National Diagnostic Protocol

Phyllosticta ampelicida

the cause of black rot on grapevine

NDP 13 V2

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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 *Phyllosticta ampelicida* is current as at the date contained in the version control box below.

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Further information

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

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1 INTRODUCTION

Black rot of grape is caused by the ascomycete fungus *Phyllosticta ampelicida* (syn. *Guignardia bidwellii*) and can lead to substantial yield losses in humid regions when it is not effectively managed. Most cultivars of *Vitis vinifera*, as well as French/American hybrids and American bunch grapes, are susceptible, while varieties of muscadine range in disease susceptibility from resistant to very susceptible.

The fungus may be spread on grapevine cuttings and fruit.

1.1 Host range

Sivanesan A and Holliday P (1981)

1.1.1 Primary hosts

Vitis arizonica	Canyon grape
Vitis labrusca	American grape
Vitis rotundifolia	Muscadine grape
Vitis vinifera	Domestic grape

1.1.2 Alternative hosts

Ampelopsis	Wild grape
Asplenium nidus	Birds nest fern
Cissus	Ornamental vine
Parthenocissus quinquefolia	Virginia creeper
Parthenocissus tricuspidata	Boston ivy
Vitis amurensis	Amur grape

2 TAXONOMIC INFORMATION

2.1 Taxonomic description

Class: Dothideomycetes

Order Incertae sedis

Family: Phyllostictaceae

Genus: Phyllosticta

Species: ampelicida

Name

Phyllosticta ampelicida (Engelm.) Aa, Stud. Mycol. 5: 28 (1973)

Synonyms

Botryosphaeria bidwellii (Ellis) Petr. 1958

Carlia bidwellii (Ellis) Magnus 1892

Carlia bidwellii (Ellis) Prunet 1898

Depazea labruscae Engelm. 1877

Guignardia bidwellii (Ellis) Viala & Ravaz 1892

Guignardia bidwellii f.sp. *bidwellii* (Ellis) Viala & Ravaz 1892

Guignardia bidwellii var. *bidwellii* (Ellis) Viala & Ravaz

Guignardia bidwellii var. *euvitis* Luttr. 1946

Guignardia bidwellii f. *parthenocissi* Luttr. 1946

Laestadia bidwelli (Ellis) Viala & Ravaz, 1888

Naemospora ampelicida Engelm. 1863

Phoma ustulata Berk. & M.A. Curtis 1873

Phoma uvicola var. labruscae Thüm. 1878

Phyllachorella bidwellii (Ellis) Theiss. 1919

Phyllosticta ampelopsidis Ellis & G. Martin 1886

Phyllostictina clemensae Petr. 1928

Phyllosticta labruscae Thüm. 1878

Phyllosticta labruscae var *labruscae* Thüm. 1878

Phyllosticta vitea Sacc. 1896

Phyllosticta viticola Thüm. 1878

Physalospora bidwelli (Ellis) Sacc., 1882

Sphaeralla bidwellii (Ellis) Ellis 1890

Sphaeria bidwelli Ellis 1880

Septoria viticola

2.2 Morphological description

The following description is taken from Sivanesan and Holliday (1981).

Pseudothecia formed as locules in a stroma, depressed globose, immersed subepidermal, 70-180µm broad with a flat or papillate ostiolar apex (Fig. 1A). The pseudothecial wall is made up of pseudoparenchymatic cells. Asci arise from a cushion shaped hyaline tissue at the base, cylindrical to clavate, 45-65 × 9-14 µm (Fig. 1B). Ascospores hyaline, one celled, ovoid to ellipsoid, 12-17 × 6-7.5 µm, often with hyaline, mucilaginous, apical caps.

Pycnidia mostly epiphyllous, solitary, unilocular, globose or depressed globose, with a flat or inconspicuous papillate ostiolar apex, 120-230 µm broad (Fig. 1C). Stroma poorly developed on leaves but well developed on fruits. Conidiogenous cells conical to cylindrical (Fig 1D). Conidia one celled, hyaline, broadly ovoid, ellipsoidal or almost globose, somewhat clavate when young and slightly indented, 5-12 × 4-7 µm, surrounded by a mucilaginous sheath and with an apical hyaline appendage as long as the conidium. Spermatia hyaline, unicellular, rod shaped 4-7 × 0.5-2 µm.

