

Diagnostics**Diagnostic****PM 7/46 (3) *Lecanosticta acicola* (formerly *Mycosphaerella dearnessii*), *Dothistroma septosporum* (formerly *Mycosphaerella pini*) and *Dothistroma pini*****Specific scope**

This Standard describes a diagnostic protocol for *Lecanosticta acicola* (formerly *Mycosphaerella dearnessii*), *Dothistroma septosporum* (formerly *Mycosphaerella pini*) and *Dothistroma pini*.¹

Specific approval and amendment

Approved in 2004–09 as PM 7/46 for *Mycosphaerella dearnessii* and PM 7/47 for *Mycosphaerella pini*.

Revised as a single Standard PM 7/46 (2) for *Mycosphaerella dearnessii* and *Mycosphaerella pini* in 2008–09.

Second revision approved in 2015–04.

1. Introduction

Lecanosticta acicola, *Dothistroma septosporum* and *Dothistroma pini* are pathogens causing foliage diseases of conifers. *Lecanosticta acicola* is the causal agent of brown spot needle blight on pines, though mainly known from North America, has also been found spreading in Europe. *D. septosporum*, the causal agent of red band needle blight, attacks mainly *Pinus* spp. (more than 80 species and subspecies) under favourable climatic conditions and high inoculum pressure, the pathogen has also been found to infect *Picea abies*, *P. omorika*, *P. pungens*, *P. sitchensis*, *P. shrenkiana*, *Larix decidua* and *Pseudotsuga menziesii*, (EFSA, 2013), but also *Pseudotsuga menziesii*, *Larix decidua* and *Picea abies* if these conifers grow next to severely diseased pines. *D. septosporum* has become one of the most damaging pathogens on pines in the Southern Hemisphere, and is also spreading in Europe on several pine species.

In 2004, the causal agent of red band needle blight was separated into two distinct species on the basis of molecular and morphological studies of the anamorphic stages (Barnes *et al.*, 2004). *Dothistroma septosporum* occurs worldwide, while *Dothistroma pini* (teleomorphic

stage unknown) is known from the North-Central U.S.A. and, more recently, also from Russia and Europe. *D. pini* is not currently recommended for regulation by EPPO.

In Europe, *L. acicola* has been found locally on *P. cembra* both in urban and forest sites. In Central Europe, it is known in forest sites from *Pinus mugo* subsp. *uncinata*, which is a pine species growing in swamps. Other hosts are *P. sylvestris*, *P. nigra*, *P. mugo*, *P. halepensis*, *P. radiata*, hybrids of *P. attenuata* X *radiata* and more recently on *P. cembra* (R. Engesser, WSL, pers. comm.). Though established on only a few sites and usually slowly spreading, eradication efforts have only been partly successful to date. *D. septosporum*, by contrast, is widespread on several pine species all over Europe, the main host being *Pinus nigra*, followed by *P. sylvestris* and *P. mugo*. *Dothistroma pini* was recently detected on *Pinus radiata* (Piou & Ioos, 2013). Epidemics are mostly known from young, dense afforestations of *P. nigra* (Austrian black pine). Occasionally, *D. septosporum* attacks spruce growing in the understory of pines. Recently, needle cast of *P. cembra* (Swiss stone pine) was reported from some alpine stands. *Dothistroma pini* was identified in Europe from a few sites only. Another *Mycosphaerella* species, *Mycosphaerella gibsonii* (anamorph: *Pseudocercospora pini-densiflorae*), is causing a serious needle blight on numerous pine species in Africa, Caribbean, Oceania, Eastern Asia, Central and South America. This species is not present in Europe, and is recommended for regulation by

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

EPPO (A1 List n°7). It is a regulated pest for many EPPO member countries (e.g. in the EU as *Cercoseptoria pini-densiflorae*). Susceptible European pine species are *P. cembra*, *P. halepensis*, *P. mugo*, *P. nigra*, *P. pinaster*, *P. pinea*, and *P. sylvestris*. Some species of *Abies*, *Larix*, *Picea* and *Pseudotsuga* are other potential hosts. Information is also provided on *M. gibsonii* in this protocol, although there is less detail than for the other species.

A flow diagram describing the diagnostic procedure for *Lecanosticta acicola*, *Dothistroma septosporum* and *Dothistroma pini* is presented in Fig. 1.

2. Identity

Name: *Lecanosticta acicola* (Thümen) H. Sydow.

Synonyms: *Cryptosporium acicola* Thüm., *Dothistroma acicola* (Thüm) Schischkina & Tsanova, *Septoria acicola* (Thüm.) Sacc., *Mycosphaerella dearnessii* Rostrup (teleomorph), *Oligostroma acicola* Dearn., *Scirrhia acicola* (Dearn.) Siggers, *Systremma acicola* (Dearn.) F.A. Wolf & Barbour, *Lecanosticta pini* Syd.

Taxonomic position: Fungi, Ascomycota: Capnodiales.

EPPO code: SCIRAC.

Phytosanitary categorization:

EPPO A2 list: No. 22; EU Annex designation: II/A1 – as *Scirrhia acicola*.

Name: *Dothistroma septosporum* (G. Doroguine) Morelet (as ‘*septospora*’).

Synonyms: *Cytosporina septospora* G. Dorog., *Septoria septospora* (G. Dorog.) Arx, *Septoriella septospora* (G. Dorog.) Sacc., *Actinothyrium marginatum* Sacc., *Dothistroma pini* var. *lineare* Thyr & C.G. Shaw, *Dothistroma septosporum* var. *lineare* Thyr & C.G. Shaw, *Dothistroma pini* var. *keniense* M. H. Ivory (as ‘*keniensis*’), *Dothistroma septosporum* var. *keniense* M.

H. Ivory., *Mycosphaerella pini* E. Rostrup, *Eruptio pini* (Rostr.) M.E. Barr, *Scirrhia pini* Funk & A. K. Parker, *Actinothyrium marginatum* Sacc., *Dothistroma pini* var. *keniense* M. H. Ivory (as ‘*keniensis*’), *Dothistroma pini* var. *lineare* Thyr & C.G. Shaw.

Taxonomic position: Fungi, Ascomycota: Capnodiales.

EPPO code: SCIRPI.

Phytosanitary categorization: EU Annex designation: II/A2 – as *Scirrhia pini*.

Name: *Dothistroma pini* Hulbary (anamorph).

Synonyms: none.

Taxonomic position: Fungi, Ascomycota: Capnodiales.

EPPO code: DOTSPI.

Phytosanitary categorization: none.

3. Detection

For detection, attention should be focused on all pine species suffering from needle cast associated with discolorations characterized by formation of cross bands. In addition, trees with needle losses and the remaining needles reduced in length, or trees with a ‘paintbrush’-like appearance, should be checked for *Mycosphaerella* needle diseases. Recommendations concerning the time of collection are not possible, since in both species time of sporulation varies with respect to local climatic conditions.

3.1 Symptoms

It should be noted that symptoms, in particular early ones, are not distinctive for the species.

3.1.1 *Lecanosticta acicola*

In *L. acicola*, symptoms first appear on needles as orange/yellow, sometimes resin-soaked spots, which later become dark brown in the centre with a yellow margin. Sometimes,

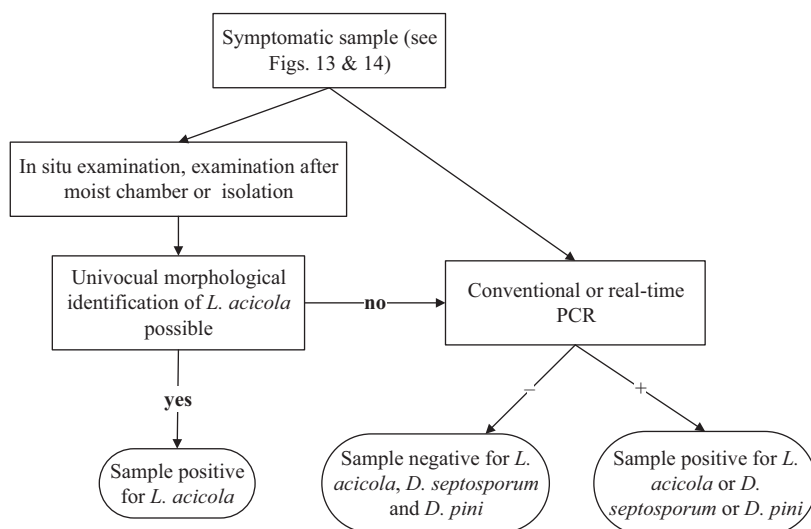


Fig. 1 Flow diagram describing the diagnostic procedure for *Lecanosticta acicola*, *Dothistroma septosporum* and *Dothistroma pini*.



Fig. 2 (A–C) Brown spots and necrotic bands (1c: resin-soaked spots) on needles of *P. mugo* caused by *L. acicola*.

these spots show a darker edge, although a yellowish halo is always visible around it (Fig. 2A–C).

The spots usually widen to bands encircling the needle and causing death of the parts distal from the band (Fig. 3A,B). Lesions are always sharply delimited from the surrounding living tissue. Diseased needles typically show dead tips, central zones with spots in green tissue, and green bases. Sometimes, diseased needles are shorter than healthy ones.

