

National Diagnostic Protocol

Roesleria subterranea

The cause of grape root rot



NDP 35 V1

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Department of Agriculture and Water Resources

Street Address 1: [18 Marcus Clarke Street](#), Canberra City ACT 2601

Street Address 2: [7 London Circuit](#), Canberra City ACT 2601

Postal Address: GPO Box 858, Canberra City ACT 2601

Switchboard Phone: 02 6272 3933

Web: <http://www.agriculture.gov.au>

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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

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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

1.1 The Pathogen

This diagnostic protocol provides technical information for the identification of *Roesleria subterranea* (Weinm.) Redhead (synonym *R. hypogaea*), the causal agent of grape root rot.

Roesleria subterranea is primarily known as a pathogen of grapevine (Gärtel 1988), but is also responsible for the death of a variety of fruit trees (Veghelyi 1987).

Roesleria subterranea is often found in combination with other root pathogens such as phyloxera, nematodes and *Cylindrocarpon* species, and is particularly serious in replant situations. Until relatively recently, it was regarded as an opportunistic secondary invader of damaged roots or weakened hosts, but is now known to be a primary pathogen, able to kill vines and fruit trees within 2-5 years from infection (Veghelyi 1989, Miles and Schilder 2009).

Roesleria subterranea is a soilborne pathogen which infects the roots of the host, causing the roots to rot and decay. As such, the aboveground symptoms are not particularly diagnostic, consisting of poor growth, leaf chlorosis, early senescence and dieback.

1.2 Host range

Relatively little is known about the full host range of *Roesleria subterranea*. It was first recorded from *Vitis* and infects several species from this genus. It is known to cause death of grapevines and fruit trees within 2-5 years of infection and has been reported on other deciduous woody hosts. Nothing is known about the susceptibility or otherwise of Australian native flora.

Recorded host plant genera: *Coryllus* (hazelnut), *Cydonia* (quince), *Juglans* (walnut), *Malus* (apple), *Paliurus Populus* (aspen), *Prunus* (almond, apricot, plum, peach, cherry, Mahaleb cherry), *Pyrus* (pear), *Rosa* (rose), *Rubus* (blackberry), *Salix* (willow), *Tilia* (lime, basswood, linden), *Vitis* (grapevine)

(Beckwith 1924, Veghelyi 1987, 1989, Gärtel 1988, Kirchmair pers. comm.).

2 TAXONOMIC INFORMATION

Kingdom: Fungi
Phylum: Ascomycota
Order: Helotiales
Family: Roesleriaceae
Genus: *Roesleria*
Species: *subterranea*

Name: *Roesleria subterranea* (Weinm.) Redhead

Basionym: *Pilacre subterranea* Weinm. 1832

Morphic status: Teleomorph (anamorph connection(s) unknown)

Taxonomic synonyms (ordered by epithet name): *Sphinctrina coremioides* Berk. & Broome 1873, *Vibrissea flavipes* Rabenh. 1852, *Pilacre friesii* Weinm. 1832, *Onygena friesii* (Weinm.) Weinm. 1834, *Roesleria hypogaea* Thüm. & Pass. 1877, *Vibrissea hypogaea* (Thüm. & Pass.) Richon 1881, *Roesleria pilacriiformis* Henn. 1895, *Coniocybe pilacriiformis* (Henn.) Rehm 1896, *Pilacre pilacriiformis* (Henn.) Boud. 1907

Roesleria subterranea is morphologically very similar to the lichen, *Sclerophora pallida* (Pers.) Y.J. Yao & Spooner (synonym *Roesleria pallida* (Pers.) Sacc.), and this has led to confusion. The name *R. pallida* had been misused frequently to refer to *R. subterranea* (as *Roesleria hypogaea*; Beckwith 1924). Dennis (1978) considered *R. pallida* as a synonym of *R. hypogaea*. However, the name *Roesleria pallida* is not applicable to the grape root parasite (Redhead 1984, Beckwith 1924), as it is based on the lichen *S. pallida* (previously known as *Calicium pallidum* Pers.). This confusion has been comprehensively discussed by Redhead (1984) and Yao and Spooner (1999).

More recently, Kirchmair *et al.* (2008) evaluated the taxonomy of *Roesleria subterranea*. Based on multiple genes, it is closely related to *Hymenoscyphus pseudoalbidus* (Helotiales), the cause of ash dieback in Europe. Furthermore, isolates of *R. subterranea* from different hosts appear to be genetically dissimilar and may represent a species complex. Analysis of additional isolates from different hosts are required to resolve the phylogeny of this species.

Common names

Grape root rot
Roesleria root rot

3 DETECTION

Identification should not be attempted by morphology as *R. subterranea* is morphologically very similar to several related and unrelated organisms. All suspect samples should be tested by PCR for rapid diagnosis (within one day) coupled with isolation and culturing (one week). Samples that were positive based on the PCR assay should be sequenced for confirmation.

3.1 Plants capable of hosting *Roesleria subterranea*

Most reports of *R. subterranea* are from grapevine, particularly self-rooted *Vitis vinifera*. There is anecdotal evidence of variation in susceptibility. In Germany, for instance, the pathogen is commonly found on *V. x riparia* rootstocks, but less often on the rootstock Börner (Kirchmair pers. comm.). In Michigan, USA, grape root rot has caused the death of *Vitis* interspecific hybrid cultivars Canada Muscat and Chardonel (Miles and Schilder 2009).

In Hungary, *R. subterranea* has been reported on a wide range of fruit trees, causing decline and early death. It occurs on quince (*Cydonia oblonga*), walnut (*Juglans regia*), apple (*Malus domestica*), pear (*Pyrus communis*), grapevine (*V. vinifera*) and numerous *Prunus* species: apricot (*P. armeniaca*), cherry (*P. avium*), sour cherry (*P. cerasus*), plum (*P. domestica*), almond (*P. dulcis*), mahaleb cherry (*P. mahaleb*) and peach (*P. persica*) (Veghelyi 1987).

Other reported hosts include deciduous shrubs and trees, such as hazelnut (*Coryllus avellana*), willow (*Salix* spp.), *Tilia* spp., *Rosa* spp., blackberry (*Rubus fruticosus*), *Paliurus* spp. and aspen (*Populus tremula*) (Beckwith 1924).

3.2 Symptoms

Roesleria subterranea infects the roots, causing root rot and slow decline over a period of 2 to 3 years. After this time, the grapevine or fruit tree usually dies. Root decay is well-advanced by the time above-ground symptoms become apparent.