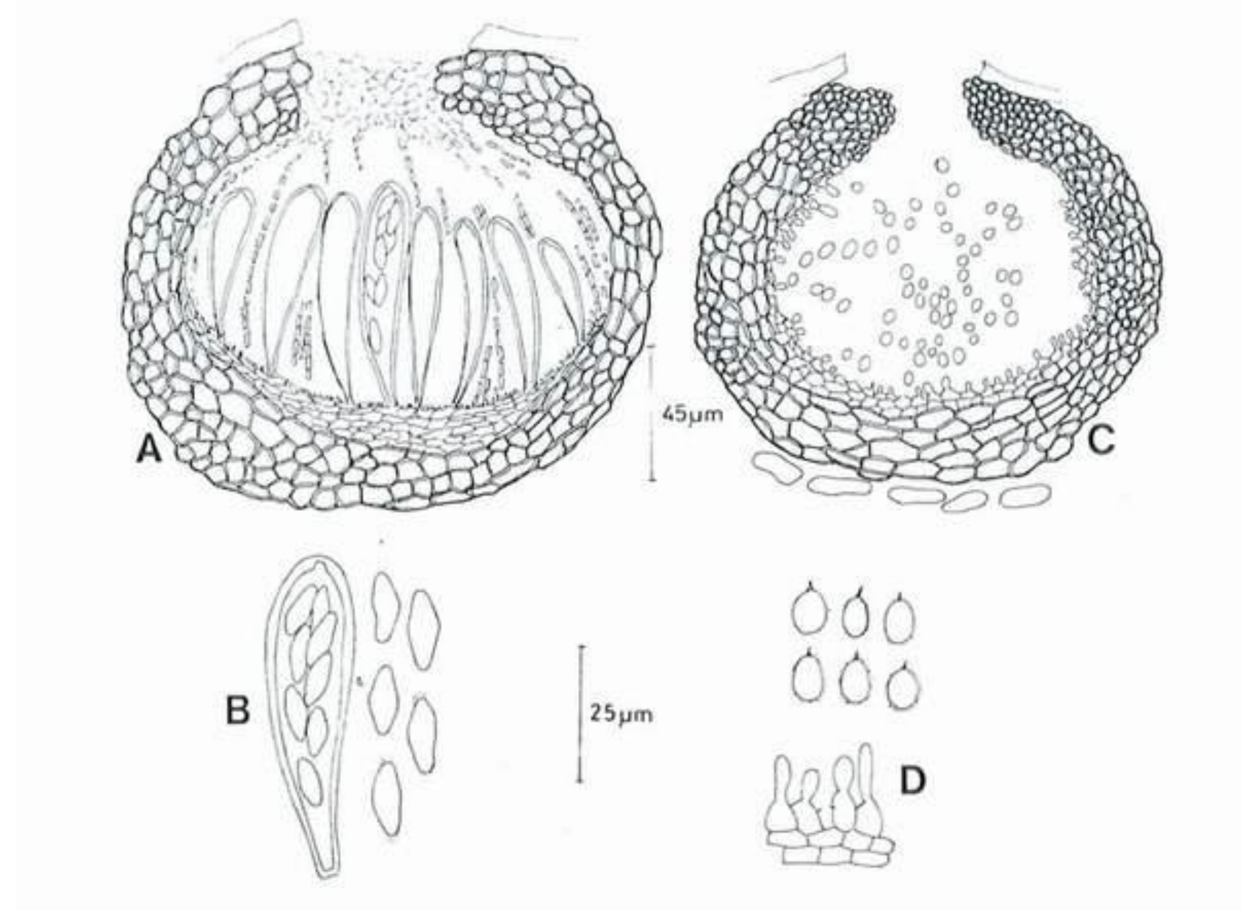

Figure 1. Microscopic description of *Guignardia bidwellii*; A. pseudothecium, B. ascus and ascospores, C. pycnidium and D. conidiogenous cells and conidia (Sivanesan and Holliday 1981).

Descriptions from Zhang et al. (2013) based on neotype.

Pycnidia black or blackish brown, globose or subglobose, 150–300 mm diam. on PDA media. Conidiogenous cells cylindrical or conical, 9–13(–15) x 3–4.5 mm. Conidia hyaline one-celled, broadly ovoid, ellipsoidal, seldomsubglobose, some with a truncate base, 8.5–12.5 x 6–7.5 mm, surrounded by a slime layer, containing small green guttules, with short appendages, 2–7 mm. Spermatia hyaline, one-celled, dumbbell shaped, 5–7 x 1.3–2.2 mm.

Culture characteristics: Colonies reaching 3 cm diam. in 7 d on PDA, pale gray to olive green when mature, gray olivaceous to greenish black in reverse. Aerial mycelium dense, white; submerged mycelium olivaceous to brown. Stromata formed after 20 d, black.

3 DETECTION

Phyllosticta ampelicida can be readily identified by macroscopic symptoms on most parts of the plant. The disease cycle of black rot is described in Appendix 1.

3.1 Leaf, stem and fruit symptoms

Phyllosticta ampelicida is most likely to be found on leaves, stems and fruit of grapevines and minor hosts. Sources could include imported fruit or cuttings.

In the vineyard, symptoms are visually most evident on leaves in the spring, leaves, stems and fruit in the summer, and on stems and fruit in autumn and winter.

Infected **leaves** can be easily detected by circular lesions which vary in size (Fig 2b) but are typically small under field conditions (Fig 2a and d), appear brown or tan with reddish margins, and characteristically contain small black pycnidia (Fig 2**Figure** c). When inspecting vines, leaf lesions may be observed associated with mummified berries from the previous or current seasons (Fig 2d).

On **shoots, petioles and tendrils,** the lesions are initially tan, brown turning purple to black, sunken, elliptical to elongated and contain pycnidia or pseudothecia observed as small black dots (Fig 3b and c). The bark may split, but stem infections remain localised, and do not usually extend more than several centimetres.

