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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/112 (1)

Diagnostics
Diagnostic

PM 7/112 (1) *Phytophthora kernoviae*

Specific scope

This standard describes a diagnostic protocol for *Phytophthora kernoviae*[1].

Specific approval and amendment

Approved in 2012-09

Introduction

Phytophthora kernoviae was discovered in 2003 in the south-west of England during surveys for *Phytophthora ramorum* (Brasier *et al.*, 2005). The pathogen was isolated concurrently from a large, bleeding lesion on a mature *Fagus sylvatica* and from *Rhododendron ponticum* in another woodland in the same area. The disease is also present in the wider environment, infecting bilberry (*Vaccinium myrtillus*) plants in heathland. The extent of the damage to trees, shrubs and heathland plants, and the apparent speed of infection at the affected sites, indicate that this disease is a serious threat to woodland and heathland environments.

Since damage caused by *P. kernoviae* was first seen in October 2003, there have been findings in woodlands, gardens and a small number of nurseries, principally in south-west England. There have also been findings in Scotland, Ireland and New Zealand.

As of February 2010, *P. kernoviae* has been found on *F. sylvatica* (European beech), *R. ponticum* (and *Rhododendron* hybrids), *Aesculus hippocastanum*, *Castanea sativa*, *Drimys winteri*, *Gevuina avellana* (Chilean hazelnut), *Hedera helix*, *Ilex aquifolium*, *Liriodendron tulipifera* (tulip tree), *Lomatia myricoides*, *Magnolia* sp., *Michelia doltsopa*, *Pieris* spp., *Podocarpus salignus*, *Prunus laurocerasus*, *Quercus ilex* (holm oak), *Q. robur* (English oak), *Sequoiadendron giganteum* and *Vaccinium myrtillus*. In New Zealand, the custard apple tree (*Annona cherimola*) is a further host.

A regularly updated host list can be found on the Defra website http://www.fera.defra.gov.uk/plants/plantHealth/pestsDiseases/phytophthora/documents/pKernoviaeHost.pdf.

Identity

Name: *Phytophthora kernoviae* Brasier, Beales & S. A. Kirk, sp. nov
Synonym: None
Taxonomic position: Chromista: Oomycota, Oomycetes, Peronosporales, Peronosporaceae
EPPO code: PHYTKE
Phytosanitary categorization: EPPO Alert list 2011

Detection

Symptoms

Symptoms caused by *P. kernoviae* can be diverse, as described below. In some cases they are very similar to those caused by *P. ramorum*. Symptoms can be seen in the Defra factsheet at http://www.fera.defra.gov.uk/plants/publications/documents/factsheets/phytophthoraKernoviaeFactsheet.pdf

Shrub hosts
Rhododendron ponticum, *R. catawbiense*, *R. yakushimanum* and hybrids. Early leaf symptoms include a blackening of the leaf petiole that often extends into the base of the leaf. This necrotic lesion may progress further into the leaf tissue, and in extreme cases may affect the whole leaf. Occasionally, however, only blackening of the leaf tip is observed. Both old and young leaves appear to be affected equally and, unusually for a *Phytophthora* infection of

[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.

rhododendron, leaves often fall within a few weeks of infection. Shoot dieback and cankers occur frequently and where these girdle the stem tissue, leaves above the lesion wilt. In severe infections the whole bush may be killed. Leaf and stem infections can be found at any height or position on a rhododendron bush.

Pieris spp., *Michelia doltsopa*, *Hedera helix*, *Ilex aquifolium* and *Prunus laurocerasus*. Leaf blight symptoms similar to rhododendron are seen. Infection on *M. doltsopa* is characterized by drip tip lesions on the leaves that progress along the leaf margins and into the tissue of the leaf blade. Necrotic leaf tissue is characteristically a dark black-brown colour. Typically, lesions on leaves of *Pieris* spp. are a light tan to rusty brown colour. Necrosis progresses directly towards the midrib and along the vein, causing a visually striking leaf blight. To date, only stem infection has been observed on *H. helix*; dark necrotic lesions have been recorded on leaves of *I. aquifolium*, and both leaf infection and stem dieback on *P. laurocerasus*.

Vaccinium myrtillus. Symptoms are early defoliation and multiple stem infections, leading to a striped appearance of green healthy parts and necrotic black parts of the stems.

Tree hosts

Fagus sylvatica (European beech). Initial symptoms are bleeding lesions on the trunk, which may be found anywhere from ground level up to 12 m. The bleeding is usually dark brown to blue-black and similar to symptoms caused by *P. ramorum*. Underneath, orange-pink to pink-brown active lesions in the inner bark are visible. Sometimes girdling of the entire tree can occur. Older lesions may appear sunken.

Quercus robur (English oak). Stem bleeding lesions are similar to those on *F. sylvatica* but may be more difficult to see both internally and externally because of the thick outer bark ridges and outer bark plates of oak. Bleeding can occur from cracks between the bark ridges from the underlying infected tissue, but the thickness of the bark prevents older cankers from becoming sunken, as in beech.

Liriodendron tulipifera (tulip tree). Only one diseased tree has been found to date, but disease symptoms occur on foliage, shoots and the trunk. Multiple bleeding lesions are formed on the trunk from ground level up to 9 m. Internal lesions range in colour from pale chocolate through dark chocolate to blue-black. Lesions tend to be limited in size (approximately 15 × 20 cm) and the bark becomes highly corrugated as a result of the multiple lesions. Lesions can also develop on leaves; these are largely restricted to leaf tips (approximately 10–15 mm long) and on leaf margins. The necrotic tissue dries out to a dark black colour. Shoot dieback also occurs and infected shoots are defoliated.

Castanea sativa/Aesculus hippocastanum (sweet chestnut/horse chestnut). Only foliar symptoms have been seen on sweet chestnut, including lesions along the mid-vein and evidence of infection coming in at the leaf margins in the form of necrotic lesions. In the UK, these symptoms were seen late in the season when the leaves started to senesce. Symptoms on horse chestnut have so far also been foliar.

Quercus ilex (holm oak). Severe necrotic leaf lesions and dieback associated entirely with epicormic shoots. No evidence of sunken or bleeding cankers has been observed.

