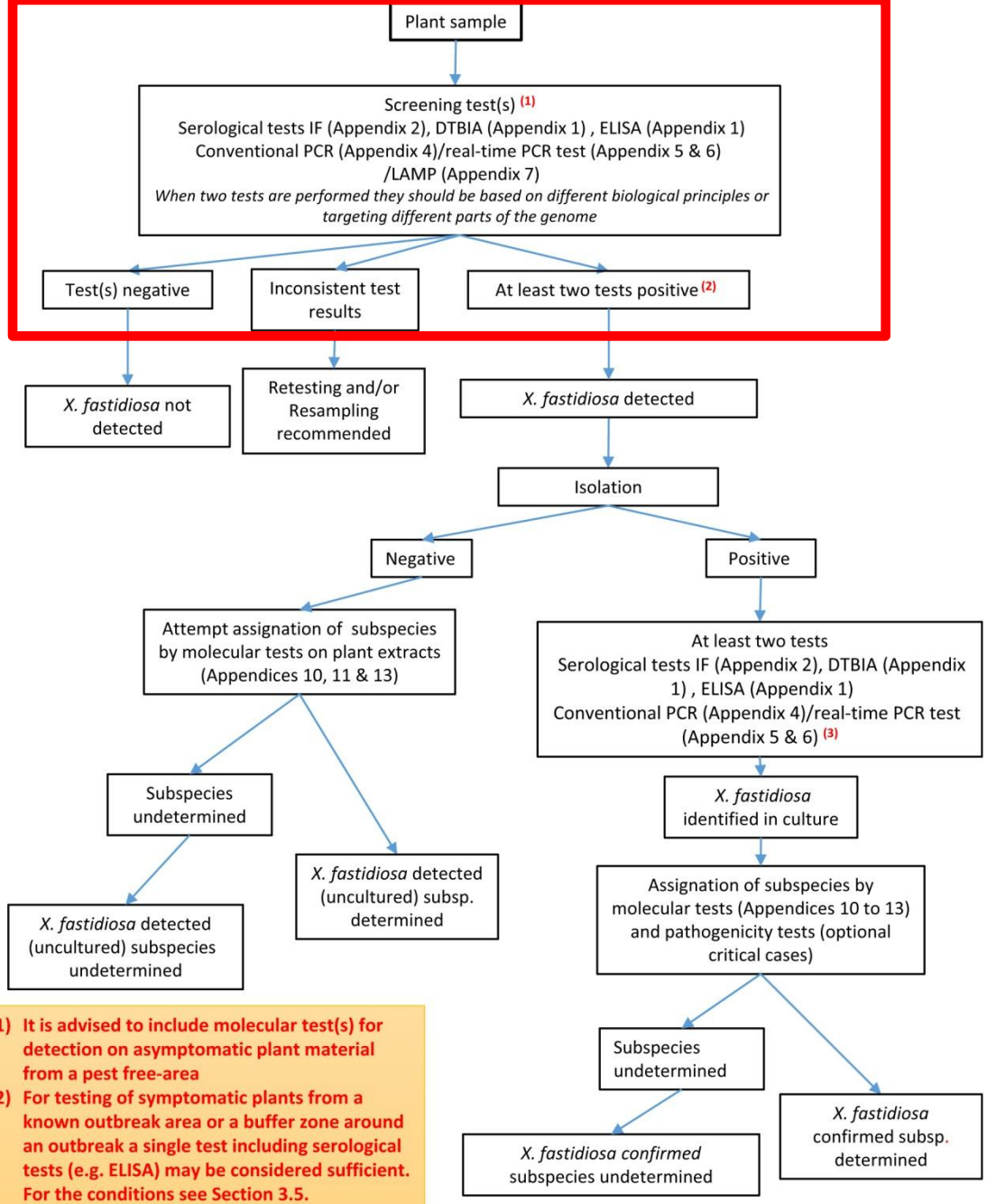


(1) It is advised to include molecular test(s) for detection on asymptomatic plant material from a pest free-area

(2) For testing of symptomatic plants from a known outbreak area or a buffer zone around an outbreak a single test including serological tests (e.g. ELISA) may be considered sufficient. For the conditions see Section 3.5.

(3) Molecular tests for assignation of subspecies can be used for confirmation of the identification of *X. fastidiosa*

Protocolo de diagnóstico de *X. fastidiosa* en material vegetal



(1) It is advised to include molecular test(s) for detection on asymptomatic plant material from a pest free-area
(2) For testing of symptomatic plants from a known outbreak area or a buffer zone around an outbreak a single test including serological tests (e.g. ELISA) may be considered sufficient. For the conditions see Section 3.5.
(3) Molecular tests for assignation of subspecies can be used for confirmation of the identification of *X. fastidiosa*

Protocolo de diagnóstico de *X. fastidiosa* en material vegetal

Plant sample

↓

Screening test(s) [1]

Serological tests IF (Appendix 2), DTBIA (Appendix 1) , ELISA (Appendix 1)

Conventional PCR (Appendix 4)/real-time PCR test (Appendix 5 & 6) /LAMP (Appendix 7)

When two tests are performed they should be based on different biological principles or targeting different parts of the genome

Protocolo de diagnóstico de *X. fastidiosa* en material vegetal

Plant sample

↓

Screening test(s) [1]
Serological tests IF (Appendix 2), DTBIA (Appendix 1) , ELISA (Appendix 1)
Conventional PCR (Appendix 4)/real-time PCR test (Appendix 5 & 6)
/LAMP (Appendix 7)
When two tests are performed they should be based on different biological principles or targeting different parts of the genome

3.5. Screening tests

Unlike other EPPO protocols for bacteria, isolation is not recommended as a screening test because the bacterium is very difficult to isolate (see Figs 1 and 2). Samples should be considered as 'samples with *X. fastidiosa* detected' when at least two screening tests are positive based on different biological principles or targeting of different parts of the genome. Subspecies determination by molecular tests included in Section 4.2 and/or sequencing analysis should then be performed. Isolation should also be attempted. For