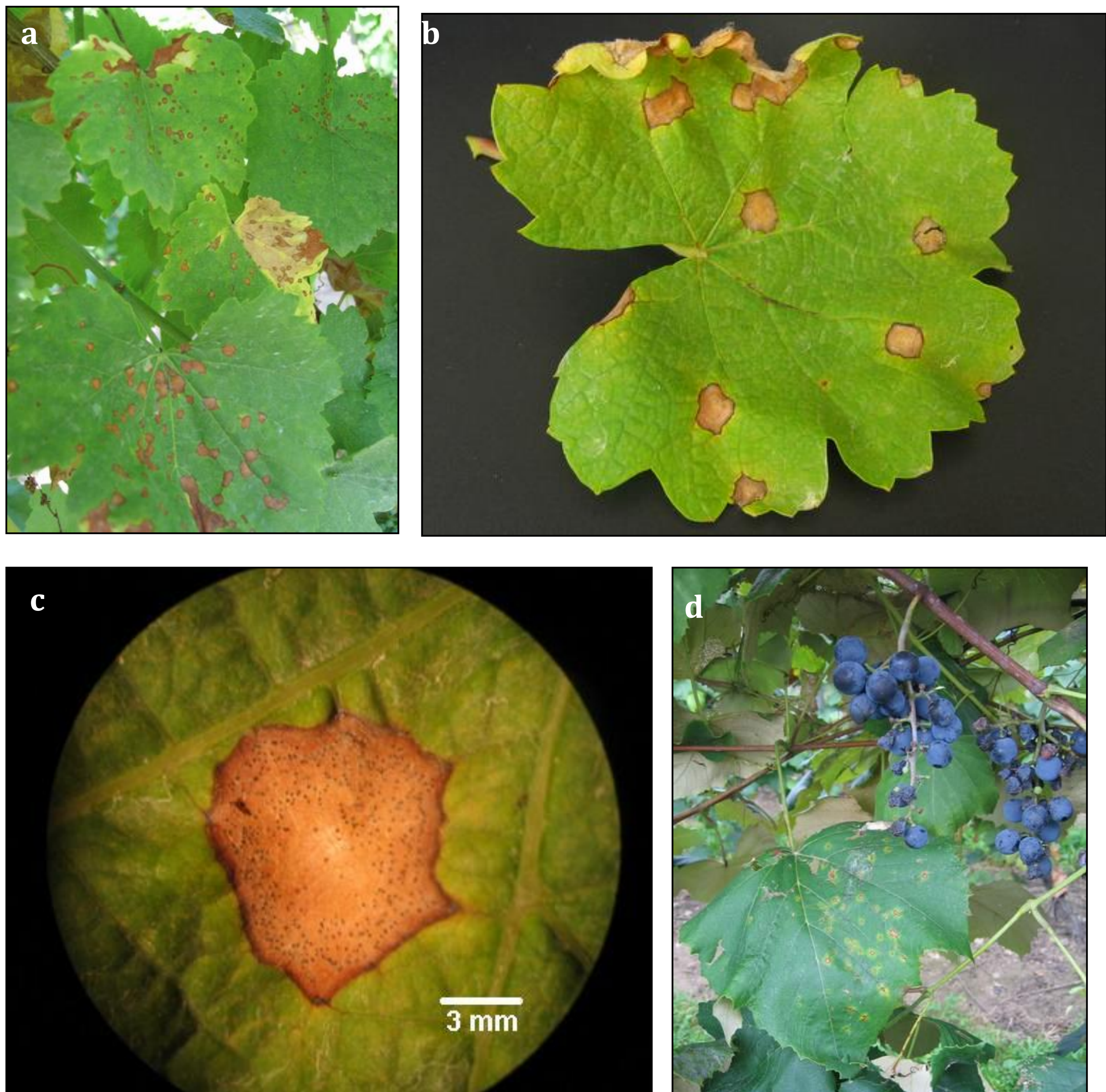

Figure 2. Symptoms of *Phyllosticta ampelicida* on leaves of *Vitis vinifera* (cv. Riesling); (a) on the vine, (b) on the laboratory bench, (c) under the dissecting microscope with pycnidia evident and (d) leaf lesions caused by conidia ejected from infected berries above. (Photos by M. Sosnowski, SARDI)

Figure 3. Symptoms of *Phyllosticta ampelicida* on stems and petioles of *Vitis vinifera* (cv. Riesling); (a) on young green shoots on the vine, (b) on the laboratory bench, (c) under the dissecting microscope, and (d) on older lignified shoots of a *Vitis* interspecific hybrid (cv. Vignoles) on the vine. (Photos a-c by M. Sosnowski, SARDI and photo d by W. Wilcox, Cornell University)

On the **developing fruit**, small, pale dots (approx. 1mm diam.) appear, rapidly becoming surrounded by a widening brown ring. If lesion expansion is halted due to an application of some fungicides or the development of age-related resistance in half grown berries, pale-brown coloured spots with a dark ring (bird's eye effect) and a sunken centre, about 6mm diam., may result (Fig 4a). More typically, however, a chocolate-brown lesion expands through the berry (Fig 4b and c) until it becomes completely rotted. Berries then shrivel and turn dark brown with numerous black pseudothecia or pycnidia developing over the surface (Fig 4d). Eventually, the fruit becomes dry and shrivelled, turning into hard, blue-black mummies that often remain firmly attached to the pedicel, although some may be shed from the vine (Fig 4e).

