

National Diagnostic Protocol for Detection of *Neonectria ditissima* (European canker)



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This version of the National Diagnostic Protocol (NDP) for *Neonectria ditissima* is current as at the date contained in the version control box on the front of this document.

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

The most current version of this document is available from the SPHDS website:
<http://plantbiosecuritydiagnostics.net.au/resource-hub/priority-pest-diagnostic-resources/>

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1 INTRODUCTION

This diagnostic protocol provides technical information for the identification of *Neonectria ditissima* (synonym *N. galligena*), the causal agent of European canker, and has been written to provide clear general operational information and references.

Neonectria ditissima (anamorph *Cylindrocarpon heteronema*) is a fungal pathogen of pome fruit and many species of hardwood forest trees worldwide. It causes economically important diseases such as European canker on apple and beech bark canker on beech. Cankers develop on the woody tissues, girdling and killing branches and, occasionally, the whole tree. The pathogen was present in apple orchards in Spreyton, Tasmania, from about 1954 but was eradicated by 1991. Australia is currently free of the disease.

European canker is present in almost all pome-producing regions of the world and losses are greatest in humid climates of northern Europe, northwest America and southern Chile. Young and old trees can be affected in apple orchards and loss of young trees due to European canker has been reported to range from 1-10% (Berrie *et al.* 2000, Lovelidge 1995), sometimes requiring replanting of the whole orchard (Grove 1990). *N. ditissima* also causes eye-rot of apple fruit, which may develop in the orchard or during storage, with losses of up to 80% of the apple crop reported in Europe and America (Berrie *et al.* 2000). Infection of the fruit occurs on the tree through open calyxes, lenticels, scab lesions or insect wounds, and may remain latent (i.e. symptomless), developing post-harvest into storage rots (Dewey *et al.* 1995). Internationally, the fungus is spread via vegetative propagation plant material, rooted plants (Howard *et al.* 1974, McCracken *et al.* 2003) and fruit (CPC 2005).

1.1 Host range

N. ditissima has a wide host range, having been recorded on more than 60 tree and shrub species from 20 genera. It is prevalent in commercial apple (*Malus* spp.) and pear (*Pyrus* spp.) orchards from most temperate growing regions of the world (Langrell 2002), and beech forests in North America. There is no data on the susceptibility or otherwise of Australian native flora. The pathogen has been recorded on three species of New Zealand native flora, however, the source of infection for these hosts is unknown.

Recorded plant families and genera (Edwards *et al.* 2006)

- Maple, *Aceraceae* – *Acer*
- Sumac, *Anacardiaceae* - *Rhus*
- Holly, *Aquifoliaceae* – *Ilex*
- Birch, *Betulaceae* - *Alnus*, *Betula*
- Dogwood, *Cornaceae* – *Cornus*
- *Corylaceae* (*Betulaceae*) - *Corylus*, *Carpinus*
- Legume, *Fabaceae* – *Cercis*
- Beech, *Fagaceae* - *Castanea*, *Fagus*, *Quercus*
- Chestnut, *Hippocastanaceae* – *Aesculus*
- Walnut, *Juglandaceae* - *Carya*, *Juglans*
- Laurel, *Lauraceae* – *Umbellularia*
- Mallow, *Malvaceae* – *Tilia*
- Mahogany, *Meliaceae* – *Swietenia*
- Sour Gum, *Nyssaceae* – *Nyssa*
- Olive, *Oleaceae* – *Fraxinus*
- Rose, *Rosaceae* - *Amelanchier*, *Crataegus*, *Cydonia*, *Malus*, *Pyrus*, *Sorbus*, *Eriobotrya*
- Citrus, *Rutaceae* – *Citrus*
- Willow, *Salicaceae* - *Populus*, *Salix*
- Elm, *Ulmaceae* – *Ulmus*

- Violet, *Violaceae* – *Viola*

2 TAXONOMIC INFORMATION

Kingdom Fungi
 Phylum Ascomycota
 Order Hypocreales
 Family Nectriaceae
 Genus *Neonectria*
 Species *Neonectria ditissima*

2.1 Names and Synonyms

Until recently, *Nectria galligena* was the accepted name of this pathogen and much of the literature regarding European canker on apple uses that name. In 1999, the fungus was reassigned to the genus *Neonectria* as *Neonectria galligena*. In 2006, Castlebury *et al.* re-examined relationships between *Neonectria* species and determined that *Neonectria ditissima* and *N. galligena* were the same species, as were their respective anamorphs, *Cylindrocarpon willkommii* and *C. heteronema*. The current accepted name for this pathogen is *Neonectria ditissima* (anamorph *Cylindrocarpon heteronema*) (Samuels *et al.* 2006; www.Mycobank.org)

Teleomorph

- *Neonectria ditissima* (Tul. and C. Tul.) Samuels and Rossman 2006
- *Nectria ditissima* Tul. and C. Tul. 1865
- *Cucurbitaria ditissima* (Tul. and C. Tul.) Kuntze 1898
- *Nectria ditissima* var. *arctica* Wollenw. 1926
- *Nectria galligena* Bres. 1901
- *Neonectria galligena* (Bres.) Rossman and Samuels 1999
- *Dialonectria galligena* (Bres.) Petch ex E.W. Mason and Grainger 1937

Anamorph

- *Cylindrocarpon heteronema* (Berk. and Broome) Wollenw. 1928
- *Fusarium heteronemum* Berk. and Broome 1865
- *Fusarium mali* Allesch. 1892
- *Cylindrocarpon mali* (Allesch.) Wollenw. 1928
- *Fusarium willkommii* J. Lindau 1909
- *Cylindrocarpon willkommii* (Lindau) Wollenw. 1928
- *Ramularia heteronema* (Berk. and Broome) Wollenw. 1916

Common names

- European canker
- Nectria canker
- Nectria eye-rot (fruit)
- Apple canker
- Target canker
- Pear canker
- Beech bark canker
- Fruit tree canker
- Perennial canker

3 DETECTION

3.1 Plants capable of hosting *N. ditissima*

Apple (*Malus domestica*) is the most susceptible host for *N. ditissima* but there is variation in susceptibility between apple varieties (Braun 1997). Other hosts of horticultural importance include European pear (*Pyrus communis*), Asian pear (*Pyrus pyrifolia*), loquat (*Eriobotrya japonica*) and walnut (*Juglans regia*). *N. ditissima* also causes perennial cankers on a range of forest broad-leaved trees in temperate regions and other trees and shrub species worldwide (Braun 1997, Flack and Swinburne 1977, Plante *et al.* 2002). These include many trees that are commonly used as windbreaks around fruit orchards in Europe such as poplars (*Populus* spp.), willows (*Salix* spp.) and ornamentals such as oaks (*Quercus* spp.), birches (*Betula* spp.), elms (*Ulmus* spp.) and maples (*Acer* spp.). Other woody plants susceptible to *N. ditissima* include crab apples (*Malus* spp.), beeches (*Fagus* spp.), ashes (*Fraxinus* spp.), mountain ash (*Sorbus aucuparia*) and walnut (*Juglans* spp.) (Anagnostakis and Ferrandino 1998, Barnard *et al.* 1989, Booth 1998, Lortie 1969, Ng and Roberts 1974).