Magnolia spp. Distinctive symptoms are found on infected foliage and buds. Infection occurs anywhere on the leaf surface, and multiple infections are evident as numerous dark brown necrotic patches, giving leaves a spotty appearance. There is a tendency for the necrotic spots to merge and develop towards the midrib. Leaves become conspicuously mottled when lesions are well developed. The mottling may have angular edges, and uninfected tissue between necrotic areas becomes chlorotic. Infections that take place at the leaf margin cause the margin to collapse and form a hard, dry rim. The petioles can be infected, and disease often progresses along the leaf base following petiole infection. Buds can also become diseased and turn light khaki grey.

Drimys winteri (winter's bark tree). Leaf symptoms are similar to those on rhododendron. Bleeding cankers on trunk are possible.

Sampling procedures

Depending on the type of material to be sampled, different methods are used as described below.

Plant material. Trees (trunk/logs): for bleeding cankers, the inner bark in the area directly around the oozing sap is cut until a canker margin is evident. Pieces of phloem and xylem are removed and placed in a sealed container, or small pieces may also be plated out directly on different media.

Shoots/twigs: a piece of stem including the leading edge (junction between diseased and healthy tissue) is removed and placed in a sealed plastic bag, with a small piece of damp tissue to prevent desiccation.

Leaves: 4–6 leaves showing a suitable range of symptoms are removed and placed in a sealed plastic bag with a piece of damp tissue.

If possible, all samples of plant material should be sent to the laboratory to arrive by the next day. Overheating or desiccation of samples prior to despatch should be prevented. To maximize the success of isolation of *P. kernoviae* from plant material, samples should be processed as soon as possible. Storage in a fridge (4–10°C) for up to 7 days can take place but may reduce the likelihood of isolating the target organism. Storage for longer periods at

low temperatures can also reduce the ease of isolating *P. kernoviae*.

Water and soil. Detecting viable *P. kernoviae* in soil or water can be done by baiting with rhododendron leaves. Soil is placed in a transparent plastic container and covered with Petri's mineral solution or demineralized water (P. Giltrap, pers. comm.; S. Werres, pers. comm., 2011). Clean rhododendron (cv. Cunningham's White or *R. ponticum*) whole leaves or leaves cut into pieces (e.g. approximately 1.5 cm × 2.0 cm) are then floated on top. The lid is replaced and the tub incubated for 3–6 days at 18–23°C in light (12–16 h day^{-1}) on the laboratory bench. After this time the leaves are recovered, then surface decontaminated and plated onto P_5ARPH or another suitable medium to allow *P. kernoviae* to grow out. The leaves can also be tested immediately by PCR or real-time PCR. It should be noted that the test is less sensitive for *P. kernoviae* than *P. ramorum* (down to 10 sporangia for *P. ramorum* but only 1000 for *P. kernoviae*; Jennings, 2008).

Water testing can be carried out in a similar manner in the laboratory by simply floating cut leaves on the water to be tested. Alternatively, on-site testing can be done using cut rhododendron leaves placed in a muslin bag, which is then lowered into the top layers of the water on a string and left for 3 days. Once recovered, the leaves are washed and plated and/or tested using molecular tests.

Isolation

Plant material. There are a number of alternative methods for surface disinfection of plant material that may be used depending on the type of substrate and the analysis to be performed. Alcohol treatments as well as rinses with sterile water may be used to reduce surface-colonizing microorganisms in cases where *P. kernoviae* is presumed to be present inside tissues. The choice of method depends on:

(1) the type of substrate: very thin root or thin leaf tissue should not be treated with alcohol as this can kill both the pathogen and the root/leaf;
(2) the type of method to be performed: culturing is prone to contamination by other microorganisms but molecular methods are less affected;
(3) the risk of false negatives: water rinses may partially remove contamination with irrelevant organisms, but the remaining organisms may suppress *P. kernoviae*, whereas alcohol treatments may remove the contaminants more completely but also remove *P. kernoviae.*

Relevant alternative methods are given for each of the tissue types involved (Appendix 1).

Media and incubation

Sometimes, using a microscope, *P. kernoviae* can be seen directly on the collected material (presence of typical sporangia). If not, material can be incubated for 3–5 days in a closed box with a small piece of damp tissue on the bottom to promote sporulation.

For isolation, a number of media may be used as listed in Appendix 2.

A commonly used medium is P_5ARP [H] (Jeffers & Martin, 1986) as it is semi-selective for *Phytophthora* spp. and characteristic features of *P. kernoviae* are readily observed. Other media may be used, for example semi-selective V8 agar (Jung *et al.*, 1996), cherry decoction agar (CHA) or carrot piece agar (CPA), the low pH of which prevents rapid bacterial development.

Growth media such as V8 agar, CPA, carrot juice agar (CJA) or corn meal agar (CMA) may be used for storage/studying morphological features (see Appendix 2).

Once samples have been decontaminated using one of the methods outlined above, at least four small pieces (approximately 2 mm^2) of symptomatic tissue are excised with a sterile scalpel and transferred aseptically onto one or more of the agar media mentioned above. To permit direct observation under a compound microscope, it is advisable to place the pieces approximately 2–3 cm from the edge of the dish.

Plates can be incubated on the laboratory bench under normal daylight conditions or in an incubator, with or without light (white) (e.g. 12 h light/12 h dark) at 18–23°C.

Identification

For a positive identification, *P. kernoviae* should have been identified at species level either by its growth characteristics in culture and morphology, or by molecular methods (see Fig. 1).

In case of uncertainty, it is recommended to carry out confirmation by a complementary method (see Fig. 1). The identity of *P. kernoviae* isolates from new hosts or isolates that do not morphologically match published descriptions can be confirmed by sequencing. Pure cultures are used for this.

Growth characteristics in culture and morphology

The most essential features on a selective and a non-selective medium are given in Table 1. These refer to features seen on fresh cultures growing either directly out of the plant material placed onto the agar, or on recently purified cultures. To maintain *P. kernoviae*'s pathogenicity and diagnostic features in culture, pure cultures should regularly (approximately every 6–8 weeks) be inoculated into fresh rhododendron leaves, reisolated once a lesion develops, and purified. At Fera, (GB), the purified culture is subcultured onto CPA and future subcultures alternate between CPA and 10% V8 agar to prolong active and abundant sporulation. Good sporulation has also been reported by Brasier *et al.* (2005) and K. Heungens (pers. comm.) when CA or V8 plugs are immersed in unsterile pond water or soil leachate. Two methods to induce sporulation are described in Appendix 3. The use of P_5ARP [H] for the study of pure cultures is not recommended as sporulation is poor after the second or third subculturing.

