Diagnostics
Diagnostic

Diaporthe vaccinii

Specific scope

This standard describes a diagnostic protocol for *Diaporthe vaccinii*[1].

Specific approval and amendment

Approved in 2008-09.

Introduction

Diaporthe vaccinii Shear (anamorph *Phomopsis vaccinii* Shear) is recorded on stems, shoots and leaves of cultivated *Vaccinium corymbosum* L. (blueberry), *V. macrocarpon* Aiton (American cranberry), *V. vitis-idaea* L. (cowberry) and autochtonous species of European *V. myrtillus* L., (blueberry), *V. oxycoccus* L. (cranberries). *D. vaccinii* causes phomopsis canker and dieback, twig blight, viscid rot (fruit rot). It is common in temperate climate areas of North America: Canada (Nova Scotia), USA (in 11 States). There are a few reports of this fungus on plants in Europe: in Romania, UK (eradicated) and Lithuania.

Identity

Name: *Diaporthe vaccinii* Shear
Anamorph: *Phomopsis vaccinii* Shear
Taxonomic position: Fungi: *Ascomycota: Diaporthales*
EPPO computer code: DIAPVA
Phytosanitary categorization: EPPO A1 list no. 211, EU Annex designation: II/A1

Detection

Blueberries can be killed by *D. vaccinii* within a few months. The first symptoms appear on the tips of non-woody shoots (Fig. 1A). Infected succulent, current-years shoots wilt in 4–6 days and become covered with minute lesions. The fungus spreads downward through vascular vessels on average 5.5 cm in two months, killing single twigs and often entire plants of a susceptible cultivar. On stems, *D. vaccinii* causes a brown discoloration of the xylem below wilt symptoms. Conidiomata appear on lesions on 1–2 year old twigs (Fig. 1B), and *ascomata* on 2–3 year old twigs. The fungus also infects leaves, buds, and fruits of cranberries (Fig. 2A, Fig. 3). Berries become brownish red, inflated and shiny.

Fig. 1 (A) Symptoms of *Phomopsis*/*Diaporthe vaccinii* on twigs of *Vaccinium corymbosum*. (B) Conidiomata on stem of blueberry.

Similar disease symptoms and morphological features can be associated with other fungi of the genus *Phomopsis* described on *Vaccinium* L., for example *P. columnaris* D.F. Farr & Castlebury, *P. myrtilli* Petr. and *P. conorum* Sac. (Died.), however these species show differences in the size of conidiomata and in the shape of conidia (Table 1). Another *Phomopsis* species regularly encountered on *Vaccinium corymbosum* in the

[1] Use of names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

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Fig. 2 (A) Symptoms caused by *Phomopsis*/*Diaporthe vaccinii* on twig and leaves of *Vaccinium macrocarpon*. (B) Conidiomata on stem of cranberry (damp chamber test).

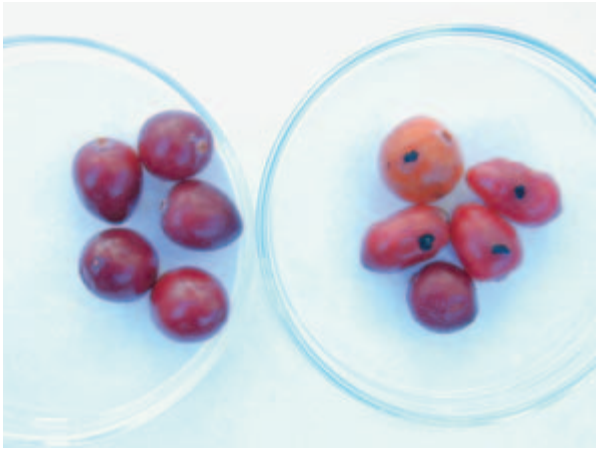

Fig. 3 Cranberries with symptoms of viscid rot caused by *Phomopsis*/*Diaporthe vaccinii* (right, artificial inoculation) and healthy cranberries (left).