3.2 Symptoms associated with *N. ditissima*

3.2.1 Apple – wood symptoms

The formation of cankers on woody tissue (twigs, branches and trunks) is the most obvious symptom of *Neonectria ditissima*. *N. ditissima* spreads within and between orchards by means of spores (asexual conidia and sexual ascospores) dispersed during rainy and windy weather conditions. Spores infect through natural wounds such as leaf scars at leaf fall (autumn) and artificial wounds such as pruning wounds.

The first sign of canker is a reddish brown spot around a leaf scar, spur or pruning wound or as small, inconspicuous dark depressions on young stems in spring and early summer, leading to twig death and spur death (Figure 1-2). If not pruned out, the disease can take over and kill the tree.



Figure 1 (a) Leaf scar infection resulting in (b) death of whole twig; apple cv. Royal Gala; Waikato, NZ. (Photo: J. Edwards).



Figure 2. (a) Leaf scar infection resulting in (b) twig and spur death; apple cv. Royal Gala; Waikato, NZ. (Photo: J. Edwards).

Young developing cankers appear as reddish brown lesions (Figure 3a and b). These lesions soon elongate into elliptical, sunken areas with the necrotic tissue inside appearing water-soaked. Young cankers are often not noticed until other symptoms develop. As cankers enlarge, they often girdle infected twigs and branches (Figure 4), killing the distal portions of the shoot tissue. Cankers on the main stem of older trees reduce the vigour and the value or productivity of the tree (Figure 5 and 6). These trees are also subject to wind breakage. Pruning to control European canker eventually results in misshapen, hard-to-manage orchard trees (Figure 7).



Figure 3. Young canker on apple (a) cv. Royal Gala, (b) cv. Braeburn, Waikato, NZ. (Photo: J. Edwards).

Figure 4. Canker on apple cv. Royal Gala, Waikato, NZ (Photo: J. Edwards).



Figure 5. Cankers developed from infected pruning wounds; apple cv. Red Delicious; County Armagh, Northern Ireland (Photo: J. Edwards).



Figure 6. Cankers on the main trunk, apple cv. Bramley Seedling, Wisbech, England (Photo: J. Edwards).



Figure 7. Pruning to manage European canker leads to misshapen, hard-to-manage orchard trees, Royal Gala, Waikato, NZ (Photo: J. Edwards).



Figure 8. Numerous cankers along a branch of apple cv. Red Delicious, County Armagh, Northern Ireland (Photo: J. Edwards).

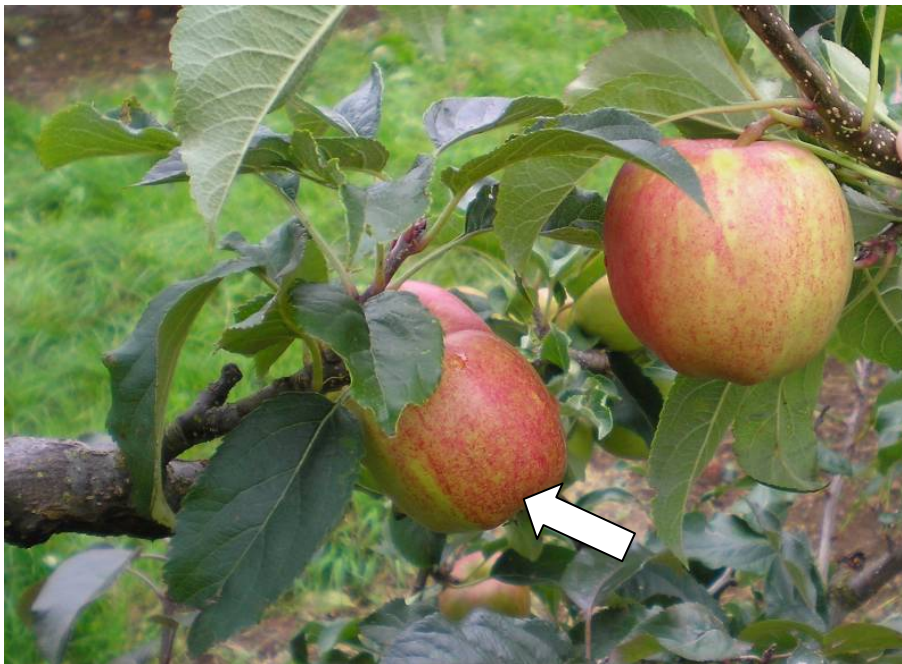


Figure 9. Smaller fruit (arrow) on a branch affected by cankers; apple cv. Red Delicious, County Armagh, Northern Ireland (Photo: J. Edwards).

Reduction in water conductivity due to distorted and narrow xylem, especially on branches and stems with numerous or large cankers (Figure 8), results in smaller fruit (Figure 9). The disease is most noticeable when the apple tree is in full foliage, as the dead twigs and spurs stand out (Figures 1, 2, 10a, 10b and 11). Twig death can be traced back to a canker girdling the stem if the cause is *N. Ditissima*.

Symptoms can be confused with woolly aphid (*Eriosoma lanigerum*) damage which kills buds, resulting in swollen dead cankers (Figure 12) and fireblight, caused by *Erwinia amylovora*, which causes twig death without cankers (Figure 13). There are also several other tree canker diseases of *Malus* and related genera which have similar symptoms. Unless the characteristic perithecia are present, this disease could be confused with several others, notably cankers caused by *Neofabraea malicorticis* (exotic), *Neofabraea alba* and *Neofabraea perennans*.

Apple cultivars differ in their susceptibility to European canker. The Delicious cultivars are highly susceptible, e.g. Red Delicious (Figures 6, 8 and 11), Golden Delicious (Figure 13) and Royal Gala, which has a Red Delicious parent (Figures 1-4). In these cultivars, the symptoms start in buds and twigs, and progress into large branches, trunks, etc., killing the tree if not regularly pruned out.

Cultivar Braeburn is moderately susceptible, and the disease remains relatively confined to killing spurs. Cultivar Granny Smith is moderately resistant. The infection is contained; killing small twigs but does not move into larger branches (Figure 13). In young apple trees infected by *N. ditissima* at the propagation phase, the disease has been shown to remain latent for up to 3 years (McCracken *et al.* 2003b), expressing as poor growth, early senescence and dieback of the young tree (Figure 14).



Figures 10. Twig death when the apple tree is in full foliage (a) cv. Golden Delicious (b) cv. Red Delicious, respectively, County Armagh, Northern Ireland (Photo: J. Edwards).



Figure 11. Bud death and swollen canker caused by woolly apple aphid (arrow); Waikato, NZ (Photo: J. Edwards).