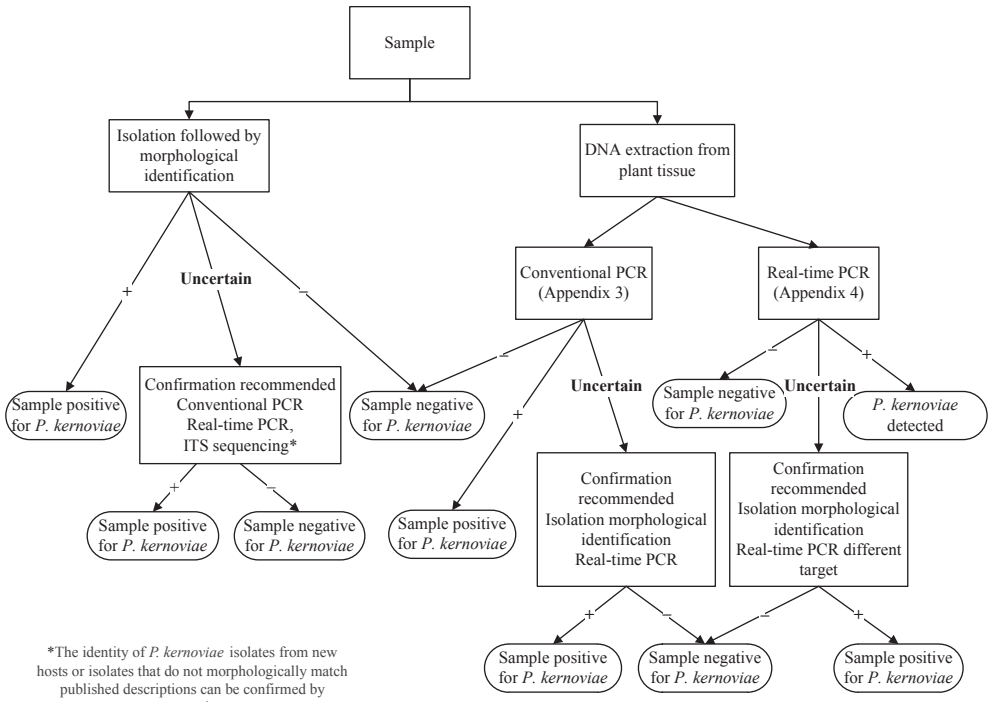

Fig. 1 Flow diagram for diagnosis of *Phytophthora kernoviae* on plants or plant products.

Table 1 Growth characteristics of fresh *Phytophthora kernoviae* cultures on a selective and non-selective medium and morphometric characteristics (sizes as in Brasier *et al.*, 2005)

Character	P_5ARP(H)* (from excised lesion)	Carrot piece agar†
Colony	Relatively fast-growing, approximately 2–4 mm per day	Weak rosette-like pattern, pronounced concentric rings, see Fig. 2A, in diurnal light, growth rate approximately 3.8–4.6 mm per day in dark
Mycelium	Weakly coralloid, growing within agar with little superficial growth, no hyphal swellings‡	Aerial mycelium sparse, generally no hyphal swellings‡
Sporangia	Produced abundantly on agar surface but sometimes in agar. Papillate, regular, ovoid or limoniform to distinctly asymmetrical or mouse-shaped. Size: range of means: 38.5–45.5 × 22.5–27 µm, common range: 34–52 × 19–31 µm; average length/width ratio 1.5 µm. Caducous/deciduous, pedicel length – range of means: 8.6–14.1 µm, common length range: 5–19 µm (Fig. 3). Many have a conspicuous vacuole.	
Chlamydospores	Not seen	Not seen
Sexual structures	Homothallic, gametangia produced readily after 10 days on CA or CPA. Oogonia: range of means: diameter 23.5–25.5 µm, common range: diameter 21–28 µm. Antheridia amphigynous. Oospores: plerotic, range of means: diameter 21.1–22.5, common range: diameter 19–25 µm (Fig. 4), wall thickness: average approximately 3.5 µm, common range: 3.5–5 µm.	

*On P_5ARP(H) characters can be observed after 4–6 days' incubation on the bench at around 18–23°C, diurnal light. Typical characteristic are best seen when *P. kernoviae* grows out of excised pieces of infected plant material placed on the media.
†On carrot agar (CPA) this is after 3–5 days' incubation at approximately 20°C.
‡Hyphal swellings have been observed on corn meal agar (N. Schenck, pers. comm., 2010).

Generally, the unique morphological features described below make *P. kernoviae* a relatively easy organism to identify when growing out of plant material on semi-selective media (e.g. P_5ARP [H] or CHA). At Fera, (GB), all the features described below have been seen on all of over 2000 isolates incubated on the laboratory bench (18–23°C) under normal daylight conditions.

The growth characteristics on agar and morphological features are described in Brasier *et al.* (2005) and can be seen in the following Figs 2A to 4.

Molecular methods

Several molecular methods have been developed and are used to detect *P. kernoviae* from culture plates, soil and

