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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/123 (1)

Diagnostics
Diagnostic

PM 7/123 (1) *Phytophthora lateralis*

Specific scope

This Standard describes a diagnostic protocol for *Phytophthora lateralis*.[1]

Specific approval and amendment

Approved in 2015–09.

1. Introduction

Phytophthora root rot was first reported from *Chamaecyparis lawsoniana* (Lawson Cypress, Cupressaceae), also called Port Orford cedar in its native locality, in Seattle (US) in 1923 (Zobel *et al.*, 1985), but the causal agent, *Phytophthora lateralis*, was only described two decades later (Tucker & Milbrath, 1942). The disease is now widely distributed in North West America, throughout the natural range of *C. lawsoniana* (Hansen *et al.*, 2000).

In Europe, *Phytophthora lateralis* was detected on *C. lawsoniana* in France in 1998 (Hansen *et al.*, 1999) and in the Netherlands in 2004 (Sansford, 2009), in nurseries. More recently, it has been identified on windbreak hedges in Brittany (France), where it can be considered as established (Robin *et al.*, 2011). Since 2010, the pathogen has also been isolated in the UK from forest, parkland and shelterbelt on symptomatic *C. lawsoniana* and from recently imported *C. lawsoniana* and *Thuja occidentalis* in nurseries (Green *et al.*, 2012). *P. lateralis* was also discovered in Taiwan in 2008 in soil underneath asymptomatic *Chamaecyparis obtusa* var. *formosana* (Brasier *et al.*, 2010) and identified in 2011 in South East Ireland on *C. lawsoniana* in a public area (EPPO, 2011). Recently, four phenotypically and phylogenetically distinct lineages of *P. lateralis* have been identified based on a wide geographic sampling (Brasier *et al.*, 2012).

The main host of *P. lateralis* is *Chamaecyparis lawsoniana*; other *Chamaecyparis* species (*C. formosensis, C. obtusa, C. pisifera*) may also be infected but the impact of the disease is limited. Within Cupressaceae *Thuja occidentalis* (Schlenzig *et al.*, 2011) and *T. plicata* have also been reported as hosts (Schlenzig, pers. comm. 2014). However, this oomycete has also been found on *Taxus brevifolia*, Taxaceae (DeNitto & Kliejunas, 1991), which might suggest a wider host range.

Root, collar and stem lesions are the main symptoms observed, seedlings are killed within weeks of infection and for larger trees, death may occur within a year after first appearance of crown symptoms (Winton & Hansen, 2001). Direct infections of the foliage have also been reported (Robin *et al.*, 2011).

A flow diagram describing the diagnostic procedure for *P. lateralis* is presented in Fig. 1.

2. Identity

Name: *Phytophthora lateralis* Tucker & Milbrath
Synonym: None
Taxonomic position: Chromista: Oomycetes, Peronosporales, Pythiaceae
EPPO code: PHYTLA
Phytosanitary categorization: EPPO A2 list no. 337

3. Detection

3.1. Symptoms

Symptoms caused by *P. lateralis* are not specific (they may be very similar to those caused by other species of *Phytophthora*) and can be diverse as described below. Damage to a *C. lawsoniana* hedge is shown in Fig. 2.

On *Chamaecyparis lawsoniana*:

– Root, collar and stem lesions are the most frequent symptoms. Fine roots are first invaded by zoospores, the hyphae of the oomycete colonize the roots and collar

