National Diagnostic Protocol

Chestnut blight

Caused by *Cryphonectria parasitica*

NDP 11 V2

Cryphonectria parasitica is now present in Australia. This protocol is being kept as a resource but will not be reviewed or updated.

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Purpose

National Diagnostic Protocols (NDPs) are diagnostic protocols for the unambiguous taxonomic identification of plant pests. NDPs:

- are a verified information resource for plant health diagnosticians
- are consistent with ISPM No. 27 – Diagnostic Protocols for Regulated Pests
- provide a nationally consistent approach to the identification of plant pests enabling transparency when comparing diagnostic results between laboratories; and,
- are endorsed by regulatory jurisdictions for use (either within their own facilities or when commissioning from others) in a pest incursion.

Where an International Plant Protection Convention (IPPC) diagnostic protocol exists it should be used in preference to NDPs although NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

https://www.ippc.int/core-activities/standards-setting/ispms

Process

NDPs are facilitated and endorsed by the Subcommittee on Plant Health Diagnostics (SPHD). SPHD reports to Plant Health Committee and is Australia's peak technical and policy forum for plant health diagnostics.

NDPs are developed and endorsed according to Reference Standards developed and maintained by SPHD. Current Reference Standards are available at
http://plantbiosecuritydiagnostics.net.au/sphd/sphd-reference-standards/

NDPs are living documents. They are updated every 5 years or before this time if required (i.e. when new techniques become available).

Document status

This version of the National Diagnostic Protocol (NDP) for *Cryphonectria parasitica* is current as at the date contained in the version control box below.

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http://plantbiosecuritydiagnostics.net.au/resource-hub/priority-pest-diagnostic-resources/

Further information

Inquiries regarding technical matters relating to this project should be sent to:
sphds@agriculture.gov.au

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1 INTRODUCTION

Cryphonectria parasitica (Murrill) Barr is a bark-inhabiting fungus causing the disease called chestnut blight. The virulent form of the disease develops quickly in chestnut (Castanea) species causing stem cankers, ringbarking of stems, mortality of the distal part of the tree, and often mortality of the whole tree (Heiniger and Rigling 1994).

Hypovirulence due to infection of the fungus by the double-stranded RNA virus, *Cryphonectria hypovirus* 1 (CHV 1), has however, enabled the regrowth of chestnut trees and stands (Hillman and Suzuki 2004; Root *et al*. 2005). Virulent and hypovirulent strains of the fungus give rise to different types of cankers and this may, in some cases, make detection and identification difficult. In more tolerant hosts (mostly *Quercus petraea* and less often *Quercus robur*, *Quercus ilex* and other oaks) or in its hypovirulent form, chestnut blight appears as perennial 'healing' cankers or superficial infections of the bark that rarely cause the death of branches, stump, sprouts or the whole tree. Information on biology and geographical distribution can be found in EPPO/CABI (1997) and OEPP/EPPO (2005). See also; Fulbright (1999), Heiniger and Rigling (1994), Roane et al. (1986).

C. parasitica can attack trees of any age. It occurs over the complete range of *Castanea dentata* and does not appear to be limited by climate. Infection rate and lesion growth was shown to be greater during summer than in winter (Guerin *et al*. 2001; Guerin and Robin 2003).

1.1 Host range

Chestnut Blight causes mild cankers on Asian chestnut trees (*C. crenata* and *C. mollissima*). European Chestnut (*C. sativa)* is also very susceptible to Chestnut Blight. Other species, e.g. *Quercus* spp., *Castanopsis, Acer. Rhus typhina* and *Carya ovata* show some susceplibilily (EPPO/CABI 1997). The occurrence on other rare hosts (e.g. *Eucalyptus*) has not been conclusively proven and are considered doubtful (Gryzenhout *et al* 2009).