Figure 12. Branch death caused by fireblight; unknown apple cv., near Blenheim, New Zealand (Photo: J. Edwards).



Figure 13. Infection is confined to killing small twigs in resistant apple cv. Granny Smith (Photo: J. Edwards).



Figure 14. Severe symptoms of dieback including leaf reddening, poor growth and early senescence caused by *N. ditissima* infection in apple cv. Royal Gala seedling; County Armagh, Northern Ireland (Photo: J. Edwards).

3.2.2 Apple – fruit symptoms

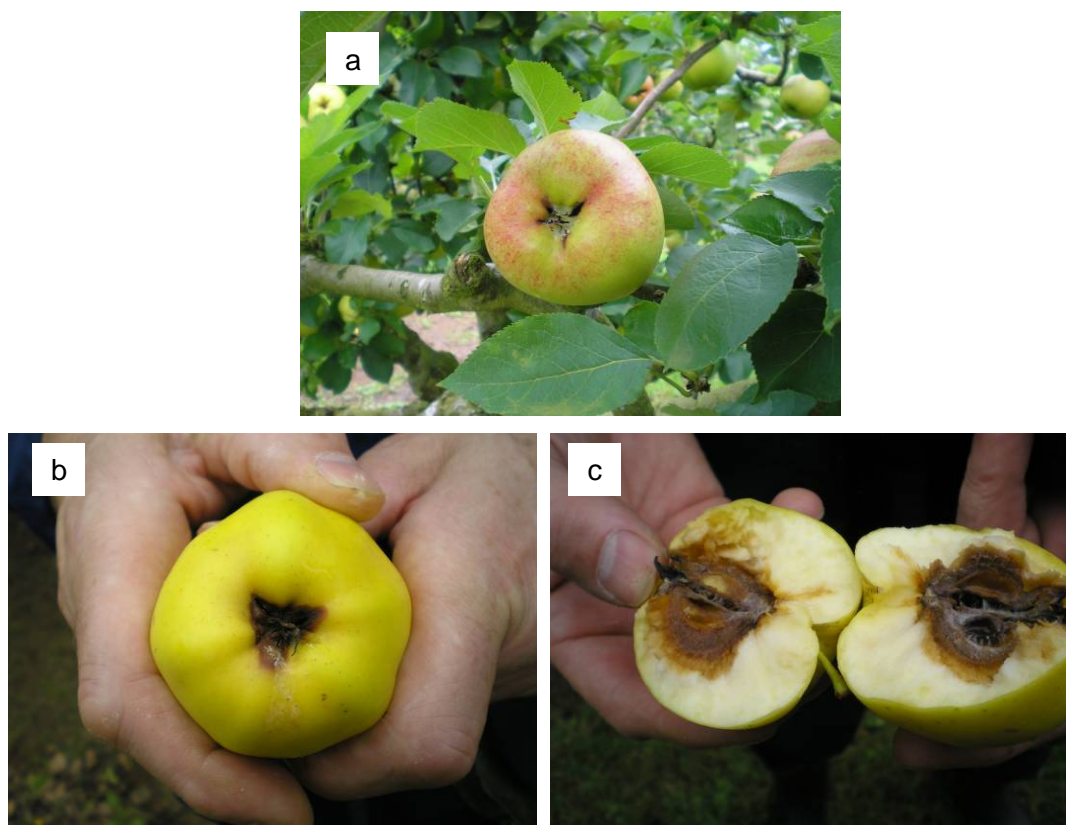
In general, fruit infection occurs on the tree when spores are dislodged from cankers and land on fruit. The spores infect through openings such as the calyx, sinus, lenticels, scab lesions and wounds caused by insects. The incidence of fruit infection depends on the amount of sporulation occurring on tree cankers and on weather conditions. Rotting of fruit can occur while the fruit is still on the tree and such fruit becomes mummified.

Depending on the variety, symptoms of fruit infection are generally not observed until shortly before or after harvest, or in storage. The most obvious symptom of fruit infection is a brown rot (known as eye rot) characterised by circular, sunken necrotic areas on the surface of the fruit that develop before harvest (Figure 17). Some varieties, particularly those with an open sinus, may develop a core rot that is difficult to detect without cutting open the fruit (Figure 16a and 16b) and that may remain latent for many months in long term cold storage. Rot generally develops at the calyx end of the fruit and is a darker brown than rots produced by *Penicillium* species. Internally, the rotted tissue is soft and may have a striated appearance (Figure 16c).

Note: Fruit rot caused by *N. ditissima* cannot be diagnosed from visual symptoms alone and should always be confirmed by other means such as culturing or PCR.



Figure 15. Eye rot on apple cv. Red Delicious, Northern Ireland (Photo: J. Edwards).



Figures 16. (a) Open sinus varieties of apple are more prone to core rots that develop post-harvest in storage. (b) Core rot in apple cv. Golden Delicious and (c) once cut, showing striations in the rotted tissue, Northern Ireland (Photo: J. Edwards).

3.2.3 Symptoms on hosts other than apple

Neonectria ditissima also causes cankers on other woody hosts (see host list above). There are two types of canker: open and closed. Open cankers are a series of concentric calluses. As infection progresses into healthy tissue around the canker, the plant responds with the formation of new closely packed ridges of callus. The fungus repeatedly invades and kills the callus tissue that forms at the edge of the lesion. The alternating fungal growth and callus ridge formation, which continues over subsequent years, results in the characteristic target-like cankers (Figures 17, 18 and 19). Open cankers are formed when conditions are favourable for the host and the fungus is slow growing.

Closed cankers occur when the conditions favour *N. ditissima*, allowing rapid growth of the fungus. Closed cankers are more irregular than the open cankers and are covered by dead bark. The outer bark is rough and cracked but does not fall off for several years in these cankers (Figure 20).



Figure 17. Open or target canker on black walnut, caused by *Neonectria ditissima* (Photo: J.H. Hart, USA). www.apsnet.org/online/Archive/1998/pdcvr15.htm



Figure 18. Open or target canker on maple (Photo: Chad Behrendt, USA). <http://www.extension.umn.edu/yardandgarden/ygbriefs/p-cankermple.html>



Figure 19. Perennial target canker on birch (*Betula* species), Michigan, USA (Photo: Robert L. Anderson, USDA Forest Service, Bugwood.org).



Figure 20. Closed canker on Asian pear (*Pyrus pyrifolia*) (Photo: Bengt Boysen).

3.2.4 Parts of the plant on which *N. ditissima* can be found

Neonectria ditissima can be found associated with symptomatic host tissue such as cankers and fruit rots. It can also be present as latent infections (i.e. symptomless) in vegetative propagating material such as cuttings and rooted plantlets, and in apple fruit.

N. ditissima is not usually found in roots. However, there are many *Cylindrocarpon* species associated with roots of trees and shrubs which should not be confused with the anamorphic state of *N. ditissima*.