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

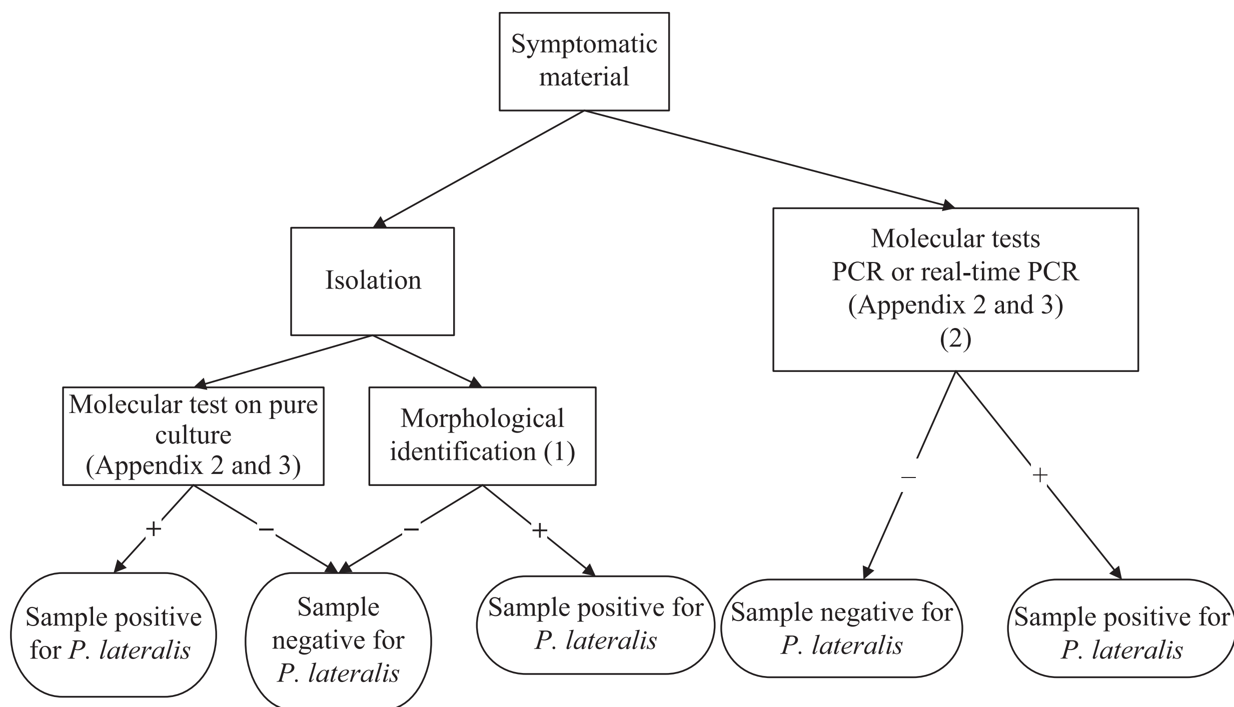


Fig. 1 Flow diagram for diagnosis of *Phytophthora lateralis* (symptomatic material).

Fig. 2 Damage due to *Phytophthora lateralis* on a *Chamaecyparis lawsoniana* hedge.

through the inner bark (Winton & Hansen, 2001). The infected roots appear water soaked and are often a deep cinnamon brown colour (Roth *et al.*, 1972). Spread to the root collar and girdling of the trunk leads to a yellowing and a browning of the foliage that finally becomes crisp and dry. The first above-ground symptoms may be a slight wilt during warm days, followed by the change in colour of the foliage.

A sharp line of demarcation between the healthy and the infected tissues can be observed by removal of the bark (Fig. 3).

– Foliar symptoms not linked to root and collar infections, due to aerial dispersal of the pathogen, can be observed: discoloration of the foliage, death of branches sometimes with small cankered areas and brown cortical lesions (Robin *et al.*, 2011). Aerial dieback of branches was also reported by Green *et al.* (2012), sometimes accompanied by resin bleeding.

On *Taxus brevifolia*:

Symptoms of root rot are similar to those observed on *C. lawsoniana* with a rapid wilting of the crown and brown discoloration of inner bark and cambium at the root collar (DeNitto & Kliejunas, 1991).

On *Thuja occidentalis* and *T. plicata*:

Discoloration of the foliage (grey olive) and dieback of branches (Schlenzig *et al.*, 2011) can be observed.

3.2. Sampling procedures

For root, collar, stem, branches and foliage lesions, a piece of wood or foliage including the margin between healthy and diseased tissue is cut out and placed in a sealed bag. For fragile samples (e.g. foliage), a small piece of damp tissue can be placed in the container to prevent desiccation.

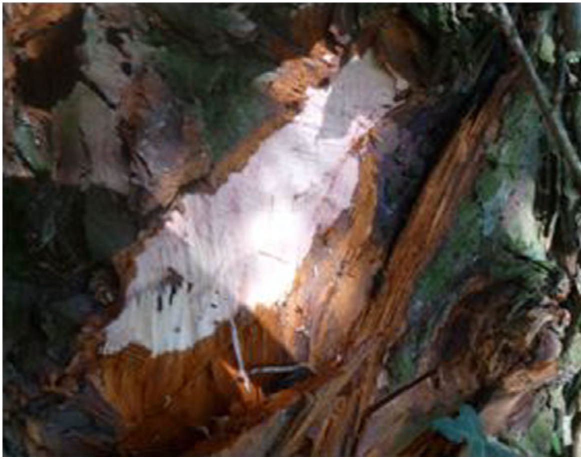


Fig. 3 Symptoms of *Phytophthora lateralis* in *Chamaecyparis Lawsoniana* wood and bark.

Fig. 4 Infection by *Phytophthora lateralis* on *Chamaecyparis lawsoniana* leaves after a 5-day baiting.

Soil for testing should also be placed in a sealed container (e.g. bag, plastic box).

If possible, all samples should be sent to the laboratory to arrive by the next day and be processed as soon as possible. Storage at 4–10°C for up to 7 days is possible but this may reduce the likelihood of isolation of the pathogen. The storage period may be longer when molecular tests are to be used.

There is no sampling procedure described for asymptomatic plants.

3.3. Baiting

The baiting procedure described here is as followed in ANSES (FR).

Bait test is used for soil, plant debris and may also be used for small roots. This material is placed in a large plastic box and osmosis water is added, *ca.* 5–10 vol/1 vol of sample. Foliage pieces of *Chamaecyparis lawsoniana* (3–4 cm long, cut from the distal 10–25 cm non-woody branches) are floated on the surface (Ostrofsky *et al.*, 1977). To avoid direct contact between soil/plant debris and the baits, a net or cheesecloth can be used to stop the debris floating (Linderman & Zeitoun,1977).

The presence of symptoms on the baits (necrosis, discoloration) is checked for every day (Fig. 4) up to 2 weeks if necessary and isolation, PCR or real-time PCR can be carried out as described below for plant material.

According to Hamm & Hansen (1984), recovery of *P. lateralis* from soil by baiting is improved by adding 25 μg mL^{-1} hymexazol to the water (to limit the growth of *Pythium* spp.).