Netherlands, is *P. viticola* (Sacc.) Sacc but its conidia are bigger (Table 1).

Symptoms similar to *Phomopsis* dieback can be associated with other fungal pathogens such as *Godronia cassandrae* Peck (anamorph *Fusicoccum putrefaciens* Shear) (Fig. 4), *Colletotrichum* spp., *Fusarium* spp. and *Botryosphaeria dothidea* (Moug.: Fr) Ces. & de Not (anamorph *Fusicoccum aesculi* Corda). In the presence of reproductive structures, these fungi can in the first instance be separated from *Phomopsis* spp. by checking for sporulating structures under a stereoscopic microscope (20×) or a compound microscope (400×–1000×). In the absence of sporulating structures, incubation in a damp chamber (see below) may stimulate formation of fruiting bodies.

Identification

The identification of the fungus is possible on the basis of morphology but in case of uncertainty given the variable nature

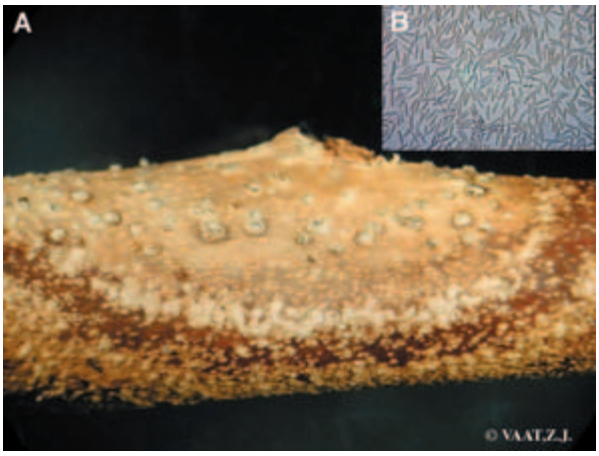

Fig. 4 (A) Typical lesions caused by *Fusicoccum putrefaciens* (×10). (B) Conidia (×400).

of *Diaporthe*/*Phomopsis vaccinii* morphology and its morphological overlap with other *Diaporthe* species it should be confirmed by internal transcribed spacer (ITS) amplicon sequencing. A flow diagram describing the appropriate tests necessary for a positive diagnosis is given in Fig. 5.

Morphology

Morphological identification in vivo

Direct examination

If suspicious symptoms of *Phomopsis* spp., e.g. fruiting bodies, are observed on diseased parts of *Vaccinium* spp., a preliminary diagnosis is possible by direct examination. Spores from fruiting bodies can be removed with the tip of a sterile needle and placed directly in a drop of distilled water on a microscope slide for examination under a compound microscope. Spores should also be transferred to an agar medium for isolation in pure culture as described in the section 'Morphological identification *in vitro*'.

Damp chamber test

In the absence of fruiting bodies on diseased twigs, leaves or fruits, the specimens should be incubated in damp chambers with damp filter papers to induce production of fruiting bodies, namely conidiomata (pycnidia) (Fig. 2B). Pycnidia are usually produced in 4–7 days. Incubation may be prolonged up to 30 days to induce the production of *ascomata* (perithecia) although this stage has only been observed in America. Portions of twigs (5–7 cm long, containing healthy parts as well as lesions) should be excised with a scalpel, then washed in distilled water before incubation in damp chambers at 22–25°C in a 12/12 h light/dark period. The chambers should be periodically moistened and examined daily under a stereoscopic microscope

Table 1 Morphology and diagnostic features of *Phomopsis vaccinii*, *P. conorum*, *P. myrtilli*, *P. columnaris* and *P. viticola*