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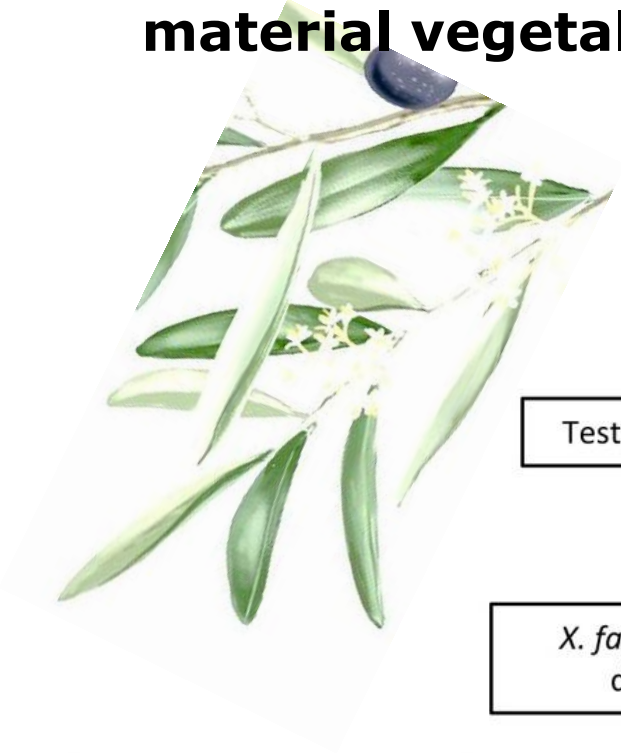
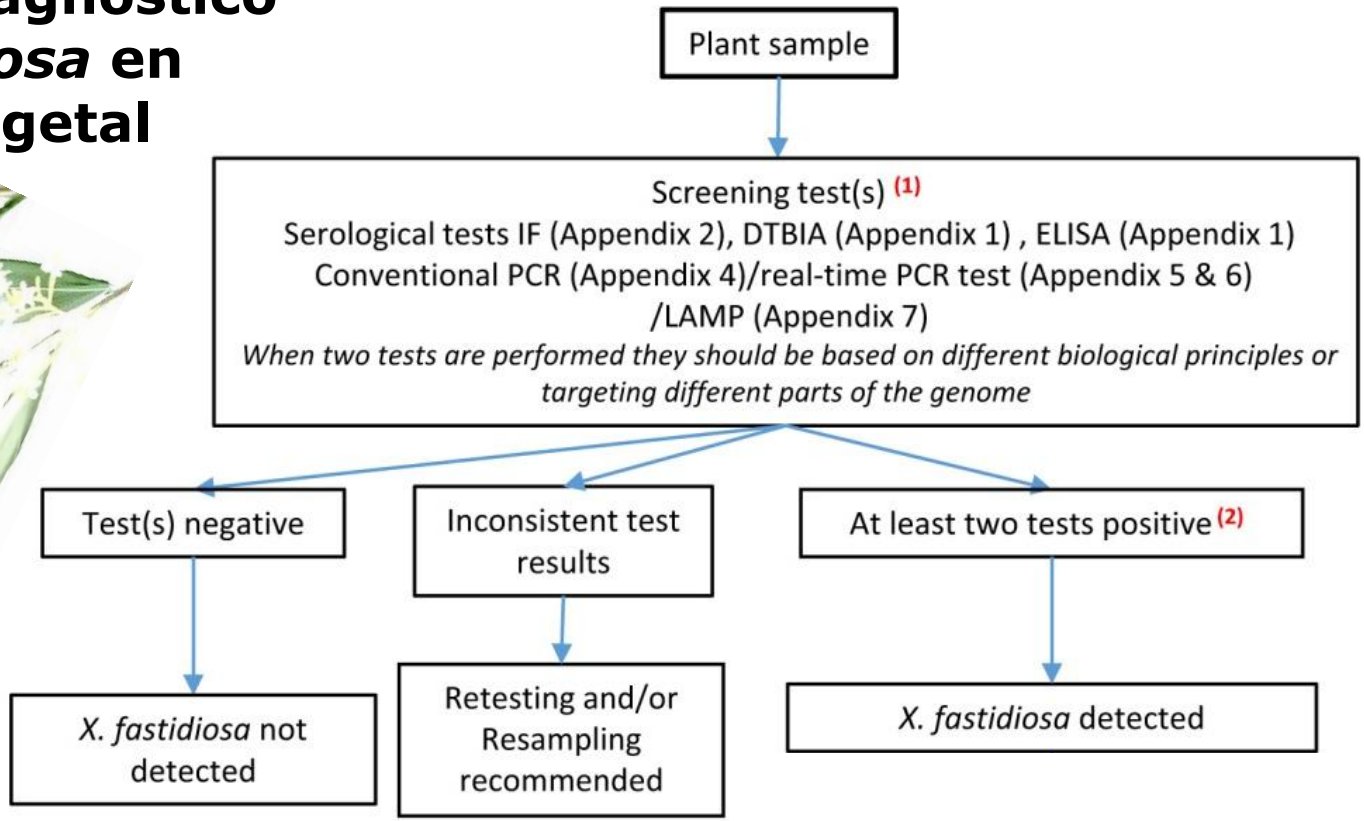
Protocolo de diagnóstico de *X. fastidiosa* en material vegetal





(1) It is advised to include molecular test(s) for detection on asymptomatic plant material from a pest free-area

(2) For testing of symptomatic plants from a known outbreak area or a buffer zone around an outbreak a single test including serological tests (e.g. ELISA) may be considered sufficient. For the conditions see Section 3.5.

