DOI: 10.1111/epp.12944

EPPO STANDARD ON DIAGNOSTICS

PM 7/1 (2) *Bretziella fagacearum* (formerly *Ceratocystis fagacearum*)

Specific scope: This Standard describes a diagnostic protocol for *Bretziella fagacearum*.[1]
This Standard should be used in conjunction with PM 7/76 Use of EPPO diagnostic protocols.
Specific approval and amendment: First approved in 2000–09. Revised in 2023–07.
Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Bretziella fagacearum is the cause of oak wilt in the United States. Although the pathogen is mainly reported on *Quercus* spp., several species in other genera of the Fagaceae (e.g. *Castanea mollissima*, *Notholithocarpus densiflorus*) are documented as hosts, either as naturally occurring infections or as the result of artificial inoculation studies (EPPO, 2023a). In 2022, the species was detected on *Castanea sativa* × *C. crenata* (Sakalidis et al., 2023). In *Quercus* spp., red oaks (subgenus *Lobatae*) are highly susceptible to the fungus, whereas American white oaks (subgenus *Quercus*) are more resistant to disease development. *Bretziella fagacearum* is a classic vascular wilt, in which the fungus is confined to the vessels of the outermost xylem ring until the trees become moribund. The most important means of short-distance spread is through natural root grafts, while above-ground spread occurs through beetles such as *Carpophilus sayi* or *Colopterus truncatus* (Coleoptera: Nitidulidae), which are the main vectors. In some areas, *Pseudopityophthorus minutissimus* and *P. pruinosus* (Coleoptera: Curculionidae) have also been involved in the spread of the pathogen. Movement of infested firewood is suspected to have been a source of infections in new areas (Davies, 1992). More information on host plants and distribution is available in the EPPO Global Database (EPPO, 2023b).

Testing of insect vectors is performed near infested areas in North America. Tests have been described in McLaughlin et al. (2022) and Bourgault et al. (2022). However, the current diagnostic protocol focuses on the testing of symptomatic plant material.

A flow diagram describing the diagnostic procedure for *Bretziella fagacearum* is presented in Figure 1.

2 | IDENTITY

Name: *Bretziella fagacearum* (Bretz) Z.W. de Beer, Marinc., T.A. Duong & M.J. Wingf.
Other Scientific names: *Ceratocystis fagacearum* (Bretz) Hunt, *Endoconidiophora fagacearum* Bretz, *Chalara quercina* Henry, *Thielaviopsis quercina* (Henry).
Taxonomic position: Fungi: Ascomycota: Microascales, Ceratocystidaceae.
EPPO Code: CERAFA.
Pest categorization: EPPO A1 list no. 6, EU Annex II A.

3 | DETECTION

3.1 | Disease symptoms

In red oaks, the first appearance of foliar wilt may occur any time from mid-spring to late summer. Early season wilt is indicative of previous season infection of the tree via root-graft spread. Early to late-summer wilting results from current season infections associated with insect transmission to wounds or via root grafts. Infected trees show sudden wilting and death of the foliage, often over the entire tree (Figure 2). Occasionally, leaves may become brown from the apex, while their base remains green (Figure 3). There may be blackened, longitudinal streaks in the outermost sapwood ring (Figures 4 and 5). These symptoms are similar to those caused by other wilting organisms. In oak wilt, however, unlike other wilt diseases, sporulating mats may form below the bark within a few months after tree death (Figure 6). These take the form of a central 'pressure cushion' surrounded by a greyish mat of mycelium and spore-bearing structures. They can be detected before the bark cracks by knocking on the trunk;[2] a hollow sound may be an

[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.

[2]A video on mat tapping is available at https://upload.eppo.int/download/1703oe7699f807.