In the brown-coloured dead parts of the needle, black stromata develop under the epidermis visible as round black spots (Fig. 4). During further development, the oval-shaped fruit bodies, arranged parallel to the long axis of the needle, break through the epidermis opening by one or

two longitudinal slits, raising a flap of epidermis and hypodermal tissue (Fig. 5A,B). Under moist conditions, mature conidiomata produce mucilaginous spore masses (Fig. 6A). Dried conidiomata show blackish-green tufts of conidia (Fig. 6B,C). After a severe attack, the whole needle turns brown, then grey (Fig. 3A), and abscises prematurely. In less severe attacks, needle cast may be delayed for 1 or 2 years. Heavily infected pines typically show twigs carrying only the current year’s needles. These take on a ‘paintbrush’ appearance as the disease develops. Over several years, the disease may result in branch and tree death (Sinclair *et al.*, 1989).

Symptoms are similar on *P. halepensis*.



Fig. 3 (A, B) Attack of *L. acicola* on *P. mugo*.

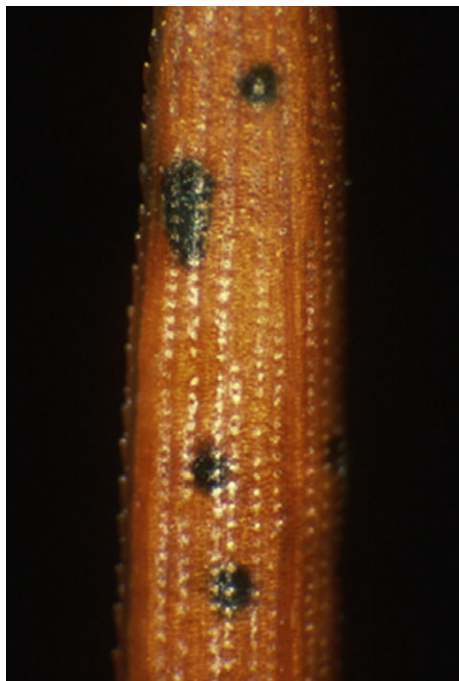


Fig. 4 Spots of black stroma of *L. acicola* developing under the needle epidermis (*P. mugo*).

3.1.2 *Dothistroma septosporum*

Disease symptoms first appear on needles as yellow spots (Fig. 7A–D) which later on widen to a sharply delimited necrotic band. As in *L. acicola*, the bands encircle the needles and cause death of distal parts. Diseased needles often show dead tips, central zones with lesions or necroses, and green bases (Fig. 8). Typically, the brown bands are encircled by a zone showing a conspicuous, often brick-like reddening

in the form of bands around the needle axis (Fig. 9A,B). This red colour is due to accumulation of dothistromin, a toxin which is produced by the fungus in needle tissue as well as in culture. However, this feature is not always present depending on host species and site. Strong light enhances reddish symptoms caused by the toxin, and shade suppresses them. In some cases, the reddening is reduced to small parts surrounding the fruiting structures (Fig. 10A,B; hand lens 10×). If the characteristic red bands are not produced or suppressed, the initial symptoms may easily be confused with those produced by *L. acicola* or *M. gibsonii*.

In the red coloured areas, as well as the brown and dead parts of the needle, black stromata develop underneath the epidermis, visible as round black spots (Fig. 11). Later on, the elliptical conidiomata, arranged in rows parallel to the longitudinal axis of the needle, break through the epidermis opening by one or two longitudinal slits, raising a flap of epidermal and hypodermal tissue. Sometimes, the conidiomata are concentrated in the red bands.

Severely attacked needles turn completely brown and drop prematurely, the older ones first. In less severe attacks, needle cast may be delayed for 1 or 2 years. As in *L. acicola*, heavily infested pines typically have twigs where only the current year's needles are alive (Fig. 12). Repeated attacks may result in branch and tree death (Gibson *et al.*, 1964; Sinclair *et al.*, 1989).

3.1.3 *Dothistroma pini*

Symptoms caused by *D. pini* can not be distinguished from *D. septosporum*.

A description and figures of the symptoms caused by *L. acicola* and *Dothistroma* spp are shown in Fig. 13 and in Fig. 14.

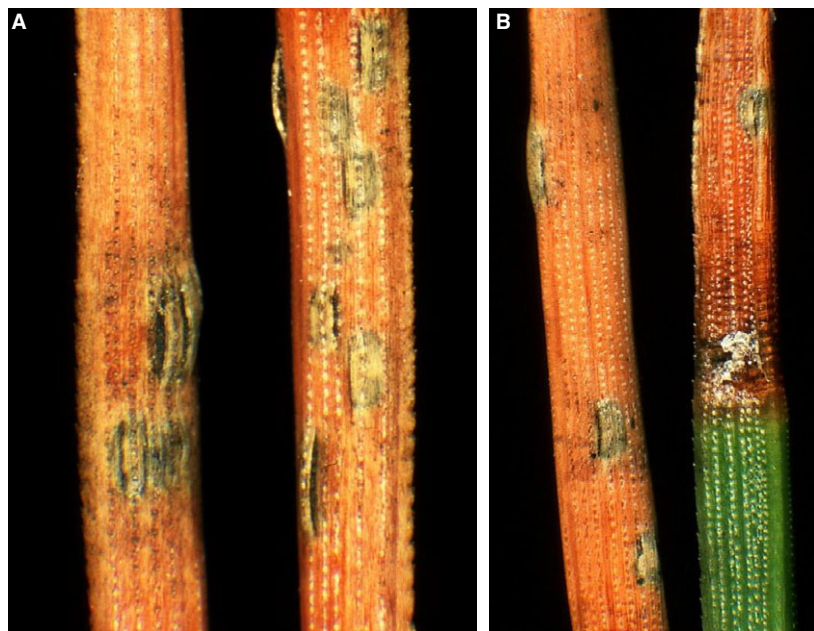


Fig. 5 (A, B) Fructifications of *L. acicola* rupturing needle epidermis of *Pinus mugo*.

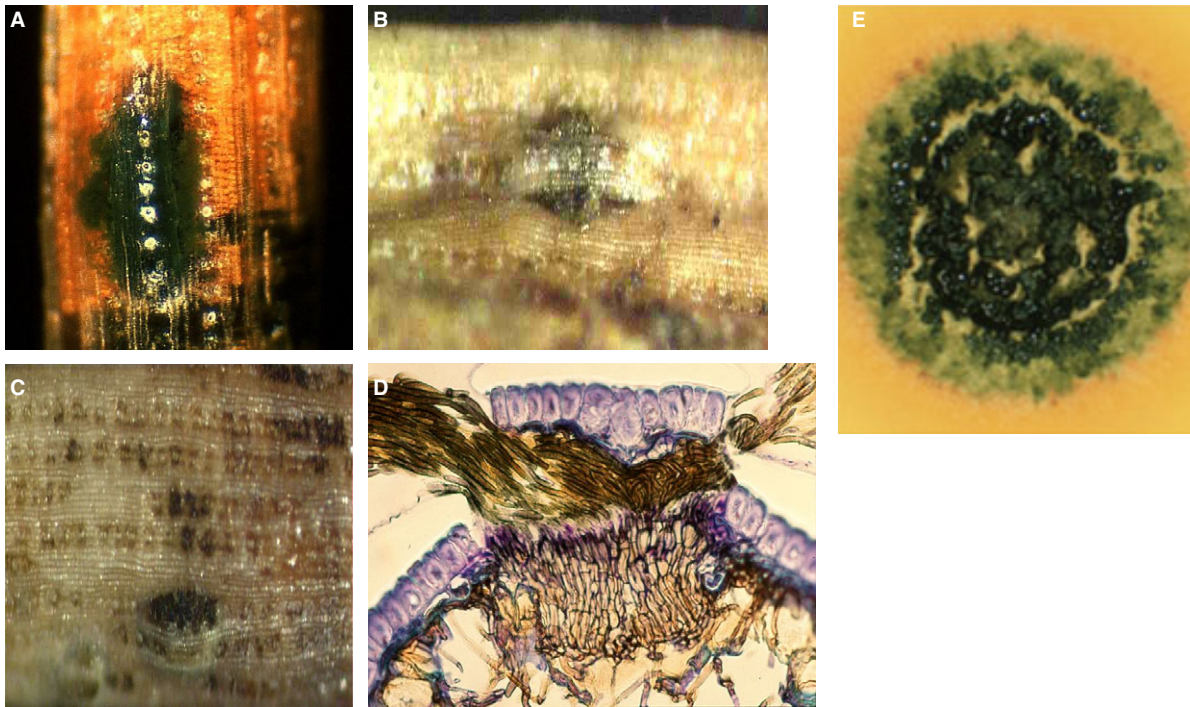


Fig. 6 (A) Slimy-dark green spore mass of *L. acicola* produced from conidiomata under moist conditions. (B, C) Dark green blackish spore tufts of *L. acicola* under dry conditions. (D) Cross section through a conidioma of *L. acicola* (staining: thionine). (E) Sporulating culture of *L. acicola* on malt extract agar.

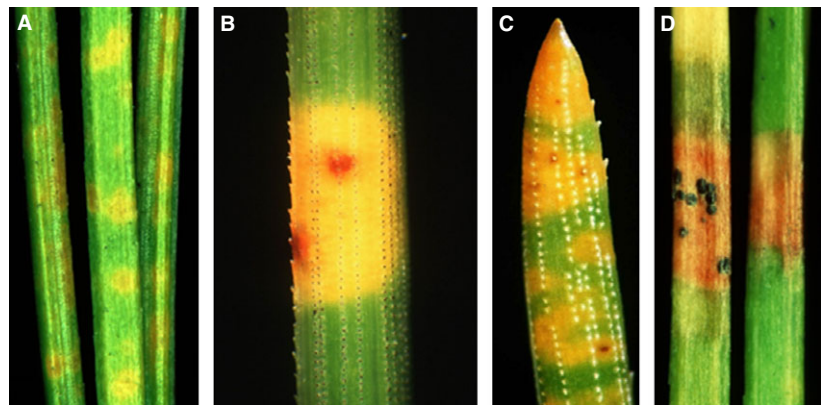


Fig. 7 (A–D) Yellow spots and necrotic bands on needles of *P. cembra* (1A, D) and *P. mugo* (1B, C) caused by *Dothistroma septosporum*.