3.4. Disinfection and isolation

As for other *Phytophthora* species, there are a number of alternative methods for surface disinfection and decontamination of plant material (alcohol or sodium hypochlorite treatments, water rinses etc.). Description of these methods can be found in the EPPO diagnostic protocols on *Phytophthora ramorum* (PM 7/66 (1)) and *Phytophthora kernoviae* (PM 7/112 (1)). The author of this protocol on *P. lateralis* performs disinfection with alcohol.

Phytophthora lateralis can then be isolated by plating small pieces of surface disinfected symptomatic plant material (2–5 mm, if possible from the leading edge) on a suitable medium. Details of media are given in Appendix 1.

Semi-selective media can be used for isolation. The pathogen is slow growing (about 2 mm a day on V8 agar) and sensitive to hymexazol (a fungicide used to inhibit most of the *Pythium* species growth) Erwin & Ribeiro, 1996; Winton & Hansen, 2001; therefore the *Phytophthora* semi-selective media should be prepared without this compound (e.g. P_5ARP instead of P_5ARP [H]). Isolation of *P. lateralis* on a medium with hymexazol is possible, but growth is slowed down (Hamm & Hansen, 1984; Schlenzig & Schenck, pers. comm.).

The optimal temperature for *P. lateralis* growth is 20°C (min. <2°C, max. approx. 25°C). The standard conditions for incubation are 18–20°C in the dark.

3.5. Molecular methods

Several species-specific molecular methods have been developed to detect the pathogen directly *in planta*:

- Conventional PCR targeting the RAS-relatedYpt1-gene (Schena *et al.*, 2008) Appendix 2
- Real-time PCR targeting the RAS-relatedYpt1 gene (N. Schenck *et al.*, submitted) Appendix 3

The conventional PCR method developed by Winton & Hansen (2001) is used as a routine test in the United States despite the fact that it shows a cross reaction with *Phytophthora ramorum*.

4. Identification

Growth media such as V8 agar, Carrot Agar (CA), Carrot Piece Agar (CPA), Corn Meal Agar (CMA) are suitable for storage and study of morphological features (Figs 5–6).

For a positive result, *P. lateralis* should have been identified at species level by cultural features and morphology (for a soil or water sample, all the morphological features listed below in the paragraph 'distinguishing characteristics' below should be observed), or by molecular methods (Fig. 1).

In case of uncertainty, it is recommended to carry out confirmation by a complementary method (Fig. 1). Identification from new hosts or isolates that do not morphologically match published descriptions can be confirmed by sequencing.

4.1. Growth characteristics in culture and morphology

The most essential features are given in Table 1.

To obtain chlamydospores, oogonia and sporangia in pure cultures:

Fig. 5 Colonies of *Phytophthora lateralis* on corn meal agar after 10 days at 22°C in the dark.

Chlamydospores (Fig. 7):
They are abundantly produced in many agar media at temperatures between 15 and 25°C (Erwin & Ribeiro, 1996); production may be suppressed by light (Englander & Roth, 1980).
Oogonia and oospores:
Vegetable (V8, carrot, etc.) agar media are commonly used to obtain *Phytophthora* oogonia, but *P. lateralis* gametangia seem to be rarely observed (Werres & Wagner, 2010).
To obtain oospores, Erwin & Ribeiro (1996) recommend growing *P. lateralis* 'on agar of Port-Orford cedar foliage [filtrate from 200 g of foliage steamed at 95°C for 1 h plus 15 g of glucose per litre of water (Trione, 1974)]'.
Sporangia:
They may be obtained by flooding 1-cm diameter plugs from the edges of actively growing colonies (e.g. grown on carrot or V8 agar) with unsterile pond water (24–36 h incubation at 20°C; Green *et al.*, 2012), soil extract (Ribeiro, 1978) or Petri solution (Erwin & Ribeiro, 1996). Duration (or amount) of light appears to be an important factor: sporangia could be obtained on V8 agar and from V8 agar plugs in tap water under natural light in summer months (A. Schlenzig, pers. comm.); so duration of light should be over 12 h/24 h.

According to Erwin & Ribeiro (1996), sporangia formation is increased by growing *P. lateralis* mycelium on a weak agar medium such as V8 juice (10 mL L^{-1}) and incubating the culture under light (continuous or for 12-h periods, mixing Blacklight Blue and Cool White).

Another method, tested by A. Belisario (pers. comm.) to induce sporangia production, is the one described by Ilieva *et al.* (1995): autoclaved organic pepper seed are incubated for 24 h at room temperature on the surface of approximately 7 old agar cultures. Seeds are then transferred on

Fig. 6 Colonies of *Phytophthora lateralis* on V8 agar after 10 days at 22°C in the dark.

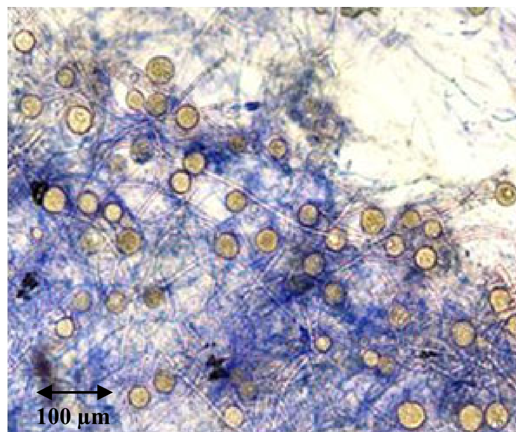


Fig. 7 Chlamydospores of *Phytophthora lateralis* after a 20-day subculture on Corn Meal Agar (in cotton-blue stain).