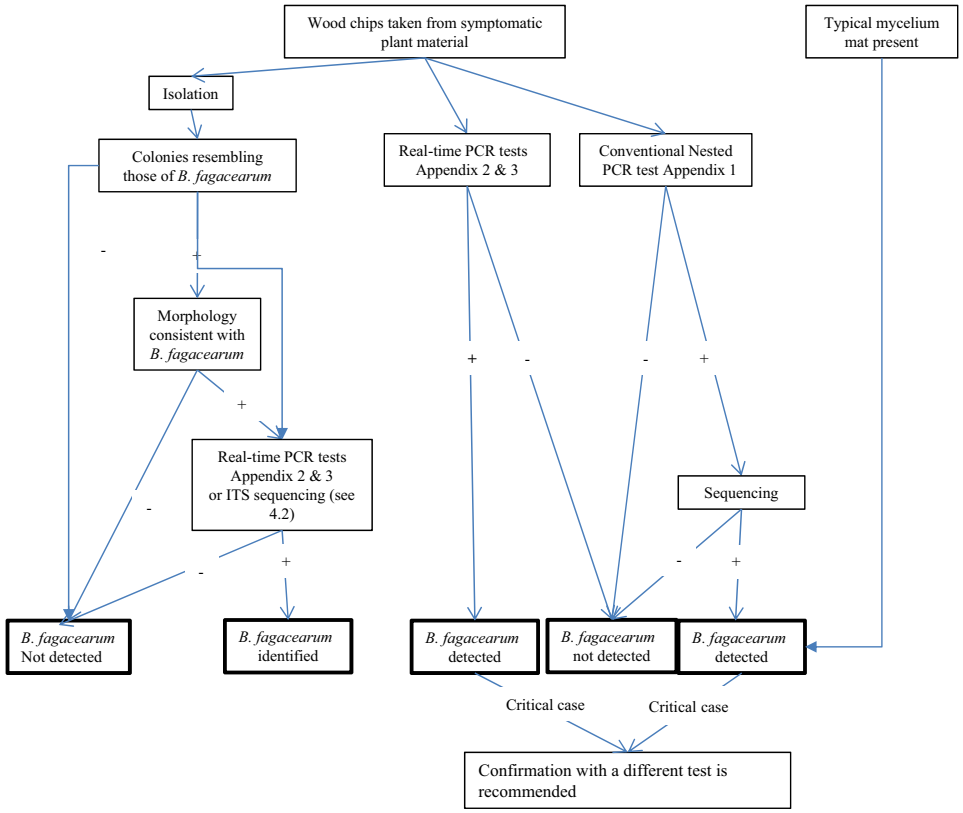

FIGURE 1 Flow diagram describing the diagnostic procedure for *Bretziella fagacearum* in symptomatic plant material. This flow diagram is intended to provide an overview of the diagnostic process and may not cover all possible scenarios.

FIGURE 2 Foliage symptoms of oak wilt caused by *Bretziella fagacearum* on oak. Courtesy: K. Chahal, Forest Pathology Laboratory—Michigan State University (US).

FIGURE 3 (left) Foliar symptoms showing bronzing starting at the leaf apices and lobes, and an area of green tissue around the base of the midrib. Evolution of foliar symptoms from left to the right. Courtesy: K. Chahal, Forest Pathology Laboratory—Michigan State University (US).

indication of the presence of pressure pads/cushions. These fruity-smelling mats occur most commonly on red oak species and may be visually detected on vertical bark cracks several months after complete tree wilt. Mats are seldom to never formed on species in the white oak group (EPPO, 2023a). On *Quercus* sp.

FIGURE 4 Xylem of red oak infected by *Bretziella fagacearum* showing diffuse stain (often there is less stain than this). Courtesy: J.N. Gibbs—Forestry Commission (GB).

FIGURE 5 Sapwood discoloration on red oak. Courtesy: K. Chahal, Forest Pathology Laboratory—Michigan State University (US).

FIGURE 6 Mycelial mat. Courtesy: K. Chahal, Forest Pathology Laboratory—Michigan State University (US).

the expression of symptoms may vary depending on the species involved.

Mycelium mats are typical for *B. fagacearum* and, when these are present, this is enough to consider that the pest is detected.

3.2 | Screening tests for symptomatic plant material

Molecular tests can be used to screen plant material showing symptoms resembling those caused by *B. fagacearum*.

No data is available to make recommendations to test for asymptomatic material.

3.2.1 | Test sample requirements

Samples are best taken from symptomatic branches of more than 2 cm in diameter, while the inner bark is still fresh and green. Several branches should be collected because the fungus may not be isolated from all symptomatic branches. Branches should be surface disinfected (by dipping in 70% alcohol and flaming), the bark removed and wood chips of a few millimetres thick of symptomatic areas of the underlying tissue (discoloured streaks) sampled (Figure 7).

Other plant material (round wood, wood chips, roots) can be sampled when symptomatic tissue is present.