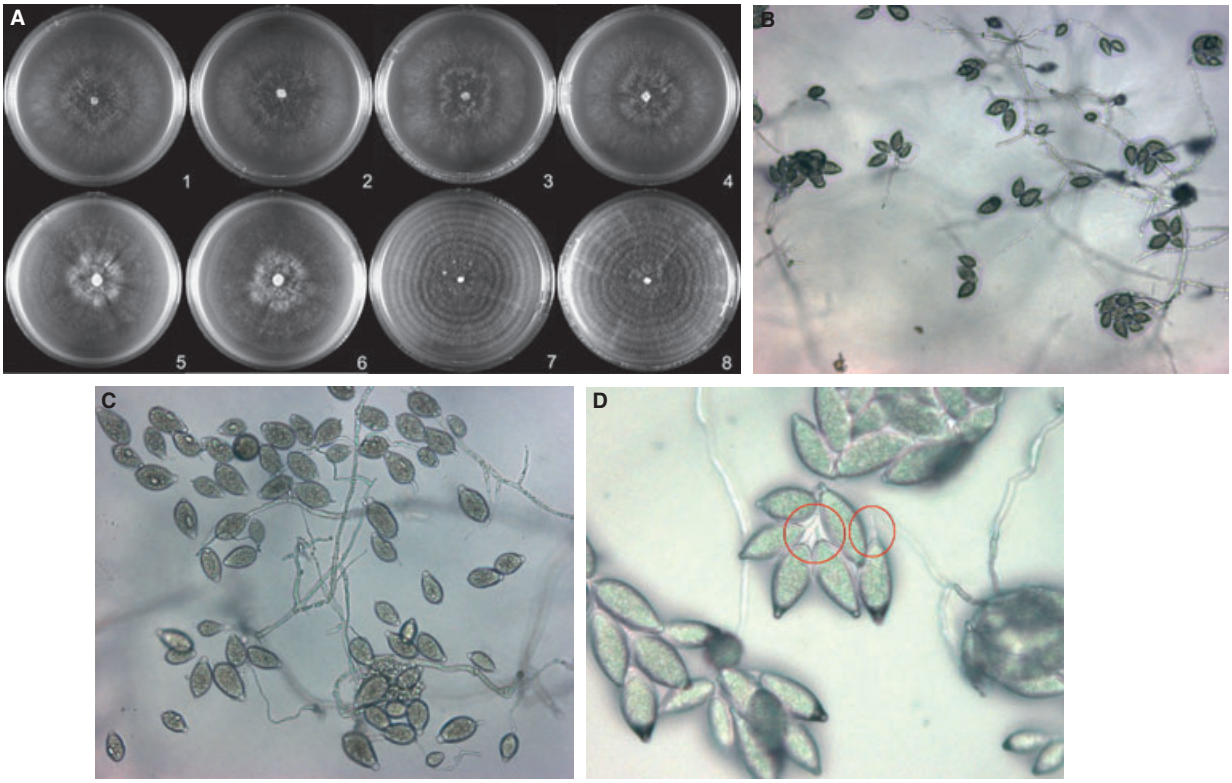

Fig. 2 (A) Colony types of *Phytophthora kernoviae*. Plates 1–4: after 10 days at 20°C on carrot agar (CA) in complete darkness. Plates 5–6: after 10 days at 20°C on CA with some exposure to light at 7 days for colony measurement. Plates 7–8: after 10 days on CA at ambient room temperature (around 23°C) in diurnal light (temperature as given in Brasier *et al.*, 2005, photos courtesy Fera, GB). (B) Abundant sporulation as seen ×100 on P_5ARP(H) from excised lesions (courtesy Fera, GB). (C) Numerous deciduous sporangia ×200 (courtesy Fera, GB). (D) Clusters of sporangia ×200 (zoomed) with deciduous ones highlighted (courtesy Fera, GB).

water samples as well as directly *in planta*. For the purposes of this protocol, only validated PCR and real-time PCR tests are described. Conventional PCR is presented in Appendix 4 and real-time PCR in Appendix 5.

Further confirmation includes sequencing of the ITS region (using isolates from pure culture) (Appendix 6). Note that the *P. kernoviae* isolates from New Zealand (Ramsfield *et al.*, 2007) sequenced to date differ by one base pair from the authentic type *P. kernoviae* accession in GenBank (AY940661).

Reference material

Isolates

p1553	ex *Fagus sylvatica* bark	Forest Research *Phytophthora* collection
2166	ex *Rhododendron* sp. Cornwall, UK	Fera *Phytophthora* culture collection
2444	ex *R. catawbiense*, Cheshire, UK	Fera *Phytophthora* culture collection
2528	ICMP 14761 Mark Braithwaite	MAF, New Zealand and Fera *Phytophthora* culture collection

GenBank accession numbers
UK isolate: AY940661
New Zealand isolate: EU909457

Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 (1) *Documentation and reporting on a diagnosis.*

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 specificity, full validation reports).

Further information

Further information on this organism can be obtained from the Food and Environment Research Agency, York YO41

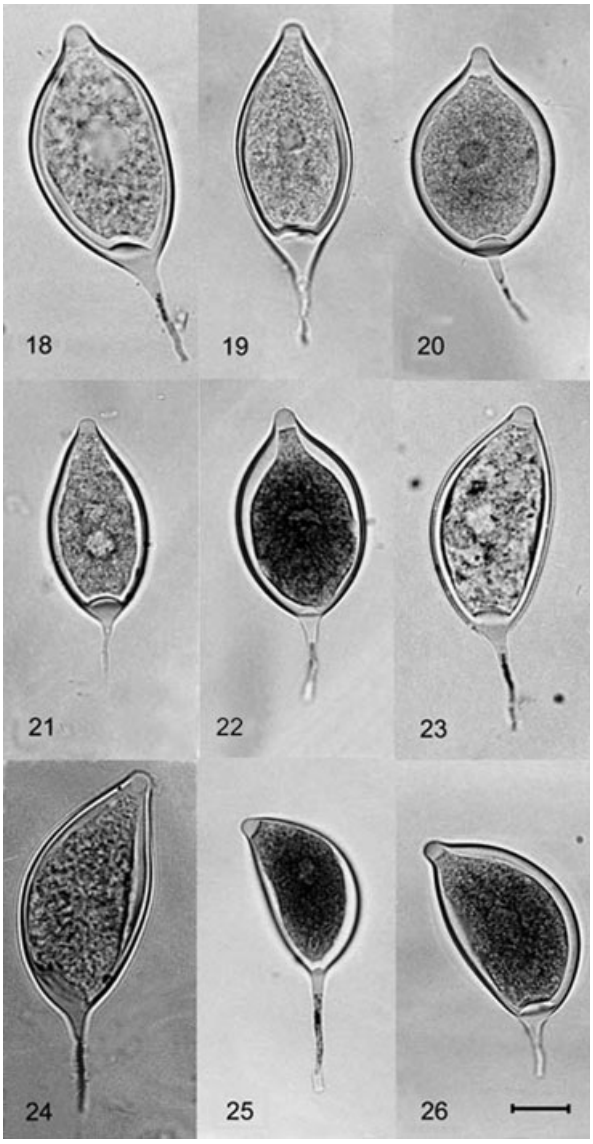

Fig. 3 Representative sporangia of *Phytophthora kernoviae*. Nos 18–22: regular, ovoid limoniform sporangia. Nos 23–26: asymmetrical or mouse-shaped sporangia. Bar = 10 mm (from Brasier *et al.*, 2005).

1LZ, England, GB (fax: 44 1904 462111, tel: 44 1904 462000, email: paul.beales@fera.gsi.gov.uk

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.int.

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.