3.2.1 Grapevine – above-ground symptoms

The above-ground symptoms are most conspicuous in summer and affected plants are usually found in patches (Fig 1). The most common symptom is poor vigour of the entire plant (Fig 2). The shoots are stunted with shortened internodes and yellow leaves (Fig 3). Leaf symptoms begin as mild chlorosis, but as the disease progresses the discoloration intensifies and the leaf edge becomes necrotic (Fig 4). The leaf margins may also become cupped or rolled (Fig 5). During very hot weather in summer, the whole vine may collapse rapidly, a symptom known as apoplexy. The leaves wither and dry suddenly and remain attached to the shoots.

NOTE: The above-ground symptoms are remote from the site of infection (the root) and are the result of root damage. As a consequence, they are NOT diagnostic and are similar to symptoms resulting from abiotic causes such as soil compaction, poor soil nutritional or moisture status, 'J'-rooting and poor graft unions, or biotic causes such as damage caused by phylloxera or nematodes, Petri disease, Eutypa dieback, esca, grapevine leafroll virus, and phytoplasmas.



Figure 1. A patch of grapevines affected by grape root rot, Germany. Photo: Lars Huber, Johannes Gutenberg-University of Mainz, Germany.



Figure 2. Poor growth due to grape root rot, Germany. Photo: Lars Huber, Johannes Gutenberg-University of Mainz, Germany.



Figure 3. The shoots of infected grapevines are stunted, with shortened internodes and yellow leaves; Austria. Photo: Helga Reisenzein, Austrian Agency for Health and Food Safety.



Figure 4. (L) Leaf symptoms of an infected vine: note the chlorosis and necrotic leaf margins; Austria.
Figure 5. (R) Apoplexy, or sudden death, of an infected grapevine; Austria. Photos: Helga Reisenzein, Austrian Agency for Health and Food Safety.

3.2.2 Grapevine – root symptoms

A characteristic symptom of grape root rot is that an infected root becomes brittle and snaps as if it was made of glass. *Roesleria subterranea* colonises the vascular tissue of the root (Fig 6). The hyphae pack the xylem vessels, blocking water transport. No mycelium is observed on the root surface.

The internal tissue is stained dark brown from the production of phenolic compounds (Figs 7, 8). Fruiting bodies (mazaedia) are produced on the surfaces of infected roots and underground portions of the trunk (Figs 9, 10, 11) in late summer to autumn, but can be found at any time of year if conditions are favourable. They are persistent and difficult to remove by washing or rubbing.

In a patch of dying vines, the fruit bodies are more likely to be found on vines near the margin of the patch than on dead vines in the centre.

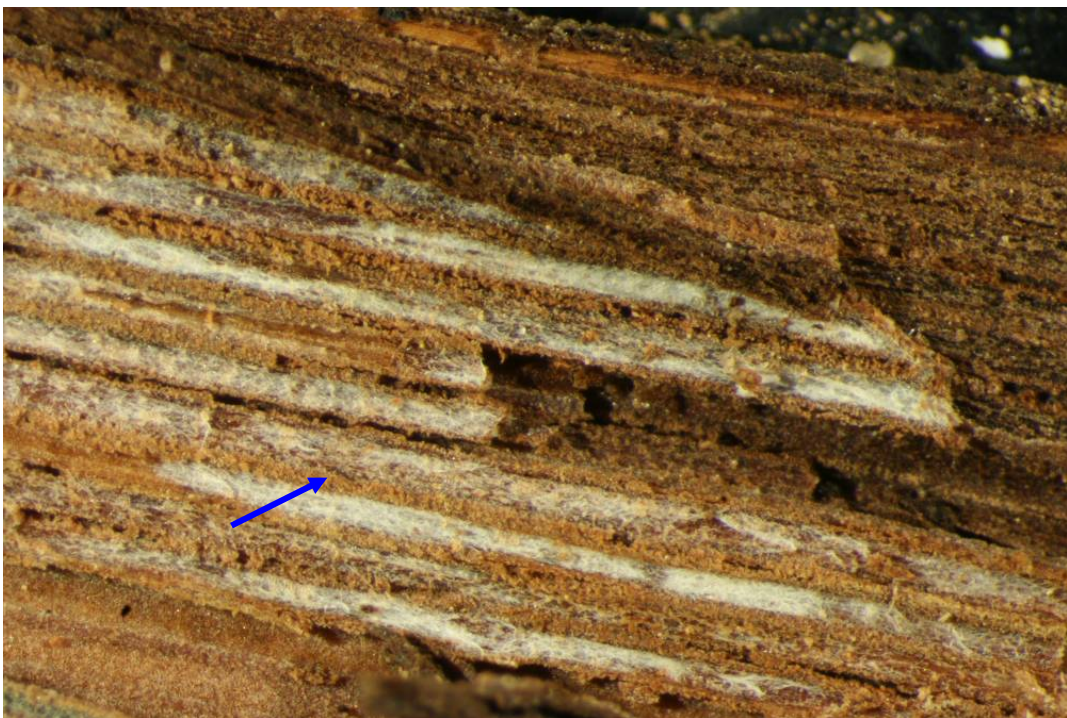


Figure 6. Longitudinal section of an infected grapevine root showing *Roesleria subterranea* hyphae packed in the xylem vessels. Photo: Martin Kirchmair, Leopold-Franzen-University of Innsbruck, Austria.



Figure 7. Grapevine root infected with *Roesleria subterranea* (right) is stained dark brown, compared with healthy root tissue (left). Photo: Martin Kirchmair, Leopold-Franzen-University of Innsbruck, Austria.



Figure 8. Cross-section of an infected root showing staining of the infected portion. Photo: Sigrid Neuhauser, Leopold-Franzen-University of Innsbruck, Austria.



Figure 9. Cross-section of infected rootstock portion of grapevine, showing internal stained wood and *Roesleria subterranea* fruit bodies on the external surface; Germany. Photo: Martin Kirchmair, Leopold-Franzen-University of Innsbruck, Austria.



Figure 10. *Roesleria subterranea* fruit bodies on infected grapevine roots; Germany. Photo: Lars Huber, Johannes Gutenberg-University of Mainz, Germany.