Samples should be processed as soon as possible.

3.2.2 | Molecular tests

The following tests can be used for testing plant material:

- Nested PCR (Yang & Juzwik, 2017), described in Appendix 1;
- Real-time PCR (Yang & Juzwik, 2017—modified by Fera), described in Appendix 2;
- Real-time PCR (Lamarche et al. 2015), described in Appendix 3.

Although there is limited experience in the region with the real-time PCR of Lamarche et al. (2015), it is included in this protocol as it targets a different part of the genome and validation data is available in the publication.

Sample processing for molecular tests is described in the Appendices.

A new onsite test has recently been published (Bourgault et al., 2022) and may be considered in a future revision of this protocol.

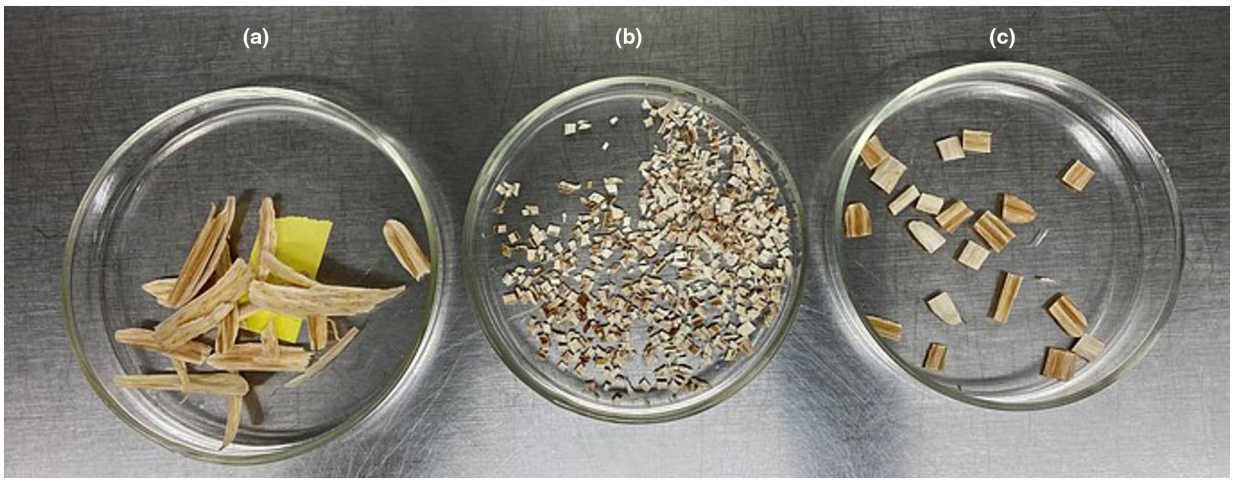

FIGURE 7 Chips sampled from the branches (a) and prepared for molecular tests (b) and culturing (c) Courtesy: K. Chahal, Forest Pathology Laboratory—Michigan State University (US).

3.3 | Isolation

Wood chips are pushed down into either 2% malt extract agar supplemented with streptomycin or potato dextrose agar with lactic acid (Appendix 4). Plates are incubated at 20–25°C in either light or dark. Plates should be examined daily from the second day and for approximately 8–10 days.

Growth characteristics in culture

Mycelium usually appears around the chips after 3–5 days (Figures 8 and 9). The conidiophores are usually formed in loose clusters and are frequently produced only on certain portions of the mycelium. Fine mycelial branches can be observed, which are frequently curved or curled. On both media the culture is greyish with a characteristic sweet, fruity smell. Endoconidiophores and endoconidia are produced.

If the two mating types of the fungus are present, perithecia appear after 7–10 days, exuding a sticky creamy-white mass of ascospores. However, in practice, isolates from the xylem are almost exclusively of one mating type only. In order to see the perithecia, it is necessary to culture the fungus together with reference isolates of the two mating types of *B. fagacearum*.

4 | IDENTIFICATION

Identification can be done either based on micro morphological features of the purified fungus on culture medium with a subsequent confirmation with a molecular test or directly based on a molecular test on the cultures.