2 TAXONOMIC INFORMATION

Kingdom: Fungi

Phylum Ascomycota

Class Sordariomycetes

Order Diaporthales

Family Cryphonectriaceae

Genus Cryphonectria

Accepted name: *Cryphonectria parasitica* (Murrill) M.E. Barr

Disease common name: Chestnut Blight

Synonyms:

- *Diaporthe parasitica* Murrill

- *Endothia gyrosa* var. *parasitica* (Murrill) Clinton

- *Endothia parasitica* (Murrill) P.J. Anderson & H.W. Anderson

- *Valsonectria parasitica* (Murrill) Rehm

Anamorph: *Endothiella parasitica* Roane

Recent taxonomic revisions have left only four species in *Cryphonectria*; *C. macrospora*, *C. nitschkei*, *C. parasitica* and *C. radicalis* (Gryzenhout *et al*. 2006c). Closely related genera are *Amphilogia*, *Chrysoporthe*, *Endothia*, *Rostraureum*, and the anamorphic genera *Aurapex* and *Ursicollum* (Gryzenhout *et al*. 2006a; Gryzenhout *et al*. 2006b; Gryzenhout *et al*. 2006c).

3 DETECTION

3.1 Symptoms

In the highly susceptible hosts, *Castanea dentata* and *Castanea sativa*, chestnut blight symptoms include girdling stem cankers, splitting bark and tree death. In more tolerant hosts (mostly *Quercus petraea* and less often *Quercus robur*, *Quercus ilex* and other oaks), chestnut blight appears as perennial 'healing' cankers or superficial infections of the bark that rarely causes the death of branches, stumps, sprouts or the whole tree . A hypovirulent form of the fungus may also cause perennial, healing cankers.

Cankers can be either swollen or sunken with stroma, the colour of which may vary from yellowish-brown or orange to red-brown. Flat, buff coloured mycelial fans under and throughout the bark. *C. parasitica* ascospores or conidia infect stems and branches via wounds and grow in the cambium, causing cankers that may eventually girdle the stem or branch and kill it. Branch points are a common site of entry as small wounds may be created by branch movements. On grafted trees, infections are most frequently found in the region of the graft, where callusing occurs. In coppices or orchards, infections are often located at the base of the stem (collars or insertion points). The cambium under the infected bark is killed and the bark appears sunken or swollen. The surface of the bark is cracked. There may be several cankers on a single plant or only one. The fungus may be visible as orange stromata, semi-immersed in the bark at a distance from the advancing edge of the canker lesion.

The characteristic orange stromatic tissue on the bark of the host is visible macroscopically, however a hand lens will facilitate visualization of fruiting structures within this. Several related species and genera produce similar orange stromata, but it is not possible to adequately differentiate these without a detailed laboratory examination.

Figure 1. Cankers caused by *Cryphonectria parasitica* on *Castanea sativa* in Victoria, showing the splitting bark and swollen appearance of the stem (photo courtesy of David Smith, DPI Victoria).

3.2 Pathogen description

From Gryzenhout et al (2009). Figure 2.

Ascomata on host gregarious or single, sometimes confluent, pulvinate, semi-immersed in bark, oramge, typically 200-350 µm high, 300-1200 µm diam., upper region eustromatic, lower region pseudostromatic, pseudoparenchymatous on edge of stroma, prosenchymatous in center. **Perithecia** usually valsoid, embedded beneath surface of bark at base of stromata, fuscous black, necke emerge at stromatal surface as black ostioles covered in orange stromatal tissue to form papillae extending up to 150 um above stromatal surface. **Asci** (30-)40-50(-60) x (7-)8(-9) µm, oblong ellipsoidal to subclavate, 8-spored. **Ascospores** (7.5-)8-9(-9.5) x 3.4-4(-4.5) µm, hyaline, ellipsoidal to fusoid, ends round, one median septum.

Conidiomata part of ascostromata as conidial locules or as separate structures, pulvinate, semi-immersed, orange uni- to multilocular structures with the same tissue morphology and structure as ascostromata, 10-390 µm high, 270-390 µm diam., with locules often convoluted, non-ostiolate. **Conidiophores** (10.5-)12-23(-34) µm long, occasionally with separating septa and branching, hyaline, conidiogenous cells 1.5-2(-2.5) µm wide, cylindrical or flask shaped with attenuated apices. **Conidia** (3-)3.5-4(-4.5) x 1-1.5 µm, hyaline, cylindrical, aseptate, exuded as orange droplets.