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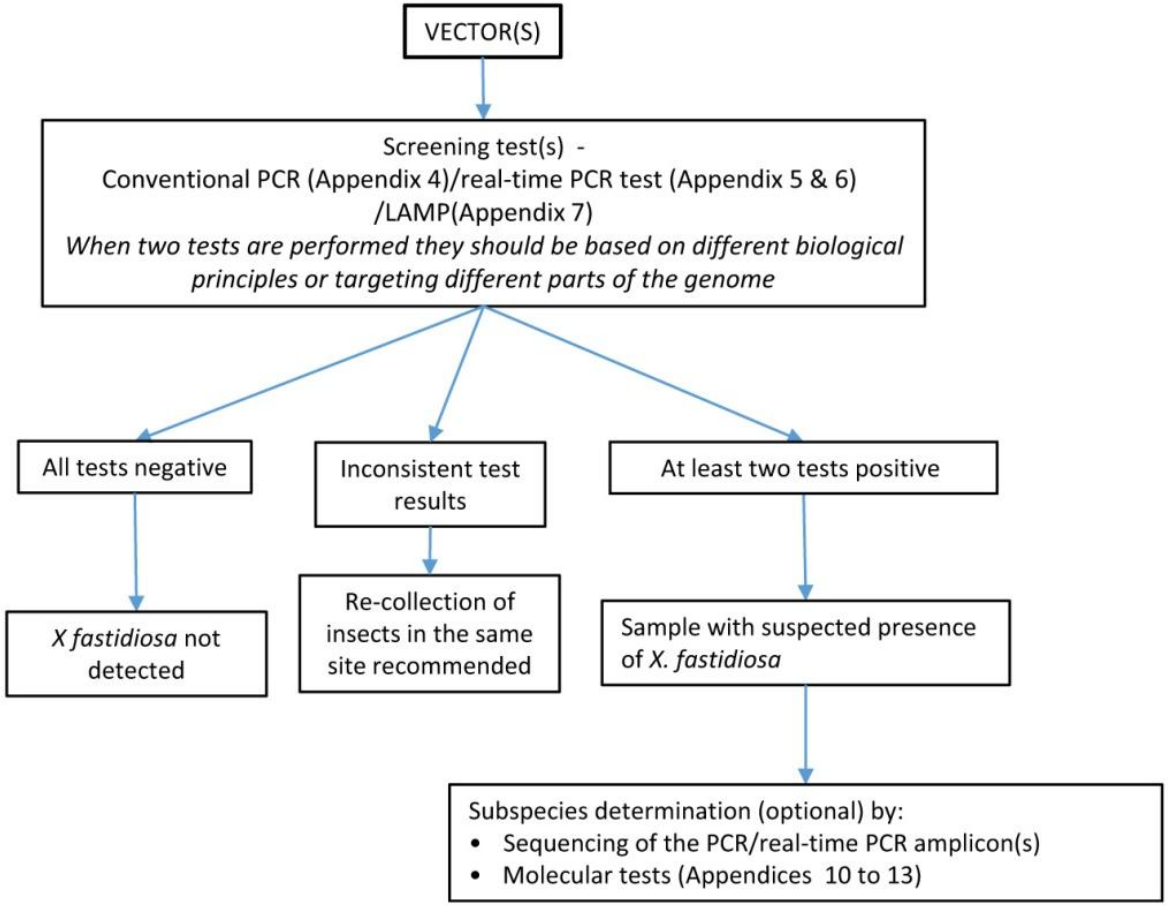
Protocolo de diagnóstico de *X. fastidiosa* en vectores



GENERALITAT VALENCIANA ivia

Muestreo

Fig. 22 Leaf scorch symptoms caused by *Xylella fastidiosa* on cherry. Courtesy D. Boscia, CNR-Institute for Sustainable Plant Protection (IT).

Polygala myrtifolia is one of the major susceptible hosts in the current European outbreaks. Infected plants show scorched leaves, with desiccation starting from the tip and progressing to the entire blade (see leaf tip desiccation in Fig. 20). An illustration of an infected plant is given in Fig. 21.

Leaf scorching symptoms have been also reported on cherry (Fig. 22) in late summer/autumn in Italy.

3.2. Sampling of plant material

3.2.1. 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.

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).

3.2.2. 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.

3.2.2.1. 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

EUROPEAN COMMISSION
DIRECTORATE-GENERAL FOR HEALTH AND FOOD SAFETY

Safety of the Food Chain
Plant health

Brussels, 16 December 2015

Guidelines for the survey of *Xylella fastidiosa* (Wells et al.) in the Union territory

1 INTRODUCTION

Xylella fastidiosa, one of the most harmful plant pathogenic bacteria worldwide, is subject to a regular survey activity within the Union territory. In this respect, in accordance with Article 3 of Decision (EU) 2015/789[1], Member States conduct annual surveys on a list of regulated plants (also known as 'specified plants') for the presence of *X. fastidiosa*.

These general survey activities consist of visual examinations and, in the case of any suspicion of infection, collection of samples and testing. They are carried out at appropriate times of the year, taking into account available scientific and technical evidence, the biology of the pest, its vectors, the presence and biology of the specified plants, as well as any appropriate information, concerning the presence of *X. fastidiosa*. For demarcated areas, where the pest is known to be present, different survey provisions apply.

At the same time, according to Article 14 of Decision (EU) 2015/789, Member States shall by 31 December of each year communicate to the Commission and to the other Member States a survey plan with the measures, including the scheduled time period of each measure, to be taken in the following year.