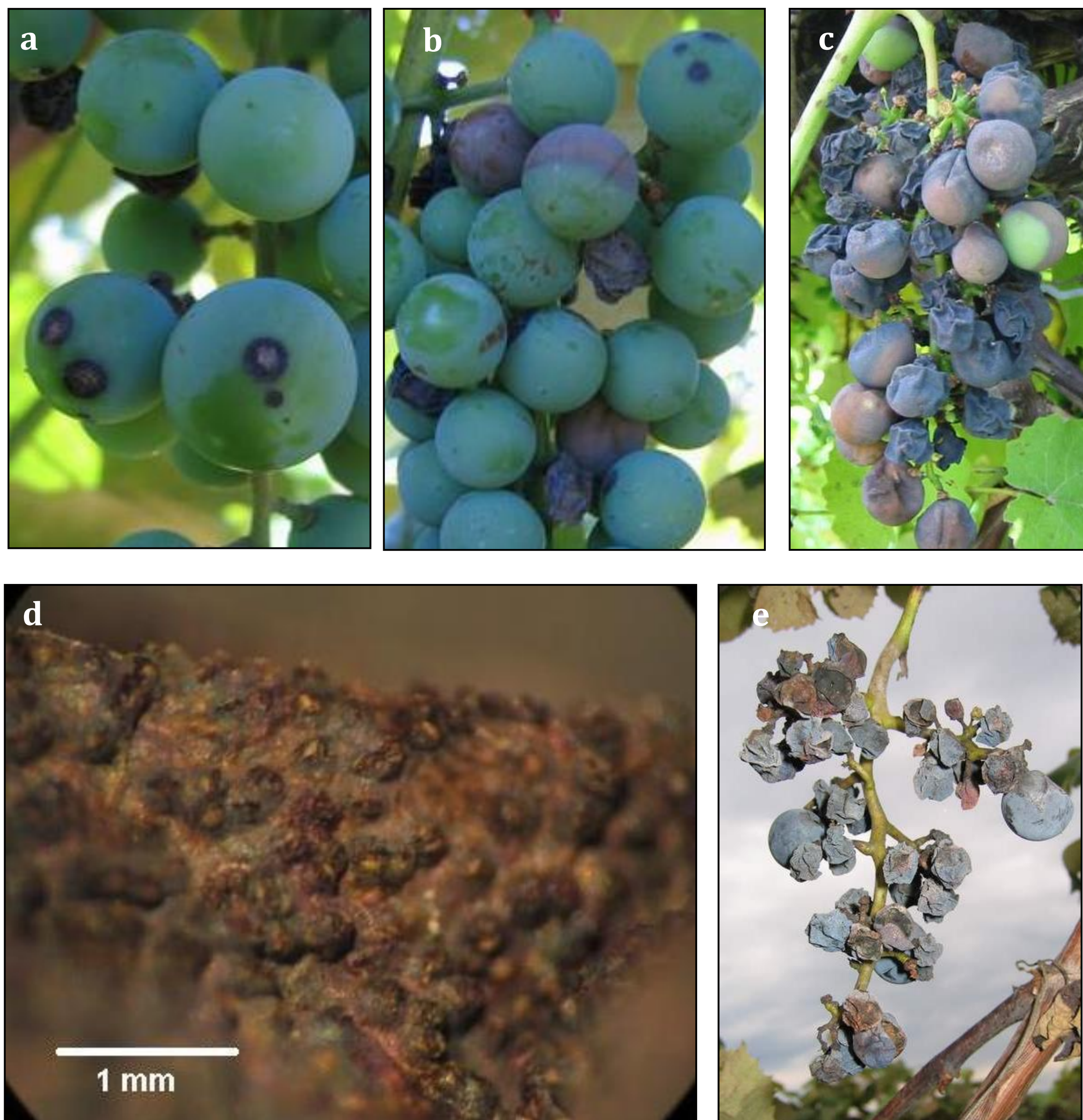

Figure 4. Symptoms of *Phyllosticta ampelicida* on grape berries of *V. labrusca* (cv. Concord); (a-c) progression from small lesions to mummified berries, (d) pseudothecia on surface of mummified berry under dissecting microscope and (e) mummified berries. (Photos a, b, d and e by M. Sosnowski, SARDI and c by W. Wilcox, Cornell University)

3.2 Confusion with other diseases

Symptoms can be confused with those of **black spot (anthracnose) disease** caused by the fungus *Elsinoë ampelina,* which is endemic in Australia. Leaf lesions are similar when viewed from a distance, but do not contain the small black pycnidia typically found in black rot lesions. Stems and petioles develop raised cankers with sunken centres, whereas black rot lesions are less "three-dimensional". Infected fruit develop similar lesions initially, but those of black spot do not usually expand to include the entire berry nor evolve into mummified fruit (Fig 5).

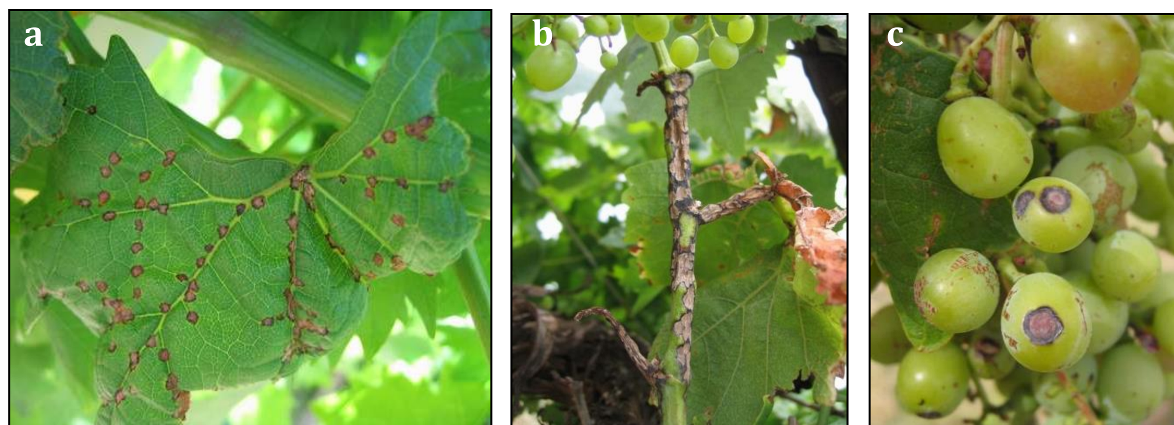

Figure 5. Symptoms of black spot (anthracnose) disease caused by the fungus *Elsinoë ampelina* on *Vitis vinifera* (table grape cv. Red globe); (a) leaf lesions, (b) stem cankers and (c) berry lesions. (Photos by M. Sosnowski, SARDI)

Symptoms of **Phomopsis cane and leaf spot**, which is caused by the fungus *Diaporthe ampelina*, may also be confused with those of black rot (Fig 6). Grapevines infected by *D. ampelina* develop leaf lesions that are smaller than those due to black rot; they are often surrounded with a translucent halo, and do not contain pycnidia. Stem lesions are sometimes smaller in size, but when larger, the two diseases are very difficult to distinguish on the basis of lesions on the green shoots. Lesions on lignified canes often have a bleached appearance and develop into larger basal cankers. Fruit symptoms can be similar to black rot, with shrivelled mummified berries; however, this phase of the disease does not usually occur with *D. ampelina* infections until shortly before harvest, whereas it typically occurs with veraison with black rot.