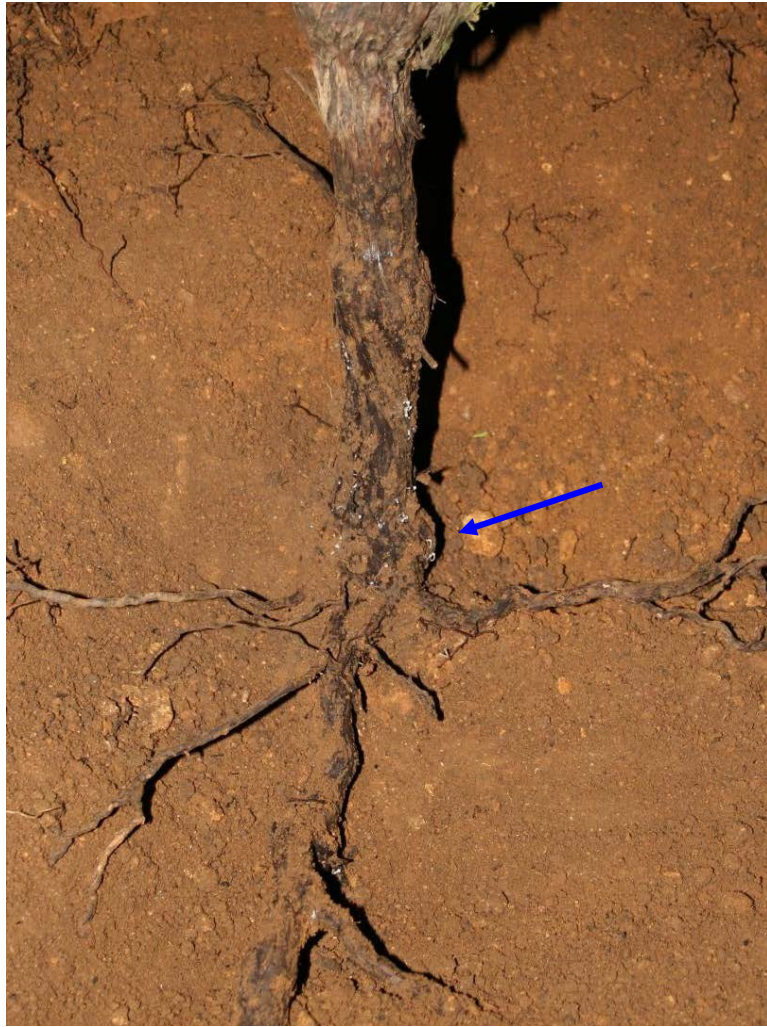


Figure 11. *Roesleria subterranea* fruit bodies (mazaedia) produced on the surface of roots and underground trunk of an infected grapevine, Germany. Photo: Lars Huber, Johannes Gutenberg-University of Mainz, Germany.

3.2.3 Symptoms on hosts other than grapevine

Roesleria subterranea also causes root rot and death on other deciduous woody hosts (see host list above). Apart from the extensive research undertaken by Veghelyi on replant diseases and early death of fruit trees in Hungary (Veghelyi 1985a, 1985b, 1985c, 1986, 1987, 1989, 1994, Molnar *et al.* 2003), there is very little published information on the effects of *R. subterranea* on hosts other than grapevine.

For fruit trees, as for grapevines, aboveground symptoms merely reflect the root damage and are not particularly diagnostic. Examination of the roots for rotting and the presence of mazaedia (Figs 9, 10, 11) is required to diagnose the disease. Veghelyi (1986) reported that infected roots have very few root hairs compared to healthy roots. Losses are mainly concerned with replant disease (i.e. the inability to establish new plantings into land that was previously planted with the same fruit tree species) and premature death of infected planting material both in the nursery and in the orchard.

3.3 Sampling

Sampling is best undertaken in autumn when the fruiting structures will be present. According to Neuhauser (pers. comm), the pathogen is more difficult to detect in warm dry soil (e.g. during summer) than in cool moist soil.

Root samples should be taken from the edge of disease hotspots. Using a spade, collect roots from the upper 50 cm of the soil horizon within 10-20 cm from the grapevine trunk. At least 15 samples per hectare should be collected from the population of suspected plants. During transport to the laboratory the roots should be kept cool (< 25°C), preferably in an esky. In the laboratory, soil should be removed as much as possible without damaging the roots, and the roots should be examined for mizeadia. If not processed immediately, the roots should be airdried overnight and stored at -20°C until use (Neuhauser *et al.* 2011)

Soil samples should be taken from the root zone with a soil corer (minimum diameter 1 cm) from 0-20 cm depth, put into plastic bags and transported to the laboratory. The samples should be airdried overnight, passed through a 2 mm mesh sieve and stored at -20°C until use (Neuhauser *et al.* 2009). Do not pool the soil samples as well-selected, individual samples give more reliable results than pooled samples (Neuhauser *et al.* 2011).

4 IDENTIFICATION

Description: The stipitate ascocarp is a mazaedium (i.e., a powdery mass of ascospores, asci with disintegrating walls and paraphyses covering the disk of an apothecium). The mazaedia are generally solitary on bark (Fig 12). Asci are slender, septate, sometimes branched paraphyses form a dry powdery head on a long stipe that can grow up to 10 mm tall. The head breaks down into a light grey powdery spore mass scarcely 1 mm across (Figs 13 & 14). The slender, smooth, light yellow stalk is composed of compact, parallel, thin-walled hyphae about 3-4 μm wide, which become loose and radiating in the head. Asci (Fig 15) are cylindrical, up to 55 μm long by 10 μm wide, 8-spored, soon vanishing; ascospores (Fig 16) are uniseriate, lens-shaped and septate across the broadest plane, colourless to light greenish-grey, 5-6 x 4-5 μm , smooth. There are slender hyphae among the asci which have been called paraphyses, but there is no compact hymenium. Ascocarps are formed on bark, especially of dead roots, often well below soil level (Dennis 1978, as *Roesleria pallida* (Pers.) Sacc.).

4.1 Microscopic examination

Prior to washing and isolation attempts, the specimen should be examined under a dissecting microscope for fungal structures such as mazaedia (Figs 12-14) which may be present on the host tissue and may assist in the selection of tissue for isolations.

If any structures are observed, they should be carefully picked with a fine needle, 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. Due to the dry, powdery nature of the spores, 3% potassium hydroxide (KOH) is the recommended mounting fluid.



Figure 12. *Roesleria subterranea* mazaedia in situ on a grapevine root (Photos: Lars Huber, Johannes Gutenberg-University of Mainz, Germany; Peter Sholberg, Agriculture and Agri-Food Canada).