Identificación de lugares de mayor riesgo

- **Carreteras, aeropuertos, puertos, en la proximidad de áreas demarcadas**

- **Viveros: plantas importadas, origen del material, lugares de crecimiento de las plantas madre, campos de producción a cielo abierto, sitios de producción en ambiente protegido**

❑ Priorización de plantas que deben ser analizadas

- **Plantas prioritarias según las subespecies y las cepas de *X. fastidiosa* que causan actualmente brotes o son interceptadas con frecuencia en la UE**

  * **En general, árboles y arbustos o plantas huéspedes perennes: *Olea europaea*, *Nerium oleander*, *Polygala myrtifolia*, *Prunus* sp., *P. dulcis*, *P. avium*, *P. cerasifera*.**

  * ***Coffea* sp.**

- **Plantas indicadoras**

  * **Que expresan clara y tempranamente los síntomas**

  * **Susceptibles para varias subespecies y cepas**

❑ Período apropiado para la inspección

- **Visual**

  * **En campo abierto: período vegetativo (no durmiente). Finales de primavera a principios de otoño**

  * **Ambientes protegidos: todo el año**

- **Toma de muestras**

Período de muestreo de plantas sintomáticas y asintomáticas

Período de crecimiento activo de la planta

- Plantas tropicales de interior: todo el año

- Plantas tropicales de exterior: final de primavera-otoño

Según el huésped

- *Polygala* sp.: final de primavera-principio de otoño

- *Olea europaea* y *Nerium oleander*: los síntomas se expresan más intensamente en verano

- Especies caducifolias (*Prunus* spp.): síntomas en verano

Plantas durmientes: ramas maduras (tacos leñosos) de las cuales se recoge y procesa el xilema

- Vid: final del verano-principio del otoño

Toma de muestras

- Deben ser ramas y esquejes con hojas

- Hojas maduras (evitar brotes jóvenes)

- Plantas pequeñas: enteras

- Hojas duras: enviar hojas y peciolos

Toma de muestras

Plantas sintomáticas

- **Ramas / esquejes con hojas sintomáticas**, o de zona cercana a los síntomas, con 10-25 hojas
- **Evitar tejidos muertos** o partes con síntomas avanzados
- **Tallos y hojas maduros** para especies herbáceas
- **Preferiblemente de una sola planta, o de varias plantas con síntomas similares**

Toma de muestras

Plantas asintomáticas

- La muestra debe ser representativa de toda la parte aérea. Tallos con hojas maduras de diferentes partes del follaje de la planta

- Preferentemente de la mitad o la parte superior de la zona aérea

- De una sola planta: 4-10 ramas

- 100-200 hojas (con peciolo) procedentes de varias plantas

Transporte de las plantas

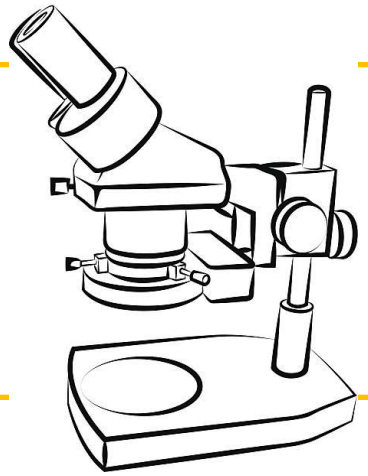
- **Agitar las muestras** para que no viajen con ella posibles vectores

- Poner en un **contenedor cerrado** (bolsa de plástico, etc.)

- **Mantener en frío**, evitando exposición al sol o a elevadas temperaturas

- **Llevar al laboratorio de diagnóstico lo antes posible**, antes de que el tejido vegetal se deteriore

LABORATORIO



Toma de muestras

3.2.2. 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.

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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).

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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.

- **Llevar las muestras al laboratorio lo antes posible**

Laboratorio

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Toma de muestras

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

- **Llevar las muestras al laboratorio lo antes posible**
- **Muestrear peciolos y nervios centrales**

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

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Análisis de las muestras

- Symptomatic plant material
  Serological and molecular tests are both suitable for screening of symptomatic plant material.
- Asymptomatic plant material
  Testing asymptomatic plants in a pest-free area
  There is limited experimental data available on testing asymptomatic plant material. Consequently, the recommendations given in this Protocol are derived from data on testing symptomatic material and test performance studies. In most situations, the concentration of *X. fastidiosa* in asymptomatic plant material is likely to be lower than in symptomatic plant material (Purcell & Saunders, 1999; Almeida & Nunney, 2015). Consequently, it is advised to include molecular test(s) for detection on asymptomatic plant material.
  Testing asymptomatic plants in other areas
  Testing for asymptomatic plants in an outbreak area or a buffer zone around an outbreak often implies that a large number of tests need to be performed. In such a situation, and given that the concentration of the bacterium is expected to be higher than in an area thought to be pest free, a single test including serological tests (e.g. ELISA) may be performed. In serological tests in Italy in the outbreak area and the buffer zone around the outbreak area, 5% of the negative samples are also tested using a molecular test.

- **Material sintomático**

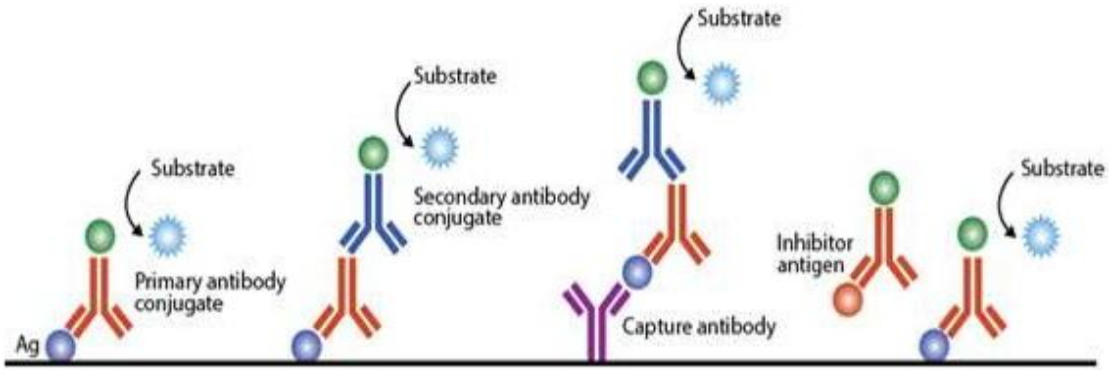
  ***Son adecuadas tanto las pruebas serológicas como las moleculares**