The pathogen is reportedly spread internationally as latent infections (Howard *et al.* 1974, McCracken *et al.* 2003, CPC 2005). Therefore, in Australia, entry of the pathogen is most likely to be discovered through suspicious rots occurring in imported fruit or suspicious symptoms such as twig dieback observed on hosts in the field.

In the field, look for cankers, sap flow and hypertrophied, roughened fissured bark, especially in branch axils. Under favourable conditions, perithecia may be present in the canker tissue as small, round, red granulations that are visible with the aid of a hand lens. When wet, the red colour of the perithecia is not very obvious and they can be hard for the untrained eye to detect (Figure 21), but they become easily visible when dry (Figure 22).



Figure 21. Perithecia present in a small branch canker on unknown apple cultivar, October 2009, Auckland, NZ. Note that the red colour is not very obvious when the perithecia are wet (Photo: J. Edwards).



Figure 22. Bright red perithecia are much easier to see if the wood is dry. October 2009, unknown apple cultivar, Auckland, NZ (Photo: J. Edwards).

3.3 Sampling procedures

The suspect plant material may consist of fruit, shoots or twigs, or cankers in larger branches or trunks. Select specimens on which there are both diseased and adjacent healthy areas (Figure 23).



Figure 23. Example of a sample collected for diagnosis (Photo: J. Edwards).

4 IDENTIFICATION

The specimen must be first microscopically examined, then assayed by direct PCR of the diseased sample for rapid diagnosis (within one day) coupled with isolation and culturing (one week) as confirmation. Cultures identified as *Neonectria ditissima* by morphology should be sequenced to confirm identification.

4.1 Microscopic examination

Prior to washing and undertaking isolations, the specimen should be examined under a dissecting microscope to observe/identify fungal structures such as sporodochia (Figure 24) or perithecia (Figure 25) which may be present in/on host tissue and assist in the selection of tissue for isolations.

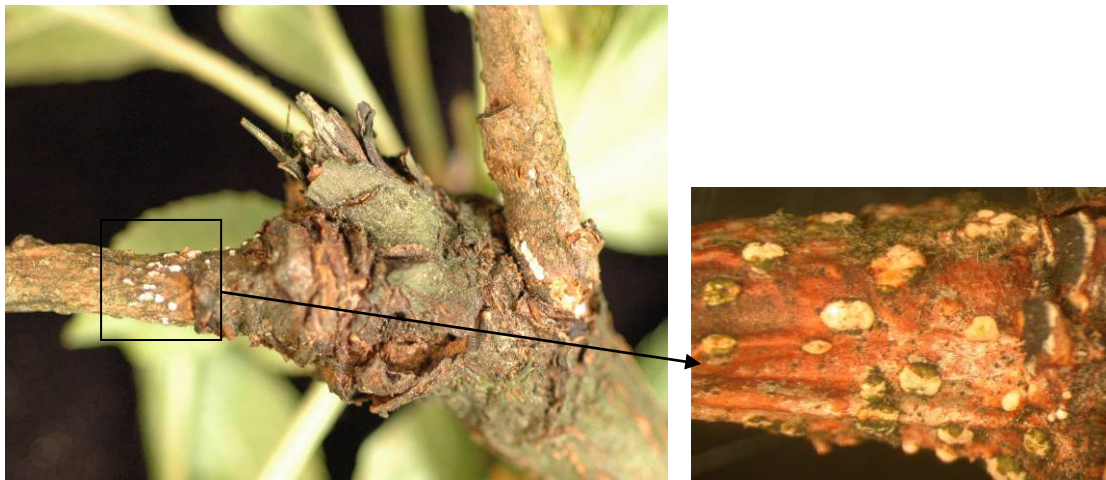


Figure 24. *Neonectria ditissima* sporodochia *in situ* on apple twig (Photo: J. Edwards).



Figure 25. *Neonectria ditissima* perithecia *in situ* on apple twig (Photo: J. Edwards).

If any structures are observed, they should be carefully picked off with a fine needle and mounted on a microscope slide and examined using a compound microscope. Gently squash the structures by tapping the coverslip to allow close examination of the asci and ascospores, or conidiophores and conidia.

Description: Perithecia (Figure 25) gregarious on surface or edge of cankers, subglobose to globose, red, KOH+ purple, 250-350 μm diam, with a slightly convex, darker ostiolar disk, not collapsing, cells at surface of perithecial wall globose to angular, thick-walled. Asci (Figure 26) clavate, 75-95 x 12-15 μm . Ascospores (Figure 27a) ellipsoidal to narrowly fusiform, one-septate, 14-22 x 6-9 μm , smooth to verrucose, hyaline becoming pale yellow-brown. *Cylindrocarpon* anamorph (Figure 24) producing macro- (Figure 28a and 28b) and microconidia. Macroconidia (Figure 27b) straight, predominantly 3-5 septate, 36-65 x 4-7 μm . Microconidia ellipsoidal, 4-8 x 2-3 μm , unicellular. (Taken from Minnis AM, Farr DF, Rossman AY. Fungal Nomenclature Database. Systemic Mycology and Microbiology Laboratory, ARS, USDA. Retrieved April 6 2010, from <http://nt.ars-grin.gov/fungaldatabases/>)

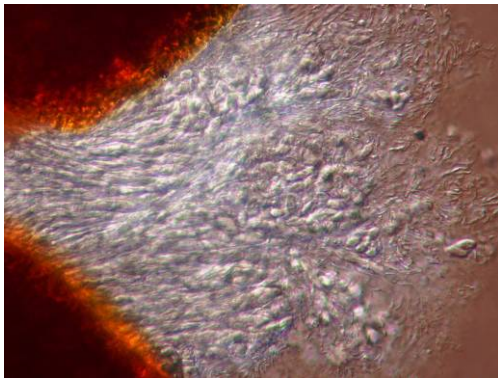
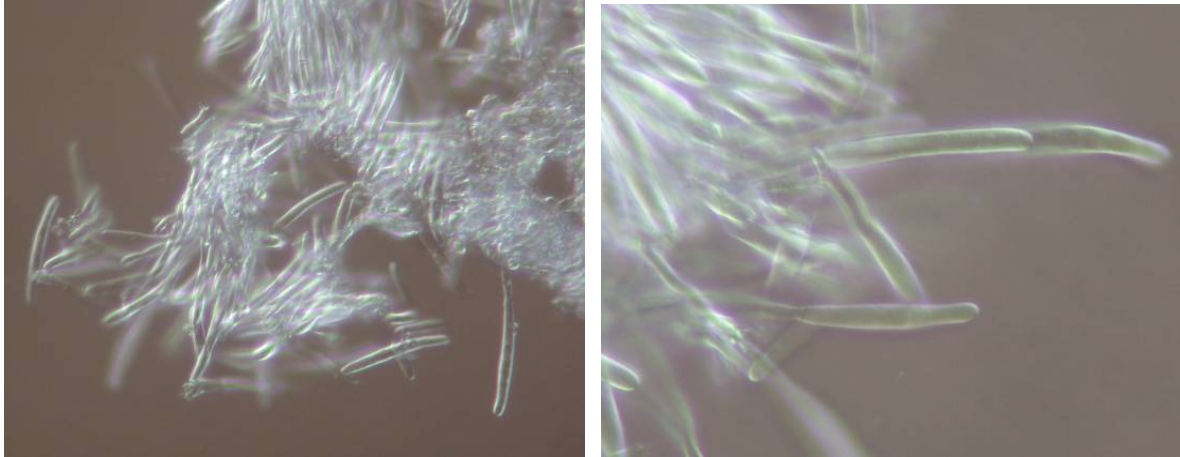


Figure 26. *Neonectria ditissima* (a) asci released from squashed perithecium (Photo: J. Edwards).