Species	Host (country of observation)	Conidiomata (measured on host plant)	Conidia (measured on host plant)	Culture on PDA
P. vaccinii Shear (Jovaišienė, 2005)	*Vaccinium macrocarpon* (Lithuania)	Pycnidia 200 µm high and 500 µm wide (average), dark, subcuticular, scattered to confluent, spherical, large with broader base, unilocular, ostiole single. Conidiophores short, septate and branched. Conidiogenous cells 12 µm enteroblastic, phialidic.	Conidia of two types: Alpha conidia 6.0–10.5 × 2.2–3.2 µm, hyaline, unicellular, fusiform, pointed at both ends, straight, biguttulate.[2]	Mycelium not compact, radiate growth pattern, slightly floccose, zonate, white, after 3 weeks sometimes greyish white around agar plug in some strains.
P. conorum Sacc. (Died.) (Jovaišienė & Kačergius 2008)	*Vaccinium myrtillus* (Lithuania)	Pycnidia 290–300 µm wide, dark, scattered, subcuticular, spherical, unilocular, ostiole single.	Conidia 6.5–8.0 × 2.3–3.2 µm, hyaline, elongated oval, some with one end obtuse, some slightly curved, with two at the ends or one large oil globule.	Mycelium white from reverse, equally cottony, minutely floccose.
P. myrtilli Petr. (Farr *et al*., 2002b)	*Vaccinium myrtillus* (Austria, Czech Republic)	Pycnidia 250–350 × 100–150 µm, scattered, black, subcuticular, conical, ostiole central, oval, 30 µm, uniloculate or occasionally multiloculate. Conidiophores absent. Conidiogenous cells laginiform to ampulliform, 6–9 × 3–4 µm, holoblastic, annellids present.	Conidia 8.5–14.5 × 3.1–4.0 µm, fusiform, sometimes obovate or tapering towards base, hyaline, non-septate, smooth, apex slightly rounded, base narrowly truncate or rounded, straight or slightly curved, with scattered, small 2–4 oil globules.	
P. columnaris Farr&Castl. (Farr *et al*., 2002b)	*Vaccinium vitis-idaea* (USA)	Pycnidia 200–330 µm high and 440–840 µm wide, subcuticular, scattered to confluent, uniloculate, black, broadly spherical to flattened, uniostiolate. Conidiophores thin-walled, dark brown, vertically aligned, multicellular. Conidiogenous cells 5–12 × 1.5–2.5 µm, obclavate to cylindric, straight or curved, developing from the apex of columnar cells.	Conidia 6.4–9.4 × 3.6–4.9 µm, ellipsoidal to oval, apex broadly rounded, base slightly truncate, hyaline, unicellular, generally with one large gutulla.	Mycelium cottony, white to olive grey, radiate growth pattern, reverse black to dark brown, without zones.
P. viticola Sacc.	*Vitis vinifera* (Australia, S-Africa) *Vaccinium corymbosum* (the Netherlands)	No measurement available.	Conidia 13.0–17.0 × 2.5–3.0 µm (on oatmeal agar) Hyaline, unicellular, fusiform, pointed at both ends, with many small guttulla.	Colony edge entire/slightly undulate; mycelium cottony, white. Sometimes sectoring visible. In older cultures occurrence of orange droplets on colony surface.

[2] Beta conidia may also been seen 15.0–24.0 × 0.8–1.5 µm, hyaline, filiform, uncinate. These conidia are not used for identification.

so that any sporulating structures can be prepared for examination as described above. Spores should be transferred to agar medium for isolation in pure culture as described in the section 'Morphological identification *in vitro*'.

Morphological characteristics

Anamorph: *Phomopsis vaccinii*

Conidiomata are dark, spherical, flat near the base, partially subcuticular, scattered to confluent, uniloculate, uniostiolate, 0.2 × 0.5 mm in diameter. Sometimes pycnidia exude a cream coloured mass of conidia. Two types of conidia are produced: alpha (α) conidia – hyaline, unicellular, ellipsoid, biguttulate, 6–11 × 2–3 µm (av. 7.8 × 2.6 µm), and beta (β) conidia – unicellular, filiform, uncinate, hyaline 15–24 × 1–1.5 µm (av. 20 × 1 µm) (according to Farr *et al*. (2002a), α conidia are 6–11 × 2–4 µm; according to Guerrero & Godoy (1989), β conidia are 14–20 × 0.5–1 µm).