- **Material asintomático**

  *** En zonas libres de la enfermedad: Se deben incluir pruebas moleculares**

  *** En otras zonas: Se puede emplear una sola prueba (incluyendo las serológicas)**

Técnicas serológicas



ELISA

MEIF
(membrane entrapment immunofluorescence)

DBIA
(dot immunobinding assay)

Western blotting

IF

DTBIA
(direct tissue blot immunoassay)

Técnicas moleculares

3.5.2. Molecular tests

Several molecular tests have been developed for *X. fastidiosa*. Only those that are commonly used in the EPPO region are described in full. Molecular tests can be performed on plants and insects. Validation data is available for testing of plants. These tests have been used for detection in insects but validation data is not available.

Although several PCR tests have been developed that effectively detect *X. fastidiosa* DNA in purified DNA extract, a recurrent problem with some matrices is the presence of inhibitors. These effects may be overcome by adequate DNA extraction protocols and dilutions of the extract.

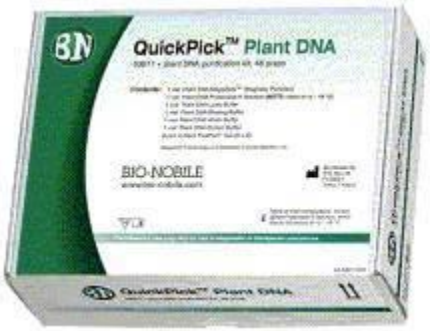
The procedures for extracting DNA from plants and insects are described in Appendix 3.

The tests listed in this section allow the detection of *X. fastidiosa* regardless of the subspecies (tests specific for subspecies are presented in Section 4).

Métodos de extracción de ADN

Appendix 3 – DNA extraction

Extraction of DNA for molecular analyses can be achieved using standard commercial kits (e.g. Bextine & Child, 2007; Huang, 2009), and CTAB buffer (Hendson *et al.*, 2001; Rodrigues *et al.*, 2003; de Souza *et al.*, 2003). The following commercial kits are widely used and validated in several European Union (EU) laboratories to process samples from different plant species: DNeasy Plant Mini Kit-based extraction (Qiagen), Modified DNeasy® mericon™ Food Standard Protocol (Qiagen), QuickPick™ SML Plant DNA Kit-based extraction (Bio-Nobile). Validation data are available in the EPPO Database on Diagnostic Expertise.



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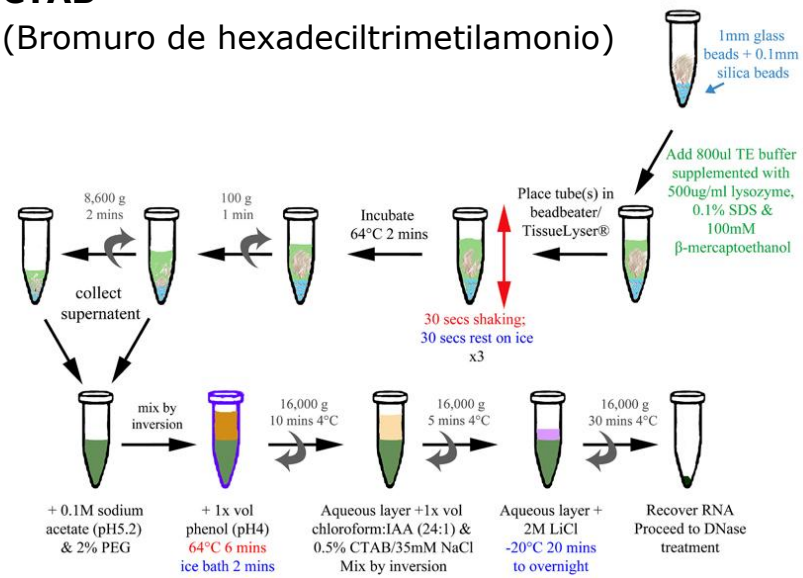
CTAB
(Bromuro de hexadeciltrimetilamonio)



CTAB-based extraction

0.5–1 g of fresh small pieces of midribs, petioles, leaf basal part or twigs (1/4 of the indicated amount, if lyophilized) should be weighed, put into the extraction bags or into suitable tubes with 5 mL of CTAB buffer and homogenized using a homogenizer (e.g. Homex, Polytron, etc.).

1 mL of extract should be transferred into a 1.5-mL micro-centrifuge tube and the sample should be heated at 65°C for 30 min and then centrifuged at 16 000 *g* for 5 min. 1 mL of the supernatant from centrifugation should be transferred to a new 2-mL micro-centrifuge tube, with care being taken not to transfer any of the plant tissue debris. 1 mL of chloroform:isoamyl alcohol (24:1) should be added and the sample should be mixed well by shaking. After centrifugation at 16 000 *g* for 10 min, 700 µL of the supernatant should be transferred to a 1.5-mL micro-centrifuge tube and 490 µL (approximately 0.7 volumes) of cold 2-propanol should be added. After mixing by inverting twice, the tube should be incubated at −20°C for 20 min. Centrifugation of the samples at 16 000 *g* for 20 min will allow recovery of a pellet that should be washed with 1 mL of 70% ethanol. An additional centrifugation at 16 000 *g* for 10 min and decantation in 70% ethanol should be performed. Sample should be air or vacuum-dried. The pellet should be resuspended in 100–150 µL of TE buffer or RNase- and DNase-free water.