4.1 | Morphological characteristics

In addition to the growth characteristics in culture (see Section 3.3), the morphology of fungal structures in culture should be as described below:

Hyphae—subhyaline to brown, septate, branched, 2.5–6 µm in diameter;
Conidiophores—subhyaline to brown, septate, simple or branched, 2.5–5 µm in diameter, and often slightly tapered towards the apex;
Conidia—hyaline, continuous, cylindrical, truncate at each end, 2–4.5 × 4–22 µm (mean 3 × 6.5 µm), endogenous and catenulate;
Sclerotia—sometimes present, tan to black, loosely knit, irregular in shape, up to 2.5 mm in diameter;
Perithecia—flask-shaped, black, spheroidal base, 240–380 µm in diameter, and with an erect beak 250–450 µm long;
Ascospores—hyaline, one-celled, elliptical, 2–3 × 5–10 µm, exuded in a sticky creamy-white mass.

Pictures of fungal structures can be seen in de Beer et al. (2017, https://mycokeys.pensoft.net/article/20657/element/2/12/).

When morphology is consistent with the description of *B. fagacearum*, molecular confirmation should be performed for a definitive identification.

Several similar fungi have been reported from oak. The *Ophiostoma* species can be distinguished from *B. fagacearum* because they have a *Leptographium*, *Graphium* or *Sporotrix* anamorph. *Ceratocystis* species are more similar to *B. fagacearum* but can be distinguished, in particular, by the size and shape of the ascospores or based on molecular tests.

FIGURE 8 Cultures of *Bretziella fagacearum* growing from chips of oak xylem onto 2% Difco malt extract agar containing 100 mg L^{-1} streptomycin.

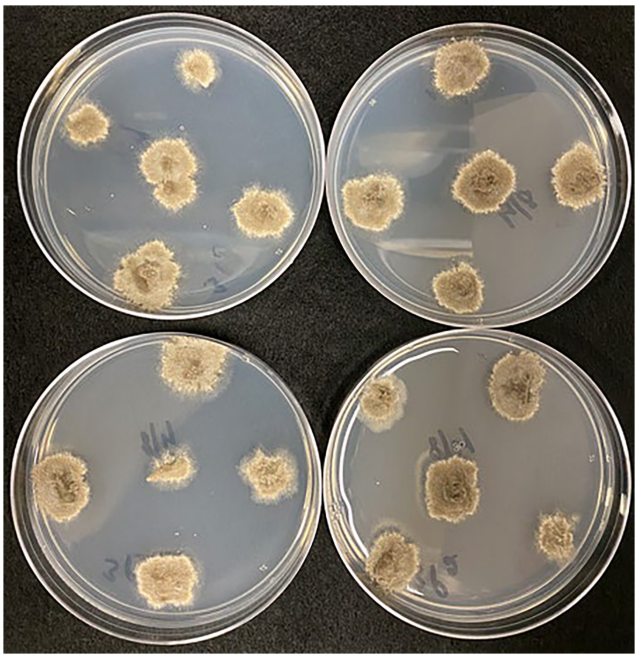

FIGURE 9 Cultures of *Bretziella fagacearum* growing from chips of oak xylem onto potato dextrose agar with lactic acid. Courtesy: K. Chahal, Forest Pathology Laboratory—Michigan State University (US).

4.2 | Molecular tests

The following tests can be used for identification

- Real-time PCR (Yang & Juzwik, 2017—modified by Fera), described in Appendix 2;
- Real-time PCR (Lamarche et al., 2015), described in Appendix 3;
- Internal transcribed spacer (ITS) sequencing (see PM 7/129 DNA barcoding as an identification tool for a number of regulated plant pests, EPPO, 2021).

5 | REFERENCE MATERIAL

American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852-1776, USA. Fax: +1 301 231 5826.

Westerdijk Fungal Biodiversity Institute, P.O. Box 85167 3508AD, Utrecht, The Netherlands.

ITS sequences are available in EPPO-Q-bank with links to specimens in collections.

A full genome sequence is available at: https://genome.jgi.doe.gov/portal/Brefa1/Brefa1.download.html.

6 | REPORTING AND DOCUMENTATION

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

7 | PERFORMANCE CHARACTERISTICS

When performance characteristics are available, these are provided with the description of the test. Validation data is 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, etc.)

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

J. Juzwik, Research Plant Pathologist, Northern Research Station, US Department of Agriculture—Forest Service, US.

M.L. Sakalidis, Assistant Professor—Michigan State University, US.

P.P. Parra Giraldo, EURL Fungi and Oomycetes, ANSES Plant Health Laboratory, Mycology Unit, Nancy, 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.int.