Teleomorph: *Diaporthe vaccinii*

Caruso & Ramsdell (1995) described the teleomorph on *V. macrocarpon* in the USA. Ascomata are localized between bark and xylem and are sometimes near conidiomata. Perithecia 0.3–0.5 × 0.2–0.4 mm diameter, spherical with black, thick, multilayered walls, and flat near the base. Necks of mature perithecia are long, and protrude through the bark. Asci are 37–51 × 7–12 µm, oblong, sessile, thickening at the top with a tight pore. They contain 8 ascospores 8–12 × 2–3 µm, bicellular, ellipsoid, slightly constricted at septum, sometimes irregularly shaped and containing an oil drop.

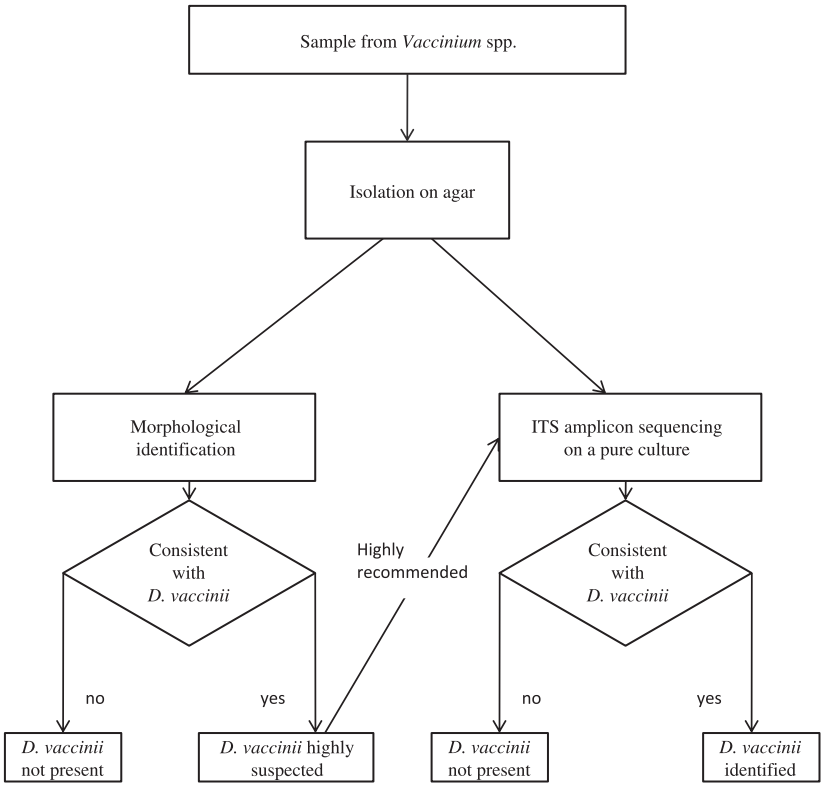

Fig. 5 Flow diagram for diagnosis of *Diaporthe vaccinii*.

Guerrero & Godoy (1989) described the teleomorph on *V. corymbosum* in Chile. Asci were longer (55–75 × 5–8 µm) and ascospores were larger (15–18 × 3.5–4.5 µm), but other characters were the same.

Morphological identification in vitro

Isolation of fungi from aerial plant parts

For identification, the pathogen should be obtained in pure culture. Plant material (leaves, twigs) showing symptoms should be cut into 1–2 cm pieces with a sterile knife. First this material is washed in sterile water, then the material is dipped for 1 min in 2% NaOCl solution and washed again in sterile water. After drying, the shredded pieces are placed onto agar medium as outlined below.

Growth characteristics in culture

P. vaccinii grows well on Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and MSM (Appendix 2). MSM is more suitable for isolation of *Phomopsis*/*Diaporthe vaccinii* from plant tissue and the size of pycnidia and conidia is closer to their natural size. In addition the isolate can be stored longer on this medium (up to half a year).