There is also a complex of pathogens, including *Alternaria* spp., *Aspergillus niger*, *Botrytis cinerea*, *Cladosporium* spp., *Penicillium* spp., and *Rhizopus arrhizus*, which may cause fruit rot of grapevines, and thus should also be considered when diagnosing black rot.

Figure 6. Symptoms of Phomopsis cane and leaf spot disease caused by the fungus *Diaporthe ampelina* on *Vitis vinifera* (cv. unknown); (a) small leaf lesions with halos, (b) green stem lesions, (c) bleached cane, (d) basal stem canker and (e) bunch symptoms. (Photos a-d by B. Rawnsley, SARDI and e by R. Emmett, DPI Victoria)

3.3 Sampling methods

As visual symptoms can be found on leaves, stems and fruit of grapevines, they should all be targeted during any inspections of vineyards, post-entry quarantine or examination of grapevine material at the border. In the vineyard, symptoms are likely to be most obvious on leaves first, but mummified fruit could also be present from infection in the previous season. Symptoms to look for are brown circular lesions (often containing small black pycnidia) with reddish margins on leaves; brown/purple elliptical or elongated lesions on stems; and brown circular lesions on fruit or shrivelled berries (details in section 3.1). In vineyards, symptoms can be present both during the growing season and dormancy, as infected, mummified fruit often remain hanging on the vine since they are not detached from the rachis as easily as healthy berries that merely shrivel on the vine through normal dehydration if not harvested at maturity.

Symptomatic leaf, stem and fruit samples should be sealed in a polyethylene bag and transported to a diagnostic facility, keeping the samples cool during the entire process. For national guidelines on response to an emergency plant pest refer to PLANTPLAN (PHA 2009).

4 IDENTIFICATION

Phllysticta ampelicida can only be confirmed by DNA sequence analysis, as morphology is unreliable for the identification of *Phyllosticta* species.

4.1 Isolation of pathogen from symptomatic plant material

Equipment

Dissecting microscope
Scalpel
Trays, polyethylene bags and paper towel
Slides and coverslips
Sterile distilled water (SDW)

Autoclave
Sterile plastic petri dishes
Laminar flow cabinet
Incubator (25ºC, white fluorescent lights)
Scalpel and forceps
Bunsen burner and alcohol
Sodium hypochlorite solution (0.5%)
Sterile distilled water (SDW)
Sterile filter paper
Parafilm

Media preparation:

Potato Dextrose Agar – half-strength (½ PDA) Hoffman *et al.* (2002)

Potato Dextrose Agar (PDA)	9.8 g
Bacto agar (granulated agarose)	3.5 g
Distilled water	500 ml

Mix ingredients and autoclave at 121ºC for 15 minutes, mixing well before pouring into sterile plastic petri dishes.

Method:

Symptomatic leaf, stem and fruit material should be placed in a tray lined with wet paper towel and sealed in a plastic bag for incubation under high humidity. The samples can be left on the bench in normal laboratory conditions. Within 1-3 days, pycnidia on lesions may exude cirrhi or tendrils containing conidia which can be viewed under the dissecting microscope (Fig 7).

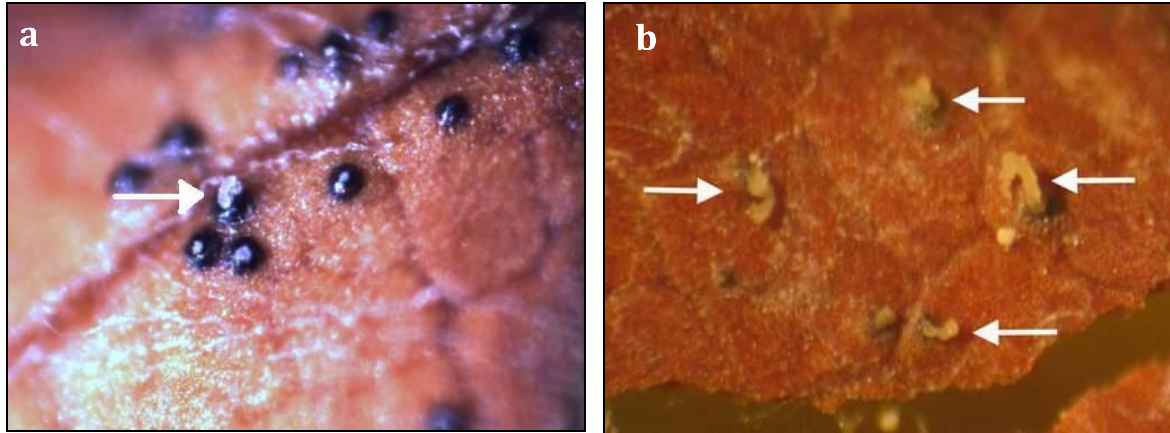

Figure 7. (a) Pycnidia of the sexual morph of *Phyllosticta ampelicida*, (b) exuding cirrhi on the leaf surface of grapevine cv. Riesling following incubation at high humidity overnight. (Photos by M. Sosnowski, SARDI)

In the laminar flow cabinet, remove sections of leaf, stem and fruit samples with lesions using a scalpel. Surface sterilise sections in 0.5% sodium hypochlorite for 60 s, rinse in sterile distilled water (SDW) and place on sterile filter paper to dry. Excise small pieces (approx. 3 mm x 3 mm) from lesions and place onto surface of agar media.

Alternatively, cirrhi exuding from pycnidia on lesions can be carefully removed with a flame-sterilised scalpel and placed directly onto agar surface.

Seal plates with parafilm strips and place into incubator at 25ºC under continuous fluorescent light. Check plates for contamination at regular intervals and subculture putative colonies of *G. bidwellii* if necessary. Pure cultures should be incubated for 15-20 days in order to induce sporulation.