10 | PROTOCOL REVISION

A regular 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.

ACKNOWLEDGEMENTS

This protocol was originally drafted by:

R. Pieters, Plantenziektenkundige Dienst (the Netherlands) (retired).

J.N. Gibbs, Forest Research (UK) (retired).

The revision was drafted by A.V. Barnes & A. Elliott, Fera Science Ltd (York, GB) and P.P. Parra Giraldo EURL Fungi and Oomycetes (Nancy, FR). It was reviewed by the Panel on Diagnostics in Mycology. The valuable contributions of M.L. Sakalidis (US) and J. Juzwik (US) to this protocol are acknowledged.

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APPENDIX 1 - NESTED PCR (YANG & JUZWIK, 2017)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. The test was originally developed for detection of *Bretziella fagacearum* from sapwood tissues of symptomatic oak species. No data is available for other hosts.

1.2. The test was developed by Yang & Juzwik (2017) using primers originally developed and validated by Wu et al. (2011).[3]

[3]The primer CF01 described in Yang & Juzwik (2017) lacks three nucleotides compared with Wu et al. (2011). The author confirmed that this was an editorial error.

1.3. The PCR test targets a region of the rDNA internal transcribed spacer (ITS).

1.4. Oligonucleotides:

- First round PCR (generic primers)

Forward primer	ITS1-F	5′-TCCGTAGGTGAACC TGCGG-3′
Reverse primer	ITS4	5′-TCCTCCGCTTATTGATATGC-3′

- Second round PCR (specific primers)

			Amplicon size
Forward primer	CF01	5′-GGCAGGGACTTCTT TCTT-3′	280 bp
Reverse primer	CF02	5′-AAGGCTTGAGTGGT GAAA-3′	

1.5. The PCR system used was a Mastercycler (Eppendorf, AG, Hamburg, Germany).

2. Methods

2.1. Nucleic acid extraction and purification

Total DNA can be extracted following the extraction protocol described by Yang & Juzwik (2017) using the commercial DNA extraction kit (see below).

2.1.1. Between 0.18 and 0.2 g of drilled shavings of symptomatic *Quercus* spp. should be transferred to a sterile 2 mL microcentrifuge tube containing two 4.5 mm metal beads. Samples are homogenized by vortexing for 60 s in the presence of 1.4 mL lysis buffer (QIAmp DNA Stool Kit; Qiagen).

2.1.2. DNA is extracted from samples using a QIAmp DNA Stool Kit (Qiagen) according to the manufacturer's instructions.

2.1.3. DNA should preferably be stored at approximately −20°C.

2.2. Conventional PCR

2.2.1. Master Mix (round 1)

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water	N.A.	9.5	N.A.
GoTaq Green Master Mix (Promega)	2×	12.5	1×
Forward primer (ITS1-F)	10 μM	0.5	0.2 μM
Reverse primer (ITS4)	10 μM	0.5	0.2 μM
Subtotal		23	
Genomic DNA extract		2	
Total		25	

2.2.2. Master Mix (round 2)

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water	N.A.	10.5	N.A.
GoTaq Green Master Mix (Promega)	2×	12.5	1×
Forward primer (CF01)	10 μM	0.5	0.2 μM
Reverse primer (CF02)	10 μM	0.5	0.2 μM
Subtotal		24	
Amplicons obtained from round one		1	
Total		25	

2.2.3. PCR conditions (for both rounds): Initial DNA denaturation/DNA polymerase activation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and elongation at 72°C for 2 min; and a final extension of 72°C for 7 min.

2.3. Sequencing

The second round PCR amplicon should be sequenced. The PCR product should be cleaned with CleanSeq (Agencourt) magnetic beads following the manufacturer's protocol. The PCR product should be sent for two-way sequencing and a trimmed consensus sequence generated. This can be compared by BLAST with *B. fagacearum* reference sequences deposited in the GenBank database (e.g. KU042044).

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 extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives owing to contamination during the preparation of the reaction mix—application of the amplification procedure to 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 not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPCs) can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- Specific amplification or co-amplification of nucleic acid from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid).

IPC primers are not included in the Master Mix table (see points 2.2.1 and 2.2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

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.

3.2. Interpretation of results: in order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC, PAC a band of 280 bp is visualized (second PCR). If relevant, a band of the expected size is visualized for the IC and IPC.