Figure 27. *Neonectria ditissima* (a) ascospores and (b) macroconidia (Photo: J. Edwards).



Figures 28. (a) Conidiogenous cells and (b) production of macroconidia in sporodochium of *Neonectria ditissima* (Photo: J. Edwards).

4.2 Method for isolation from symptomatic plant tissue

Equipment and/or material needed:

- Secateurs
- Scalpel
- Forceps
- 70% ethanol
- Sterile water
- Paper Tissues
- Crucible
- Beaker
- Potato Dextrose Agar amended with antibiotic (PDA+A) plates for routine isolations. (NB. the antibiotic concentration required varies with type of antibiotic used. Refer to manufacturer's recommendations).
- Incubator

Method

This method of recovery is used when there are symptoms but no obvious fungal development such as hyphae, spores, fruiting bodies etc. Standard aseptic technique should be followed.

Only use recently collected material which has not dried out or severely rotted. The tissue for isolation should be selected from where diseased tissue meets healthy, i.e. the active "front" of the infection. Young, recently affected areas should be selected, avoiding dead tissue which usually contains many saprophytic organisms. The external tissue over the canker should not be removed.

Following the isolation procedure, any fungus recovered which requires further examination can be sub cultured and maintained in pure culture.

Fruit

- Gently wash off any adhering extraneous matter from the fruit surface.
- Spray the affected area of the fruit with 70% ethanol, rinse in sterile water, wipe dry.

- Using a scalpel, lift the skin away and cut small sections of affected tissue (approx. 5 mm x 2 mm) mainly from the margin of the lesion.
- Using sterile forceps, aseptically transfer sections to PDA+A media plates (approximately 6/plate and 4 plates of media). Do not place sections too close together or too near the edge of the plate.
- Label and incubate plates at 25°C.

Wood

Note: It is quite difficult to isolate from old cankers. Isolation is more successful when the sap is flowing and the canker is actively growing. It is also important not to remove the bark from the canker or necrotic lesion.

- Gently wash off any adhering extraneous matter from the plant tissue.
- Cut the affected twigs or wood chips (if canker on trunk) into small pieces 2-4 cm long. Do not remove the bark.
- Place the pieces into a beaker containing 95% ethanol and leave for 5 mins if twig pieces, or 1 minute if wood chips. Remove and place on sterile paper tissue.
- Using secateurs soaked in 95% ethanol, cut small sections of affected tissue (approx. 5 mm x 2 mm) mainly from the margin of the lesion or from an area where vascular discolouration is obvious. If the wood is thin (i.e. less than 5 mm), then cut into cross-sections 2 mm thick. If the wood is thicker, split it longitudinally first, then cut into 2 mm sections.
- Using sterile forceps, aseptically transfer sections to PDA+A media plates (approximately 6/plate and 4 plates of media). Do not place sections too close together or too near the edge of the plate.
- Label and incubate plates at 25°C.



Figure 29. *Neonectria ditissima* isolated from infected apple twig, NZ (Photo: J. Edwards).

Check the plates daily for fungal growth (Figure 29) and aseptically transfer any such growth onto fresh PDA+A plates to obtain pure cultures. On PDA+A @ 25°C, *N. ditissima* grows approx 47 mm in two weeks. Colony growth is pale cream in colour and fluffy on plain PDA (Figure 30), but produces a soluble orange-brown pigment in the presence of antibiotic (e.g achromycin 50 mg/ml) (Figure 31).

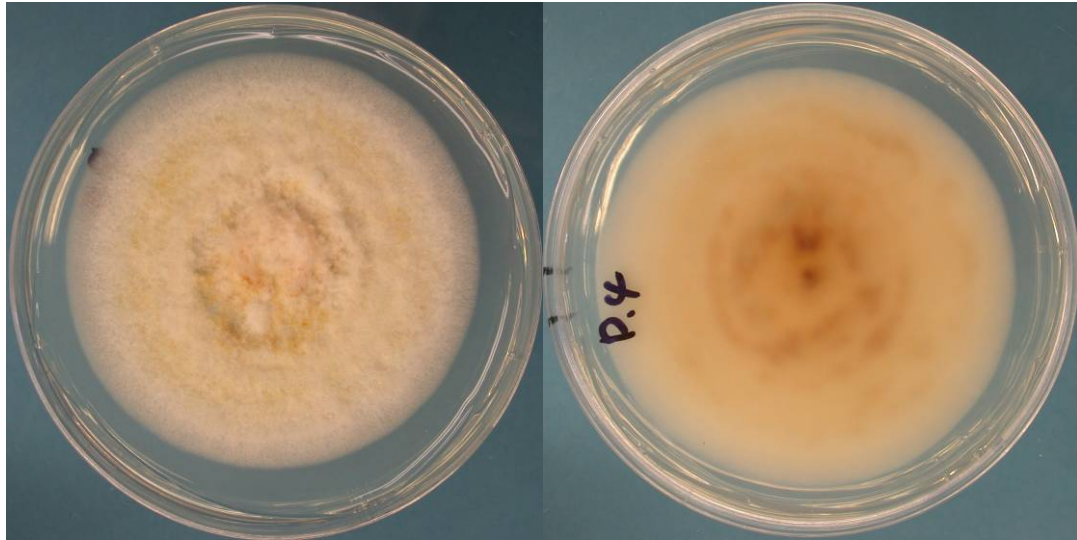


Figure 30. *Neovectria ditissima* grown on PDA for 7 days at 25°C (Photo: J. Edwards).