3.1.4 Confusion with other species

Macroscopic symptoms and morphological features of *L. acicola* and *D. septosporum* can easily be confused, especially at initial disease stages, but even later, if typical red bands are lacking (Evans, 1984; Pehl & Wulf, 2001). Similar symptoms may also be caused by insects. Information on species commonly found on needles of various pine species in Central European countries (Cech, 2012) is available on the UK Forestry Commission website.

- *Mycosphaerella gibsonii*

Some symptoms and features of *M. gibsonii* (anamorph: *Pseudocercospora pini-densiflorae*) are also very

close to *L. acicola* (Evans, 1984). *M. gibsonii* initially infests older leaves in young saplings (1–2 years old), forming lesions on the needles. The infection starts as light yellow-green bands (5–10 mm long) on the distal parts of the needles and spreads from the lower crown to the tips of branches. Any reddish discoloration of the lesions is lacking. The lesions fade to yellow, then brown and finally to a grey-brown colour coalescing to destroy the distal part of the needles, which become colonized by various saprophytic fungi, whereas the proximal portions remain alive for some time. Dead foliage usually remains on the tree for many months, but can be shed



Fig. 8 Needles of *P. mugo* infected by *Dothistroma septosporum*.

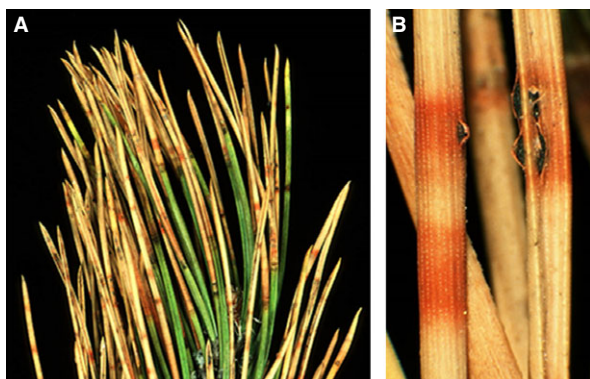


Fig. 9 (A, B) Typical red bands on needles of *P. nigra* caused by *Dothistroma septosporum*.

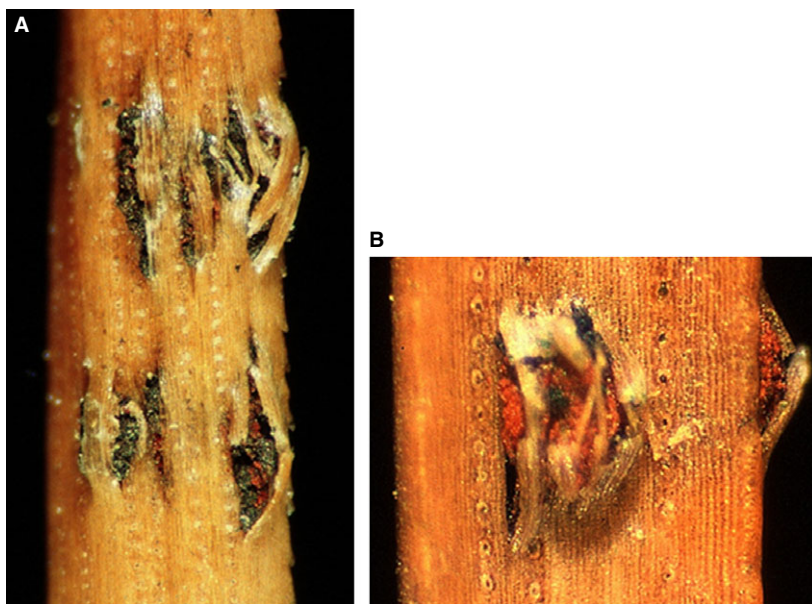


Fig. 10 (A, B) Fructifications of *Dothistroma septosporum* rupturing needle epidermis of *P. mugo*. Red pigments are visible (lens).

during strong winds or heavy rain. In the lesions the anamorphic stage of *M. gibsonii* is formed on fungal stromata, which erupt through the stomata, producing dark olive brush-like sporodochia with elongated conidia. Spermata may also be extruded in tiny, clear droplets from spermagonia (Sullivan, 2010).

- *Lophodermella sulcigena* & *Lophodermella conjuncta*

Another needle cast fungus, which may be mistaken for *L. acicola*, is *Lophodermella sulcigena*. It causes a needle disease (Swedish pine cast) on *Pinus mugo* in Europe (CMI, 1978). *L. sulcigena*, is characterized by an initial reddish-violet discoloration of the distal half of the current year's needles. Later on, the discoloured parts die and the colour changes to yellow-brown and finally grey. On the dead parts of the needles, elongated, blackish ascomata (hysterothecia) appear. Another species, *Lophodermella conjuncta* (Darker) Darker, also causes an initial brownish (becoming greyish later on) discoloration of the distal half of the needles, however, this symptom develops on the previous year's needles (Mitchell *et al.*, 1978). Ascomata are shorter than with *L. sulcigena* and usually clustered at the base of the discoloured needle part.

3.2 Isolation

If no mature conidiomata are present, isolation of both *L. acicola* and *D. septosporum* is possible, but identification requires the production of conidia. Samples should be taken from infected needles with brown dead tissue (dead needle tips, brown spots, bands or dead parts with black stroma spots). Needles are sterilized in 70% ethanol for 30 s, or in NaOCl (commercial bleach, 2% of active chlorine) for 60 s, or in 96% ethanol for 10 s, then rinsed in sterile water. After surface sterilization, needles are cut into segments 4–6 mm

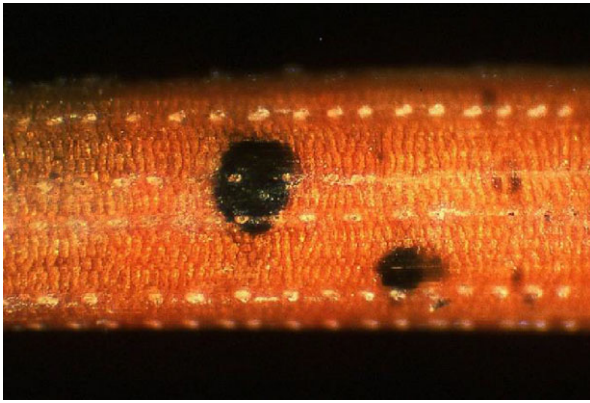


Fig. 11 Spots of black stroma of *D. septosporum* developing under the needle epidermis (*Pinus mugo*).



Fig. 12 Attack of *D. septosporum* on *Pinus mugo* over several years showing only last years needles (paintbrush-looking).

long under sterile conditions, placed on malt extract agar medium (MEA: 2% malt extract, 2% agar agar or MEA+ chloramphenicol 1 mg mL⁻¹) in 9 cm Petri dishes. After an incubation period of 1–3 weeks (2–4 weeks for *D. septosporum*) at room temperature (20°C) in daylight, isolates can be examined. Mycelium usually appears on the two cut surfaces of the needle segment. If both fast and slow-growing fungi are visible, the slow-growing mycelia should be subcultured onto fresh MEA. Because of the slow growth in culture and the presence of endophytic fungi in pine needles, isolation (especially of *D. septosporum*) is not always successful. Other fungi or bacteria present in the needles may grow rapidly on the culture medium and mask any possible colonies of the pathogen. Therefore, it might be useful to produce reference cultures from clearly identified samples. A practicable method for a successful culturing of *D. septosporum* from viable conidia is given by Barnes *et al.* (2004):

Infected needles collected from the field are deposited in –70°C freezers (minimum 1 h), in paper bags to kill possible contaminants (insects or mites). Mature conidiomata from the needles are then scraped from the needle surfaces under sterile conditions and rolled across the surface of 2% malt extract agar plates to release the conidia

(Barnes *et al.*, 2004). From areas with many conidia but no contaminating debris blocks of agar are cut. These blocks are then lifted and transferred to new MEA plates to yield pure cultures.

3.2.1 *L. acicola*

On MEA, firstly a white aerial mycelium appears which turns greenish-olive to dark olive, forming stromatic and erumpent colonies. At 20°C in daylight, the mycelium grows 2.5–3 mm a week. Colonies produce a yellow stain on MEA. Full morphological and diagnostic features are summarized in section 4.1.1 Table 2.

3.2.2 *D. septosporum*

On MEA, firstly a white aerial mycelium appears which turns greyish-brown (Fig. 20). As in *L. acicola*, colonies are stromatic and erumpent. Growth rate of mycelium at 20°C is 1.5–2 mm week⁻¹. Conidia of the anamorph appear as whitish-hyaline slimy masses. Colonies produce a light reddish-brown stain on MEA. Full morphological and diagnostic features are summarized in section 4.1.2 Table 3. See also Butin (1984).