Colonies on PDA, MEA and MSM grow up to 10–12 mm in diameter/day.

Characteristics of colonies on PDA

P. vaccinii colonies are initially white, circular and grow up to 35 mm in diameter over three days. Aerial mycelium is not compact, the colony is regular in outline with a thinner margin (Fig. 6). After 7 days colonies reach approximately 60 mm diameter, become slightly floccose with indistinct concentric rings. At senescence (after 3–4 weeks) the colour of the mycelium is greyish-white around the agar plug in some strains, but becoming thinner, more heterogeneous and noticeably floccose. In some (fresh) isolates, colony colour on PDA in time turns isabelline to fuscous black (Rayner, 1970), starting after approximately 3 weeks incubation in the dark (Fig. 7). The fungus grows well between 20–28°C (optimum 25°C), in the light as well as in the dark. For sporulation cultures should be cultivated under a 12/12 h light/dark regime, preferably on MSM or oatmeal agar. Conidiomata start to form after 7–10 days, maturing within 20–28 days.

Morphological characteristics

Anamorph: *Phomopsis vaccinii*

Conidiomata are black, uniloculate, spherical to irregular, large, with broader base, with two thirds protruding above the surface of the medium, 0.5–1.0 mm high and 1.2–1.3 mm wide. They form one at a time or sometimes close together in time and

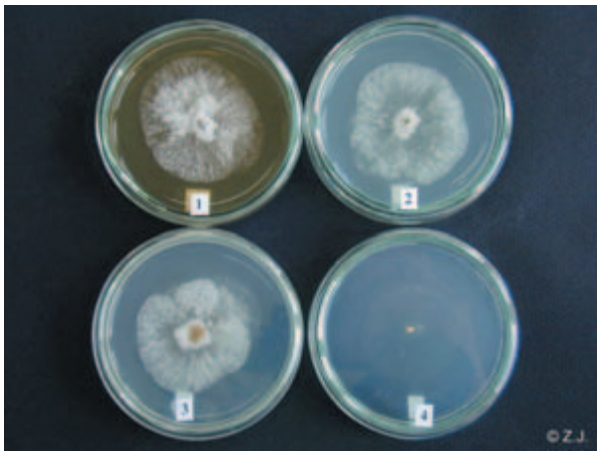

Fig. 6 Colonies of *Phomopsis vaccinii* on the different media: no. 1, MEA; no. 2, PDA; no. 3, MIX; no. 4, water agar (WA) after 3 days, under 12/12 h light/dark regime.

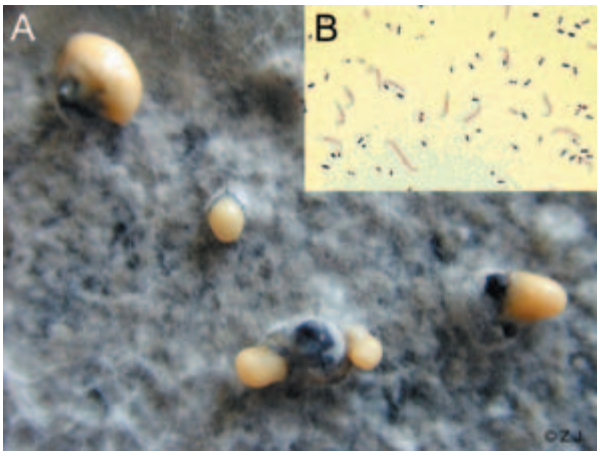

Fig. 8 (A) Spore masses erupting from conidiomata of *Phomopsis vaccinii* (×20). (B) Alpha and beta conidia (×200).