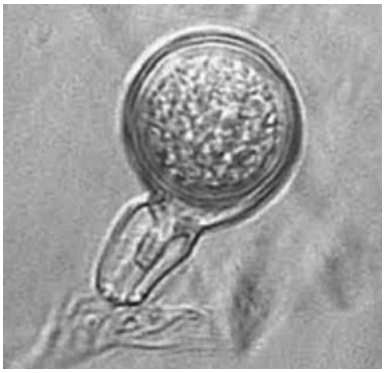

Fig. 4 Sexual structures of *Phytophthora kernoviae* on carrot agar (CA) after 10 days. Oogonia (diameter 23.5–25.5 µm); amphigynous antheridia and plerotic oospores (diameter common range approximately 19–25 µm), wall thickness average 3.5 µm, common range 3.5–5 µm (courtesy A. Schlenzig, Science and Advice for Scottish Agriculture, GB).

Acknowledgements

The protocol was initially drafted by P. Giltrap, K. Webb and J. Tomlinson, Food and Environment Research Agency, York, GB.

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Appendix 1 – Disinfection/cleaning techniques

Disinfection/cleaning techniques for aerial plant parts

Alcohol swabbing

Quickly wipe the surface of selected plant tissue with cotton wool impregnated with 70% ethanol, then excise small pieces of material and transfer aseptically to selective medium. For woody stems, remove the bark prior to plating out.

Rinsing in water

Select appropriate plant parts (to contain the growing edge of the lesion), place in a plastic bag and add about 20–50 mL distilled water. Allow to soak for several minutes, agitate the sample within the bag for 10 s, drain off the water, repeat washing, then transfer the plant material onto selective media.

Sodium hypochlorite treatment

Place in active sodium hypochlorite solution (suggested range 0.5–1%) for 2–5 min in a laminar flow cabinet, wash twice in sterile distilled water, dry carefully (on filter paper) and transfer aseptically to one of the media listed below under 'Media and incubation'. For twigs: cut out at least three pieces per twig, one from the dark brown area, one from the edge of the lesion and one from the healthy-looking tissue just under the lesion. For leaves: cut out pieces approximately 0.5 × 0.5 cm from the edge of a necrosis or spot.

Disinfection/cleaning techniques for stem base material/roots or heavily contaminated samples

Flushing with water

This method produces large quantities of contaminated water and should be used only if large volumes of wash water can be sterilized following washing.

Excise suitable pieces of tissue to include the leading edge, place in a 250 mL side-arm conical flask, and place over the neck a porous cover (e.g. muslin, fine wire mesh, Parafilm with small holes pierced in it). Connect the flask to the tap and flush with water for at least 2 h. Transfer aseptically at least four pieces of tissue to an appropriate medium.

Washing in a bag

Excise suitable pieces of tissue to include the leading edge, place in a small plastic bag and add approximately 50 mL water. Agitate vigorously, then pour off water, retaining plant pieces in the bag. Add a further 50 mL water and rinse again. Continue rinsing until wash water is clear. Aseptically transfer the plant pieces to a selective medium.

Alcohol treatment

For stems, cut out larger pieces (minimum 10 × 10 cm) to send to the laboratory. In the laboratory, dip these pieces into 70% alcohol, let them dry, and cut out small pieces to stick into (as opposed to placing on top of) the selective medium. Alternatively, cut out small pieces from the cambium area and (in the field) stick them directly on carrot piece agar or selective medium.

Appendix 2 – Media

Media for isolation of *Phytophthora kernoviae*

All media are sterilized by autoclaving at 121°C for 15 min.

P_5ARP[H] (Jeffers & Martin, 1986)

Cornmeal agar	17.0 g
Pimaricin	5.0 mg
Ampicillin (Na salt)	250 mg
Rifampicin (dissolved in 1 mL 95% ethanol)	10 mg
PCNB	100 mg
Hymexazol (30% active substance) (final concentration 22.5 ppm)	75 mg
Distilled water to	1 L

Cool media to 50°C in a water bath. Prepare pimaricin, ampicillin (Na salt), rifampicin, PCNB, hymexazol and dissolve all in 10 mL sterile distilled water. Add to cooled media, pour onto plates. For optimal use, store at 4°C in the dark up to 7 days.

P_5ARP: if hymexazol is unavailable, then PARP can be used.

PARB [H] (Robin *et al.*, 1998)

Agar	20.0 g
Malt	15.0 g
Pimaricin	10 mg
Ampicillin	250 mg
Rifampicin (dissolved in 1 mL 95% ethanol)	10 mg
Benlate (50% benomyl)	15 mg
Tachygaren (75% hymexazol)	50 mg
Distilled water to	1 L

Cool media to 50°C in a water bath. Then prepare pimaricin, ampicillin, rifampicin, benlate, tachygaren and dissolve in 10 mL sterile distilled water. Add to cooled media.

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

Agar	20.0 g
$CaCO_3$	3.0 g
V8 juice	100 mL
Pimaricin	0.02 g
Ampicillin	0.2 g
Rifampicin	0.01 g
PCNB	0.025 g
Nystatin	0.05 g
Hymexazol	0.05 g
Distilled water	900 mL

After cooling down to 50°C, add pimaricin, ampicillin, rifampicin, PCNB, nystatin, hymexazol.

Cherry decoction agar (CHA)

Agar	60.0 g
Cherry juice	400 mL
Distilled water	3600 mL

Filter cherry juice through muslin and adjust the pH to 4.4 with KOH. Dissolve the agar thoroughly first, then add cherry juice. Autoclave at 102°C for 5 min.

Carrot agar (CA)

Agar	20.0 g
Carrots	400 g
Distilled water	900 mL

Wash carrots and cut up into small pieces. Autoclave for 10 min in 400 mL water. Blend carrots and liquid to produce an even mixture and add 500 mL water. Add 20 g agar and autoclave.

Carrot piece agar (CPA) (Werres *et al.*, 2001)

Agar	22.0 g
Carrots	50 g
Distilled water	1000 mL

Petri's mineral solution

Calcium nitrate ($Ca(NO_3)2$)	0.4 g
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0.15 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.15 g
Potassium chloride (KCl)	0.06 g
Distilled water	1000 mL

Media for further study or subculturing

All autoclaved at 121°C for 15 min.