Figure 13. Fruiting bodies of *Roesleria subterranea* growing on a grape root. Photo: Michael Weis, Agriculture & Agri-Food Canada.



Figure 14. *Roesleria subterranea* mazaedia demonstrating the disintegration of the powdery grey heads. Photo: Martin Kirchmair, Leopold-Franzen-University of Innsbruck, Austria.

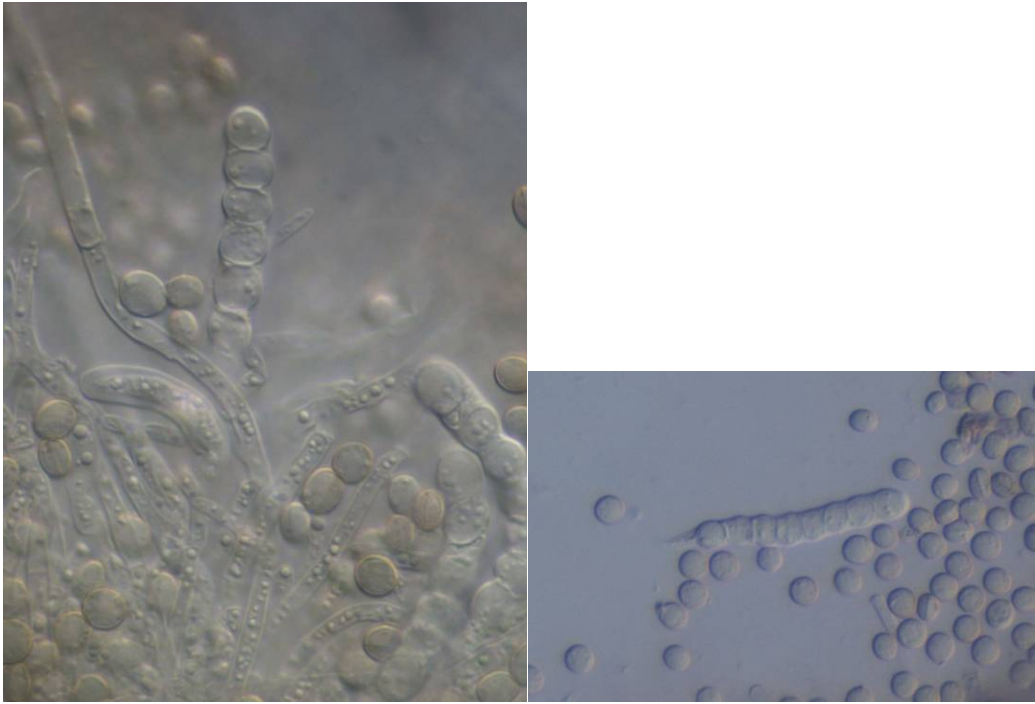


Figure 15. *Roesleria subterranea* asci and ascospores. Photos: Martin Kirchmair, Leopold-Franzen-University of Innsbruck, Austria.

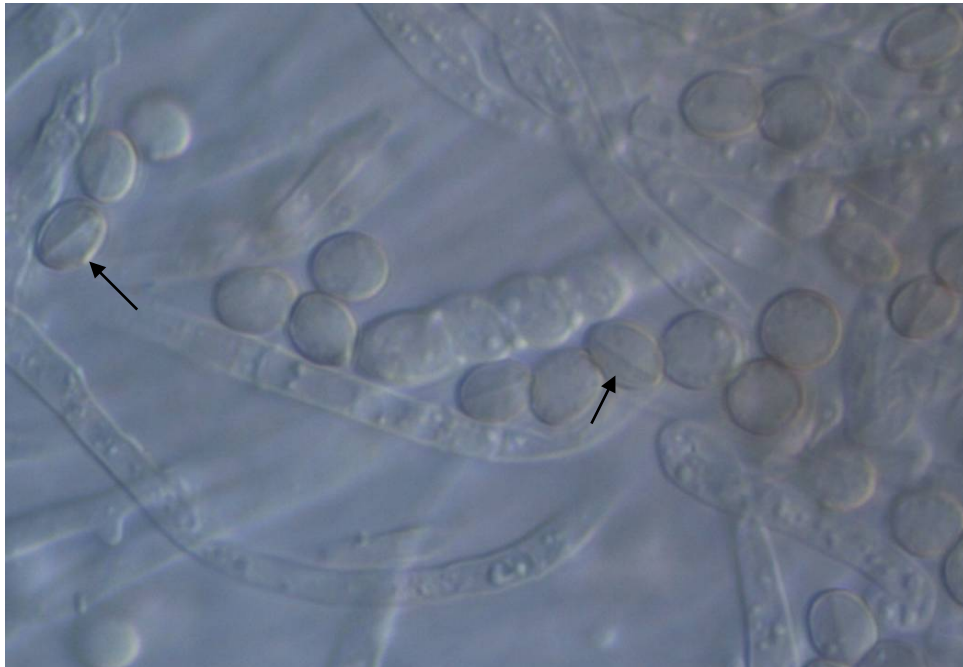


Figure 16. Lens-shaped ascospores of *Roesleria subterranea*, septate across the broadest plane (arrows). Photo: Martin Kirchmair, Leopold-Franzen-University of Innsbruck, Austria.

4.2 Method for isolation from infected roots

Equipment and/or material needed:

- Secateurs
- Scalpel
- Forceps
- Burner (e.g. small gas camping stove)
- General purpose fungal medium plates (e.g. PDA, MEA, OMA)
- Dissecting microscope
- Incubator

Method

If there is a suspect infection, but no fruiting bodies are present, molecular techniques should be used for identification. Isolation directly from root tissue is rarely successful, so the remaining roots should be incubated in a moist, sealable bag maintained at 10–15°C. If roots are infected, fruit bodies may form within 3 days to 2 weeks and spores can be transferred directly to isolation media.

Isolation onto media can be performed on the laboratory bench. Create an upward pull of air by lighting a small gas camping stove or burner next to a dissecting microscope. This minimises the risk of contamination (Neuhauser and Kirchmair pers. comm.). The fungus grows equally and readily on potato dextrose agar (PDA), malt extract agar (MEA), cornmeal agar (CMA) or oatmeal agar (OMA).

Examine the diseased root piece under the dissecting microscope. Using sterile forceps, carefully pick a fruiting body and dot ascospores onto an agar plate. The green pigmentation of *R. subterranea* is suspected to be a potent antibiotic that suppresses growth of other organisms.