3.2.3 Comparison with similar species

L. acicola, *D. septosporum* and *M. gibsonii* can also be distinguished by cultural characteristics (Table 1). On MEA, colonies of *D. septosporum* are slow-growing, grey-brown-black, stromatic and produce a whitish conidial slime. A reddish-brown diffusate is present in the agar. *L. acicola* on the same media grows significantly quicker and shows green-black stromatic colonies with an olive-green conidial slime. Typically a yellow diffusate is visible. *M. gibsonii* is the fastest-growing species of the three and forms grey to greyish-green or black colonies, attaining 1.8–2.2 cm after 15 days on potato carrot agar under near UV at 25°C; the aerial mycelium is low, compact and grey becoming pulvinate in centre, with a black reverse (Sullivan, 2010).

4. Identification

There are three morphological methods available (*in situ* morphology of fungal organs present on the sample as it is received in the laboratory; moist chamber incubation followed by examination for fungal organs produced during incubation of the sample, isolation of the fungus followed by examination of fungal organs produced on agar media) and two molecular methods (a conventional and a real-time PCR). Classical identification methods can only be used for distinguishing between Brown spot disease (*L. acicola*) and Redband disease (*D. septosporum* and *D. pini*). The essential distinguishing features of *L. acicola* are those of the conidia produced on mature conidiomata on infected needles and in culture. Because of these limitations, the use of the molecular methods is recommended for a differentiation of all three species.

<i>Lecanosticta acicola</i> – Brown spot disease	<i>Dothistroma septosporum</i> and <i>D. pini</i> – Redband disease
<i>Early infestation stages</i>	
Needle cast only slight or absent Distinct reddening or browning of needle tips Infected needles shortened	Needle cast only slight or absent Reddening or browning of needle tips Needles of normal length
<i>Later infestation stages (1- several years)</i>	
Cast of needles of different ages Paint-brush-like appearance due to lack of older needles with only the youngest remaining Conspicuous reddening or browning of needle tips Infected needles shortened	Cast of needles of different ages Paint-brush-like appearance due to lack of older needles with only the youngest remaining No reddening or browning of needle tips Needles of normal length
<i>Symptoms on needles, early and later stages with conidiomata</i>	
Needles with typical yellow bordered brown spots or bands especially on current-year's needles in spring and mid summer. Any reddish discoloration missing.	Needles with yellow spots or bands especially on current-year's needles in spring and mid summer.
Needles with brown necrotic bands, showing black, usually not shining spots of stromata developing under the epidermis. No reddish discoloration.	Needles with brown necrotic bands, showing black, usually shining spots of stromata developing under the epidermis. Reddish (often brick-red) discoloration around the bands
Conidiomata breaking through the epidermis by longitudinal slits, raising a flap of the epidermis. No reddish discoloration.	Conidiomata in the bands breaking through the epidermis by longitudinal slits, raising a flap of the epidermis. Reddish (often brick-red) discoloration around the bands

Fig. 13 Description of the main distinguishing symptoms for *L. acicola* and *Dothistroma* spp.

4.1 Morphological identification

By use of classical morphological methods, *L. acicola* and *D. septosporum* can be identified by microscopic examination of the conidial stage on symptomatic needles or by culturing. Sporulation of the anamorphs on host material can be induced by incubating needles with mature conidiomata in a moist chamber for 1–2 days. A compilation of morphological features of the anamorphic as well as the teleomorphic stages of *L. acicola*, *D. septosporum*/*D. pini* as well as *Lophodermella sulcifera*, *Lophodermella conjuncta* and *Pseudocercospora pini-densiflorae* (*Mycosphaerella gibsonii*) is given in Table 1 (A, B, C).

4.1.1 *L. acicola*

Mature conidiomata produce a slimy olive spore mass under moist conditions. Upon drying, the spore masses turn into a blackish-green, not shining tuft (Fig. 5B–D, hand lens 10×).

The conidia are subhyaline to light brown-olive, thick-walled, spinulose to verrucose, straight to curved, fusiform to cylindrical, 1–5 septate, with a rounded apex and trun-

cate base (Fig. 15, Table 2). The best view is obtained using differential interference contrast optics at a magnification greater than ×400.

Ascstromata are produced irregularly on fallen needles and therefore are not particularly useful for identification.

4.1.2 *D. septosporum*

A quick presumptive indication of the presence of *D. septosporum* in pine needles is a reddish cross band in the necrotic needle tissue (Fig. 9A,B). Final confirmation, however, can only be obtained by microscopic identification of the conidial stage. Ascstromata (Fig. 16) are produced irregularly on fallen needles and can only be used for identification if red bands are also present. Asci and ascospores are shown in Fig. 17.

Mature conidiomata extrude slimy whitish-hyaline spore masses under moist conditions (Fig. 18, hand lens 10×, cross section Fig. 19A). The conidia are hyaline, thin-walled, smooth, straight to curved, fusiform to slightly clavate, 1–5 septate, with a rounded apex and sometimes a truncate base (Fig. 19B, Table 3).

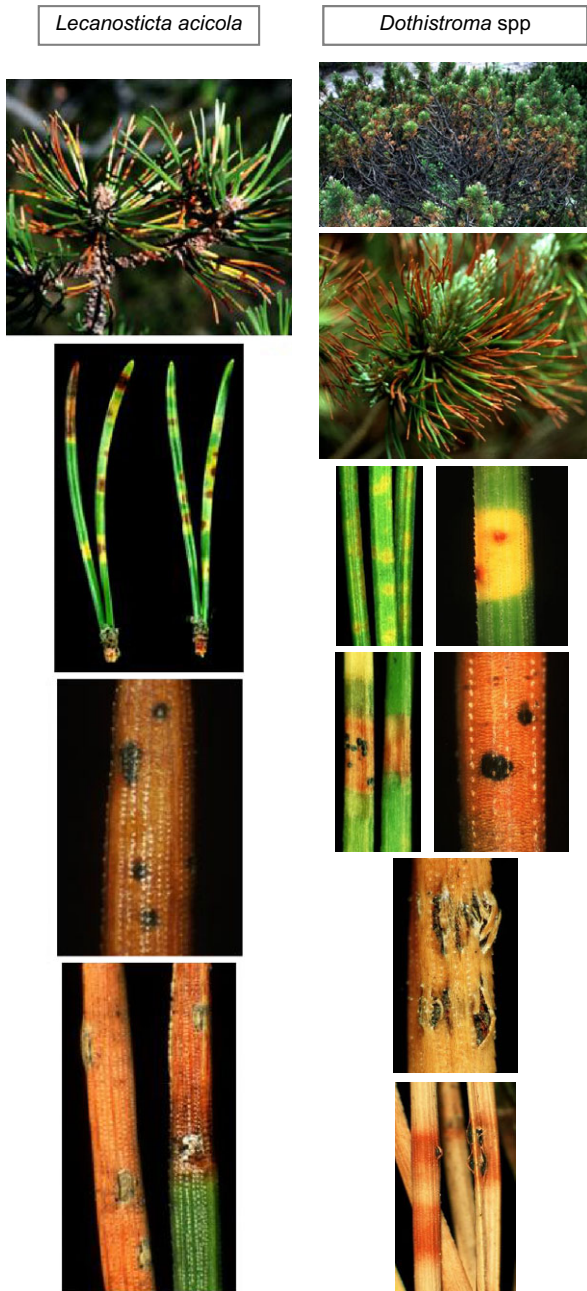


Fig. 14 Symptoms on pines infected by *L. acicola* and *Dothistroma* spp.

4.1.3 Comparison with similar species

L. acicola, *D. septosporum* and *Pseudocercospora pini-densiflorae* are consistently different and provide the best characters to separate the species (Table 1). The conidiomata known as *Lecanosticta* and *Dothistroma* vary in form between acervuli and pseudopycnidia, according to host and climate, but differ clearly from the sporodochia of *Pseudocercospora*. The conidia are initially hyaline, then grey-green-pale brown, thin-walled, cylindrical, 1–6 (–10)

septate, rounded to pointed at the apex with a truncate base, measuring (12–) 20–60 (–80) × 2–4 μm. They appear as grey-green conidial tufts developing from minute substomatal stomata or initiating from an extensive, deep-seated stroma composed of dark, thick-walled pseudoparenchyma which ruptures the epidermis with a median or two longitudinal slits. Conidiogenous cells develop directly on this stroma. The conidia of *Pseudocercospora* are smooth. Conidia appear to be produced in dry fascicles, but a faint mucilaginous covering is sometimes evident (Sullivan, 2010).

The most important and consistent character to distinguish *L. acicola* from *D. septosporum* is the structure and colour of the conidial wall: conidia of *Lecanosticta* are characterized by melanin granules integrated in their outer wall. Viewed under the microscope, the spores are light to dark brown and thick-walled with a verrucose surface. The best view is obtained using differential interference contrast optics at a magnification greater than ×400 or a scanning electron microscope. In contrast, conidia of *Dothistroma* are hyaline, thin walled and smooth. *L. acicola* from *D. septosporum* can not however, be distinguished from the size of the conidia, since sizes vary greatly in both species.

D. pini can only be distinguished from *D. septosporum* by use of molecular techniques (Barnes *et al.*, 2004; Ioos *et al.*, 2010). Therefore, all classical techniques described under *D. septosporum* as follows refer to both *D. septosporum* and *D. pini*.

4.2 Identification by molecular methods

A conventional and a real-time PCR test have been developed for the identification of *L. acicola*, *D. septosporum* and *D. pini* using species-specific PCR primers by Ioos *et al.* (2010) and are described in Appendix 1 to 3.