Table 1. Growth and morphological characteristics of *Phytophthora lateralis*

Character	According to Erwin & Ribeiro (1996)	According to Gallegly & Hong (2008)	Isolate BBA 368 on Carrot Piece Agar (n = 50; Werres & Wagner, 2010)	Information available in the Q-bank fungi database
Vegetative growth				On CMA
Temperature (°C)				
Minimum	3	–	2	3
Optimum	20	–	20	15–24
Maximum	<26	25	25	27
Growth rate at optimum temperature (mm/24 h)	–	–	1.7	1.4–1.7
Sporangia	Nonpapillate Noncaducous* Ovate, obovate, obpyriform, often elongate or distorted in shape	Nonpapillate Noncaducous* Mostly ellipsoid	Nonpapillate Noncaducous* Mostly ovate-ellipsoid, often elongate in shape	Nonpapillate Noncaducous* Ovoid, obpyriform, obovoid (variable), tapered base
Length × width (range)	20–60 × 12–20 μm	50 × 25 μm	44–68 × 24–36 μm	
Length × width (average)	26 × 15 μm	–	54.6–28.4 μm	
Length/width (range)	1.6–1.91	–	1.5–2.2	
Length/width (average)	1.71	–	1.9	
Sporangiophores	Simple sympodial	Simple sympodial	Simple sympodial	
Chlamydospores	Lateral, terminal or intercalary, often sessile, cinnamon brown when produced in foliage agar	Lateral, terminal or intercalary, cinnamon brown when mature	Lateral, terminal or intercalary, cinnamon brown when mature	Lateral, terminal or intercalary
Diameter (range)	20–77 μm	30–50 μm	32–60 μm	
Diameter (average)	40 μm	–	47.8 μm	
Gametangia (rarely observed on agar media)	Homothallic	Homothallic	Homothallic	
Oogonia	Smooth, spherical and terminal	Spherical	–	
Diameter (range)	33–50 μm	31–55 μm	–	
Diameter (average)	–	–	–	
Antheridia	Paragynous	Paragynous	–	
Length × width (range)	12–18 × 13–16 μm	–	–	
Length × width (average)	–	–	–	
Oospores	Plerotic, pigmented (colour depends on the medium)	Plerotic, pigmented (colour depends on the medium)	–	
Diameter (range)	28–46 μm	28–50 μm	–	
Diameter (average)	40 μm	–	–	
Wall thickness	6 μm	5–6 μm	–	

Adapted from Werres & Wagner (2010).
Information available in the Q-bank fungi database is also provided.
–, no data available.
*Deciduous sporangia with short pedicels were observed in culture by Robin *et al.* (2011). This was confirmed by Brasier *et al.* (2012) who observed short preformed pedicels on the sporangia of the four lineages. Q-bank includes the information that sporangia of some isolates appear to be caducous with short pedicels, a rare characteristic for non-papillate species.

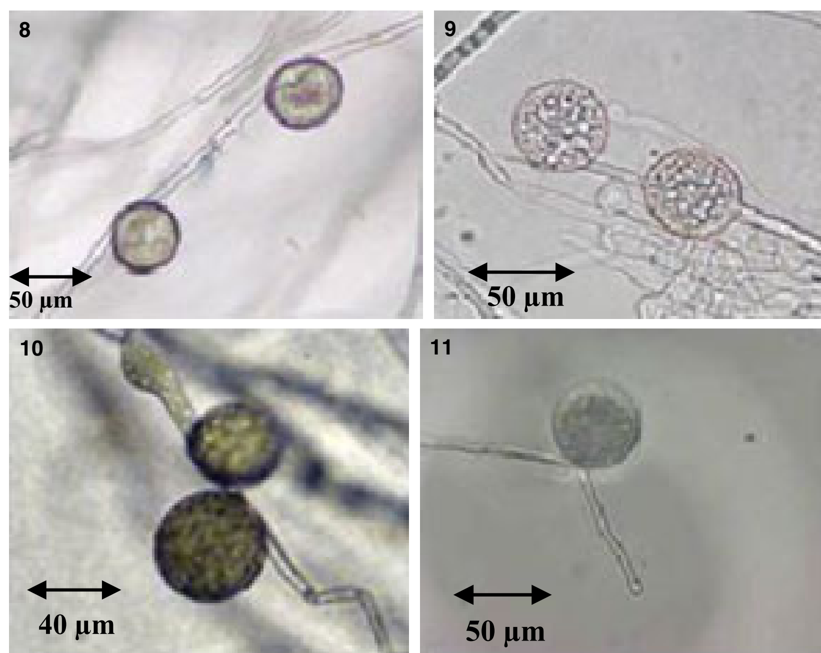
water-agar and flooded with pond/well water or with autoclaved soil extract. Sporangia can be seen after 48 h up to 10 days.

4.1.1. Distinguishing characteristics

Laterally-attached chlamydospores (Figs 8–11) are very characteristic of *P. lateralis* and can be considered as the main criterion for morphological identification. They are often sessile on the mycelium or may be supported by two hyphae. Sporangia are non-papillate, persistent on the stalk; although some may be caducous with a short pedicel (Figs 12–13; Robin *et al.*, 2011). *P. lateralis* is homothallic but oogonia seem to be difficult to obtain on agar media (Werres & Wagner, 2010); the antheridia are paragynous.