When these conditions are met:

- A test will be considered as positive if a single amplicon of 280 bp is visualized for the second PCR.[4]
- A test will be considered negative if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

[4]However, for confirmation of the identity of *B. fagacearum* sequencing should be performed.

4. **Performance characteristics available**

Data from Wu et al. (2011).

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section on validation data, https://dc.eppo.int/validation_data/validationlist).

4.1. Analytical sensitivity data: the analytical sensitivity was determined by Wu et al. (2011) using a serial dilution between 100 and 0.01 pg of *B. fagacearum* DNA from culture. The analytical sensitivity was 1 pg per reaction for the nested PCR.

4.2. Analytical specificity data:

Analytical specificity of the primers (CFO1, CF02) was assessed by Wu et al. (2011).

Inclusivity 100%, evaluated on three isolates of *B. fagacearum*.

Exclusivity 100%—the primers were evaluated on 18 fungal isolates including closely related *Ceratocystis* spp. and other species which could be isolated from infected wood samples. But see also 4.4.

4.3. Diagnostic sensitivity

100% based on eight samples of wood.

4.4. Additional information

When using this nested PCR on DNA extracted from the bark of an oak-wilt suspect tree, an amplicon (~300 bp) which is close in size to the one observed for *B. fagacearum* (280 bp) was observed for isolates of *Cladosporium* spp. in different laboratories in the US (L. Miles & B. Arenz, personal communication, 2023).

APPENDIX 2 - REAL-TIME PCR (YANG & JUZWIK, 2017—MODIFIED)

The test below differs from the one described in the original publication (see 1.2).

The test below differs from the one described in the original publication and was modified by Fera Science Ltd.

It is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. The test was originally developed for detection of *B. fagacearum* from sapwood tissues of diseased *Quercus* spp. It can also be used for pure cultures. No data is available for other hosts.

1.2. The test was developed by Yang & Juzwik (2017) based on a test originally developed by Kurdyla & Appel and presented at a conference in 2011 (Kurdyla & Appel, 2011), but not published. This test was subsequently validated by Fera Science Ltd, with minor modifications.
1.3. The PCR test targets a region of the ITS.
1.4. The amplicon length is 100 bp.
1.5. Oligonucleotides:

Forward primer	CfP2-01	5′-TGGCAGGGACTTCTTTC TTCA-3′
Reverse primer*	CfP2-03	5′-TTGTTAAATGCAACTCA GCAATGA-3′
Probe	CfP2	5′-FAM-ATGTTTCTGCCAGT AGTATT-BHQ1-3′

* CfP2-03 = CfP2-02 with mismatch between the target sequence and the reverse primer CfP2-02 (detected at the second base from the 5′ end). The primer has a G in this position, which resulted in a mismatch to the A in the target sequence. As the mismatch is at the 5′ end, it was not expected to have a great impact on the performance of the test. However, the mismatching base was replaced by a T in the modified primer CfP2-03 in this study.

1.6. The test was validated on multiple real-time PCR systems.

2. Methods

2.1. Nucleic acid extraction and purification

Total DNA can be extracted following the in-house extraction protocol of Fera Science Ltd (see below). Samples of the sapwood of infected *Quercus* spp. are taken, the bark removed and symptomatic areas of the underlying tissue (discoloured streaks) sampled (approximately 0.1–0.5 g) and added to a 5 mL screw-top tube. DNA is extracted from infected plant material using the cetyl trimethylammonium bromide (CTAB)-based method.

2.1.1. 5 mL of CTAB buffer 2% (Appendix 4) is added to the tube, and the sample is ground using a Geno Grinder for 10 min (SPEX Sample Prep). The ground sample is centrifuged at 6000***g*** for 3 min. A 700 µL aliquot of supernatant is added to 200 µL chloroform in an Eppendorf tube (1.5 mL) and mixed by vortexing, then centrifuged at 13 000***g*** for 5 min. The top aqueous layer is mixed with 500 µL of isopropanol and 50 µL of Magnesil Paramagnetic Particles (MPPs, Promega). DNA is extracted using a KingFisher (mL) instrument by transferring the MPPs sequentially through 1 mL GITC Buffer (5.25 M guanidine thiocyanate; 50 mM Tris–HCl pH 6.4, 20 mM EDTA, 1.3% [w/v] Triton X-100) and two washes in 1 mL of 70% ethanol, followed by elution in 200 µL 1× TE buffer.