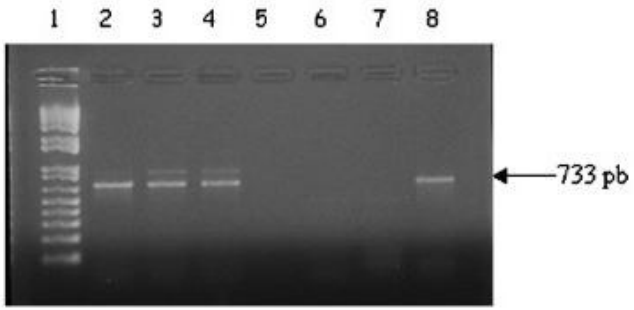
Pruebas moleculares

PCR convencional
(Minsavage *et al.*, 1994)

PCR en tiempo real

LAMP

Diana: extremo 3' del gen *rpoD* (733 pb)



Pruebas moleculares

PCR convencional
(Minsavage *et al.*, 1994)

PCR en tiempo real

LAMP

Francis *et al.*, 2006

-Diana: gen de la proteína hipotética conservada HL
-Iniciador directo HL5:
5'-AAGGCAATAAACGCGCACTA-3'
Iniciador reverso HL6:
5'-GGTTTTGCTGACTGGCAACA-3'

Harper *et al.*, 2010 (and erratum 2013)

-Diana: gen de la proteína rimM
- Iniciador directo XF-F:
5'-CACGGCTGGTAACGGAAGA-3'
Iniciador reverso XF-R:
5'-TCGCATCCCGTGGCTCAGTCC-BHQ-1-3'

Técnicas moleculares

3.5.2. Molecular tests

Several molecular tests have been developed for *X. fastidiosa*. Only those that are commonly used in the EPPO region are described in full. Molecular tests can be performed on plants and insects. Validation data is available for testing of plants. These tests have been used for detection in insects but validation data is not available.

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Inhibidores

↓

Diluir la muestra

Aislamiento

3.7. 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. Media are described in Appendix 8.

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.

Procedures for isolation from plant material are presented in Appendix 9.

As a control, whenever possible a suspension of a *X. fastidiosa* strain (see Section 5) 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.

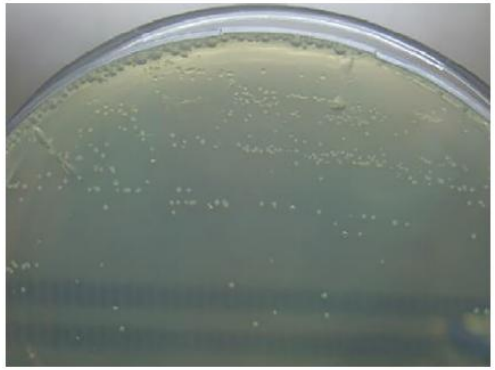


Fig. 23 Colonies of *Xylella fastidiosa* subsp. *fastidiosa* on PD2 (size < 2 mm after 3 weeks).

Fig. 24 Collection strain of *Xylella fastidiosa* subsp. *fastidiosa* ATCC 35879 on BCYE (size < 2 mm after 3 weeks).

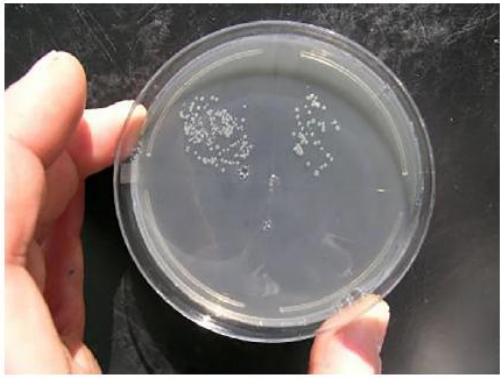


Fig. 26 *Xylella fastidiosa.* subsp. *fastidiosa* isolated from *Coffea canephora* on modified PWG (size < 2 mm after 3 weeks).

Identificación de los aislados y determinación de la subespecie

4. Identification and subspecies determination

For this fastidious pathogen, subspecies determination on plant extracts is performed after positive screening test(s) using PCR-based molecular tests described in Appendices 10, 11, 12 and 13, respectively.

When a pure culture is obtained, the identification of *X. fastidiosa* should be performed using at least two tests, based on different biological principles or targeting two different parts of the genome for molecular tests. Relevant tests are described below.

4.1. Identification of pure cultures as *X. fastidiosa*

4.1.1. Serological tests

Serological tests can be used to identify a pure culture of *X. fastidiosa*; however, as no polyclonal antibodies are available the test cannot be used for the assignment of subspecies.

Instructions for performing ELISA are provided in the EPPO Standard PM 7/101 *ELISA tests for plant pathogenic bacteria* (EPPO, 2010). Recommended antisera and validation data are given in Appendix 1.

Instructions for performing an IF test are provided in EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009). Recommended antisera and validation data are given in Appendix 2.

4.1.2. Molecular tests

The following molecular tests can be used for confirmation of a pure culture.

4.1.2.1. Conventional PCR. The test based on Minsavage *et al.* (1994) is described in Appendix 4.

4.1.2.2. Real-time PCR. Three real-time PCR tests are recommended and have been validated.

Two tests based on Francis *et al.* (2006) are described in Appendix 5.

The test based on Harper *et al.* (2010, erratum 2013) is described in Appendix 6.

Determinación de la subespecie

4.2. Molecular tests for the identification of *X. fastidiosa* and assignment of isolates to *X. fastidiosa* subspecies

Although different tests are available for subspecies assignment, MLST analysis is recommended for new findings. In other cases, subspecies assignment may be performed using subspecies-specific molecular markers (Pooler & Hartung, 1995; Hernandez-Martinez *et al.*, 2006); however, in the case of atypical/new patterns MLST should be performed.

The following tests are described:

- The MLST test based on Yuan *et al.* (2010) is described in Appendix 10 and allows an isolate to be assigned to the subspecies.
- The PCR test based on the primers described by Hernandez-Martinez *et al.* (2006) allows the subsp. *fastidiosa*, *multiplex* and *sandyi* to be assigned. This test can be performed either as a simplex test *in planta* or on isolates (Appendix 11) or a multiplex on isolates (Appendix 12).
- The PCR test based on Pooler & Hartung (1995) is described in Appendix 13. It allows assignment of an isolate to subsp. *pauca*.