An EPPO Standard PM 7/XX on DNA barcoding as an identification tool for selected plant pests is in preparation. In Q-bank, multilocus analysis is recommended for *L. acicola* (*M. dearnessii*) based on the combination of ITS and TUB2 and for *D. septosporum* based on the combination of ITS and EF1α (<http://www.q-bank.eu/Fungi/>).

5. Reference material

Source of reference material is also identified in the Fungi database of Q-bank <http://www.q-bank.eu/Fungi/>.

6. Reporting and documentation

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

7. Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data are also

Table 1 Morphological features that can be used to distinguish *L. acicola*, *D. septosporum/pini*, *L. sulcigena*, *L. conjuncta* and *P. pini-densiflorae*

(A) Anamorphic stage: Morphological features		<i>Lecanosticta acicola</i>	<i>Dothistroma septosporum/pini</i>	<i>Lophodermella sulcigena</i> (CMI 1978; Kowalski, 1988)	<i>Lophodermella conjuncta</i>	<i>Pseudocercospora pini-densiflorae</i> (Sullivan, 2010)
Anamorph						
Type	Variable, pseudopycnidial to acervular	Variable, pseudopycnidial to acervular	Variable, pseudopycnidial to acervular	Variable, pseudopycnidial to acervular	Sporodochia	Grey-green conidial tufts developing from minute substomatal stomata or initiating from an extensive, deep-seated basal stroma which ruptures the epidermis with a median or two longitudinal slits.
Occurrence	Irregularly dispersed on necrotic areas, arranged parallel to the long axis of the needle, Subepidermal becoming erumpent and rupturing the epidermis by one or two longitudinal slits when mature	Irregularly dispersed on necrotic areas, arranged parallel to the long axis of the needle, Subepidermal becoming erumpent and rupturing the epidermis by one or two longitudinal slits when mature	Irregularly dispersed on necrotic areas, red bands and parts of dead needle tissue, or aggregated in red bands, arranged parallel to the long axis of the needle; subepidermal becoming erumpent and rupturing the epidermis irregularly or by one lateral or median longitudinal slit, seldom by two slits, when mature	Irregularly dispersed on necrotic areas, red bands and parts of dead needle tissue, or aggregated in red bands, arranged parallel to the long axis of the needle; subepidermal becoming erumpent and rupturing the epidermis irregularly or by one lateral or median longitudinal slit, seldom by two slits, when mature	Irregularly dispersed on necrotic areas, red bands and parts of dead needle tissue, or aggregated in red bands, arranged parallel to the long axis of the needle; subepidermal becoming erumpent and rupturing the epidermis irregularly or by one lateral or median longitudinal slit, seldom by two slits, when mature	Irregularly dispersed on necrotic areas, red bands and parts of dead needle tissue, or aggregated in red bands, arranged parallel to the long axis of the needle; subepidermal becoming erumpent and rupturing the epidermis irregularly or by one lateral or median longitudinal slit, seldom by two slits, when mature
Shape	Elliptical	Elliptical	Elliptical	Elliptical	Irregular	Irregular
Size	200–800 x 150–200 µm	200–800 x 150–200 µm	300–650 x 150–300 µm	300–650 x 150–300 µm	150 µm x 60 µm	150 µm x 60 µm
Color	Black to olive-green	Black to olive-green	Black	Black	Silvery-grey to dark green or black	Silvery-grey to dark green or black
Anatomy	Basal stroma (thick-walled pseudoparenchymatic cells)	Basal stroma (thick-walled pseudoparenchymatic cells)	Basal stroma (thick-walled pseudoparenchymatic cells)	Basal stroma (thick-walled pseudoparenchymatic cells)	Composed of dark, thick-walled pseudoparenchyma	Composed of dark, thick-walled pseudoparenchyma
Conidia						
Color	Exuded in an olive mucilaginous mass, conidia subhyaline to light brown-olive	Exuded in an olive mucilaginous mass, conidia subhyaline to light brown-olive	Slimy whitish-hyaline spore masses, conidia hyaline	Slimy whitish-hyaline spore masses, conidia hyaline	Hyaline, then grey-green-pale brown, developing in dry fascicles, sometimes with a faint mucilaginous covering	Hyaline, then grey-green-pale brown, developing in dry fascicles, sometimes with a faint mucilaginous covering
Morphology						
	Straight to curved, fusiform to cylindrical, thick-walled, rounded apex and truncate base	Straight to curved, fusiform to cylindrical, thick-walled, rounded apex and truncate base	Straight to curved, fusiform to short clavate, thin-walled, rounded apex and sometimes a truncate base	Straight to curved, fusiform to short clavate, thin-walled, rounded apex and sometimes a truncate base	Thin-walled, cylindrical, rounded to pointed at the apex with a truncate base	Thin-walled, cylindrical, rounded to pointed at the apex with a truncate base
Septate	1–5 septate, most 2–3 septate, Echimulate to verrucose	1–5 septate, most 2–3 septate, Echimulate to verrucose	1–5 septate, most 2–3 septate	1–5 septate, most 2–3 septate	1–6 (-10) septate	1–6 (-10) septate
Surface						
Size	(11)–31–(64) x (1)–3.4–(6) µm	(11)–31–(64) x (1)–3.4–(6) µm	(12)–28–(48) x 2–3 µm	(12)–28–(48) x 2–3 µm	Smooth	Smooth
(B) Culture: Morphological features						
Culture						
	On malt extract agar (2% malt extract, 2% agar)	On malt extract agar (2% malt extract, 2% agar)	On malt extract agar (2% malt extract, 2% agar)	On malt extract agar (2% malt extract, 2% agar)	On Potato-Carot agar and UV at 25°C	On Potato-Carot agar and UV at 25°C
Growth	2.5–3 mm week ⁻¹ at 20°C	1.5–2 mm week ⁻¹ at 20°C	1.5–2 mm week ⁻¹ at 20°C	Difficult to obtain, very slow growing (up to 2 cm diam. after several weeks incubation)	Difficult to obtain	1.8–2.2 cm after 15 days

(continued)

Table 1 (continued)

(B) Culture: Morphological features		<i>Lecanosticta acicola</i>	<i>Dothistroma septosporum/pini</i>	<i>Lophodermella sulcigena</i> (CMI 1978; Kowalski, 1988)	<i>Lophodermella conjuncta</i>	<i>Pseudocercospora pini-densiflorae</i> (Sullivan, 2010)
Morphology	Stromatic, green-olive to olive-black, producing dark olive conidial slime, agar coloured yellow by diffusates	Stromatic, brown to grey-black, producing whitish conidial slime, agar coloured light reddish-brown by diffusates	Greyish-pinkish mycelium, composed of hyaline hyphae, 3–5 µm in size with irregular swellings up to 10 µm in size and short, thickened lateral hyphae; revers grey-brown with light brown rim, with reddish patches. Agar coloured dark brown after longer incubation	Pinkish growth from cut ends of surface sterilized infected needles	Grey to greyish-green or black; low, compact, grey mycelium becoming pulvinate in centre, black reverse	
(C) Teleomorphic stage: Morphological features						
Type	Pseudothecium (ascolocular development), ascostroma	Pseudothecium (ascolocular development), ascostroma	Apothecium	Apothecium	Pseudothecium (ascolocular development), ascostroma	
Occurrence	Rare; irregularly dispersed on dead needle tissue, mostly on fallen needles; subepidermal, becoming erumpent and splitting the epidermis when mature	Irregularly dispersed on dead needle tissue, typically aggregated in red bands, mostly on fallen needles, seldom developed; Subepidermal development, becoming erumpent and splitting the epidermis irregularly or by one or two longitudinal slits when mature	Amphigenous on whitish dead parts of green living needles; below epidermis, no stromatic cross-lines except for a brown band between the living portion and the whitish dead portion of the needle; lips absent	Embedded in greyish-brown regions of otherwise green needles, on abaxial and adaxial or radial sides, scattered; zone lines absent	Occasionally uniting laterally in bands; innate to erumpent, linear, discrete, subepidermal,	
Shape	Globose to flask-shaped, ostiolate	Globose to pear-shaped, ostiolate	Elliptical to elongate	Elliptical to elongated, single longitudinal slit	Globose but highly variable in shape ostiolate	
Size	400–1200 x 120–250 µm	400–1000 x 300–400 µm	Usually >2000 µm long	<3750 µm long, sometimes coalescing laterally to form broad ascocarps up to 840 µm wide,	(50-) 70–90 (-120) µm to 150–800 (-1400) µm x 70–125 (-160) µm, 90–150 µm deep	
Color	Black	Black	Grey	The same colour as, or slightly darker than the needle, disc in mature stage cream to pale brown	Dark brown to black	
Anatomy	Pseudoparenchymatic, thick-walled cells, uni- to multiloculate	Pseudoparenchymatic, thick-walled cells, uni- to multiloculate	Pseudoparenchymatic, thick-walled cells, uni- to multiloculate	Black hypodermal clypeus covering only the central part of the hymenium	Stroma uni- to multiloculate, pseudoparenchymic, thickwalled cells, 3–8 (-12) µm diam	

(continued)

Table 1 (continued)