Carrot piece agar (CPA) (see above)

Carrot juice agar 5% (CJA) (Kröber, 1985)

Agar	15 - 22 g
Carrot juice (without honey)	50 mL
Distilled water	950 mL

Dark carrot agar (DCA) (Fera recipe)

Carrots	200 g
Agar Oxoid No. 3	15 g
Distilled water	1000 mL

Slice fresh carrots and comminute in a blender with 500 mL distilled water at high speed for 1 min. Filter through four layers of cheesecloth and squeeze out the juice from the residue. Make up the resultant filtrate to 1L and add the agar. Heat to dissolve the agar, pour into bottles and autoclave.

V8 agar (10% V8) (Fera recipe)

V8 juice	100 mL
Agar Oxoid No. 3	20 g
0.1M KOH (0.14 g in 25 mL H_2O)	25 mL
$CaCO_3$	1.0 g
Distilled water	875 mL

Appendix 3 – Methods for zoospore induction

Method used in Julius Kühn Institut (S. Werres, pers. comm., 2012)

Place a piece from the edge of a growing *P. kernoviae* colony in the centre of a Petri dish with carrot piece agar.

Incubate at 16 h light and approximately 20°C.

After 7 days of incubation, check using a microscope whether sporangia have developed. If sufficient numbers of sporangia are present, add 0.5 mL sterile demineralized water on the colony surface and distribute carefully with a Drigalski spatula over the complete colony and agar surface (it is important that the sporangia are well distributed).

Incubate again at approximately 20°C, 16 h light

After a further 7 days of incubation, open the Petri dish and apply 5–7 mL sterile demineralized water on the surface. Incubate the Petri dish in the fridge (at 4–8°C) in the dark for approximately 1 h.

Incubate further at room temperature (approximately 20°C) for 30 min to 1 h (check the increasing turbidity visually to monitor the progress of zoospore release: maximum release is usually after 1 h). Note that released zoospores start to germinate very quickly, thus incubation for longer than 1 h at room temperature is not favourable if motile zoospores are required.

Collect the supernatant and calculate the number of zoospores.

An average number of approximately 1×10^6 zoospores mL^{-1} is usually recovered (in first studies [$n = 9$] the number of zoospores varied between 37 500 and 2.45×10^6).

Method used in Fera (P. Giltrap, pers. comm., 2012)

1. Start with a plug-inoculated culture of sporulating *P. kernoviae* grown on 10% V8 agar in 12 h light at approximately 20°C.
2. Subculture from this by adding 10 mL sterile demineralized water to the plate and dislodging the sporangia with a sterile spreader. Transfer 100 µL of the sporangia suspension to new a 10% V8 agar plate and spread.
3. Incubate at approximately 20°C, 12 h light for 72 h. (Tests at Fera have shown that as sporangia age, they become less likely to release zoospores. This method allows for the inoculated sporangia to germinate, producing sporangia of a similar age from which zoospores can be obtained. Do not use spread plates older than 5 days.)
4. After 3–5 days' incubation, flood the spread plate with 15 mL sterile demineralized water, chill plates at −20°C for 5 min only, and incubate at room temperature for 1 h.
5. Check for zoospore release under the microscope, then filter the suspension through Whatman no. 113V (retention size >30 µm). If this is unavailable, use eight thicknesses of lens tissue.

An average number of 3.4×10^5 zoospores mL^{-1} per plate is usually recovered.

Appendix 4 – Identification at species level by conventional PCR (Schlenzig, 2011)

1. General information (Schlenzig, 2011)

1.1 The PCR primers and probe are designed to target the internal transcribed spacer region of *P. kernoviae*.

1.2 Nucleic acid source: pure culture. The test has not been fully validated on plant tissue (see point 4).

1.3 Amplicon size is 469 base pairs.

1.4 Oligonucleotides for *P. kernoviae* detection:
Forward primer Pkern60F: 5′-TCCTCGTTGGCAGTTTCGAC-3′
Reverse primer PkernR1: 5′-CACAACTACATTCTGCACAGC-3′

1.5 PCR can be carried out using *Taq* DNA polymerase (Sigma Aldrich) at 0.65 U per 20 µL reaction (a final concentration of 0.0325 U μL^{-1}).

1.6 Nucleotides should be used at a final concentration of 0.125 mM each nucleotide.

1.7 PCR buffer supplied with *Taq* should be used at a final concentration of 1×.

1.8 PCR can be carried out on any suitable thermal cycler.

2. Methods

2.1 Nucleic acid extraction
DNA extraction from pure cultures can be performed using commercial kits, for example the NucleoSpin Plant extraction kit (Macherey & Nagel, Düren, Germany) or the DNeasy Plant kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.
DNA can be stored at −20°C.

2.2 Polymerase chain reaction (PCR)
Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	14.82	NA
PCR buffer (including 15 mM $MgCl_2$)	10×	2	1×
dNTPs	10 mM	0.25	0.125 mM
Pkern60F	10 µM	0.4	0.2 µM
PkernR1	10 µM	0.4	0.2 µM
Taq DNA polymerase (Sigma-Aldrich, Poole, UK)	5 U μL^{-1}	0.13	0.65 U
Subtotal		18	
DNA		2	
Total		20	

2.2.2 PCR cycling parameters: initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 30 s; and a final elongation step at 72°C for 5 min.

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 isolation 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 of clean extraction buffer.
- Positive isolation control (PIC) to ensure 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 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).

Interpretation of results:

Verification of controls:

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of 469 bp.

When these conditions are met:

- A test will be considered positive if amplicons of 469 bp are produced.
- A test will be considered negative if it produces no band, or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical sensitivity data: testing a dilution series of DNA extracted from *P. kernoviae* in pure culture indicated a limit of detection of approximately 2 pg DNA per reaction. The analytical sensitivity has not been established so far for plant extracts, and further validation should be conducted to establish it.

4.2 Analytical specificity data: no cross-reactivity was observed when testing DNA extracted from cultures of the following species: *P. citrophthora*, *P. heveae*, *P. citricola*, *P. nicotianae*, *P. cryptogea*, *P. gonapodyides*, *P. cactorum*, *P. drechsleri*, *P. syringae*, *P. ideai*, *P. cambivora*, *P. cinnamomi*, *P. infestans*, *P. rubi* and *P. ramorum*.