4.2 Morphological examination

Half-strength PDA is the optimal medium for identification and conidia production (Fig 8). Mycelium growth rate ranges from 1-2 mm/day and appears speckled and irregular. Pycnidia formation can occur within 7 days for some isolates and after 15 days all isolates produce fruiting bodies, from which conidia can be harvested.

Conidial ooze can be scraped from pycnidia on agar cultures or leaf lesions using a scalpel, and mounted on a glass slide in a drop of SDW with a cover slip placed on top.

Viewed under a compound light microscope, conidia appear as one celled, hyaline, broadly ovoid, ellipsoidal or almost globose, somewhat clavate when young and slightly indented; spores are 5-12 × 4-7 µm in size (Fig 9).

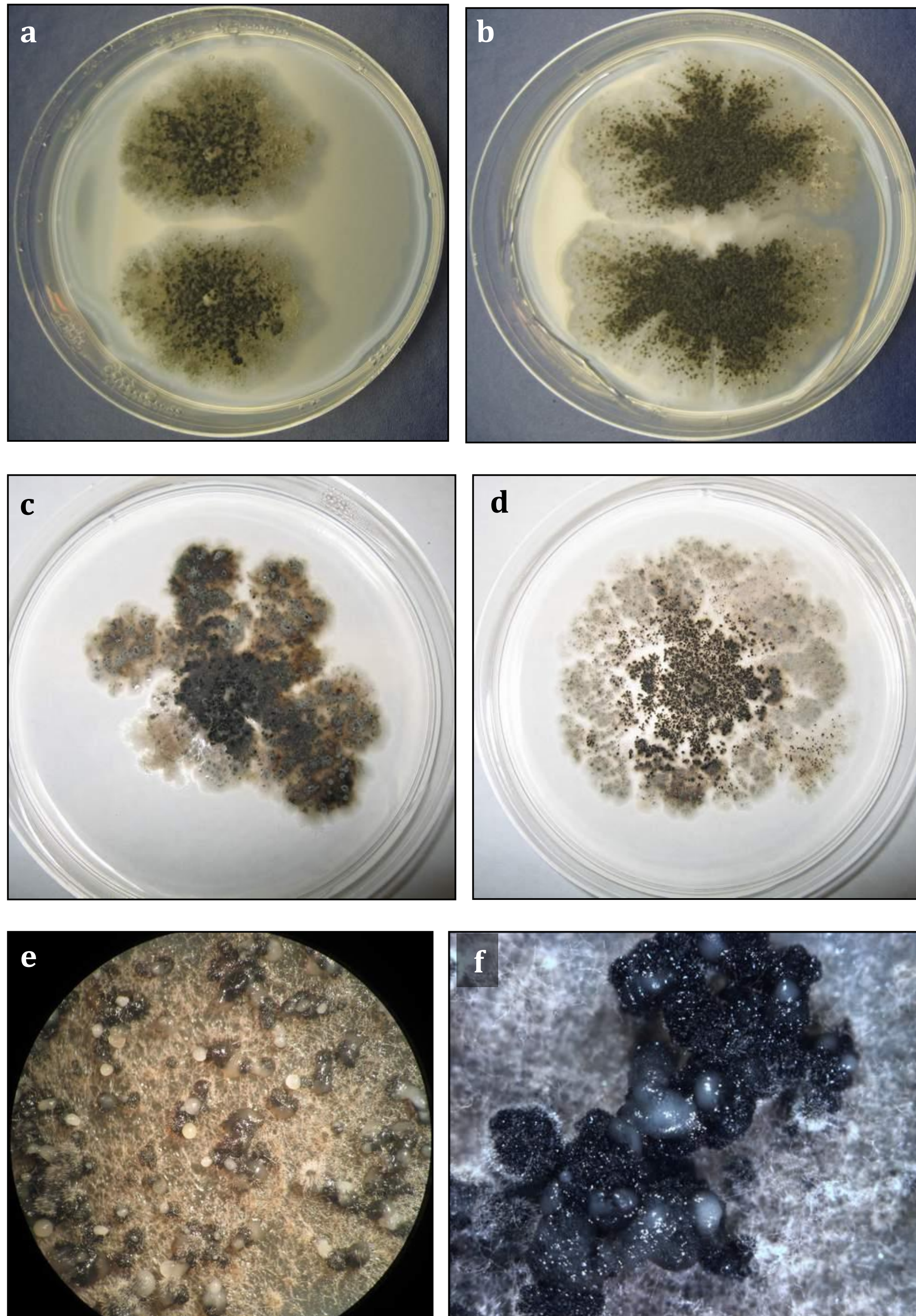

Figure 8. *Phyllosticta ampelicida* cultures on ½ Potato Dextrose Agar; (a) 15 days and (b-f) 20 days of incubation at 25ºC under continuous fluorescent light. (e & f) Under a dissecting microscope, conidia are observed oozing from pycnidia. (Photos by M. Sosnowski, SARDI)