(C) Teleomorphic stage: Morphological features		<i>Lecanosticta acicola</i>	<i>Dothistroma septosporum/pini</i>	<i>Lophodermella sulcigena</i> (CMI 1978; Kowalski, 1988)	<i>Lophodermella conjuncta</i>	<i>Pseudocercospora pini-densiflorae</i> (Sullivan, 2010)
Loculi	Periphysate, 50–70 × 50–80 µm	Periphysate, 70–110 × 70–90 µm	Paraphyses filiform, unbranched, 100–120 µm long, sometimes slightly swollen at the tip	Paraphyses filiform, unbranched, septate, hyaline, straight, smooth, slightly swollen at the tip	Globose to flask-shaped, (45–) 50–75 (-95) × 55–75 µm, periphysate, often with an apical stromatic shield, 70–90 µm diam, in longitudinal series	
Asci	Saccate to cylindrical, bitunicate, 8-spored, hyaline, with a rounded apex, 25–55 × 6.5–10.5 µm	Saccate to cylindrical, bitunicate, 8-spored, hyaline, with a rounded apex, 35–55 × 6–9 µm	Unitunicate, clavate, 4-spored or 8-spored, 110–140 × 13–15 µm	Unitunicate, cylindrical to clavate, 100–160 µm × 15–16 µm, containing eight spores fasciculate arranged	Clavate to cylindrical, bitunicate, 8-spored, obliquely biseriolate, (33–) 35–38 × 5.5–7 µm, apex thickened, bluntly rounded, rarely saccate, 32–36 × 6–8 µm, interthelial tissue present or absent	
Ascospores						
Colour	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline
Morphology	Elliptic, typically 4-guttulate	Elliptic, typically 4-guttulate	Clavate, enveloped in a gelatinous sheath 3–4 µm thick	Filiform or elongate-clavate, enveloped in a gelatinous sheath 1.5–3 µm thick	Elliptic to cuculate, guttulate	
Septae	1-septate	1-septate	Aseptate	Aseptate	1-septate	
Surface	Smooth	Smooth	Smooth	Smooth	Smooth	
Size	7.5–13.5 × 2–3.5 µm	10–15 × 3–4 µm	27–40 µm × 4–5 µm	75–90 µm × 3–3.5 µm	(7.5–) 8.5–11(-12.5) × (1.8) 2.2–2.8 µm	
Spermatia						
	Rod-shaped, hyaline to pale green, 2–4 × 0.8–1.3 µm	Rod-shaped, hyaline, 1.5–2.5 × 0.5–1 µm			Rod-shaped, hyaline, 2–3 × 1 µm	

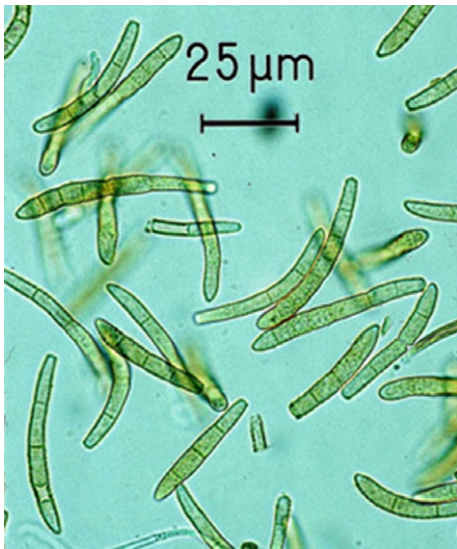


Fig. 15 Conidia of *Lecanosticta acicola*.

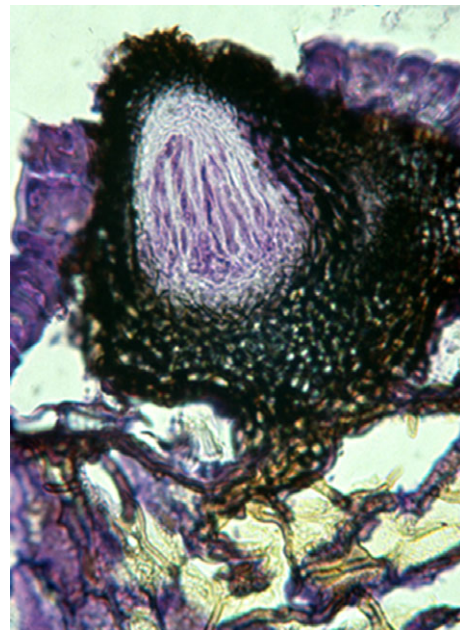


Fig. 16 Cross section through an ascoma of *Dothistroma septosporum* sexual stage (staining: thionine).

Table 2 Morphology and diagnostic features of *L. acicola*

Teleomorph	
Occurrence	Irregularly dispersed on dead needle tissue, mostly on fallen needles, seldom developed
Ascstromata	Subepidermal, becoming erumpent and splitting the epidermis when mature, composed of pseudoparenchymatic, thick-walled cells, uni- to multiloculate, black, 400–1200 × 120–250 µm
Fructification	Pseudothecium (ascolocular development)
Loculi	Globose to flask-shaped, ostiolate, periphysate, 50–70 × 50–80 µm
Asci	Saccate to cylindrical, bitunicate, 8-spored, hyaline, with a rounded apex, 25–55 × 6.5–10.5 µm
Ascospores	Elliptic, 1-septate, hyaline, typically 4-guttulate, 7.5–13.5 × 2–3.5 µm
Spermatia	Rod-shaped, hyaline to pale green, 2–4 × 0.8–1.3 µm
Anamorph	
Occurrence	Irregularly dispersed on necrotic needle tissue
Conidiomata	Subepidermal becoming erumpent and rupturing the epidermis by one or two longitudinal slits when mature, elliptical, arranged parallel to the long axis of the needle, black to olive-green, basal stroma composed of thick-walled pseudoparenchymatic cells, 200–800 × 150–200 µm (Fig. 6D)
Fructification	Acervulus (Fig. 6D)
Conidia	Exuded in an olive mucilaginous mass, straight to curved, fusiform to cylindrical, subhyaline to light brown-olive, 1–5 septate, most 2–3 septate, thick-walled, echinulate to verrucose, rounded apex and truncate base (11)–31–(64) × (1)–3.4–(6) µm (Fig. 15)
Culture	On malt extract agar (2% malt extract, 2% agar agar) 2.5–3 mm growth per week at 20°C, colonies stromatic, green-olive to olive-black, producing dark olive conidial slime, agar coloured yellow by diffusates (Fig. 6E)



Fig. 17 Asci and ascospores of *Dothistroma septosporum*/sexual stage (staining: cotton blue in lactic acid).

available in the EPPO Database on Diagnostic Expertise (<http://dc.eppo.int>), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8. Further information

Further information on those organisms can be obtained from:



Fig. 18 Slimy-hyaline spore mass of *D. septosporum* produced from conidiomata under moist conditions.

Ms Barnes, University of Pretoria, Forestry and Agricultural Biotechnology Institute (FABI), Department of Genetics, Pretoria (ZA).

Ms Brown, Forest Research, Alice Holt Lodge, Farnham, Surrey GU10 4LH, UK.

Mr Cech, Bundesamt und Forschungszentrum für Wald, Institut für Forstschutz, Abteilung für Phytopathologie, Seckendorff-Gudent-Weg 8, A-1131 Wien (AT).

Mr Ottmar Holdenrieder, ETH-Zentrum, Department Wald- und Holzforschung, Rämistr. 101, 8092 Zürich (CH).

Mr Iooos, ANSES Laboratoire de la Santé des Végétaux - Unité de Mycologie, Domaine de Pixérécourt, Malzéville, France.

Mr Kowalski, Akademia Rolnicza, im. H. Ko44ataja, Wydział Lesny, Katedra Fitopatologii Lesnej, Al. 29 Listopada 46, 31-425 Kraków (PL).

9. Feedback on this diagnostic protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide



Fig. 19 (A) Cross section through a conidioma of *D. septosporum* (staining: thionine). (B) Conidia of *Dothistroma septosporum*.

Table 3 Morphology and diagnostic features of *D. septosporum*

Teleomorph	
Occurrence	Irregularly dispersed on dead needle tissue, typically aggregated in red bands, mostly on fallen needles, seldom developed
Ascstromata	Subepidermal development, becoming erumpent and splitting the epidermis irregularly or by one or two longitudinal slits when mature, composed of pseudoparenchymatic, thick-walled cells, uni- to multiloculate, black, 400–1000 × 300–400 μm (Fig. 16)
Fructification	Pseudothecium (ascolocular development)
Loculi	Globose to pear-shaped, ostiolate, periphysate, 70–110 × 70–90 μm
Asci	Saccate to cylindrical, bitunicate, 8-spored, hyaline, with a rounded apex, 35–55 × 6–9 μm (Fig. 17)
Ascospores	Elliptic, 1-septate, hyaline, typically 4-guttulate, 10–15 × 3–4 μm (Fig. 17)
Spermatia	Rod-shaped, hyaline, 1.5–2.5 × 0.5–1 μm
Anamorph	
Occurrence	Irregularly dispersed on necrotic areas, red bands and parts of dead needle tissue, sometimes aggregated in red bands
Conidiomata	Subepidermal development, becoming erumpent and rupturing the epidermis irregularly or by one lateral or median longitudinal slit, seldom by two slits, when mature, elliptical, arranged parallel to the long axis of the needle, black, basal stroma composed of thick-walled pseudoparenchymatic cells, 300–650 × 150–300 μm (Figs 10A,B and 19A)
Fructification	Variable, from pseudopycnidial to acervular
Conidia	Exuded in a whitish mucilaginous mass; fusiform to short-clavate, hyaline, smooth, thin-walled, 1–5 septate, most 2–3 septate, rounded apex and truncate base, 12–28–48 × 2–3 μm (Figs 18 and 19B)
Culture	On malt extract agar (2% malt extract, 2% agar) 1.5–2 mm growth per week at 20°C, colonies stromatic, brown to grey-black, producing whitish conidial slime, agar coloured light reddish-brown by diffusates (Fig. 20).