MLST

PCR Hernández-Martínez *et al.*, 2010

PCR Pooler & Hartung, 1995

Appendix 10 – Multilocus sequence typing (MLST) (Yuan *et al.*, 2010)

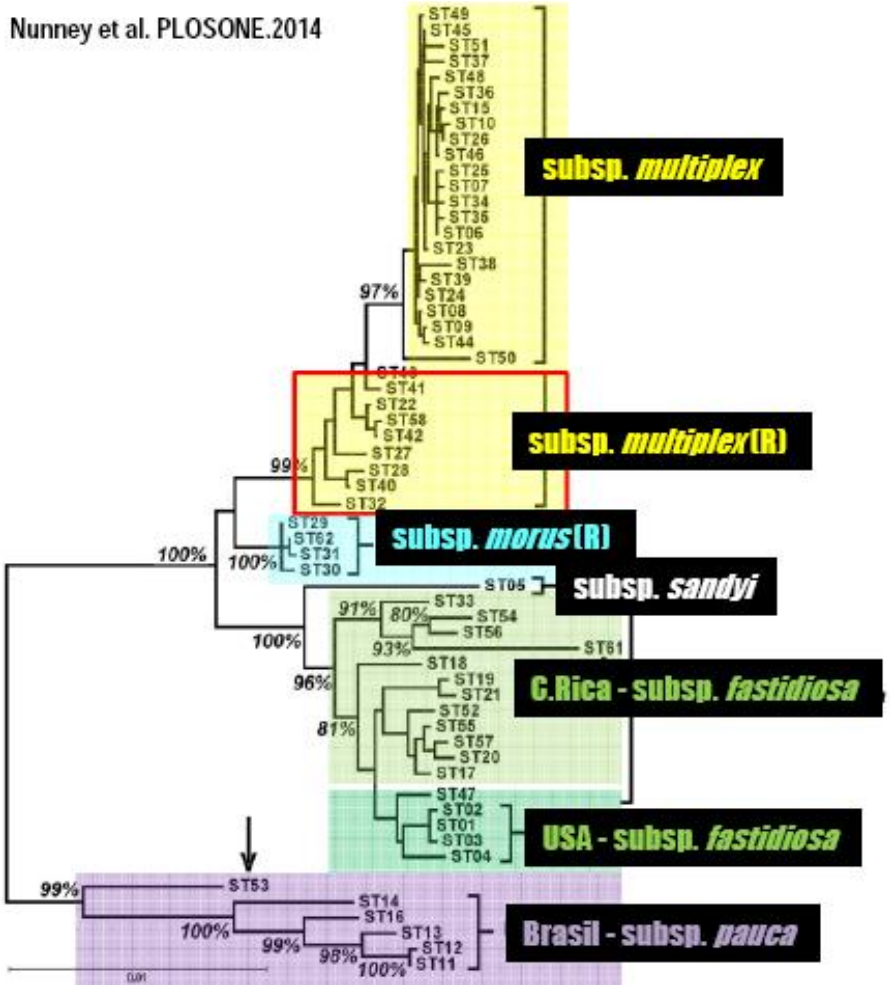
1. General information

1.1 This test is suitable for the assignment of an isolate to one of the known subspecies from DNA from a pure bacterial culture. It has also been used with DNA from plant extracts (Loconsole *et al.*, 2016) although it is recognized that the quantity and quality of target DNA may not always be suitable for obtaining all amplicons.

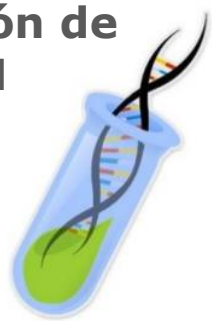
1.2 The test is based on Yuan *et al.* (2010).

1.3 The target sequences are those of seven house-keeping genes amplified individually: 2-isopropylmalate synthase (*leuA*) gene; ubiquinol cytochrome *c* oxidoreductase C1 subunit (*petC*) gene; ABC transporter sugar permease (*malF*) gene; sirohaem synthase (*cysG*) gene; DNA polymerase III holoenzyme chi subunit (*holC*) gene; NADH-ubiquinone oxidoreductase NQO12 subunit (*nuoL*) gene; and glutamate symport protein (*gltT*) gene.

1.4 Amplicon size: 708 bp for *leuA*, 533 bp for *petC*, 730 bp for *malF*, 600 bp for *cysG*, 379 bp for *holC*, 557 bp for *nuoL*, 654 bp for *gltT*.



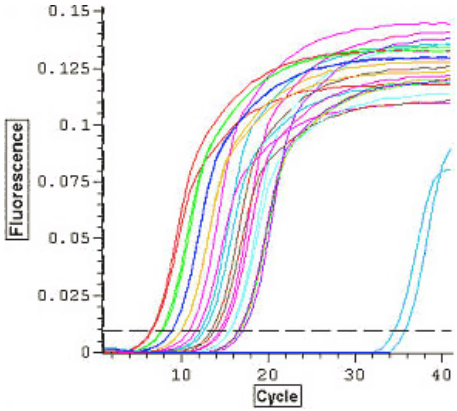
Extracción de ADN



PCR en tiempo real

Harper *et al.*, 2010 (and erratum 2013)

Francis *et al.*, 2006



Aislamiento en medios de cultivo

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Consideraciones

Limitaciones de las técnicas

- Baja cantidad de material vegetal muestreado.
- Distribución desigual del patógeno en la planta.
- Concentración de la bacteria: depende de factores ambientales, de la cepa en cuestión, de la especie vegetal hospedadora.