Incubate the plates at 23–25°C. Check daily for spore germination, and transfer single germinated spores onto fresh media. The fungus grows slowly (approximately 2 mm per day), white to buff at first, then becoming brilliant green at the centre (Fig 17). It takes 2 to 3 weeks for growth to reach the edge of a 9 cm-diameter plate.



Figure 17. *Roesleria subterranea* grown on MEA at 24°C. Photos: Sigrid Neuhauser, Leopold-Franzen-University of Innsbruck, Austria.

4.3 Molecular identification of *Roesleria subterranea*

Molecular techniques must be used to confirm the identity of *Roesleria subterranea*. If a pure culture is obtained from diseased plant material, sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA can provide confirmation. If a pure culture cannot be obtained, DNA extraction of potentially-infected plant material followed by PCR using species-specific primers can be used to detect the presence of *R. subterranea*. The methods are described below.

4.3.1 Identification of fungus by sequencing the ITS region

Material needed: A pure culture of the suspect fungus.

Universal ITS Primers: (Neuhauser *et al.* 2009; White *et al.* 1990)

- Forward ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3')
- Reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3')

Note: Alternative universal primers for sequencing the ITS region may be used (such as ITS1 and ITS4)

Methods

- Extract DNA (refer to 4.3.1.1) for use as template
- Set up PCR (refer to 4.3.1.2) using the following cycles:
 - 94°C for 120 seconds
 - 30 cycles of 94°C for 30 seconds, 54°C for 45 seconds, 72°C for 60 seconds
 - Final extension for 10 minutes at 72°C
 - Hold at 10°C
- Examine PCR products using electrophoresis gel documentation system. Expected product size range approximately 600 bp.
- Sequence PCR products with primers ITS4 and ITS5, using the laboratory's own methods.
- Using a similarity search program, e.g. BLAST, compare sequences generated from samples with sequences for *R. subterranea* in the GenBank database (www.ncbi.nlm.nih.gov). The ITS region should be at least 95% similar to GenBank sequence EF060308 (CBS 339.96).

DNA extraction

Equipment and/or material needed

- Approximately 1 cm² fungal mycelium from culture
- DNA extraction kit (DNeasy® Plant Mini Kit, QIAGEN)
- Microcentrifuge
- Waterbath (to 65°C)
- Ice and ice tray
- 20, 200 and 1000 µL pipettes and sterile pipette 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 approximately 6 glass beads, add appropriate amount of fungal material, and macerate
- Continue following subsequent steps in the *Protocol*
- Clearly label tubes containing the final elution volume of DNA template and maintain tubes at -20°C

Polymerase Chain Reaction (PCR) for fungi

Equipment and/or material needed

- Thermocycler
- ITS Primers: Forward ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'), Reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3')
- Nuclease-free water
- 10× PCR reaction buffer + Mg
- dNTP mix (10 mM of each dNTP)
- Primers ITS4 and ITS5 (10 µM)
- Taq DNA Polymerase (5 Units/µL)
- 2, 20 and 300 µL pipettes and sterile pipette tips
- 0.2 mL PCR tubes

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

(NB. For generic PCR kit – adapt for the specific kit used)

- 1.0 µL DNA template
- 18.3 µL nuclease-free water
- 2.5 µL 10× PCR reaction buffer (includes 25 mM MgCl₂*)
- 1.0 µL dNTP mix (10 mM of each dNTP)
- 1.0 µL of primer ITS4 (10 µM)
- 1.0 µL of primer ITS5 (10 µM)
- 0.2 µL Taq (or equivalent) DNA Polymerase (5 Units/µL)

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

Methods

- In a laminar flow cabinet, clearly label 0.2 mL tubes according to samples to be used in PCR reaction, plus a positive control of fungal DNA and a blank water sample. Keep tubes on ice while making up master mix.
- Make up master mix from appropriate volumes of PCR reagents in the following order:
 - Nuclease-free H₂O
 - PCR reaction buffer
 - MgCl₂
 - dNTP mix
 - Primers

- Taq DNA polymerase (NB: Taq should remain refrigerated until required. Add Taq last.).
- Quickly vortex and spin at 10,000 x g.
- Add 24.0 µL master mix to each tube
- Add 1.0 µL of DNA template (including the positive control and water negative control) to each respective tube
- Place tubes in thermocycler
- Set thermocycler to desired cycle parameters, run PCR

Check for PCR product using laboratory electrophoresis procedure or equivalent methodology.

Sequencing and analysis

Purify and sequence PCR product using standard laboratory methods. Conduct Blast search of Genbank (e.g. <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). *Roesleria subterranea* is likely a species complex with isolates from *Vitis* genetically different than isolates from other hosts (Kirchmair *et al.* 2008). As the referenced culture is from a deciduous shrub the match is not ideal. However, the ITS region should be at least 95% similar to GenBank sequence EF060308 (CBS 339.96). The default blast phylogenetic analysis should yield a clearly defined clade containing only *R. subterranea* sequences.

4.3.2 Molecular detection of *Roesleria subterranea* in infected root tissue and infested soil

Molecular detection of *Roesleria subterranea* is the preferred method of identification as morphological identification requires the presence of fruiting bodies. Molecular detection directly from diseased root tissue should be used (a) when no fruiting bodies are present, and also (b) to confirm diagnosis when fruiting bodies are present. The high content of polyphenolic compounds in grapevine roots can interfere with the DNA extraction process as well as in later processing steps such as PCR, therefore the method described was developed and published by Neuhauser *et al.* (2009).

During the review process the Qiagen DNeasy® Plant Mini Kit (QIAGEN, Inc.) was used successfully to extract DNA from the plant roots following the manufacturer's instructions. If the chloroform-based DNA extraction method is not routinely used by the laboratory, commercial DNA extraction kits are readily available and sufficient for purposes of pathogen detection.

It may be useful to test soil directly for the presence of *R. subterranea*. This may be needed to investigate the distance the pathogen has spread from an infected host or for surveillance purposes during an incursion.

The presence of various impurities co-extracted with soil DNA, such as metal ions or humic acids, can inhibit PCR reactions. In addition, clay minerals in soil can immobilise DNA and prevent it from being extracted. Therefore, the protocol for DNA extraction from soil was considerably optimised to overcome these problems (Neuhauser 2007, Neuhauser *et al.* 2009).

DNA extraction from roots

Examine the roots and cut the diseased portions into 2 cm pieces. Roots may not need to be washed of adhering soil if this method is used; however, if an alternative method is used (i.e. a commercial kit), then it is necessary to wash the roots of adhering debris.