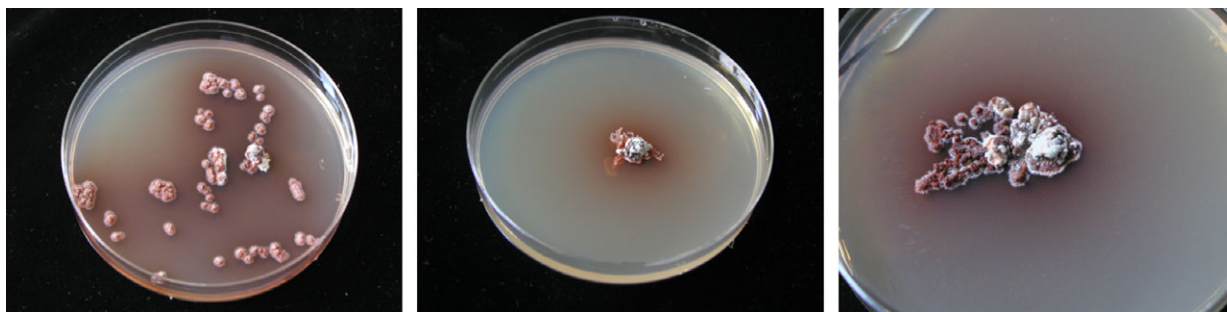


Fig. 20 Cultures of *D. septosporum* on 3% malt extract (Photos, courtesy D. Palovcikova, Mendel University in Brno, Czech Republic).

additional validation data for tests included in this protocol that you wish to share please contact diagnostics@epo.int.

10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

11. Acknowledgements

This protocol was originally drafted by Ms Pehl, BBA (now JKI), Braunschweig (DE). It was revised by Mr Thomas L. Cech, Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), Department of Forest Protection, Unit of Phytopathology, Vienna (AT) and Mr Ioos Laboratoire de la Santé des Végétaux - Unité de Mycologie Malzéville, France.

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Appendix 1 – DNA extraction for *in planta* detection

Five mm length needle pieces (one up to 15) with red band or brown spot symptoms bearing conidiomata are transferred into an individual 2-mL microcentrifuge tube. Different grinding methods can be used such as mortar and pestle with liquid nitrogen, bead mills or other equipment such as the FastPrep (MP Biochemicals), providing they produce a homogenous ground sample. DNA extraction can be performed using several commercial kits for example those that proved to be efficient in Ioos *et al.* (2010): DNeasy plant mini kit (Qiagen, Hilden, Germany), Nucleospin plant II kit (Macherey-Nagel, Düren, Germany), or PureLink Plant Total

DNA purification kit (Invitrogen, Carlsbad, CA, USA). DNA should not be extracted using a home-made CTAB lysis/Phenol-chloroform purification protocol (See Ios *et al.*, 2010). The samples are ground with the appropriate lysis buffer for 3 min and total DNA is further extracted following each corresponding manufacturer's instructions. Total DNA is eluted in the elution buffer provided by the manufacturer and kept at $<-17^{\circ}\text{C}$ until analysis.

Appendix 2 – Identification at species level by conventional PCR (Ios *et al.*, 2010)

1. General information

- 1.1 This protocol was developed by Ios *et al.*, 2010.
- 1.2 The nucleic acid source is infected plant tissues (needles).
- 1.3 Name of targeted genes are Beta-Tubulin 2 (β -tub2) for *Dothistroma septosporum*, and Translation Elongation Factor I alpha (EF1- α) for *Dothistroma pini* and *L. acicola*.
- 1.4 Position of the *D. septosporum* forward primer considering as reference sequence GenBank accession AY808228: 24
Position of the *D. pini* forward primer considering as reference sequence GenBank accession AY808271: 52
Position of the *L. acicola* forward primer considering as reference sequence GenBank accession AY808273: 36
- 1.5 Amplicon size with these primers is 231, 193, and 237 bp, for *Dothistroma septosporum*, *Dothistroma pini*, and *L. acicola*, respectively.
- 1.6 Oligonucleotides:
 - 1.6.1 *Dothistroma septosporum*
Forward primer DStub2-F: 5'- CGAACATGG ACTGAGCAAAC -3'
Reverse primer DStub2-R: 5'- GCACGGCTCTT TCAAATGAC -3'
 - 1.6.2 *Dothistroma pini*
Forward primer DPtef-F: 5'- ATTTTTTCGC TGCTCGTCACT -3'
Reverse primer DPtef-R: 5'- CAA TGTGA-GATGTTTCGTCGTG -3'
 - 1.6.3 *L. acicola*
Forward primer LAtef-F: 5'- GCAAATTT TCGCCGTTTATC -3'
Reverse primer MAtef-R: 5'- TG TGTCCCA AGAGTGCTTGC -3'

2. Methods

- 2.1 Nucleic acid extraction and purification: see Appendix 2.
- 2.2 PCR reaction:

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular grade water*	N.A.	To make up to 20 (12.7)	N.A.
PCR buffer (Hotgoldstar, Eurogentec)	10 X	2	1 X
MgCl_2 (Eurogentec)	50 mM	0.8	2 mM
dNTPs (Eurogentec)	10 mM	0.8	0.2 mM
Forward primer (DStub2-F, or DPtef-F, or LAtef-F)	10 μM	0.8	0.4 μM
Reverse primer (DStub2-R, or DPtef-R, or LAtef-R)	10 μM	0.8	0.4 μM
DNA polymerase (Hotgoldstar)	5 U μL^{-1}	0.1	0.5 U
Subtotal		18	
DNA	0.8–20 ng μL^{-1}	2	0.08–2 ng μL^{-1}
Total		20	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 μm filtered) and nuclease-free.

2.3 Thermocycler conditions

The PCR reaction conditions are carried out in a thermocycler equipped with a heated lid and include initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 1 min. A final elongation is done at 72°C for 10 min. The PCR product is kept frozen until separation by electrophoresis in agarose gel (1–2%) followed by gel staining (with ethidium bromide or similar product).

3. Essential Procedural Information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor cross-reactions with the host tissue and/or contamination during nucleic acid extraction: nucleic acid extraction and subse-

quent amplification of a sample of uninfected host tissue or clean extraction buffer

- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism)
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of PCR grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA) amplification of samples spiked with exogenous nucleic acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results

In order to assigning results from the PCR-based test the following criteria should be followed:

Verification of the controls:

- NIC and NAC should produce no amplicons
- PIC, PAC should produce amplicons of 231, 193, and 237 bp, for *Dothistroma septosporum*, *Dothistroma pini*, and *L. acicola*, respectively.
- IC (if used) should yield an amplicon of the expected size, depending of the type of control chosen.

When these conditions are met:

- A test will be considered positive if amplicon of 231, 193, and 237 bp for *Dothistroma*

septosporum, *Dothistroma pini*, and *L. acicola*, respectively, are produced

- A test will be considered negative, if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 *Dothistroma pini*

Fourteen isolates from France (6), the United States (6), Ukraine (1) and Russia (1) were included in the validation (*in silico* or *in vitro* assessments).

4.1.1 Analytical sensitivity data

The analytical sensitivity has been established by the authors at 0.1 pg μL^{-1} .

4.1.2 Analytical specificity data

The analytical specificity has been checked against 0.5 ng μL^{-1} DNA extracts from 20 different fungal species, including the needle pathogen *Dothistroma septosporum* and *L. acicola*.

4.1.3 Data on repeatability

Not available.

4.1.4 Data on reproducibility

Not available.

4.2 *Dothistroma septosporum*

Thirty five isolates from Germany, Belgium, France, the United States, Canada, Austria, Slovakia, Poland, Ecuador, Chile, Australia, New-Zealand, Kenya, South Africa, Hungary and Buthan were included in the validation (*in silico* or *in vitro* assessments).

4.2.1 Analytical sensitivity data

The analytical sensitivity has been established by the authors at 0.1 pg μL^{-1} .

4.2.2 Analytical specificity data

The analytical specificity has been checked against 0.5 ng μL^{-1} DNA extracts from 20 different fungal species, including the needle pathogen *Dothistroma pini* and *L. acicola*.

4.2.3 Data on repeatability

Not available.

4.2.4 Data on reproducibility

Not available.

4.3 *L. acicola*

Eight isolates from France, United States, and China were included in the validation (*in silico* or *in vitro* assessments).

4.3.1 Analytical sensitivity data

The analytical sensitivity has been established by the authors at 0.1 pg μL^{-1} .

4.3.2 Analytical specificity data

The analytical specificity has been checked against 0.5 ng μL^{-1} DNA extracts from 20 different fungal species, including the needle pathogen *Dothistroma pini* and *Dothistroma septosporum*.

4.3.3 Data on repeatability

Not available.

4.3.4 Data on reproducibility

Not available.

Appendix 3 – Identification at species level by real-time PCR using hydrolysis probes

1. General information

1.1 This protocol was developed by Ioos *et al.*, 2010. This real-time PCR test is set up with a quadruplex format, enabling the simultaneous detection of *Lecanosticta acicola*, *Dothistroma septosporum*, and *D. pini*, and includes an internal DNA extraction/inhibition control targeting plant and fungal DNA.