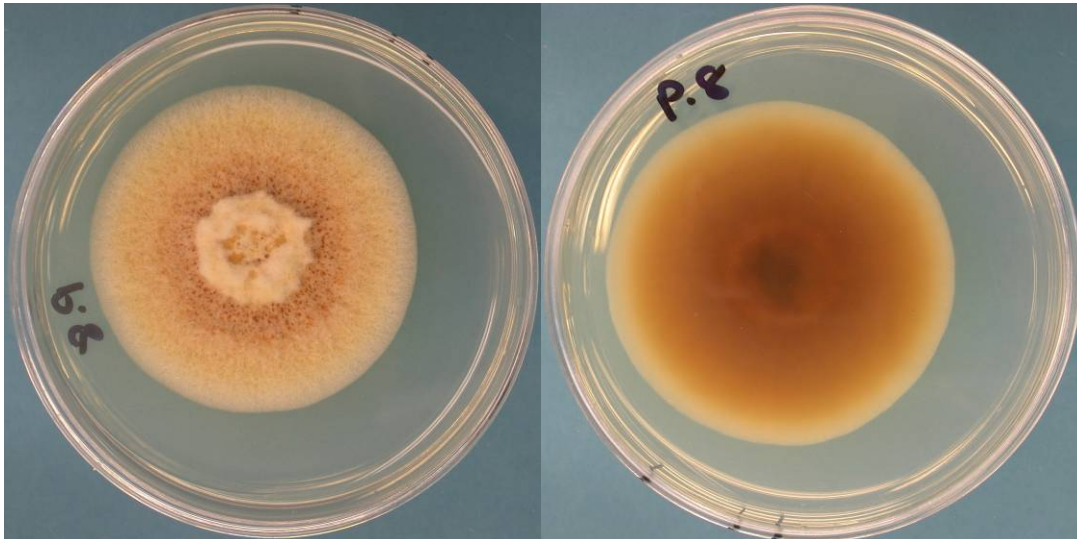


Figure 31. *Neovectria ditissima* on PDA+A produces an orange-brown soluble pigment, 7 days at 25°C (Photo: J. Edwards).

4.3 Molecular confirmation of *N. ditissima*

Molecular techniques should be used to confirm the identity of *Neonectria ditissima* as there are many *Neonectria* and *Cylindrocarpon* species. In the case of pure cultures isolated from diseased plant material, sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA will provide unequivocal identity. The method is described below. It may be possible to sequence using fungal hyphae picked directly off the culture plate for a rapid identification, but DNA should be also extracted for future reference and verification.

NB: Many *Cylindrocarpon* species are found on the roots of apple trees and other hosts. It is not necessary to sequence these.

4.3.1 Identification by ITS Sequencing

Identification by ITS sequencing requires extraction of DNA from the fungal culture, followed by PCR to amplify the ITS region, gel electrophoresis of the amplicon, sequencing of the region (approximately 700bp) and comparison of the sequence to a reference sequence. Using a similarity search program, e.g. BLAST, compare sequences generated from samples with sequences for *N. ditissima* or *N. galligena* in the GenBank database (www.ncbi.nlm.nih.gov).

The reference to use is CBS 316.34; AY677278.1. The sequence should differ by no more than 2 base pairs.

4.3.1.1 DNA extraction

Equipment and/or material needed

- Adequate fungal mycelium from culture
- Extraction kit (e.g. DNeasy[®] Plant Mini Kit, QIAGEN)
- Microcentrifuge
- Waterbath (to 65°C)
- Ice and ice tray
- 20, 200 and 1000 µL pipettes and sterile plugged (filter?) tips
- 2.0 mL screw-cap tubes
- Small glass beads
- 1.5 mL microcentrifuge tubes
- Scalpel
- 70% ethanol
- Bunsen burner attached to gas supply
- Parafilm

Method

- Follow instructions under the heading *DNA Preparation* within the *Isolation of Total DNA from Plant Tissue Using the DNeasy[®] Plant Mini Kit Protocol* in the *DNeasy[®] Plant Mini and DNeasy[®] Plant Maxi Handbook* associated with the kit
- For Step 1, add Buffer AP1 and Rnase A stock solution to screw-cap tubes containing c. 6 glass beads → add appropriate amount of fungal material → macerate (other maceration methods may be used provided they have been shown to provide adequate DNA yields)
- Continue following subsequent steps in the *Protocol*
- Clearly label tubes containing the final elution volume of DNA template → tubes to freezer (-20 or -80 °C)

4.3.1.2 Polymerase Chain Reaction (PCR) for fungi

Equipment and/or material needed

- Thermocycler
- PCR reagents
- 2, 20 and 300 µL pipettes and sterile plugged (filter) tips
- 0.2 mL PCR tubes
- Primers: from White *et al.* 1990 (Amplified fragment approx. 700bp)
Forward ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3')
Reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3')

Procedure

This protocol is intended as a generic protocol to suit most commonly used kits. Ensure you follow the directions contained within your kit and modify the volumes accordingly.

Table 1. Volumes of PCR Reagents (for generic PCR kit – adapt for the specific kit used)

Reagent	Volume	Final Conc.
DNA Template	1 µl	-
Primer ITS4 10 µM	1 µl	0.4 µM
Primer ITS5 10 µM	1 µl	0.4 µM
10 mM dNTPS (dATP, dTTP, dCTP, dGTP)	1 µl	0.4 mM
10x buffer (includes 25 mM MgCl ₂ [*])	2.5 µl	1x
Taq (or equivalent) DNA Polymerase 5 U/ µL	0.2 µl	0.04 U
Nuclease free water	18.3 µl	
Total	25.0 µl	

* If the kit supplies MgCl₂ separately, add to a final concentration of 2.5 mM and adjust the volume of water appropriately.

1. Clearly label 0.2 mL tubes according to samples to be used in PCR reaction, plus a positive control and a blank.
2. Make up a Master Mix of all reagents except the template. Add Taq DNA polymerase last (NB: Taq should remain refrigerated until required). Make enough for all samples and controls plus one extra.
3. Add 24.0 µl of the Master Mix to each tube.
4. Add 1.0 µl of DNA template (including the +ve control) to each respective tube, and add 1.0 µl of nuclease free water as template to the blank control.

Alternatively, add hyphae directly to the PCR reaction (If no product results from PCR reaction, then extract DNA (refer to 4.3.1.1) for use as template in subsequent reactions).

5. Close the tubes and vortex to mix contents, spin briefly in a microfuge (2 -3 seconds).
6. Place tubes in thermocycler.
7. Set up PCR using the following cycles:
 - 94°C for 30 s
 - 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 2 min
 - Hold at 10°C

4.3.1.3 Gel Electrophoresis

Equipment and/or material needed

- Protective gloves
- 2.0, 20 and 300 µL pipettes and sterile plugged filter tips
- 0.2 mL PCR tubes
- Gel tray with suitable comb/s, electrophoresis tank and powerpack
- UV transilluminator
- Camera/gel documentation system
- Microwave oven
- LE Analytical Grade Agarose (Promega)
- TBE electrophoresis buffer
- SYBR[®] Safe DNA gel stain
- DNA Ladder (e.g. 100 bp ladder Invitrogen)
- Gel loading dye
- 75% ethanol
- Kimwipes

Components of 0.5× TBE (Tris/borate/EDTA) electrophoresis buffer

- 54 g Tris base
- 27.5 g Boric acid
- 20 mL 0.5 M EDTA pH 8.0
- Dissolve in 1 litre sterile water and store at room temperature

Components of 6× gel loading dye

- 0.25% bromophenol blue
- 0.25% xylene cyanol FF
- 30% glycerol in water
- Store at 4°C

Procedure

- Determine size of gel and gel comb available (Table 2).