Hyphal swellings may also be observed on some isolates (Fig. 14; Gallegly & Hong, 2008).

Some more information on the phenotypic characteristics of the four lineages of *P. lateralis* can be found in the paper of Brasier *et al.* (2012).



Figs 8–11 Laterally-attached or intercalary chlamydospores of Phytophthora lateralis (in water) (Photo: A. Schlenzig).

Fig. 12 Short-stalked sporangium with zoospores (Photo: A. Schlenzig).

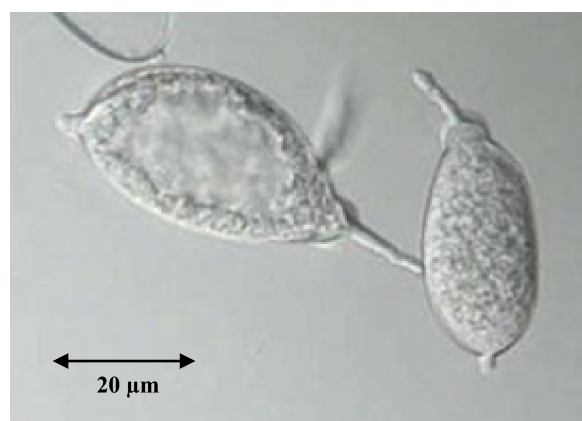


Fig. 13 Short-stalked germinating sporangia (Photo: A. Schlenzig).

4.2. Molecular methods

Several species-specific molecular methods have been developed to confirm the identity of *P. lateralis*:

- Conventional PCR targeting the RAS-relatedYpt1-gene (Schena *et al.*, 2008) Appendix 2.
- Real-time PCR targeting the RAS-relatedYpt1 gene (Schenck *et al.*, submitted) Appendix 3.

As already stated above, the conventional PCR method developed by Winton & Hansen (2001) is used as a routine test in the United States despite the fact that it shows a cross reaction with *Phytophthora ramorum*: the two species can easily be distinguished in culture (see PM 7/66 for morphological features of *P. ramorum*).

Further confirmation can be done by DNA Barcoding. An EPPO Standard PM 7/XX on DNA barcoding as an identification tool for plant pests is in preparation. In Q-bank, multilocus analysis is recommended for this pest based on a combination of Internal Transcribed Spacers (ITS) and Cytochrome oxydase I (http://www.q-bank.eu/Fungi/).

5. Reference material

LSVM 486 (Genbank Accession Number KM975318).

Sources of reference material can be identified via the Fungi database of Q-bank http://www.q-bank.eu/Fungi/.

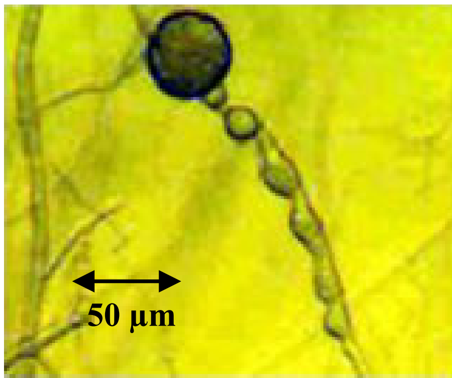


Fig. 14 Chlamydospore and hyphal swellings.

6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM7/77 (1) *Documentation and reporting on a diagnosis.*

7. Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int) and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical sensitivity, full validation reports etc.

8. Further information

Further information on this organism can be obtained from:

N. Schenck, French National Agency for Food Environmental and Occupational Health & Safety (Anses), Plant Health Laboratory, Mycology Unit, Domaine de Pixérécourt – Bât. E, BP 90059, F54220 Malzéville, France.

9. Feedback on this diagnostic protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.fr.

10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press this will also be marked on the website.

11. Acknowledgements

The protocol was initially drafted by N. Schenck, French National Agency for Food Environmental and Occupational Health & Safety (Anses), Plant Health Laboratory, France.

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Appendix 1 – Media for isolation, study and subculturing of *Phytophthora lateralis*

All media are sterilized by autoclaving at 121°C for 15 min, except when stated otherwise.

1. Semi-selective media

For these media, antibiotic and fungicides should be all dissolved apart in 10 mL sterile distilled water (for 1 L medium) and added aseptically to the other autoclaved compounds after cooling them to 50°C in a water bath.

P_5ARP (Jeffers & Martin, 1986)

Cornmeal agar	17.0 g
Pimaricin	5.0 mg
Ampicillin (Na salt)	250.0 mg
Rifampicin (dissolved in 1 mL 95% Ethanol)	10.0 mg
PCNB (pentachloronitrobenzene)	100.0 mg
Distilled water to	1 L

Store the prepared medium at 2–8°C in the dark up to 7 days.

PARB (Robin *et al.*, 1998)

Agar	20.0 g
Malt	15 g
Pimaricin	10.0 mg
Ampicillin (Na salt)	250.0 mg
Rifampicin (dissolved in 1 mL 95% Ethanol)	10.0 mg
Benlate (50% benomyl)	15.0 mg
Distilled water to	1 L

Store the prepared medium at 2–8°C in the dark up to 7 days.