Limitaciones de la PCR

Table 1. Comparison of the current methods for detecting plant diseases resulting from bacterial pathogens.

Techniques	Advantages	Limitations
PCR	Mature and common technology, portable, easy to operate.	Effectiveness is subjected to DNA extraction, inhibitors, polymerase activity, concentration of PCR buffer and deoxynucleoside triphosphate.
FISH	High sensitivity.	Autofluorescence, photobleaching.
ELISA	Low cost, visual color change can be used for detection.	Low sensitivity for bacteria.
IF	High sensitivity, target distribution can be visualized.	Photobleaching.
FCM	Simultaneous measurement of several parameters, rapid detection.	High cost, overwhelming unnecessary information.

PCR: polymerase chain reaction; FISH: fluorescence in-situ hybridization; ELISA: enzyme-linked immunosorbent assay; IF: immunofluorescence; FCM: flow cytometry; CFU: colony forming unit.

Biosensors **2015**, *4*, 537-561; doi:10.3390/bios5030537

- Método de extracción de ADN.
- Inhibidores.
- Calidad de los reactivos.

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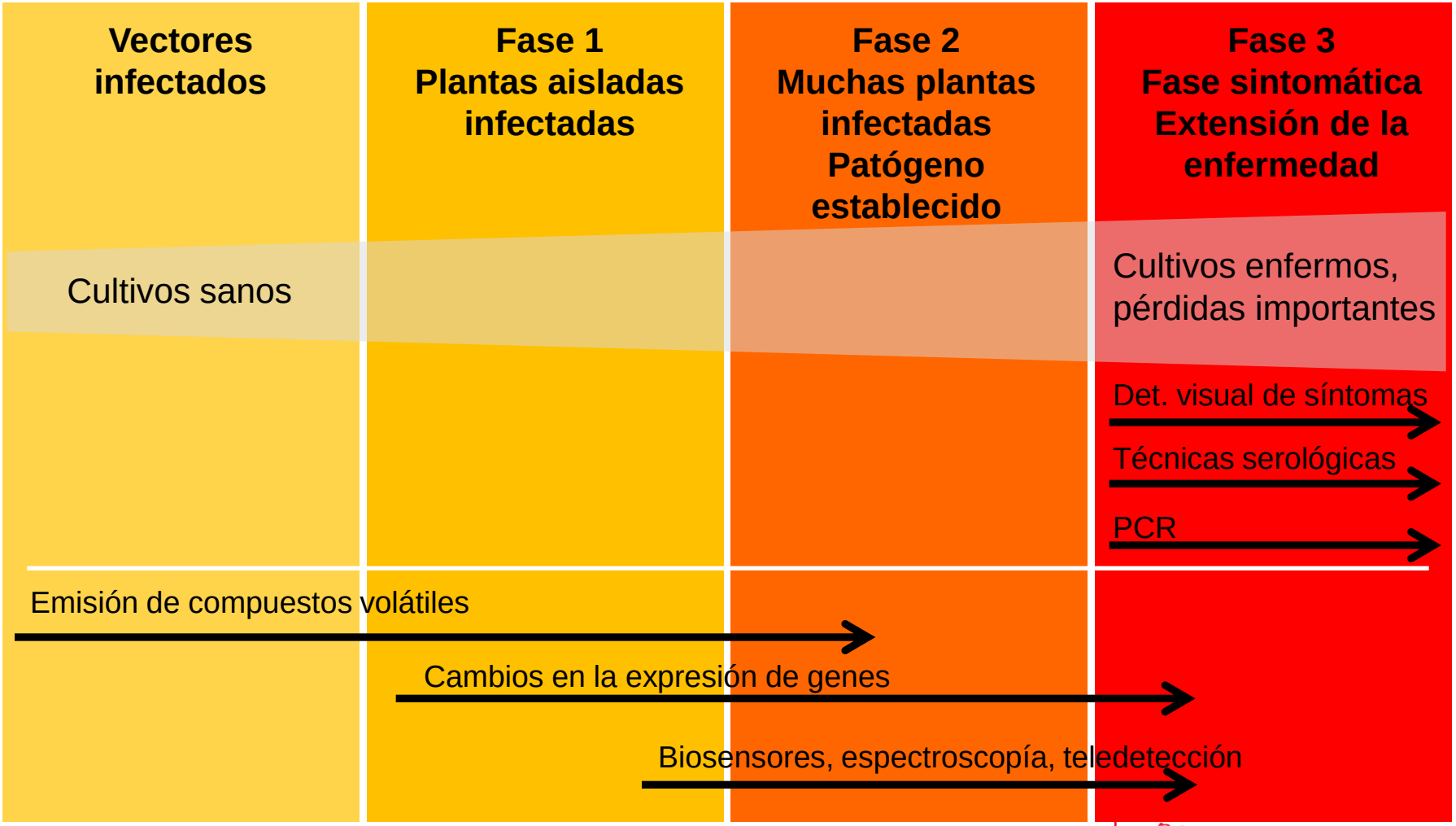
Agron. Sustain. Dev. (2015) 35:1–25
DOI 10.1007/s13593-014-0246-1

REVIEW ARTICLE

Advanced methods of plant disease detection. A review

Federico Martinelli · Riccardo Scalenghe · Salvatore Davino · Stefano Panno · Giuseppe Scuderi · Paolo Ruisi · Paolo Villa · Daniela Stroppiana · Mirco Boschetti · Luiz R. Goulart · Cristina E. Davis · Abhaya M. Dandekar



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Conclusiones

- **El protocolo europeo de diagnóstico de *X. fastidio*sa establece con claridad los pasos a seguir desde la toma de muestras hasta el análisis de resultados en el laboratorio.**

- **En zonas sin enfermedad deben utilizarse al menos dos técnicas, y una al menos debe ser molecular.**

- **En zonas con enfermedad se puede utilizar una sola técnica.**

- **¡¡ Las técnicas son limitadas !!**

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