Table 2. Volume of gel solution required for specific gel tray sizes.

Gel tray size (cm)	Volume (mL) of gel solution required (for 0.7 cm gel thickness)
7.7 × 9.5	50
14.2 × 15.0	150
18.3 × 25.0	300

- Place gel tray with appropriate comb in tray holding device
- Weigh agarose (1.0 g/100 mL of buffer) and place into suitable heat proof container.
- Add required amount of gel running buffer (0.5× TBE)
- Heat agarose/buffer solution until agarose is completely dissolved then allow to cool to approximately 50–55°C
- Add required amount of SYBR Safe gel stain → agitate gently to mix
- Pour agarose solution into gel tray and allow to set
- While gel is setting, label appropriate number of 0.2 mL PCR tubes → add 1 µL of 6× loading buffer to each tube → add 5 µL of PCR product to appropriate tube
- Place set gel in electrophoresis tank and submerge with 0.5× TBE to a depth of at least 1 mm above the gel surface
- Remove comb from set gel → load 5 µL of DNA Ladder into the first and last well of the gel → put loading buffer/PCR product solution for the blank sample into the second well → put the loading buffer/PCR product solution for remainder of samples into successive wells
- Place lid on gel tray → set powerpac settings to run at 100 Volts
- After c. 20 minutes observe travel distance of samples in the gel
- When travel distance of samples in the gel has reached approximately half the length of the gel, remove gel tray from the tank → slide gel from gel tray onto centre of surface of the UV transilluminator → place hood of gel doc. system onto surface of transilluminator → switch on camera/gel documentation system → set to 0.4 sec., adjust size, contrast and focus of image → take picture
- Switch off gel doc system → remove gel from transilluminator → place gel in gel bin → spray surface of transilluminator with 70% ethanol → wipe surface with Kimwipes

The PCR product should be approximately 700 bp in size. The remaining product may be sent directly for sequencing or purified prior to sequencing.

4.4 Molecular detection in diseased tissue

Molecular identification of *N. ditissima* directly from diseased host tissue (fruit and wood) is possible, depending on the extent of infection and fungal biomass in the tissue. The methods are provided below. However, there are no cost-effective methods for detecting *N. ditissima* infections in symptomless wood and fruit tissue due to the inability to target the infected area.

4.4.1 Fruit

1.1.1.1. DNA extraction

This protocol is intended for use with the Promega Kit: Wizard[®] SV Genomic DNA Purification System which is reliable for detecting the pathogen in a range of host tissue. Other kits may not be suitable for the purification of DNA from fruit or woody tissue.

Equipment and/or material needed

- autoclaved 1.5 µL microcentrifuge tubes
- a microcentrifuge tube opener
- autoclaved mortar and pestle, wrapped in foil
- 70% ethanol
- Sharp knife
- Scalpel and blades
- Spatula
- Foam box

- Ice box (e.g. Eski™)
- Liquid nitrogen
- Another foam box filled with ice
- Forceps
- 30 mL Falcon tube, without lid
- Benchcote
- Tissues
- Disposable gloves
- Balance
- Promega Kit: Wizard® SV Genomic DNA Purification System
- Water bath
- Microcentrifuge
- Microcentrifuge tube rack
- Vortex
- Timer

Procedure

- Set water bath temperature to 55°C. Place Wizard® SV Lysis Buffer in the water bath.
- Place foam box inside the ice box. Carefully pour liquid nitrogen into the foam box. Place the spatula and the Falcon tube into the liquid N. Replace the lid of the foam box, then close the lid of the ice box.
- Wash apple, spray with 70% ethanol.
- Flame knife and cut open apple exposing symptomatic tissue.
- Weigh and record weight of a microcentrifuge tube. Label tube.
- Flame scalpel blade. Cut small pieces of symptomatic apple tissue and place into the microcentrifuge tube. Weigh and record weight. Aim for approx 200 mg apple tissue.
- Immediately place the microcentrifuge tube in the liquid nitrogen to prevent DNA degradation due to apple tissue oxidisation.
- Place three layers of benchcote onto the work surface. Unwrap the mortar and pestle and place on the benchcote. Keep the foil handy to use as a cover.
- With flamed forceps, use a 30 mL Falcon tube to ladle approx 30 mL liquid N from the foam box and pour into the mortar and pestle. Allow to boil away. Repeat two more times to thoroughly cool mortar and pestle.
- Remove the microcentrifuge tube containing the apple tissue from the liquid N in the foam box using forceps.
- Using a tissue, hold the hinge of the lid and open the microcentrifuge tube with a tube opener. Use a sterile scalpel blade to scrape the tissue out into the mortar.
- Return the empty microcentrifuge tube into the liquid N.
- Using folded tissue to hold the pestle (as very cold), carefully grind the apple pieces to powder. Use the foil as a cover over the mortar if necessary to prevent the pieces from jumping out.
- Carefully remove the spatula and the microcentrifuge tube from the liquid N with forceps. Using folded tissue to hold the spatula (to prevent burning hands), scrape the ground powder off the mortar and place into the cold microcentrifuge tube. (The cold tube makes it possible to get the powder into the tube without it melting.)

- Close the microcentrifuge tube, wipe with tissue, then replace into liquid N.
- Repeat the process from different portions of infected apple to ensure a representative number of samples.
- Repeat with an uninfected apple (of the same cultivar if possible) as a negative control.

Follow the procedure for 'Isolation of genomic DNA from mouse tail clippings' detailed in the Promega Kit: Wizard® SV Genomic DNA Purification System. Begin at Step 4, and add 250 µL of Wizard® SV Lysis Buffer warmed to 55°C to each sample. Vortex to mix.

Follow Section III.C for purification of genomic DNA using a spin protocol. Label the minicolumns and assemble with the collection tubes in a microcentrifuge tube rack. Increase temperature of water bath to 65°C and place the nuclease-free water in the bath.

4.4.1.1 Polymerase Chain Reaction (PCR)

Primers specific for *N. ditissima*:

- Langrell Ch1 (5'-AAC CCC TGT GAA CAT ACC CAT C-3')
- Langrell Ch2 (5'-GTG GCC GCG CTG CTC TTC CG-3')

(Amplified fragment size = 412 bp)

Langrell S.R.H. (2002) Molecular detection of *Neonectria galligena* (syn. *Nectria galligena*). *Mycological Research* 106: 280-292. The primers were designed from the ITS region. The limit of detection indicated in this paper was 25 pg of target template. Individual laboratories planning on using this protocol should determine the limit of detection under their specific conditions.