Hansen *et al.* (2008)

Cornmeal agar	17.0 g
Delvocid (50% natamycin salt)	20.0 mg
Ampicillin (Na salt)	200.0 mg
Rifampicin (dissolved in 1 mL 95% Ethanol)	10.0 mg
Benlate (50% benomyl)	30.0 mg
Distilled water to	1 L

Store the prepared medium at 2–8°C in the dark up to 7 days.

Semi-selective V8 medium (Jung *et al.*, 1996, slightly adapted)

Agar	20.0 g
$CaCO_3$	3.0 g
V8 juice	100.0 mL
Pimaricin	20.0 mg
Ampicillin (Na salt)	200.0 mg
Rifampicin (dissolved in 1 mL 95% Ethanol)	10.0 mg
PCNB (pentachloronitrobenzene)	25.0 mg
Nystatin	50 mg
Distilled water to	1 L

Store the prepared medium at 2–8°C in the dark up to 7 days.

2. Non-selective media

Corn Meal Agar (CMA)

Cornmeal agar	17.0 g
Distilled water to	1 L

V8 agar (ANSES recipe)

Clarified vegetable juice (V8 if possible)	200.0 mL
Agar agar	15.0 g
Distilled water to	1 L

Clarified vegetable juice: add 4.0 g $CaCO_3$ to 350 mL vegetable juice, shake 15 min, centrifuge (5000 rpm) 15 min, use the supernatant.

V8 agar (FERA recipe)

V8 juice	100 mL
Agar Oxoid tec No.3	20.0 g
0.1 M KOH (0.14 g in 25 mL H_2O)	25.0 mL
$CaCO_3$	1.0 g
Distilled water to	1 L

Carrot Piece Agar (CPA) (Werres *et al.*, 2001)

Agar	22.0 g
Carrot pieces (grown without fungicide treatment)	50.0 g
Distilled water to	1 L

Carrot Agar (CA) (Erwin & Ribeiro, 1996)

Agar	15.0 g
Carrots (grown without fungicide treatment)	200.0 g
Distilled water to	1 L

Washed and sliced fresh carrots are mixed in 500 mL distilled water and filtered, the filtrate and the agar are brought to 1 L with distilled water and autoclaved at 121°C for 15 min.

Appendix 2 – Identification at species level by conventional PCR (Schena *et al.*, 2008) following DNA extraction adapted from Schena *et al.* (2006)

1. General Information

1.1 Scope of the test: detection and identification of *Phytophthora lateralis* in plant samples or pure culture using primers and protocol as described by Schena *et al.* (2006, 2008).
1.2 This protocol was established by the authors in 2008.
1.3 The PCR primers are designed to target the intron regions 3–4 of the *Ypt*1 nuclear gene in *P. lateralis*.
1.4 The amplicon size is 133 base pairs.
1.5 Oligonucleotides for *P. lateralis* detection:
Forward primer Ylat3F: 5′-ACTGCTGATGACGG-GATCG-3′
Reverse primer Ylat2R: 5′-AAAAATCTCCCGCAGA-CATAC-3′
1.6 Cycler: no details are given by Schena *et al.* about the device used.

2. Methods

2.1. Nucleic acid extraction and purification

• Nucleic acid extraction from pure culture

Standard extraction methods for fungal DNA (i.e. commercial plant DNA extraction kits) can be used for this purpose.

• Nucleic acid extraction from plant material

The following method has been used by Schena *et al.* (2006) but other methods are possible: approximately 10 mg of freeze-dried leaf tissues were transferred to 2-mL screw-cap tubes containing an equal volume of polyvinylpyrrolidone (PVP) together with 5-mm stainless steel ball bearings, 0.2 g each of 0.1-mm-diameter zirconia/silica beads, 1.0-mm-diameter glass beads and 1.5 mL of extraction buffer [200 mM Tri-HCl (pH 7.7), 250 mM NaCl, 25 mM EDTA, 0.5% SDS]. The extraction mixture was blended in a Mini-BeadBeater (Bio-Spec Products, Bartlesville, OK, USA) at 5000 rpm for 60 s and centrifuged at 13 000 ***g*** for 5 min. After centrifugation the upper phase (approximately 800 µL) was extracted twice with 1 mL of phenol/chloroform (1:1) and 700 µL of chloroform, respectively. DNA was precipitated with an equal volume of isopropanol for 1 h at 5°C, washed with 70% cold ethanol (−20°C), dried and resuspended in 100 µL of sterile distilled water (Sambrook *et al.*, 1989). Before amplification, DNA extracted from leaves was purified using polyvinylpolypyrrolidone (PVPP) spin columns as described by Schena & Ippolito (2003). All DNA samples were kept at −20°C for long-term storage and at 5°C for routine amplifications.

Experience with this extraction method in laboratories shows that this is also suitable for other matrices (e.g. soil, woody tissues).

2.2. Polymerase chain reaction – PCR (Schena *et al.*, 2008)

2.2.1. Master Mix.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water* (to make up to a 15 µL reaction volume)			na
Tris-HCl (pH 9)	np	np	10 mM
KCl	np	np	50 mM
Triton X-100	np	np	0.1%
$MgCl_2$	np	np	1 mM

(continued)

Table (continued)

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
dNTPs	np	np	100 μM
BSA			50 μg/15 μL
Forward primer (Ylat 3F)	np	np	6 μM
Reverse primer (Ylat 2R)	np	np	6 μM
Polymerase (*Promega Corporation*)	np	np	1 U/15 μL
DNA template or			1.5 μL/15 μL
Dilution of the amplicons derived from a first PCR if appropriate			1 μL/15 μL

np, not given in the publication; na, not appplicable.
*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 μm filtered) and nuclease-free.