Appendix 5 – Identification at species level by real-time PCR

A) Test targeting a region of the ras-related protein (*Ypt*1) (Schena *et al.*, 2006)

1. General information

1.1 Schena *et al.* (2006) developed a multiplex real-time PCR based on the ras-related protein gene (*Ypt*1) to detect *P. ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* in infected plant material. Regarding *P. kernoviae*, the test produces a 78 bp amplicon with primers Yptc3F (forward) and Yptc4R (reverse), and uses a Taqman (fluorescent) probe (YptcP) labelled with a fluorophore at the 5′ end and a black hold quencher (BHQ) at the 3′ end.

1.2 Nucleic acid source: plant material and pure cultures.

1.3 Sequences of the primers and probe:
Yptc3F: 5′ GCT CCA AAT TGT ACG TCT CCG 3′
Yptc4R: 5′ AAC CAA TTA GTC ACG TGC TGA TAT AAA 3′
YptcP: Fluorophore -5′ ATC ATA GCC CTT CCC AGA AGC TGT CAC A 3′-BHQ

1.4 Real-time PCR can be carried out using reagents in commercially available ready-to-use master mixes or as separate ingredients, and on any real-time PCR thermocycler. A variety of fluorophores can be used to label the probe, such as FAM, Yakima Yellow®.

2. Method

2.1 Nucleic acid extraction

Small pieces of plant tissue are excised from the leading edge of suspect lesions and homogenized. Different grinding methods may be used, such as mortar and pestle with liquid nitrogen, bead mills or the Homex grinder from Bioreba, provided a homogeneous ground sample is produced. DNA extraction can be performed using commercial kits, for example the NucleoSpin Plant extraction kit (Macherey & Nagel) or the DNeasy Plant kit (Qiagen), following the manufacturer's instructions. For DNA isolation from cultured isolates, the same procedure may be followed. DNA can be stored at −20°C.

2.2 Real-time PCR

Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	7.915	NA
Reaction buffer (qPCR™ Core Kit, Eurogentec, Belgium)	10×	1.5	1×

(continued)

Table *(continued)*

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
$MgCl_2$	25 mM	3.0	5 mM
dNTPs	10 mM	0.3	0.2 mM
Yptc3F F	10 µM	0.495	0.33 µM
Yptc4R	10 µM	0.495	0.33 µM
YptcP	10 µM	0.195	0.13 µM
Taq polymerase (qPCR Core Kit)	5 U μL^{-1}	0.1	0.5 U
Subtotal		14	
DNA		1	10 ng μL^{-1}
Total		15	

2.2.2 The real-time PCR conditions are set as an initial denaturation at 95°C for 10 min, followed by 40 cycles for denaturation at 95°C for 20 s and annealing/extension at 62.5°C for 60 s.

3. Essential procedural information

3.1 Controls

Different controls should be included in the procedure to ensure the reliability of the results:

- 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 nucleic acid of sufficient quantity and quality is isolated (e.g. *Rhododendron* leaves artificially infected with *P. kernoviae* stored at around −20°C).
- Negative amplification control (NAC) to rule out false positives due to contamination during preparation of the reaction mix (e.g. molecular-grade water).
- Positive amplification control (PAC) to monitor the efficiency of the amplification (e.g. total DNA from infected material or cloned PCR product at a defined concentration).

The absence of PCR inhibitors in the DNA extract should be assessed by using an internal positive control, for example by testing the extract with universal plant primers described in the literature, or by spiking the DNA extract to analyse with a known amount of target DNA (preferably at the limit of detection).

3.2 Test interpretation

The cycle cut-off value for *P. kernoviae* is set at 36, and was obtained using the equipment/materials and chemistry described in Schena *et al.* (2006). The cycle cut-off value needs to be verified in each laboratory when implementing the test for the first time.

Verification of controls

- The PIC and PAC amplification curves should be exponential.
- NIC and NAC should be negative (Ct > cut-off).
- PIC, PAC and internal positive control (IPC) should have a Ct value below the cut-off value.

When these conditions are met

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

4. Performance criteria available (from Schena *et al.*, 2006)

4.1 Analytical sensitivity data

Testing a dilution series of DNA extracted from *P. kernoviae* in culture indicated a limit of detection of around 100 fg DNA per reaction.

4.2 Analytical specificity data. No cross-reaction when testing DNA extracted from mycelium of the following species: *P. alni*, *P. cactorum*, *P. cambivora*, *P. capsici*, *P. cinnamomi*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, *P. drechsleri*, *P. gonapodyides*, *P. europaea*, *P. fragariae*, *P. ilicis*, *P. infestans*, *P. inundata*, *P. lateralis*, *P. nemorosa*, *P. nicotianae*, *P. pseudosyringae*, *P. quercina*, *P. ramorum* and *P. sojae*

B) Test targeting the ITS region (Hughes *et al.*, 2011)

1. General information

1.1 The Taqman real-time PCR primers and probe are designed to target the internal transcribed spacer region of *P. kernoviae* (accession number DQ002008, bases 615-702).

1.2 The amplicon size is 88 bp.

1.3 Oligonucleotides for *P. kernoviae* detection:

Forward primer *Pkern* 615F: 5′-CCGAACAATCTG CTTATTGTGGCT-3′

Reverse primer *Pkern* 722R: 5′-GTTCAAAAGCCAA GCTACACACTA-3′

Probe *Pkern* 606T: FAM-5′-TGCTTTGGCGTTTGCG AAGTTGGT-3′ TAMRA

1.4 Oligonucleotides for plant cytochrome oxidase (COX) detection (internal control assay)

Forward primer COX F: 5′-CGTCGCATTCCAGAT-TATCCA-3′

Reverse primer COX RW: 5′-CAACTACGGATAT ATAAGRRCCRRAACTG-3′

1.5 COX probe: VIC-5′-AGGGCATTCCATCCAGCGTA AGCA-3′ TAMRA

Real-time PCR can be carried out using AmpliTaq Gold DNA polymerase (Applied Biosystems) at 0.625 U per

25 µL reaction (a final concentration of 0.025 U per µL).

1.6 Nucleotides should be used at a final concentration of 0.2 mM each nucleotide.

1.7 Buffer A supplied with AmpliTaq Gold should be used at a final concentration of 1×.

1.8 Real-time PCR can be carried out on a suitable real-time PCR instrument such as an ABI Prism 7500 or 7900HT (Applied Biosystems).