Equipment and/or material needed

- Thermocycler
- PCR reagents (see comments in section 4.3 re: manufacturer)
- 2, 20 and 300 µL pipettes and sterile plugged (filter) tips
- 0.2 mL PCR tubes

Volumes of PCR Reagents (to make up total volume of 25 µL)

- X µL DNA template
- 2.5 µL 10× PCR reaction buffer
- 0.5 µL of each 100 µM primer Ch1 and Ch2
- 0.5 µL dNTP mix (10 mM of each dNTP)
- 0.75 µL 50 mM MgCl₂
- X µL Taq DNA Polymerase (5 Units/µL)
- 19.15 µL nuclease-free water

PCR conditions

- 94°C for 3 min
- 94°C for 60 s, 63°C for 60 s, 72°C for 60 s; 30 cycles
- 72°C for 10 min
- 10°C hold

1) Procedure

1. In a laminar flow cabinet, clearly label 0.2 mL tubes according to samples to be used in PCR reaction, plus a positive fungal control, a lysis buffer only sample (from DNA extraction kit) and a blank water sample. Keep tubes on ice while making up master mix.
2. Make up master mix from appropriate volumes of PCR reagents in the following order: nuclease-free H₂O, 10× PCR reaction buffer, MgCl₂, dNTP mix, primers and Taq DNA polymerase (NB: Taq should remain refrigerated until required. Add Taq last.). Quickly vortex and spin at 10,000 x g.
3. Add 24.0 µL master mix to each tube.
4. Add 1.0 µL of DNA template (including the positive control and lysis buffer and water negative controls) to each respective tube.
5. Place tubes in thermocycler.
6. Set thermocycler to desired cycle parameters → run PCR.

Follow gel electrophoresis steps as described in the previous section.

In the event of a positive result (a band at 412 bp, corresponding to a band seen for the positive control), the PCR product should be sequenced using the same primers (Ch1 and Ch2) for confirmation.

4.4.2 Wood

The same protocol is used for wood as for fruit. It is important to target the area most likely to be infected (i.e. discoloured wood). Aim for 80 – 100 mg wood tissue. Transferring the sample plus lysate buffer to the minicolumn (Step 1) will need a wide-bore (i.e. cut the end off) pipette tip as fibrous material gets caught. The process may need to be repeated with an extra 250 µL lysate buffer.

As there are compounds in wood that can inhibit PCR reactions, optimise the process by making serial dilutions of 1/10 and 1/100 of the pure DNA samples. For the PCR reaction, use all three concentrations of DNA i.e. 1, 1/10 and 1/100 and bovine serum albumin (BSA) may also be added to the PCR mix to precipitate inhibitory substances to maximise the chance of success.

Modify the PCR conditions by increasing Step 2 to 35 cycles.

In the event of a positive result (a band at 412 bp corresponding to a band seen for the positive control), the PCR product should be sequenced using the same primers (Ch1 and Ch2) for confirmation.

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- Ministry of Agriculture and Lands, Government of British Columbia - Pest Management - European Canker (Nectria Canker). www.agf.gov.bc.ca/cropprot/tfipm/european.htm
- Plant Health Australia Fact sheet - Exotic Threat to Apple: European Canker.
www.planthealthaustralia.com.au/project_documents/uploads/European%20Canker.pdf
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8 APPENDIX

8.1 Disease cycle

Neonectria ditissima overwinters as mycelium in twigs and callus tissue of cankers, growing slowly while its host is dormant, or as perithecia (sexual fruiting structures) in cankered wood (Figure 32, Agrios 1997). Spore production is initiated during periods of cool, wet weather. On younger cankers asexual conidia are generally produced in cream-coloured sporodochia in spring/early summer. On older cankers, ascospores (sexual spores produced in the perithecia) and conidia are produced, both of which can cause infection. Production and release of spores is largely climate dependant, and is most common in spring and autumn. However, spore production and infection of host tissue can occur at any time of year as long as there is sufficient moisture and the temperature is above 5°C.

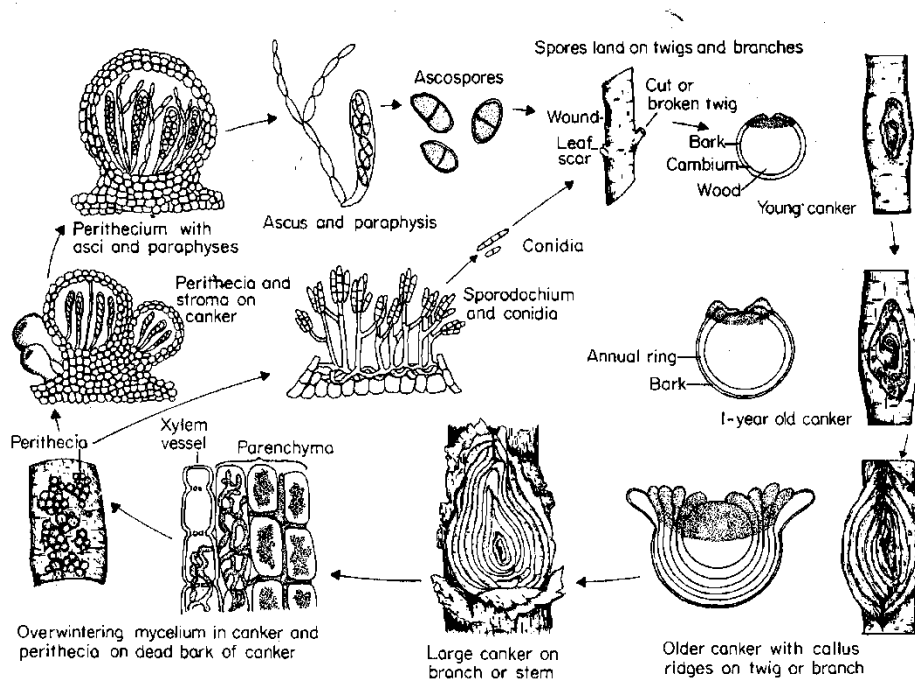


Figure 32. Disease cycle of *Neonectria* canker caused by *Neonectria ditissima* (Agrios, G. N. Plant Pathology Fourth Edition, Academic Press Ltd, London 1997).

Conidia are dispersed by moist wind currents and rain splash, and in some cases carried by insects to susceptible tissue (Houston 1994). Release of ascospores is dependent on rainfall quantity and duration of wet periods. Ascospores can be dispersed by rainsplash but are generally regarded to be aerially disseminated. The airborne ascospores are capable of long distance dispersal, while the conidia serve to spread the disease short distances and to intensify the disease in trees that are already infected.

The fungus enters its host through leaf scars or wounds caused by pruning, insect feeding, winter injury or invasion by other pathogens. Frost and crotch cracks are also common sites of entry. Plants that are stressed by cold, drought, mechanical injuries or other disease are especially susceptible. *N. ditissima* can also infect apple and pear fruit via the calyx, lenticels and wounds.