2.2.2. PCR cycling parameters. 1 Cycle of 95°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s; and a final cycle of 72°C for 10 min.

Note: Schena *et al.* (2006) recommend enhancing the sensitivity of the method by performing a first PCR run with *Phytophthora*-genus-specific primers (nested PCR):

YPh1F: CGACCATKGGTGTGGACTTT

YPh2R: ACGTTCTCMCAGGCGTATCT

The reaction mix is similar to the *P. lateralis*-specific test (as described by Schena *et al.* (2008), just replacing the primers).

Amplification conditions: 1 Cycle of 95°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 45 s and 72°C for 30 s; and a final cycle of 72°C for 10 min.

About 1 μL of the amplified product is then used as a template for the *P. lateralis* specific primers.

Warning from the authors: '…the use of nested PCR implies a greater risk of false positives arising from cross contaminations, as well as increasing the time and labour requirements of the procedure'.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer;
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism);
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix;
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCR tests not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism or DNA fragment (size different from the target size) amplified by the primers, introduced into the Master Mix.

3.2. Interpretation of results

In order to assign results from the PCR-based test the following criteria should be checked:

Verification of the controls

- NIC and NAC should produce no amplicons;
- PIC and PAC should produce an amplicon of 133 bp;
- If present, IC should produce an amplicon of the relevant size (in the case where a DNA fragment of a different size is introduced into the Master Mix, the presence of the amplicon is required only for negative *P. lateralis* results).

When these conditions are met

- A test will be considered positive if 133 bp PCR amplicons are produced;
- A test will be considered negative if no bands or a band of a different size are produced;
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical sensitivity data from Schena *et al.* (2008): testing a dilution (in water) series of total DNA extracted from *P. lateralis* in pure culture indicated a limit of detection of approximately 100 pg μL^{-1} (final concentration) for the single-round PCR and 100 fg μL^{-1} for the nested PCR.

4.2 Analytical specificity data from Schena *et al.* (2008): no cross reactivity was observed when testing DNA extracted from cultures of the following species (no data provided about DNA concentration):

Pythium spp.: *P. pyrilobum, P. catenulatum, P. torulosum, P. intermedium, P. dissotocum, P. aphanidermatum, P. ultimum, P. undulatum, P. splendens.*

Phytophthora spp: *P. alni* subsp. *alni, P. alni* subsp. *multiformis*, *P. alni* subsp. *uniformis*, *P. boehmeriae*, *P. cactorum, P. cambivora, P. capsici, P. cinnamomi, P. citricola, P. citrophthora, P. cryptogea, P. drechsleri, P. erythroseptica, P. europaea, P. fragariae* var. *fragariae, P. fragariae* var. *rubi, P. ideae, P. ilicis, P. infestans, P. insolita, P. inundata, P. katsurae, P. kernoviae, P. medicaginis, P. megasperma, P. nemorosa, P. nicotianae, P. palmivora, P. pistaciae, P. pseudosyringae, P. psychrophila, P. quercina, P. ramorum, P. sojae.*

Appendix 3 – Identification at species level by real-time PCR

1. General information

1.1 This protocol was developed by N. Schenck *et al.* (submitted).
1.2 Nucleic acid source is plant tissues or pure cultures.
1.3 Name of targeted gene is the Ras-related protein YPT region.
1.4 Position of the *P. lateralis* primers and probe considering as reference sequence GenBank accession JN182997.1: Forward primer: 51–70/Reverse primer: 201–220/Hydrolysis probe: 172–190.
1.5 Amplicon size: 170 bp.
1.6 Oligonucleotides

Primers/probe sequences for *Phytophthora lateralis* (5′-3′):

qPlat-F: ACGGGATCGTGTTCTAGCAG
qPlat-R: TAGCTGCACGTCGTTGCTAC
qPlat-P: FAM-TTTTCCCGCTTTCCTTGGGG-BHQ1.

Primers/probe sequences for the plant internal control (18S rDNA gene) (5′-3′)

18S-UniF: GCA AGG CTG AAA CTT AAA GGA A
18S-UniR: CCA CCA CCC ATA GAA TCA AGA-3′
18S-UniP: JOE-ACG GAA GGG CAC CAC CAG GAG T-BHQ1.

1.7 Real-time PCR system: Rotor-Gene 6500 (Corbett Research, Mortlake, Australia).
1.8 Software and settings for data analysis.

The run was analysed with an autogain optimization which was performed before the first fluorescence acquisition. The C_t value for each reaction was determined using the Rotor-Gene software, version 1.7.75, setting the threshold line at 0.02.