2.1.2. DNA should preferably be stored at approximately −20°C.

2.2. Real-time polymerase chain reaction—real-time PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9	N.A.
iTaq Supermix DNA Master Mix (BioRad)	2×	12.5	1×
Forward primer (CfP2-01)	7.5 µM	1	0.3 µM
Reverse primer (CfP2-03)	7.5 µM	1	0.3 µM
Probe 1 (CfP2)	5 µM	0.5	0.1 µM
Subtotal		24	
DNA extract		1	
Total		25	

2.2.2. PCR conditions: initial DNA denaturation/DNA polymerase activation at 95°C for 2 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 extraction and amplification of the target organism and target nucleic acid, respectively:

- 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.
- 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 extract spiked with the target organism).
- NAC to rule out false positives owing to contamination during the preparation of the reaction mix—application of the amplification procedure to molecular-grade water that was used to prepare the reaction mix.
- 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 not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, IPCs can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- Specific amplification or co-amplification of nucleic acid control from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid.

IPC primers are not included in the Master Mix table (see point 2.2.1). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Other possible controls

- IC to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results. In order to assign results from PCR-based tests the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation was carried out at Fera Science Ltd in accordance with PM7/98.

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section on validation data https://dc.eppo.int/validation_data/validationlist).

4.1. Analytical sensitivity data

To determine the analytical sensitivity, a serial dilution of DNA from two isolates of *B. fagacearum* was tested in the presence of host DNA (2.4 µg per real-time PCR reaction). The limit of detection of the test was 125 fg per real-time PCR reaction. The standard curve slope was 3.34 and the linear correlation r^2 was 0.99.

4.2. Analytical specificity data

Inclusivity 100% evaluated on two cultures of the target species.

Exclusivity 100% evaluated on 14 non-target fungal isolates commonly present on oak including *Armillaria mellea*, *Biscogniauxia mediterranea*, *Botrytis cinerea*, *Botryosphaeria dothidea*, *Botryosphaeria obtusa*, *Diplodia corticola*, *Eutypa lata*, *Ganoderma lucidum*, *Heterobasidion annosum* and *Stereum hirsutum*. No cross-reactions were observed.

In addition to *in vitro* tests, *in silico* analyses were conducted. ITS reference sequences corresponding to nine species (*Apiognomonia errabunda*, *Botryosphaeria stevensii*, *Cytospora ambiens*, *Cytospora chrysosperma*, *Erysiphe alphitoides*, *Inonotus hispidus*, *Neonectria ditissima*, *Phytophthora cinnamomi* and *Phytophthora plurivora*) were retrieved from GenBank and were scrutinized for possible oligo binding sites. None of the tested species would be expected to have cross-reactions.

4.3. Data on repeatability and reproducibility

The repeatability and reproducibility of the limit of detection were evaluated with three replicate real-time PCR reactions and the experiment was repeated five times by one user and twice by a second user on different real-time PCR machines. 100% repeatability and reproducibility were obtained at the limit of detection (125 fg per real-time PCR reaction).

4.4. Diagnostic sensitivity

To assess diagnostic sensitivity, three samples from naturally infected trees and 11 samples from artificially infected trees were tested along with three samples from healthy control trees. All naturally infected and artificially infected samples tested positive for *B. fagacearum*.

4.5. Diagnostic specificity

To assess diagnostic specificity, three samples from naturally infected trees and 11 samples from artificially infected trees were tested along with three samples from healthy control trees. All healthy control trees tested negative with for *B. fagacearum*.

APPENDIX 3 - REAL-TIME PCR (LAMARCHE ET AL., 2015)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This test was originally developed for the identification of *B. fagacearum* in culture and for detection in sapwood of symptomatic *Quercus* spp. No data is available for other hosts.
1.2. The test was developed by Lamarche et al. (2015).
1.3. The PCR test targets a region of the elongation factor-1 (EF-1) gene.
1.4. The amplicon length is 92 bp.
1.5. Oligonucleotides:

Forward primer	Cfagacearum_F315	5′-GTCTGTAGAAGGGGG-3′
Reverse primer	Cfagacearum_R406	5′-CTCCATTCTTTACTACAACC-3′
Probe	Cfagacearum_T357	5′-6-FAM-AGAAGTAAC/ZEN/TGGACAACCGTCT-IFBQ-3′

This probe with an internal ZEN and an Iowa Black FQ quencher can be ordered at Integrated DNA Technologies, Inc.
1.6. Real-time PCR was performed with an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies).