2. Methods

2.1 Nucleic acid extraction

Small pieces of plant tissue are excised from the leading edge of suspect lesions and homogenized. Different grinding methods may be used, such as mortar and pestle with liquid nitrogen, bead mills or the Homex grinder from Bioreba, providing they produce a homogeneous ground sample. DNA extraction can be performed using commercial kits, for example the NucleoSpin Plant extraction kit (Macherey & Nagel) or the DNeasy Plant kit (Qiagen), following the manufacturer's instructions. For DNA isolation from cultured isolates, the same procedure may be followed. DNA can be stored at −20°C.

2.2. Real-time Polymerase Chain Reaction

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	11.875	N.A.
Reaction buffer A	10×	2.5	1×
$MgCl_2$	25 mM	5.5	5.5 mM
dNTPs	10 mM	0.5	0.2 mM
Pkern 615F	10 µM	0.75	0.3 µM
Pkern 722R	10 µM	0.75	0.3 µM
Pkern 606T	10 µM	0.25	0.1 µM
COX F	10 µM	0.75	0.3 µM
COX RW	10 µM	0.75	0.3 µM
COX probe	10 µM	0.25	0.1 µM
Taq polymerase	5 U μL^{-1}	0.125	0.625 U
Subtotal		24	
DNA		1	
Total		25	

2.2.2 PCR cycling parameters: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min.

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 isolation 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 of a sample of uninfected host tissue (when working with plant samples) or clean extraction buffer (when working with pure cultures).
- Positive isolation control (PIC) to ensure 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 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 PCRs performed on plant material, the PAC should preferably be near to the limit of detection.

In addition to the positive controls described above, when testing plant material an internal positive control (IPC) for detection of plant DNA (cytochrome oxidase gene, COX) should be used to verify that DNA extraction was successful for samples that give negative real-time PCR results for *P. kernoviae*.

3.3 Interpretation of results

The test interpretation is based on the cycle cut-off: the PCR cycle number above which any sample response value (Ct) is considered as a negative. The Ct value for *P. kernoviae* is set at 30 using the equipment/materials and chemistry as described in this Appendix. The cycle cut-off value needs to be verified in each laboratory when implementing the test for the first time.

Verification of controls

- The PIC and PAC amplification curves should be exponential.
- NIC and NAC should be negative (Ct > cut off)
- PIC, PAC and IPC should have a Ct value below the cut-off value.

When these conditions are met

- A test will be considered positive for *P. kernoviae* if it produces a Ct value of <30 provided the negative controls are negative.
- Tests with Ct values >30, and also tests with a Ct of 40 and a COX Ct ≥ 28, require isolation onto appropriate media such as P_5ARP-H for confirmation[2].

[2]It was noted that in some laboratories a dilution of the DNA extract is performed when the COX C_t is too high.

- A test will be considered negative if it produces a Ct >40. For plant material, a COX Ct <28 is required for a sample to be considered negative for *P. kernoviae.*
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available (from Hughes *et al.,* 2011)

4.1 Analytical sensitivity data: testing a dilution series of DNA extracted from *P. kernoviae* in culture indicated a limit of detection of around 1.2 pg DNA per reaction.

4.2 Analytical specificity data: no cross-reaction was observed with DNA extracted from the following species of *Phytophthora*: *P. boehmeriae*, *P. botryosa*, *P. cactorum*, *P. cambivora*, *P. cinnamomi*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, *P. erythroseptica*, *P. europaea*, *P. rubi*, *P. gonapodyides*, *P. heveae*, *P. hibernalis*, *P. insolita*, *P. ilicis*, *P. lateralis*, *P. macrochlamydospora*, *P. megasperma*, *P. nemorosa*, *P. nicotianae*, *P. palmivora*, *P. parasitica*, *P. pseudosyringae*, *P. quercina*, *P. ramorum*, *P. richardiae*, *P. syringae* and *P. uliginosa.*

4.3 Diagnostic sensitivity data

The test was found to have a diagnostic sensitivity of 93.3% in a direct comparison with culturing for 468 samples.

4.4 Diagnostic specificity data

The test was found to have a diagnostic specificity of 99.8% in a direct comparison with culturing for 468 samples.

Appendix 6 – Identification at species level by sequencing part of the ITS region

1. General information

1.1 The identity of *P. kernoviae* isolates from new hosts, or isolates that do not morphologically match published descriptions, can be confirmed by sequencing. Only DNA from pure isolates can be tested using this method, otherwise sequences from multiple organisms may be amplified in the same reaction.

1.2 Guidance on sequencing of fungi will be provided in the EPPO Standard PM 7/XXX on *DNA barcoding as an identification tool for plant pests*, in particular Appendix 4

1.3 The primer sequences used for PCR sequencing are:

1.3.1 ITS 1: 5′-TCC GTA GGT GAA CCT GCG G-3′ and

1.3.2 ITS 4: 5′-TCC TCC GCT TAT TGA TAT GC-3′ (White *et al.*, 1990).

2. Amplification and analysis

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	60.5	N.A.
Reaction buffer containing 15 mm $MgCl_2$ (Applied Biosystems)	10×	10	1×
dNTPs	10 mM	8.0	0.8 mM
ITS 1	5 µM	10	0.5 µM
ITS 4	5 µM	10	0.5 µM
AmpliTaq (Applied Biosystems)	5 U μL^{-1}	0.5	2.5 U
Subtotal		99	
DNA		1	
Total		100	

2.1. Amplification is performed in thin-walled PCR tubes in a PCR thermocycler programmed as follows: 2 min at 94°C; then 30 cycles of 1 min at 94°C, 1 min at 53°C, 1.5 min at 72°C. One cycle for 10 min at 72°C must be conducted after the 30 cycles. Samples are resolved on a 1.5% agarose gel as previously described. Using this method, samples containing *Phytophthora* DNA produce single amplicons of approximately 900 bp in size.

Sequencing and database searching

Guidance on sequencing and Q-bank database searching will be provided in Appendix 7 of EPPO Standard PM 7/XX on *DNA barcoding as an identification tool for plant pests*.

Consensus sequences can also be compared for test samples with type culture AY940661 (www.ncbi.nlm.nih.gov) found on GenBank: a 100% match is required for a positive identification. Other databases will be indicated in the above-mentioned Standard on DNA barcoding. It is important to note that the *P. kernoviae* isolates from New Zealand sequenced to date differ by 1 base pair from the authentic type *P. kernoviae* accession in GenBank (AY940661). Therefore it is important to compare findings with both the type culture AY940661 and the New Zealand type culture on GenBank (accession number EU909457.1).