1.2 The nucleic acid source is infected plant tissues (needles).

1.3 Name of targeted genes are Beta-Tubulin 2 (β -tub2) for *Dothistroma septosporum*, and Translation Elongation Factor I alpha (EF1- α) for *Dothistroma pini* and *L. acicola*.

1.4 Position of the *D. septosporum* forward primer considering as reference sequence GenBank accession AY808228: 24

Position of the *D. pini* forward primer considering as reference sequence GenBank accession AY808271: 178

Position of the *L. acicola* forward primer considering as reference sequence GenBank accession AY808273: 218

1.5 Amplicon size with these primers is 89, 76, and 79 bp, for *Dothistroma septosporum*, *Dothistroma pini*, and *L. acicola*, respectively.

1.6 Oligonucleotides and probes:

1.6.1 *Dothistroma septosporum*

Forward primer DStub2-F1: 5'- CGAA-CATGGACTGAGCA AAA -3'

Reverse primer DStub2-R1: 5'- TG CCTT CGTATCTGCATTTC -3'

Hydrolysis probe DStub2-P1: 5'- ROX-TGGAATCCACAGACGCGTCA-BHQ2-3'

1.6.2 *Dothistroma pini*

Forward primer DPtef-F1: 5'-ACA-GCAATCACACCCTTGC -3'

Reverse primer DPtef-R1: 5'- TCATGTGCT-CAATGTGAGATGT -3'

Hydrolysis probe DPtef-P1: 5'- FAM-CCCCAGCCGATTACACGACG-BHQ1-3'

1.6.3 *L. acicola*

Forward primer LAtef-F1: 5'- CC TCCTTCATCTTCCCCTTC -3'

Reverse primer LAtef-R1: 5'- TGTGGGA-GATAGCGTTGTCA -3'

Hydrolysis probe LAtef -P1: 5'- Cy5-CAAGCACTCTTGGAACACACCCGC-BHQ3 -3'

1.6.4 Plant/fungus

Forward primer 18S uni-F: 5'- GC AAGGCTGAAACTTAAAGGAA -3'

Reverse-primer 18S uni -R: 5'- CCACCACCCATAGAATCAAGA -3'

Hydrolysis probe 18S uni -P: 5'- JOE-AC-GGAAGGGCACCACCAGGAGT-BHQ1-3'

1.7 Real-time PCR system: Rotor-Gene 6500 (Corbett Research, Mortlake, Australia).

1.8 Software and settings for data analysis: The quadruplex test was analysed with an autogain optimization for each channel, which was performed before the first fluorescence acquisition. The Ct value for each reaction was determined using the Rotor-Gene software, version 1.7.75, setting the threshold line at 0.02

2. Methods

2.1 Nucleic acid extraction and purification: see Appendix 2

2.2 Real-time PCR reaction:

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular grade water*	N.A.	To make up to 20 (10.7)	N.A.
qPCR buffer (qPCR core kit No ROX, Eurogentec)	10 X	2	1 X
MgCl ₂ (qPCR core kit No ROX, Eurogentec)	50 mM	2	5 mM
dNTPs (qPCR core kit No ROX, Eurogentec)	5 mM	0.8	0.2 mM

(continued)

Table (continued)

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Forward primer DStub2-F1	30 μM	0.2	0.3 μM
Reverse primer DStub2-R1	30 μM	0.2	0.3 μM
Probe DStub2-P1	10 μM	0.2	0.1 μM
Forward primer DPtef1-F1	30 μM	0.2	0.3 μM
Reverse primer DPtef1-R1	30 μM	0.2	0.3 μM
Probe DPtef1-P1	10 μM	0.2	0.1 μM
Forward primer LAtef-F1	30 μM	0.2	0.3 μM
Reverse primer LAtef-R1	30 μM	0.2	0.3 μM
Probe LAtef-P1	10 μM	0.2	0.1 μM
Forward primer 18S uni-F	30 μM	0.2	0.3 μM
Reverse primer 18S uni-R	30 μM	0.2	0.3 μM
Probe 18S uni-P	10 μM	0.2	0.1 μM
DNA polymerase (Hotgoldstar, qPCR core kit No ROX, Eurogentec)	5 U μL^{-1}	0.1	0.5 U
Subtotal		18	
DNA	0.8–20 ng μL^{-1}	2	0.08–2 ng μL^{-1}
Total		20	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 μm filtered) and nuclease-free.

2.3 Thermocycler conditions

The PCR reaction conditions are carried out in a thermocycler adapted to real-time reactions and include initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation and annealing/elongation, 15 s at 95°C and 55 s at 60°C respectively. The Ct value for each reaction is determined using the software provided with the thermocycler.

3. Essential Procedural Information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor cross-reactions with the host tissue and/or contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated 18S

uni-F/-R/-P, systematically used in multiplex with DStub2-F1-R1-P1, DPtef1-F1-R1-P1 and LAtef1-F1-R1-P1 in order to check the quality of DNA extraction. This 18S uni-F/-R/-P combination targets a conserved region within the 18S rDNA gene from a wide range of plants (Ioos *et al.* 2009a).

- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of PCR grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

3.2 Interpretation of results

Verification of the controls:

- The PIC and PAC amplification curves should be exponential.
- NIC and NAC should give no amplification

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Dothistroma pini

Fourteen isolates from France (6), the United States (6), Ukraine (1) and Russia (1) were included in the validation (*in silico* or *in vitro* assessments).

4.1.1 Analytical sensitivity data

The analytical sensitivity has been established by the authors at 0.01 pg μL^{-1} .

4.1.2 Analytical specificity data

The analytical specificity has been checked against 0.5 ng μL^{-1} DNA extracts from 20 different fungal species, including the needle pathogen *Dothistroma septosporum* and *L. acicola*. See Table 1 in Ioos *et al.* (2010). No cross reaction observed.

4.1.3 Data on repeatability

The coefficient of variance is:

0.2% for a target DNA concentration of 1 ng μL^{-1} ;
 0.6% for a target DNA concentration of 100 pg μL^{-1} ;
 0.9% for a target DNA concentration of 10 pg μL^{-1} ;
 1.2% for a target DNA concentration of 1 pg μL^{-1} ;
 0.5% for a naturally infested needle sample.

4.1.4 Data on reproducibility

The coefficient of variance is:

0.1% for a target DNA concentration of 1 ng μL^{-1} ;
 1.1% for a target DNA concentration of 100 pg μL^{-1} ;
 1.0% for a target DNA concentration of 10 pg μL^{-1} ;
 1.1% for a target DNA concentration of 1 pg μL^{-1} ;
 0.6% for a naturally infested needle sample.

4.2 *Dothistroma septosporum*

Thirty five isolates from Germany, Belgium, France, the United States, Canada, Austria, Slovakia, Poland, Ecuador, Chile, Australia, New-Zealand, Kenya, South Africa, Hungary and Buthan were included in the validation (*in silico* or *in vitro* assessments).

4.2.1 Analytical sensitivity data

The analytical sensitivity has been established by the authors at 0.01 pg μL^{-1} .

4.2.2 Analytical specificity data

The analytical specificity has been checked against 0.5 ng μL^{-1} DNA extracts from 20 different fungal species, including the needle pathogen *Dothistroma pini* and *L. acicola*. See Table 1 in Ioos *et al.* (2010). No cross reaction observed.

4.2.3 Data on repeatability:

The coefficient of variance is:

0.7% for a target DNA concentration of 1 ng μL^{-1} ;
 0.6% for a target DNA concentration of 100 pg μL^{-1} ;
 1.0% for a target DNA concentration of 10 pg μL^{-1} ;
 1.1% for a target DNA concentration of 1 pg μL^{-1} ;

1.0% for a naturally infested needle sample.

4.2.4 Data on reproducibility

The coefficient of variance is:

0.3% for a target DNA concentration of 1 ng μL^{-1} ;
 0.5% for a target DNA concentration of 100 pg μL^{-1} ;
 1.4% for a target DNA concentration of 10 pg μL^{-1} ;
 1.5% for a target DNA concentration of 1 pg μL^{-1} ;
 1.3% for a naturally infested needle sample.

4.3 *L. acicola*

Eight isolates from France, the United States, and China were included in the validation (*in silico* or *in vitro* assessments).

4.3.1 Analytical sensitivity data

The analytical sensitivity has been established by the authors at 0.01 pg μL^{-1} .

4.3.2 Analytical specificity data

The analytical specificity has been checked against 0.5 ng μL^{-1} DNA extracts from 20 different fungal species, including the needle pathogen *Dothistroma pini* and *Dothistroma septosporum*. See Table 1 in Ioos *et al.* (2010). No cross reaction observed.

4.3.3 Data on repeatability

The coefficient of variance is:

0.4% for a target DNA concentration of 1 ng μL^{-1} ;
 0.7% for a target DNA concentration of 100 pg μL^{-1} ;
 1.1% for a target DNA concentration of 10 pg μL^{-1} ;
 1.3% for a target DNA concentration of 1 pg μL^{-1} ;
 0.9% for a naturally infested needle sample.

4.3.4 Data on reproducibility

The coefficient of variance is:

0.6% for a target DNA concentration of 1 ng μL^{-1} ;
 1.0% for a target DNA concentration of 100 pg μL^{-1} ;
 1.5% for a target DNA concentration of 10 pg μL^{-1} ;
 1.7% for a target DNA concentration of 1 pg μL^{-1} ;
 1.4% for a naturally infested needle sample.