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. Cultures: DNA from fungal culture can be extracted using the phenol/chloroform extraction protocol in Lamarche et al. (2015). In preliminary studies, Fera Science Ltd evaluated this test using routine DNA extraction protocols. Plant material: Lamarche et al. 2015 used in their study purified DNA from environmental samples provided by J. Juzwik, DNA was extracted using routine DNA extraction protocols in the different laboratories. It is recommended to follow the procedure described in Appendix 1 point 2.1.

2.1.2. DNA should preferably be stored at approximately −20°C.

2.2. Real-time polymerase chain reaction – real-time PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	2.6	N.A.
QuantiTect Multiplex PCR NoROX Master Mix	2×	5	1×
Forward primer (Cfagacearum_F315)	10 µM	0.6	0.6 µM
Reverse primer (Cfagacearum_R406)	10 µM	0.6	0.6 µM
Probe (Cfagacearum_T357)	5 µM	0.2	0.1 µM
Subtotal		9	
DNA extract		1	
Total		10	

2.2.2. PCR conditions

Initial DNA denaturation/DNA polymerase activation at 95°C for 15 min, followed by 50 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 90 s.

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:

- 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.
- 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 extract spiked with the target organism).
- NAC to rule out false positives owing to contamination during the preparation of the reaction mix—application of the amplification procedure to molecular-grade water that was used to prepare the reaction mix.
- 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 not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, IPCs can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- Specific amplification or co-amplification of nucleic acid from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid.

IPC primers are not included in the Master Mix table (see point 2.2.1). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Other possible controls

- IC to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results. In order to assign results from a PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation data is reported in Lamarche et al. (2015).

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section on validation data https://dc.eppo.int/validation_data/validationlist).

4.1. Analytical sensitivity data

The limit of detection (LOD) of the test was 10 copies of the target gene; this was the lowest DNA concentration with 95% successful amplification from 20 replicates of the TaqMan real-time PCR reactions.

4.2. Analytical specificity data

Inclusivity 100% evaluated on six isolates of *B. fagacearum*.

Exclusivity 100% evaluated on 62 isolates of 25 closely related species.

4.3. Repeatability data

The test was found to be highly repeatable by comparing the *Ct* values from different real-time PCR runs on DNA samples at a concentration of 5000 copies of the target gene region.

4.4. Diagnostic sensitivity

Diagnostic sensitivity was evaluated using 23 environmental samples (supplied as purified DNA samples) including sapwood from infected oak trees and three species of sap feeding beetles found on oak wilt mats. The test successfully detected the target pathogen from all the positive environmental samples. No negative environmental samples were available to be included.

APPENDIX 4 - MEDIA AND BUFFERS

1. Media

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

Potato dextrose agar (PDA) with lactic acid

PDA	39 g*
Distilled water	1 L

* Or according to manufacturer's instructions.

After autoclaving, PDA is amended with 1 mL of lactic acid.

2% malt extract agar (MEA) with streptomycin

Malt extract (BD-Difco)	20 g
Agar	20 g
Distilled water	1 L

After autoclaving, MEA is amended with 100 mg of streptomycin.

2. Buffers

CTAB buffer with sodium phosphate buffer (Bell et al., 1999)

Sodium phosphate buffer (see below)	120 mL
CTAB (final concentration 2%)	20 g
NaCl (final concentration 1.5 M)	87.66 g
Make up to 1 L with distilled water	

Add 500 mL of the distilled water to a beaker, add required CTAB, mix well, then add the required NaCl and mix well. Once ingredients are fully dissolved add the 1 M sodium phosphate buffer, stirring continuously before making up to the required volume with distilled water.

1 M sodium phosphate buffer (pH 8)

1 M solution of Na_2HPO_4	71 g in 500 mL of water
1 M solution of NaH_2PO_4	6 g in 50 mL of water

Make 500 mL of sodium phosphate buffer by mixing 466 mL of 1 M Na_2HPO_4 and 34 mL of 1 M NaH_2PO_4 and adjust pH to 8.