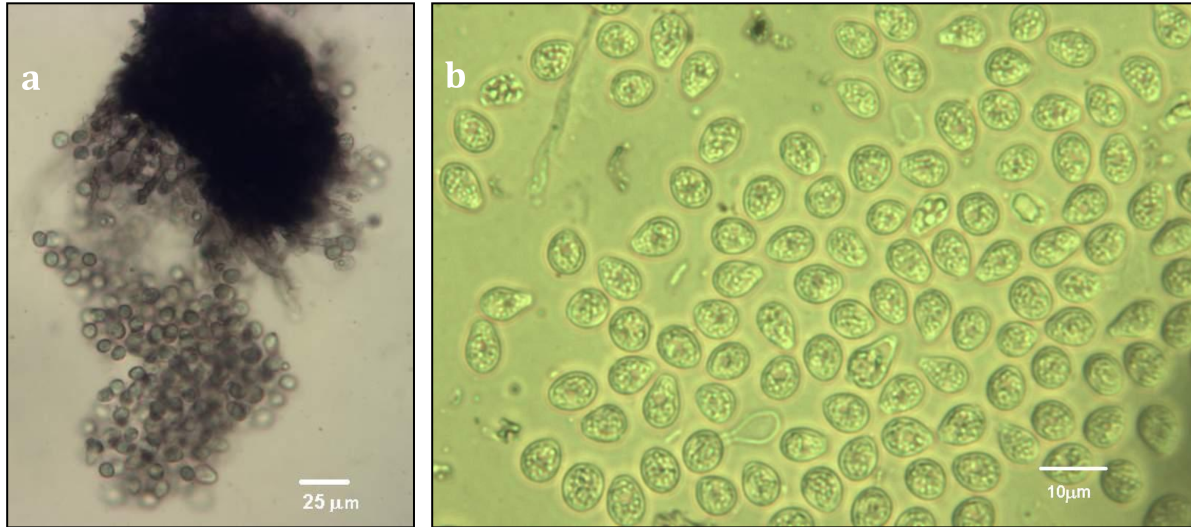

Figure 9. (a) Pycnidia and (a & b) conidia of *Phyllosticta ampelicida* from cultures on ½ PDA incubated for 15 days at 25ºC under continuous fluorescent light. (Photos by M. Sosnowski, SARDI)

4.3 Molecular method

Laboratory supplies

Protective gloves
2.0, 200, and 1000 µL sterile barrier pipette tips
2.0, 20, 200, and 1000 µL pipette tips
Microcentrifuge
Microcentrifuge tubes 1.5 mL
0.2 mL PCR tubes
Thermocycler
Gel tray with suitable comb/s, electrophoresis tank and powerpack
UV transilluminator
Camera/gel documentation system

4.3.1 DNA extraction

Genomic DNA (gDNA) may be extracted from pure fungal cultures and infected plant tissues using commercially available plant DNA extraction kits; e.g. QIAGEN Plant DNeasy kit, Bioline ISOLATE II Plant DNA extraction kit, etc. The preference is for gDNA to be eluted in nuclease-free H_2O. Store gDNA at -20°C.

4.3.2 Conventional PCR

Primers and expected product sizes

For amplification and sequencing of the ITS region:

V9G (forward) 5'-TTACGTCCCTGCCCTTTGTA-3' (de Hoog & Gerrits 1998)

ITS4 (reverse) 5'-TCCTCCGCTTATTGATATGC-3' (White et al. 1990)

Expected product size: approx. 750bp

For amplification and sequencing of partial region of the actin (ACT) gene (Carbone & Kohn 1999):

ACT512F (forward) 5'- ATGTGCAAGGCCGGTTTCGC -3'

ACT783R (reverse) 5'- TACGAGTGCCTTCTGGCCCAT -3'

Expected product size: approx. 280bp

For DNA sequencing analysis, it is preferred (but not essential) that a high-fidelity PCR master mix is used. Using the high-fidelity master mix will avoid the introduction of PCR errors into the product for DNA sequencing purposes, and to minimise pipetting errors in the preparation of PCR master mix. High-fidelity PCR master mixes can be purchased on a commercial basis, e.g. Phusion® High-Fidelity PCR Master Mix, Platinum® Taq DNA polymerase High Fidelity, etc.

DNA gel running buffer – TBE Buffer

Tris-Borate-EDTA (TBE) gel running buffer can be purchased commercially in concentrated liquid format. Follow the manufacturer's instructions to dilute it to a 1X concentration. Alternatively, it can be made up from the following components:

	Per 1L	Final conc.
Tris base	54.0 g	0.4 M
Boric acid	27.5 g	0.05 M
0.5M EDTA pH 8.0	20.0 mL	0.001 M

Dissolve components in 1L distilled water. Store at room temperature.

Note: Alternatively TAE (1×Tris-acetate-EDTA) buffer can be used.

Agarose gel

	Per 100 mL	Conc.
DNA grade agarose	1.0 g	1%
	1.5g	1.5%
TBE	100 mL	1x

Dissolve the molecular biology-grade agarose in TBE buffer in a heat-proof glass container (e.g. beaker or Schott bottle) by heating in a microwave. Once the bottle is slightly cool to the touch, pour into the gel tray with comb. It will take approximately 30 mins to set at room temperature (20-22^{0}C).

WARNING: The container and the content are extremely hot. Handle the container with care using heat-proof gloves.

DNA Loading dye and DNA stain

Certain dye powders are hazardous in concentrated forms. Therefore due to the workplace health and safety considerations of dye powders, DNA loading dye should be purchase from commercial companies.

Either Ethidium Bromide or GelRed® may be used to stain double-stranded DNA to visualise it on an agarose gel.

PCR

Prepare the following PCR mix as described below for the number of test samples, a blank, and one extra.

Reagents	Volume (µL) per reaction
Phusion ® Master Mix (2X)	12.5
Forward Primer (10µM)	0.5
Reverse Primer (10µM)	0.5
Nuclease-free H_2O	10.5
Total	24.0

Add 1 µL of gDNA from pure fungal culture as template, place in a thermocycler using the following program:

Initial denaturation	98°C	30 secs	1 cycle

Denaturation	98°C	10 secs	30 cycles
Annealing	55 or 60°C*	30 secs	
Extension	72°C	30 secs	
Final Extension	72°C	5 mins	1 cycle
Cool	12°C	10 mins	1 cycle

* Anneal temperatures for the following PCR: ITS at 55°C and ACT at 60°C.

When the PCR is complete, mix 2µl of each PCR sample with 1 µl of DNA loading dye.

Load the samples into a 1% TBE agarose gel.

Run the gel in TBE buffer at 100V for 40 minutes.