Equipment and/or material needed

- Autoclaved 1.5 mL and 2.0 mL microcentrifuge tubes
- Autoclaved glass beads: 0.1 - 0.25 mm, 2 mm and 5 mm
- Fume hood
- Ball mill (e.g. Retsch MM301)
- Bench-top incubating shaker
- Forceps
- Spatula with 8 mm diam. spoon
- Sharp knife
- Scalpel and blades
- Pipette and tips (300-500 µL)
- Microcentrifuge
- Microcentrifuge tube racks
- Vortex
- Timer
- -18 to -20°C Freezer
- Absorbent, waterproof bench-top liner
- Tissues
- Disposable gloves
- Balance

Additional equipment and/or material needed for the chloroform extraction method

- CTAB-PVPP-extraction buffer
- TE Buffer
- Chloroform:isoamyl alcohol (24:1)
- M ammonium acetate
- Chloroform waste bottle
- Ice-cold (<0°C) isopropanol (in sterile 50 mL centrifuge tubes)
- Ice-cold (<0°C) 70% ethanol (in 50 mL centrifuge tubes)

(Recipes for buffers are provided below)

Method

The root pieces should be placed (one 2 cm piece per tube) into 2 mL tubes filled with glass beads of different sizes: 2–3 glass beads of 5 mm diameter; approximately 0.14 g of 2 mm diameter beads, and approximately 0.07g of 0.2 mm beads.

First set up the 2 mL tubes with glass beads as follows:

- Label the tubes

- Fill to 0.5 ml mark with 2 mm glass beads
- In the fume hood, add one spatula of small glass beads (0.1–0.25 mm). This must be done in the fume hood to prevent inhalation of the tiny glass particles.
- Add 2–3 5 mm glass beads per tube with forceps.

Add one 2 cm root piece to each tube.

- Homogenise the samples without any buffer in a ball-mill (e.g. Retsch MM301) at maximum speed for 2 minutes.
- Set the shaker to 60°C.
- Add 1 mL CTAB-PVPP-extraction buffer to each tube. When opening the tubes, be careful of any root material adhering to the lid.
- Vortex gently to mix
- Incubate at 60°C with shaking at 800 rpm for 30 minutes.
- Transfer the tubes to a centrifuge and spin at 4000g for 5 minutes.
- Label 4 sets of 1.5 mL microcentrifuge tubes.
- Carefully remove the samples from the centrifuge without disturbing the precipitate.
- In a fume hood, transfer the supernatant into one set of the labelled 1.5 mL tubes. Use a pipette set at 0.5 mL.
- Still in the fume hood, add 0.5 mL chloroform:isoamyl alcohol (24:1) to each tube. **NB This step is tricky as the solvent runs out of the pipette tip very quickly.**
- Vortex briefly to mix the two phases.
- Centrifuge at 16,000g for 5 minutes.

From here on, leave all used tips, tubes, etc. in the fumehood due to the chloroform. Dispose of appropriately.

- Transfer the aqueous top layer (approximately 400 μ L) to a new 1.5 mL tube without disturbing the chloroform layer. Dispose of the chloroform correctly into a labelled waste container in the fume hood.
- Repeat the chloroform addition step, vortex, and centrifuge again
- While centrifuging, add 300 μ l 5M ammonium acetate into new 1.5 mL tubes. Take into the fume hood.
- Transfer the aqueous supernatant from the spun tubes into the new tubes (approximately 300 μ L)
- Close the tubes and mix gently by inversion
- Incubate in the freezer at -18 to -20°C for 15 minutes
- Cool the centrifuge to 4°C
- Take the tubes from the freezer to the centrifuge and spin at 16,000g for 5 minutes
- This time, the two phases are not visible. Transfer most of the solution (approximately 500 μ l) into new 1.5 mL tubes. Do this directly out of the centrifuge to keep as cold as possible.
- Add 500 μ L ice-cold (<0°C) isopropanol (direct from freezer)
- Incubate in the freezer for at least 30 minutes (can be overnight)
- Centrifuge at 4°C, 16,000g for 10 minutes
- Set shaker to 45-50°C to dry pellets
- Remove from centrifuge and discard the supernatant carefully without dislodging the DNA pellet

- Add 400 µL ice-cold (<0°C) 70% ethanol. Spin at 16,000g for 5 minutes at 4°C.
- Discard supernatant and dry pellets.
- When the pellets are dry, redissolve in 200 µL TE buffer and resuspend on the shaker at 60°C, 800 rpm, for 30 minutes
- Store in the freezer

Proceed to the PCR step below (0) using the extracted DNA.

DNA extraction from soil

Take 20 grams of soil from the rootzone (as previously described), and sieve to remove stones and roots. Then take 3 x 0.5 g samples for DNA extraction.

Equipment and/or material needed

- Autoclaved 2.0 mL microcentrifuge tubes, with lids (for initial step with glass beads)
- Autoclaved 2.0 mL microcentrifuge tubes, without lids (for purification step)
- Autoclaved 1.5 mL microcentrifuge tubes
- Autoclaved 0.5 mL microcentrifuge tubes, with pre-made holes in lids (for purification step)
- Autoclaved glass beads: 0.1 - 0.25 mm and 2 mm
- 50 ml centrifuge tubes
- Fume hood
- Ball mill (e.g. Retsch MM301)
- Bench-top incubating shaker
- Needle
- Spatula with 8 mm diameter spoon
- Skim milk buffer
- TE-PVPP solution
- TE buffer
- Chloroform:isoamyl alcohol (24:1)
- 2.5 M ammonium acetate
- Ice-cold (<0°C) isopropanol (in sterile 50 mL centrifuge tubes)
- Ice-cold (<0°C) 70% ethanol (in 50 mL centrifuge tubes)
- 200 and 1000 µL pipettes and tips, including sterile filtered tips
- Chloroform waste bottle
- Microcentrifuge
- Microcentrifuge tube racks
- Vortex
- Timer
- -18 to -20°C Freezer
- Absorbent, waterproof bench-top liner
- Tissues
- Disposable gloves
- Balance

Buffer recipes (see below)

Method

First set up the 2 mL tubes with approximately 0.5 g (2 mm:0.2 mm at 3:1) glass beads as follows:

- Label the tubes
- Fill to 0.5 mL mark with 2 mm glass beads
- In the fume hood, add one spatula of small glass beads (0.1-0.25 mm). This must be done in the fume hood to prevent inhalation of the tiny glass particles.