2. Methods

2.1. Nucleic acid extraction and purification

• Nucleic acid extraction from plant material

The recommended DNA extraction method is described in N. Schenck *et al.* (submitted). But other methods are possible: small pieces of plant tissue are excised from the leading edge of suspect lesions, cut into small pieces (maximum 2 mm) and homogenised. A volume of approximately 250–500 μL of this plant material is transferred in a sterile 2-mL microcentrifuge tube. Different grinding methods can be used, the most efficient tested by Schenck *et al.* being FastPrep (MP Biochemicals) with ceramic spheres. Total DNA extraction is carried out using commercial DNA extraction kits according to the manufacturer's instructions. The use of DNeasy plant mini kit (Qiagen, Hilden, Germany) is recommended in N. Schenck *et al.* (submitted). The DNA extract (200 μL) is tested after a 10-fold dilution in 1× Tris-EDTA buffer (Sigma-Aldrich, Lyon, France) or in molecular grade water (immediate or short-term use only). For DNA isolation from cultured isolates, the same procedure can be followed.

The DNA samples are kept at −20°C for long-term storage, and at 4°C for immediate or short-term use.

• Nucleic acid extraction from pure culture

Standard extraction methods for fungal DNA (i.e. commercial plant DNA extraction kits) can be used for this purpose.

2.2. PCR reaction

2.2.1. Master Mix.

P. lateralis detection:

Reagent	Working concentration*	Volume per reaction (μL)	Final concentration
Molecular grade water	N.A.	12.5	N.A.
Core kit No ROX buffer (Eurogentec)	10×	2.0	1×
$MgCl_2$	50 mM	2.0	5 mM
Primer qPlat-F	30 μM	0.2	0.3 μM
Primer qPlat-R	30 μM	0.2	0.3 μM
Hydrolysis probe qPlat-P	10 μM	0.2	0.1 μM
dNTPs	5 mM each	0.8	0.2 mM each
DNA polymerase (Hot GoldStar Eurogentec)	5 U μL^{-1}	0.1	0.025 U μL^{-1}
Subtotal		18	
DNA	0.5–15 ng μL^{-1}	2	0.05–1.5 ng μL^{-1}
Total		20	

18S-Uni detection:

Reagent	Working concentration*	Volume per reaction (μL)	Final concentration
Molecular grade water	N.A.	12.5	N.A.
Core Kit No ROX buffer (Eurogentec)	10×	2.0	1×
$MgCl_2$	50 mM	2.0	5 mM
Primer 18S-Uni-F	30 μM	0.2	0.3 μM
Primer 18S-Uni-R	30 μM	0.2	0.3 μM
Hydrolysis probe 18S-Uni-P	10 μM	0.2	0.1 μM
dNTPs	5 mM each	0.8	0.2 mM each
DNA polymerase (Hot GoldStar Eurogentec)	5 U μL^{-1}	0.1	0.025 U μL^{-1}
Subtotal		18	
DNA	0.5–15 ng μL^{-1}	2	0.05–1.5 ng μL^{-1}
Total		20	

*These figures are indicative. They can be modified provided that the final concentration in the PCR reaction is respected.

2.2.2. PCR conditions. The qPlat and the 18S-Uni tests are run separately as monoplex reactions. The real time reaction conditions include initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation and annealing/elongation, 10 s at 95°C and 45 s at 60°C respectively. The Ct value for each reaction is determined using the software provided with the thermocycler.

3. Essential procedural information

DNA extracts that do not produces a positive result with the 18S-Uni test should be diluted and tested again by PCR. If the dilution does not overcome the inhibition effect, the DNA extract should be considered as not suitable for PCR analysis.

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor cross-reactions with the host tissue and/or contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: a 18S uni-F/-R/-P PCR run (monoplex) is systematically performed as well as the Plat-F/-R/-P run in order to check the quality of DNA extraction. This 18S uni-F/-R/-P combination targets a conserved region within the 18S rDNA gene from a wide range of plants (Ioos *et al.* 2009).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of PCR grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

3.2. Interpretation of results

In order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC amplification curves should be exponential;
- NIC and NAC should be negative.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve, a C_t value below the cut off value;
- A test will be considered negative, if it produces no exponential amplification curve and a C_t value equal or above the cut off value;
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Fifty-five isolates of *P. lateralis* from France (21), United States (22), Canada (6), Scotland (3) and the Netherlands (3) were included in the validation (*in silico* or *in vitro* assessments). Other species (18 of *Phytophthora/Pythium*, 17 of non-oomycetes) were also included.

4.1. Analytical sensitivity data

The analytical sensitivity has been established by the authors at 47.2 plasmidic copies of the DNA target region per PCR tube.

4.2. Analytical specificity data

The test specificity has been assessed with 0.5 ng μL^{-1} DNA extracts from 35 different fungal species (18 oomycetes, 17 non-oomycetes), including *Phytophthora ramorum*, genetically very close to *P. lateralis*, at a high level of concentration. No cross reactions were observed.

4.3. Data on repeatability

The coefficient of variance is:

- 0.83% for a target DNA concentration of 472 plasmidic copies per PCR tube;
- 1.05% for a target DNA concentration of 4720 plasmidic copies per PCR tube;
- 0.58% for a target DNA concentration of 47 200 plasmidic copies per PCR tube;
- 0.85% for a naturally infested wood sample.

4.4. Data on reproducibility

The coefficient of variance is:

- 1.78% for a target DNA concentration of 472 plasmidic copies per PCR tube;
- 2.01% for a target DNA concentration of 4720 plasmidic copies per PCR tube;
- 2.51% for a target DNA concentration of 47 200 plasmidic copies per PCR tube;
- 2.36% for a naturally infested wood sample.