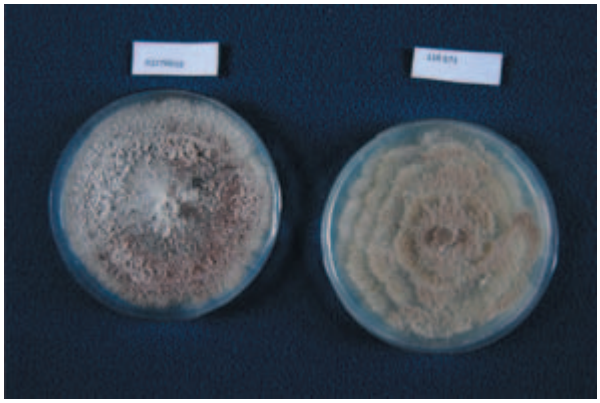

Fig. 7 Colonies of some fresh isolates of *Phomopsis vaccinii* on PDA (17 days old, incubation in the dark).

place. Cream coloured spore masses erupt from the broad irregular pore in the top of mature pycnidia (Fig. 8A). Alpha conidia are dominant in these exudates. Alpha conidia are unicellular, ellipsoid, hyaline, pointed at both ends, biguttulate, 6–11 × 2.5–4 µm (average 8.3 × 2.9 µm). Beta conidia are unicellular, hyaline, filiform, uncinate, 16–24 × 1–1.5 µm (average 20.0 × 1.0 µm) (Fig. 8B). Conidiophores are short, septate and branched. Conidiogenous cells are simple, narrow at top, sometimes spindle-shaped, 12 µm long in young pycnidia but longer in old pycnidia. With repeated sub-culturing only alpha conidia may be produced in some isolates.

Teleomorph: *Diaporthe vaccinii*
Wilcox (1940) described the teleomorph in culture. In cornmeal-agar cultures, the *ascomata* were elongated and thick-walled, with ostioles copiously supplied with upward-directed hairs. Asci are 32–48 × 5.8–9.6 µm oblong, ovate, without stalk, with small pore. Ascospores 6.4–12.8 × 2.5–4.2 µm bicellular, with oil drops.

Identification by sequencing part of the ITS region (KJD Hughes CSL-GB)
The identity of obtained isolates, resembling cultures of *P. vaccinii*, can be confirmed by DNA-sequencing. Only DNA from pure isolates can be tested using this method, otherwise sequences from multiple organisms may be amplified in the same reaction. The method is described in Appendix 1.

Reference material

The type strain (CBS 160.32) of *D. vaccinii* is available from CBS, Utrecht, the Netherlands. Material can also be obtained from Mycology Section, Plant Protection Service, P.O. Box 9102, 6700 HC Wageningen (NL).

Infected host material (*Vaccinium* spp.) and isolates in pure culture are deposited in:

1. the Collection of Regulated Fungi in the Phytosanitary Research Laboratory of the State Plant Protection Service of Lithuania, Sukilėlių str. 9A, LT-11351 Vilnius (LT)
2. the Herbarium of Institute of Botany (BILAS) and Collection of Pure Cultures of Microorganisms of Institute of Botany, Žaliųjų ežerų str. 47, LT-2021 Vilnius (LT).

Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 (1) *Documentation and reporting on a diagnosis*.

Further information

Further information on this organism can be obtained from:

Z Jovaišienė; Phytosanitary Research Laboratory, State Plant Protection Service, Sukileliu 9a LT-11351 Vilnius (LT);

A Kačergius; Laboratory of Phytopathogenic Microorganisms, Institute of Botany, Žaliųjų ežerų str. 47, LT-2021 Vilnius (LT);

KJD Hughes & AV Barnes; Central Science Laboratory, Sand Hutton, York (GB).

J de Gruyter & G van Leeuwen, Plant Protection Service, National Reference Laboratory, PO Box 9102, 6700 HC Wageningen (NL).

Acknowledgements

This protocol was originally drafted by: Z Jovaišienė, State Plant Protection Service of the Republic of Lithuania, Vilnius (LT), KJD Hughes & AV Barnes, Central Science Laboratory, Sand Hutton, York (GB). Revised by: G.C.M. van Leeuwen, K. Rosendahl & J. de Gruyter, National Reference Laboratory (NRL) Plant Protection Service, Wageningen (NL).