Holocryphia eucalypti (M. Venter & M.J. Wingf.) Gryzenh. & M.J. Wingf. is a morphologically similar fungus that occurs on chestnuts in Australia (J. Cunnington, pers. comm.). A full description can be found in Gryzenhout et al (2009). In contrast to *C. parasitica* the ascospores are single celled, and the conidia of *H. eucalypti* are about 0.5 µm thinner and 0.5 µm longer than the conidia of *C. parasitica*.

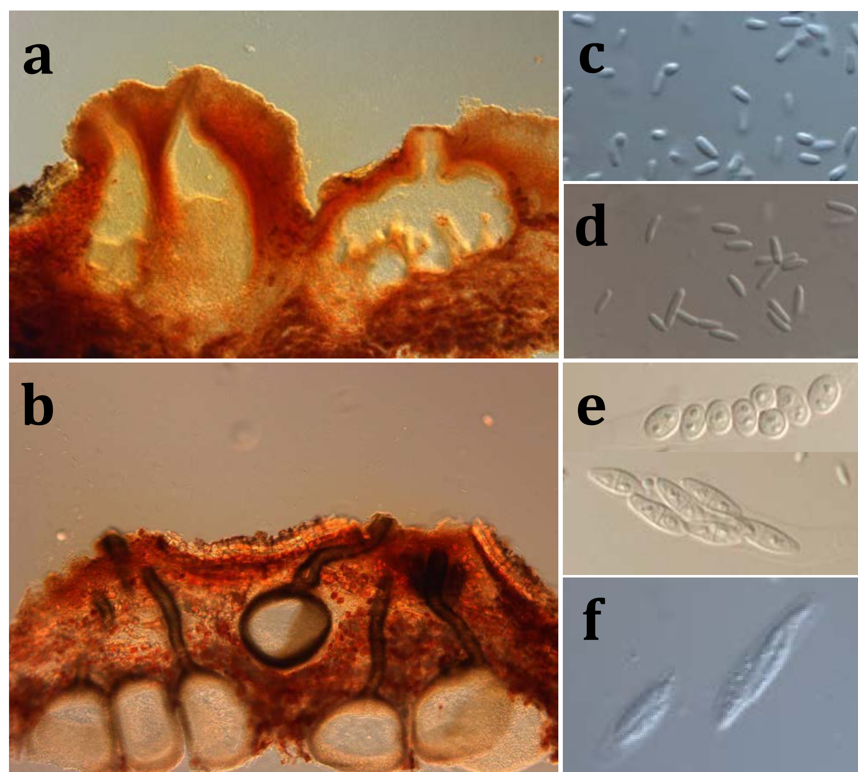

Figure 2. *Cryphonectria parasitica* conidiomata (a) and ascostromata (b). *C. parasitica* conidia (c) and ascospores (e). *Holocryphia eucalypti* conidia (d) and ascospores (f) (photos courtesy Quang Dinh, DPI Victoria)

4 IDENTIFICATION

As the *Endothiella* states of *Cryphonectria* and its relatives have overlapping morphological characters, detection of *C. parasitica* requires isolation into pure culture and DNA sequencing. This is especially important in Australia where the morphologically similar fungus *Holocryphia eucalypti* is common on chestnut.

4.1 Isolation

Equipment

1. Scalpel
2. Sterile scalpel blades
3. Petri dishes
4. Bunsen burner or spirit burner

Reagents

1. Potato Dextrose Agar (PDA) medium with antibiotic such as tetracycline
2. Sodium hypochlorite
3. 70% ethanol

Method

1. With a knife, axe or borer, take a piece of symptomatic bark from the advancing edge of the lesion. Alternatively, if mycelial fans are observed under the bark, the fungus can be readily cultured from these. If the sample is debarked wood, take a piece of the sapwood.

Figure 3. Isolation from a typical chestnut blight canker (photo courtesy Ramez Aldaoud, DPI Victoria).

2. In the laboratory, remove small chips (up to a few millimetres) from the margin of the tissue with a scalpel, or extract a core of bark, including outer bark, with a borer. Surface-sterilize the sample for up to 2 min in a solution of sodium hypochlorite (commercial bleach, 2- 6% active chlorine), and rinse in sterile distilled water.
3. Transfer aseptically onto PDA and incubate at room temperature for up to two weeks. Incubate cultures at room temperature either under natural light or with an alternating cycle of 12 h fluorescent light and 12 h darkness to induce colony pigmentation and pycnidia formation.