Weigh 0.5 g soil into each tube (there should be 1:1 volume of soil:beads). There should be three replicates per soil sample. Prevent cross-contamination by sterilising the spatula used for weighing soil by dipping in a beaker of ethanol between each soil sample.

- Add 300 μ L SDS buffer to each tube
- Add 300 μ L skim milk buffer to each tube, changing the pipette tip after every sample (i.e. after 3 tubes)
- Close lids
- Transfer the tubes to a fume hood. Add 400 μ L 24:1 chloroform:isoamyl alcohol to each tube. Close lids and mix by inversion. Do not vortex.
- Homogenise the samples in a ball-mill at maximum speed for 40 seconds
- Centrifuge at 16,000 g for 5 minutes

From here on, leave all used tips, tubes, etc. in the fumehood due to the chloroform. Dispose of appropriately.

- Label 1.5 mL microcentrifuge tubes.
- Carefully remove the samples from the centrifuge without disturbing the precipitate
- In a fume hood, transfer the supernatant into one set of the labelled 1.5 ml tubes. Use a pipette set at 0.5 ml using filtered tips.
- Add 200 μ L 2.5M ammonium acetate into each tube. Invert 2 to 3 times. Should be cloudy due to proteins.
- Centrifuge at 16,000 g for 5 minutes
- Label new 1.5 mL tubes
- In the fume hood, pipette supernatant into tubes (approximately 300-400 μ L)
- Add an equivalent volume of ice-cold (<0°C) isopropanol to each tube (approximately 400 μ L)
- Mix by inversion a couple of times
- Leave for at least 15 minutes on the bench top

Prepare the PVPP (poly [vinyl-polypyrrolidone]) solution for purification of the DNA. The polymer needs time to swell.

- Make two lots of diluted TE buffer by adding 5 mL TE buffer to each of two 50 mL centrifuge tubes and adding 45 mL sterile water
- In two more centrifuge tubes, fill PVPP powder up to the 10 mL mark.
- Add the 50 mL diluted TE buffer and mix well
- Leave for at least 30 minutes to swell

Set the centrifuge at 21°C and the incubating shaker at 45°C.

- Place the tubes in the centrifuge with the hinges at the top. This ensures the DNA pellet is not dislodged when discarding the isopropanol.
- Centrifuge at 16,000 g for 5 minutes
- Discard the isopropanol into a beaker. Dispose of appropriately.
- Add 1 mL ice-cold (<0°C) 70% ethanol to the DNA pellet in each tube.
- Centrifuge again at 16,000 g for 5 minutes
- Discard the ethanol. Pipette out the last bit.
- Place in the incubating shaker at 45°C til DNA pellets are dry.

Purification step:

- Use 2 ml microcentrifuge tubes without lids
- Using a needle, prick a hole in the bases of the 0.5 mL microcentrifuge tubes with pre-made holes in lids
- Place the 0.5 mL tubes inside the 2 ml tubes
- Fill the 0.5 mL tubes with TE-PVPP solution and close the lids
- Spin gently in the centrifuge at 800 g for 3 minutes to dry the polymer. Long and slow is best, or else the polymer spins through the hole. If not dry, spin again for another 3 minutes.
- Label 1.5 mL tubes
- Resuspend the dry DNA pellets by adding 120 µL TE buffer to each tube
- Mix on a vortex to redissolve the pellet
- Load each 120 µL onto the PVPP columns i.e. the 0.5 mL tubes inside the 2 mL tubes
- Spin at 1,400 g for 5 minutes
- Discard the columns and store the purified DNA samples at -20°C

Proceed to the PCR step below (4.3.2.3) using the extracted DNA.

Buffer recipes for DNA extraction buffers

CTAB-PVPP-extraction buffer

- 200 mM Tris (pH 7.5)
- 20 mM EDTA
- 1.4 M NaCl
- 2% CTAB
- 4.5% PVPP wt/vol (i.e. 4.5 g to 100 mL)
- Make up 200 mL. Autoclave buffer before adding PVPP.

TE Buffer

- 100 mM NaCl
- 10 mM Tris
- 1 mM EDTA
- Make up 100 mL. Adjust pH to 8 with 1 M HCl. Autoclave, then divide into 50 mL aliquots in sterile 50 mL centrifuge tubes and store in the freezer.

24:1 chloroform:isoamyl alcohol

- 240 mL chloroform
- 10 mL isoamyl alcohol
- Store in a labelled Schott bottle

Ammonium acetate

Make up 100 mL in autoclaved distilled water. Make 5 M for use with roots and 2.5 M for use with soil. Store in the fridge.

SDS Buffer (sodium dodecyl sulphate)

- 100 mM NaCl
- 500 mM Tris
- 10% SDS wt/vol
- Make up 200 mL. Adjust pH to 8 with 1 M HCl. Autoclave, then divide into 50 mL aliquots in sterile 50 mL centrifuge tubes.

Skim Milk Buffer

- (Oxoid Skim Milk Powder)
- 2% wt/vol with autoclaved distilled water.
- Add 4 g powder to 200 mL water in an Erlenmeyer flask. Heat and stir the liquid (with a stirrer bar) until the powder is dissolved. This buffer spoils very quickly, so divide into 10 mL aliquots and store frozen. Thaw in microwave as required.

Polymerase Chain Reaction (PCR)

For soil, a single PCR reaction using the species-specific primer pair Rs1R / Rs2F should be sufficient.

For root tissue, nested PCR is required. The first round of PCR should use primer pair ITS 5 / ITS 4 (or equivalent universal primers for fungi). The product from this reaction is then used as the template DNA for the second round of PCR using the species-specific primer pair, Rs1R / Rs2F. In addition, the primer pair, Cox-R / Cox-F, designed by Weller *et al.* (2000) to amplify plant DNA should be included to serve as an internal control for plant DNA. This will ensure the DNA extraction and PCR were successful in each reaction.

A separate master mix is required for each primer pair.

Universal ITS Primers: (White *et al.* 1990)

- Forward ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3')
- Reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3')

Note: Alternative universal primers for sequencing the ITS region may be used (such as ITS1 and ITS4)

Primers specific for *Roesleria subterranea*: (Neuhauser *et al.* 2009)

- Rs1R (5'-TCC GGA ACG TCT ATA GCG AGG AGA-3')
 - Rs2F (5'-TCG CGG GCA ACC GGC TCA CGC-3')
- (Amplified fragment size = 360 bp, Neuhauser pers.comm.)