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Appendix 1

Identification by sequencing internal transcribed spacer (ITS) regions 1 and 2 of the nuclear ribosomal gene

1. General information

1.1. This protocol has been developed by sequencing the rDNA ITS region
1.2. DNA is extracted from a pure culture of the test isolate
1.3. The target region is the internal transcribed spacer region (ITS) of the fungal ribosomal RNA gene
1.4. Amplicon location (start of ITS 1 and end of ITS 2)
1.5. Amplicon size (including primer sequences) is 582 bp for *Diaporthe vaccini*
1.6. Oligonucleotides:
The forward primer is ITS 1 : 5′ TCC GTA GGT GAA CCT GCG G 3′ and the reverse primer is ITS4: 5′ TCC TCC GCT TAT TGA TAT GC 3′ (White *et al*., 1990)
1.7. Taq DNA polymerase at a concentration of 5 U/µL (AmpliTaq, Applied Biosystems) is used for amplification
1.8. Molecular grade water is used for all reactions
1.9. The method was validated on an ABI 9600

2. Methods

2.1. Nucleic Acid Extraction and Purification
- 2.1.1. DNA should be extracted from a 1 cm² plug taken from a pure culture of the test isolate
- 2.1.2. A suitable DNA extraction kit such as a NucleoSpin plant extraction kit (Macherey-Nagel, Düren, Germany, Cat. ref. 740 570.250) is used or DNA is extracted following a more traditional method such as described in Hughes *et al*. (2000)
- 2.1.3. Extracted DNA should then be stored (neat) at 4°C for immediate use or at –20°C if testing is not to be performed on the same day

2.2. Polymerase Chain Reaction (PCR)
- 2.2.1. Total reaction volume of a single PCR reaction is 100 µL
- 2.2.2. 60.5 µL of molecular grade water
- 2.2.3. 10 µL 10 X of PCR buffer (Applied Bioscience)
- 2.2.4. 8.0 µM 10 mM dNTPs
- 2.2.5. 2.5 units of Taq polymerase
- 2.2.6. 0.5 µM primer ITS1
- 2.2.7. 0.5 µM primer ITS4
- 2.2.8. 1.0 µL of DNA extract
- 2.2.9. Perform amplification in PCR tubes in a thermocycler programmed as follows: 2 min at 94°C; then 30 cycles of 1 min at 94°C, 1 min at 53°C, 1.5 min at 72°C. Finally perform one cycle for 10 min at 72°C.

2.3. Sequencing of amplicons

Run 10 µL of the amplified mixture on a 1.5% agarose gel to check for positive test reactions. Purify the remaining 90 µL

from positive test reactions using a suitable PCR purification kit such as QIAquick PCR purification kit (Qiagen, Crawley, UK, Cat. ref. 28106) following the manufacturers instructions. Sequencing with forward primer ITS1 and reverse primer ITS4

3. Essential Procedural Information

3.1. Amplification and analysis
Defrost extracted DNA if necessary, prepare enough reaction mix for testing at least one sample of the unknown isolate, a positive control containing amplifiable DNA and negative control reactions of reaction mix loaded with water rather than DNA.

3.2. Resolve samples on a 1.5% agarose gel

3.3. Compare consensus sequences for test samples (excluding primer sequences) with a confirmed strain for *D. vaccinii* such as CBS 160.32, (GenBank ref AY952141) on the NCBI database GenBank (http://www.ncbi.nlm.nih.gov). The level of identity should be between 99–100%.

Appendix 2

Composition of the MSM – Sweet clover (*Melilotus* sp.) culture medium (modified by Jovaisiene, 2009)

Dry stems of sweet clover	40 g
Agar technical no. 3 (Oxoid)	12 g
Distilled water up to	1.0 L

The medium is stirred and heated to 100°C for 60 min (~500 turns/min).
It is sterilized 15 min at 110°C.