Visualise and photograph the gel under UV-light.

Sequence PCR product

Once it is confirmed that there is a single PCR product of expected size, prepare the PCR product for sequencing. Refer to the sequencing facility's guidelines for sample preparation and shipment.

DNA sequence analysis

Sequences from the ITS region and partial region of the ACT gene should only be compared to *P. ampelicida* ex-neotype strain ATCC 200578, GenBank accession KC193586 (ITS) and KC193581 (ACT). For a positive identification, the ITS sequence of the sample must be 100% match to the reference sequence, while ACT must be 99-100% match.

5 CONTACTS FOR FURTHER INFORMATION

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The protocol was reviewed by Sri Kanthi de Alwis, Victorian DPI (2010) and Yu Pei Tan, Qld DAF (2016). The molecular section was reviewed by Jeyaseelan Baskarathevan, Plant Health and Environment Laboratory, MPI, New Zealand

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8 APPENDICES

8.1 Disease cycle

The black rot disease cycle is illustrated in Fig 10. The black rot fungus overwinters primarily in mummified fruit within the vine and on the ground, although it also can overwinter for at least 2 years within lesions of infected shoots that are retained as canes or spurs. Spring rains trigger release of ascospores (airborne sexual spores) that form within mummies on the ground and in the trellis, and these can be blown for moderate distances by wind. Conidia (asexual spores) can also form, both within cane lesions or on mummies that have remained within the trellis, and these are dispersed short distances within the vine or to neighbouring vines by splashing rain drops. This process can start within 1 hour of the onset of rain (at least 0.3 mm) and may continue for up to 8 hours after rainfall ceases (Ferrin and Ramsdell 1977). Infection occurs when either spore type lands on susceptible green tissue such as leaves, blossoms and young fruit and it remains wet for 6-24 h depending on temperature (Spotts 1977).

Ascospores slowly germinate, often taking 36 to 48 hours, but eventually penetrate the young leaves and fruit stems (pedicels). The period of time required for symptoms to appear after the occurrence of infection depends on both the temperature and the age of the tissue at the time of infection, usually taking 8 to 25 days. The period during which these overwintering spores are available to cause infections depends on their source. From mummies on the ground, significant discharge of ascospores begins about 2 to 3 weeks after bud break and is virtually complete within 1 to 2 weeks after the start of flowering. In contrast, mummies within the trellis can continue to release both conidia and ascospores from the early pre-flowering period through veraison. From overwintering cane lesions, conidia can be dispersed from bud break through mid-summer.

Pycnidia, produced within the lesions, continue to release conidia during wet weather throughout the season. The conidia will germinate and infect leaves, blossoms and young fruit and can cause substantial spread of the disease under warm and rainy conditions, particularly if berries are still susceptible to infection after conidia develop. Most berries that become infected near the end of their period of susceptibility do not show symptoms until at least 3 weeks later, and the majority do not begin to rot until 4 to 5 weeks after the infection event. Fruit are most susceptible to infection by the fungus from mid-flowering to about 6 weeks after flowering, and become resistant to infection at maturity. Some older literature has reported that berries become resistant when they reach 5% to 8% sugar, while research in New York indicates that berries become resistant much earlier, 3 to 4 weeks after flowering.

In summary, spore production, dispersal, infection and continued disease are favoured by warm, humid conditions, summer rainfall and persistent dew. .

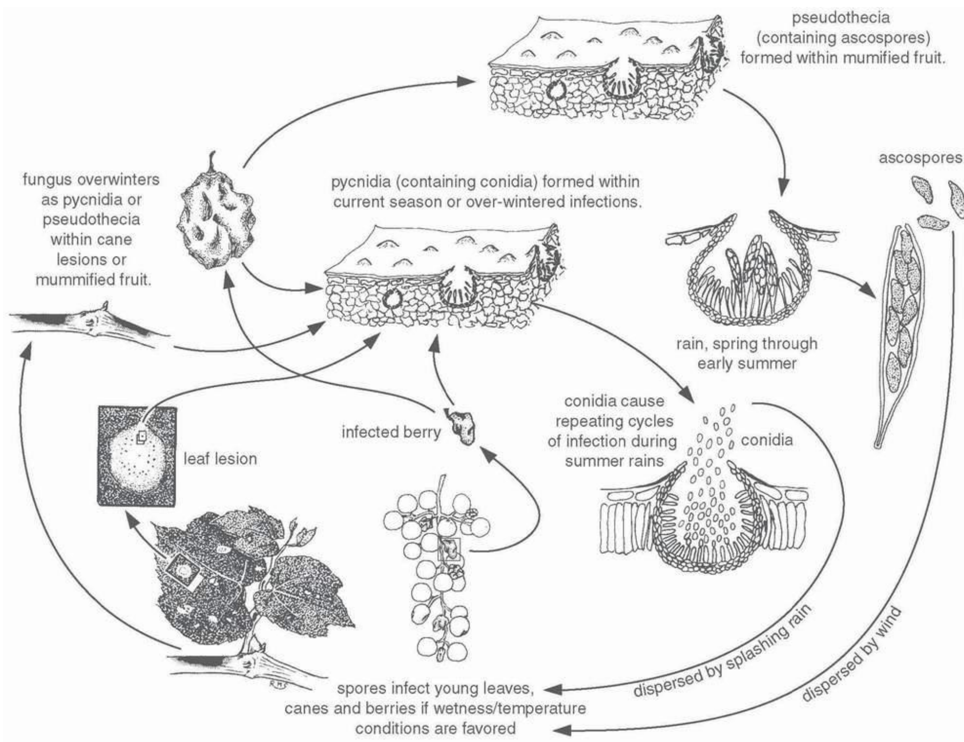

Figure 10. The disease cycle of black rot, caused by *Phyllosticta ampelicida* (Wilcox 2003)