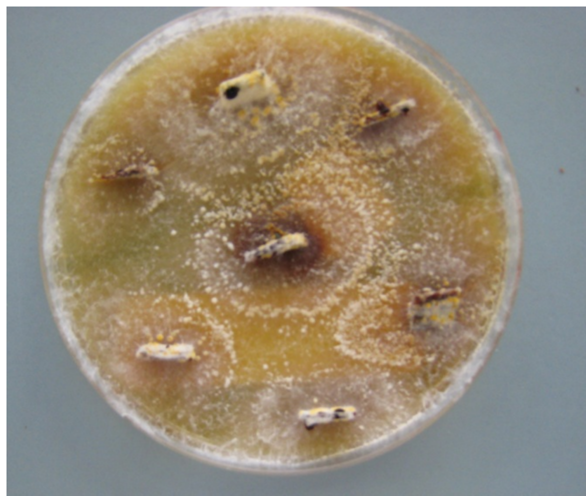

Figure 4. Primary isolation culture of *Cryphonectria parasitica* on PDA with tetracycline (photo courtesy Ramez Aldaoud, DPI Victoria).

4. Cultures of virulent isolates of *C. parasitica* can grow at 5mm per day. Young mycelium is white, turning light yellow then orange-yellow, becoming red-orange to violet after some months. Hypovirulent isolates generally remain white.
5. Subculture any suspect colonies onto PDA and use beta tubulin gene sequencing for identification.

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4.2 Beta tubulin gene sequencing

Given the small (or non-existent) differences in conidial dimensions between members of the Cryphonectriaceae, all suspect cultures should be tested by DNA sequencing. The ribosomal DNA ITS region will correctly identify *C. parasitica*, but this region is prone to long strings of single nucleotides that might interfere with sequencing chemistry (J. Cunnington, pers. comm.). The β-tubulin gene exons 6-7 region used by Myburg et al (2004a) is a more reliable target for sequence based identifications.

4.2.1 DNA extraction

DNA should be readily extracted from mycelium using most commercial column or silica based kits designed for filamentous fungi. The mycelium can be disrupted using standard grinding or bead beating methods.

4.2.2 PCR amplification and sequencing

Equipment and reagents

1. Pipettes and aerosol resistant tips
2. Thermocycler
3. Taq polymerase and PCR components
4. PCR product detection system

Methods

Each 25 µl reaction, contains 1 X Polymerase buffer, 1.5mM $MgCl_2$, 0.2 mM each dNTP, 0.25 mM each primer (Forward Bt1a 5'-TTCCCCCGTCTCCACTTCTTCATG-3', Reverse Bt1b 5'-GACGAGATCGTTCATGTTGAACTC-3', Glass and Donaldson 1995), 0.5 U DNA polymerase and 1 µl of the DNA extract. Thermocycler program is 94° x 5 min, 35 cycles of 94° x 30s, 55° x 30s, 72° x 30s, followed by 72° x 5 min. Run a 5 µl aliquot on a 1-1.5% agarose gel to confirm successful amplification (540 bp product) before proceeding with sequencing.

Clean the PCR product using an appropriate kit and sequence the product directly in both directions using standard laboratory procedures with primers Bt1a and Bt1ab.

4.2.3 Comparison with known *Cryphonectria parasitica* beta tubulin gene sequences

Final sequences should be determined using the chromatograms from both directions. Sequences should be compared to GenBank sequences using Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or similar software. *Cryphonectria parasitica* beta tubulin gene sequences should be 99-100% similar to typical *C. parasitica* sequences such as AY697949. There are some GenBank sequences that are only 97% similar to AY697949, but these are likely to be *C. parasitica* as well. An automated phylogenetic tree can also be constructed from the NCBI Blast results.

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Global Invasive Species Database
http://www.issg.org/database/species/ecology.asp?si=124&fr=1&sts
EPPO Data Sheet on Quarantine Pests
http://www.eppo.org/QUARANTINE/fungi/Cryphonectria_parasitica/ENDOPA_ds.pdf
PaDIL:
http://www.padil.gov.au/..

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This protocol was reviewed by Dr I Pascoe and NSW DPI (EMAI).

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