Primers for plant DNA (internal control): (Weller *et al.* 2000)

- Cox-R (5'- CAA CTA CGG ATA TAT AAG AGC CAA AAC TG-3')
- Cox-F (5'- CGT CGC ATT CCA GAT TAT CCA-3')

Equipment and/or material needed

- Thermocycler
- 10X PCR reaction buffer
- Primers Rs1R and Rs2F, ITS 4 and ITS 5, Cox-R and Cox-F
- 25 mM MgCl₂
- 20 mg/ml Bovine Serum Albumen (BSA)
- Taq DNA Polymerase (5 Units/μL)
- Nuclease-free water
- 2, 20 and 300 μL pipettes and sterile pipette tips
- 0.2 mL PCR tubes
- Ice
- Foam box
- Disposable gloves
- Tissues

Volume of PCR reagents per primer pair (to make up total volume of 20 μL)

- 2.0 μL DNA template
- 2.0 μL PCR reaction buffer
- 2.0 μL of each primer (ITS4/ITS5 or Rs1R/Rs2F or Cox-R/Cox-F)
- 2.0 μL dNTP mix (0.2 mM of each dNTP)
- 2.0 μL 25 mM MgCl₂
- 2.5 μL 20 mg/ml Bovine Serum Albumen (BSA)
- 0.1 μL Taq DNA Polymerase (5 Units/μL)
- 5.4 μL nuclease-free water

PCR conditions

- Step 1: denaturation 94°C for 2 minutes; 1 cycle
- Step 2: denaturation 94°C for 20 seconds, annealing 61°C for 25 seconds, extension 72°C for 50 seconds; 30 cycles
- Step 3: extension 72°C for 300 seconds; 1 cycle
- Step 4: 10°C hold

Method

- In a laminar flow cabinet, clearly label 0.2 mL tubes according to samples to be used in PCR reaction, plus a positive control of *Roesleria subterranea* DNA and a blank water sample. Keep tubes on ice while making up master mix.
- Make up master mix from appropriate volumes of PCR reagents in the following order:
 - nuclease-free H₂O
 - PCR reaction buffer
 - MgCl₂
 - dNTP mix
 - primers
 - Taq DNA polymerase (NB: Taq should remain refrigerated until required. Add Taq last.)
 - Quickly vortex and spin at 10,000 x g

- Add 18.0 µL master mix to each tube
- Add 2.0 µL of DNA template (including the positive control and water negative control) to each respective tube
- Place tubes in thermocycler
- Set thermocycler to desired cycle parameters and run PCR

Check for PCR product using laboratory electrophoresis procedure or equivalent methodology.

Interpretation of the results

The PCR test will only be considered valid if:

- The positive control produces the correct size product (approximately 600 bp, Neuhauser pers.comm.)
- The internal control produces the correct band size in each lane
- No bands are produced in the negative control.

In the event of a positive result, the PCR product should be sequenced using Rs1R and Rs2F for confirmation (Rs1R and Rs2F were designed from the ITS region of *R. subterranea*).

5 CONTACTS FOR FURTHER INFORMATION

Dr Jacqueline Edwards
Department of Economic Development, Jobs, Transport and Resources
AgriBio, 5 Ring Road, La Trobe University,
Bundoora, Vic 3083, Australia
Jacky.Edwards@ecodev.vic.gov.au

Drs Sigrid Neuhauser and Martin Kirchmair
Institute of Microbiology
Leopold-Franzen-University of Innsbruck
Technikerstraße 25
6020 Innsbruck, Austria
Ph: +43/(0)512/507-6013
Fax: +43/(0)512/507-2938
Sigrid.Neuhauser@uibk.ac.at
Martin.Kirchmair@uibk.ac.at
www.uibk.ac.at/mycologi
www.uibk.ac.at/bipesco

Helga Reisenzein
Austrian Agency for Health and Food Safety (AGES)
Institute of Plant Health
Head of Dept. for Phytopathology, Fruit Production & Viticulture
Spargelfeldstr. 191, 1226 Wien
Ph: ++ 43-0-50 555-33340
Fax: ++43-0-50555-33303
helga.reisenzein@ages.at
www.ages.at

Dr Peter Sholberg
Pacific Agri-Food Research Centre
Agriculture and Agri-Food Canada
PO Box 5000, 4200 Hwy 97
Summerland
British Columbia V0H 1Z0
Canada
Ph: 250-494-6383
Fax: 250-494-0755
Peter.Sholberg@AGR.GC.CA

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8 APPENDIX

8.1 Disease cycle

Roesleria subterranea is a soilborne facultative pathogen that can exist as a saprobe on dead material in the soil for many years (Hofer 1992). This fungus used to be considered of minor importance as it is difficult to detect and easily confused with the morphologically similar lichen, *Sclerophora pallida*. Additionally, it is often associated with other root pests such as phylloxera, nematodes and soilborne pathogenic fungi, e.g. *Cylindrocarpon* species. Therefore it was dismissed as a weak secondary invader.

During the past 20 years, it has become apparent that *R. subterranea* is a serious primary pathogen, and can cause death of a variety of fruit trees (Veghelyi 1987) and grapevines (Miles and Schilder 2009; Hofer 1992 and Huber *et al.* 2006) within 2 to 5 years of infection. The disease is particularly severe on heavy, wet soils in cool climates with soil temperatures around 15–20°C. However, the fungus is able to tolerate sandy soils, growing at temperatures ranging from -3°C to 35°C, in pH ranges from 2.5 to 8, and with soil moisture ranging from 10–80% water holding capacity of soils (Hofer 1992 in Neuhauser 2007).

Roesleria subterranea infects plant root and grows through the cortex to the vascular tissue, colonising the root and causing decay. Only teleomorphs (sexual reproductive structures) have been found. Fruiting bodies or ascomata of *R. subterranea* are usually formed in late summer to autumn on decayed roots and underground parts of the trunk from 0.2–1.5 m depth. The ascomata can be present at any time of the year when conditions are favourable.

Roesleria subterranea forms ascomata consist of ‘mazaedia’ (i.e. an ascomata in which the asci walls disintegrate leaving a powdery mass of ascospores at maturity) with paraphyses covering the disc of apothecia. Ascospores are not discharged actively, but disperse passively by soil water, soil fauna and movement of infected plant material, contaminated soil, or contaminated machinery. For example, earthworms are known to ingest the spores and are suspected of being capable of vectoring *R. subterranea* (Beckwith 1924, Kirchmair pers. comm.).