Normes OEPP
EPPO Standards

Diagnostics
Diagnostic

PM 7/46
Approval

EPPO Standards are approved by EPPO Council. The date of approval appears in each individual standard. In the terms of Article II of the IPPC, EPPO Standards are Regional Standards for the members of EPPO.

Review

EPPO Standards are subject to periodic review and amendment. The next review date for this EPPO Standard is decided by the EPPO Working Party on Phytosanitary Regulations.

Amendment record

Amendments will be issued as necessary, numbered and dated. The dates of amendment appear in each individual standard (as appropriate).

Distribution

EPPO Standards are distributed by the EPPO Secretariat to all EPPO member governments. Copies are available to any interested person under particular conditions upon request to the EPPO Secretariat.

Scope

EPPO Standards on Diagnostics are intended to be used by NPPOs in their capacity as bodies responsible for the application of phytosanitary measures. Standards on diagnostic protocols are concerned with the diagnosis of individual pests and describe different methods which can be used to detect and identify pests of phytosanitary concern for the EPPO region. General Standards on diagnostics are in preparation on: (1) the purpose of diagnostic protocols (which may differ according to the circumstances of their use); and (2) reporting and documentation of diagnoses.

In 1998, EPPO started a new programme to prepare diagnostic protocols for the regulated pests of the EPPO region (including the EU). The work is conducted by the EPPO Panel on Diagnostics and other specialist Panels. The objective of the programme is to develop an internationally agreed diagnostic protocol for each regulated pest. The protocols are based on the many years of experience of EPPO experts. The first drafts are prepared by an assigned expert author(s). They are written according to a ‘common format and content of a diagnostic protocol’ agreed by the Panel on Diagnostics, modified as necessary to fit individual pests. As a general rule, the protocol recommends a particular means of detection or identification which is considered to have advantages (of reliability, ease of use etc.) over other methods. Other methods may also be mentioned, giving their advantages/disadvantages. If a method not mentioned in the protocol is used, it should be justified.

The following general provisions apply to all EPPO Standards on Diagnostics:

- laboratory tests may involve the use of chemicals or apparatus which present a certain hazard. In all cases, local safety procedures should be strictly followed
- 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
- laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated or that proper positive and negative controls are included.

References


Definitions

Regulated pest: a quarantine pest or regulated non-quarantine pest. Quarantine pest: a pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled.

Outline of requirements

EPPO Standards on Diagnostics provide all the information necessary for a named pest to be detected and positively identified by an expert (i.e. a specialist in entomologist, mycology, virology, bacteriology, etc.). Each protocol begins with some short general information on the pest (its appearance, relationship with other organisms, host range, effects on host, geographical distribution and its identity) and then gives details on the detection, identification, comparison with similar species, requirements for a positive diagnosis, list of institutes or individuals where further information on that organism can be obtained, references (on the diagnosis, detection/extraction method, test methods).

Existing EPPO Standards in this series

Forty-one EPPO standards on diagnostic protocols have already been approved and published. Each standard is
numbered in the style PM 7/4 (1), meaning an EPPO Standard on Phytosanitary Measures (PM), in series no. 7 (Diagnostic Protocols), in this case standard no. 4, first version. The existing standards are:

PM 7/14 (1) Ceratocystis fimbriata f. sp. platani. *Bulletin OEPP/EPPO Bulletin* 33, 249–256
PM 7/16 (1) Fusarium oxysporum f. sp. albedinis. *Bulletin OEPP/EPPO Bulletin* 33, 265–270
PM 7/22 (1) Xanthomonas arboricola pv. corylina. *Bulletin OEPP/EPPO Bulletin* 34, 179–182
PM 7/23 (1) Xanthomonas axonopodis pv. dieffenbachiae. *Bulletin OEPP/EPPO Bulletin* 34, 183–186
PM 7/26 (1) Phytophthora cinnamomi. *Bulletin OEPP/EPPO Bulletin* 34, 201–208
PM 7/30 (1) Beet necrotic yellow vein benyvirus. *Bulletin OEPP/EPPO Bulletin* 34, 229–238

Some of the Standards of the present set result from a different drafting and consultation procedure. They are the output of the DIAGPRO Project of the Commission of the European Union (no. SMT 4-CT98-2252). This project involved four ‘contractor’ diagnostic laboratories (in England, Netherlands, Scotland, Spain) and 50 ‘inter-comparison’ laboratories in many European countries (within and outside the European Union), which were involved in ring-testing the draft protocols. The DIAGPRO project was set up in full knowledge of the parallel activity of the EPPO Working Party on Phytosanitary Regulations in drafting diagnostic protocols, and covered regulated pests which were for that reason not included in the EPPO programme. The DIAGPRO protocols have been approved by the Council of EPPO as EPPO Standards in series PM 7. They will in future be subject to review by EPPO procedures, on the same terms as other members of the series.
**Mycosphaerella dearnessii**

**Specific scope**
This standard describes a diagnostic protocol for *Mycosphaerella dearnessii*.

**Specific approval and amendment**
Approved in 2004-09.

**Introduction**
*Mycosphaerella dearnessii*, the cause of brown spot needle blight of pine (EPPO/CABI, 1997), kills the foliage and retards the growth of many pine species. Attack over several years can kill the trees. Only *Pinus* spp. are attacked. The fungus is mainly known in North America, but has also been found spreading in Europe (Pehl, 1995).

**Identity**

**Name:** *Mycosphaerella dearnessii* M. E. Barr  
**Synonyms:** *Scirrhia acicola* (Dearness) Siggers, *Systremma acicola* (Dearness) F. A. Wolf & Barbour  
**Anamorph:** *Lecanosticta acicola* (Thümen) H. Sydow  
**Synonyms:** *Lecanosticta pini* H. Sydow, *Septoria acicola* (Thümen) Saccardo  
**Taxonomic position:** Fungi: Ascomycota: Dothideales  
**EPPO computer code:** SCIRAC  
**Phytosanitary categorization:** EPPO A2 list: No. 22; EU Annex designation: II/A1 – as Scirrhia acicola

**Detection**

**Symptoms**
Symptoms first appear on needles as orange/yellow, sometimes resin-soaked spots, which later become dark brown in the centre with a yellow border. Sometimes, these spots have a darker edge, although a yellowish halo is always visible around it (Web Fig. 1a–c). They usually enlarge to bands that encircle needles and cause death of parts beyond (Web Fig. 2a,b). Diseased needles typically show dead tips, central zones with spots in green tissue, and green bases. Diseased needles are sometimes shorter than healthy ones.

The transition from lesions to healthy green tissue is abrupt. In the brown-coloured dead parts of the needle, the black stroma of the fructification develops under the epidermis visible as round black spots (Web Fig. 3). During further development, the oval-shaped fruit bodies, arranged parallel to the long axis of the needle, break through the epidermis opening by a longitudinal slit, or two slits, raising a flap of epidermis and hypodermal tissue (Web Fig. 4a,b). Mature conidiomata produce under moist conditions mucilaginous olive-green spore masses (Web Fig. 5). After severe attack, the whole needle turns brown, then grey (Web Fig. 2a), and abscises prematurely. In less severe attacks, needle fall may be delayed for one or two years. Heavily infected pines typically show twigs carrying only last year’s needles. These take on a ‘paintbrush’ appearance as the pathogen develops, and the needles may shed. Over several years, this may result in branch and tree death. See Sinclair et al. (1989).

**Isolation**
The fungus is most easily identified by examination of mature conidiomata (and can also be isolated directly from them). If none are present, the fungus should be isolated from affected needles with brown dead tissue (brown spots, bands or dead parts with black stroma spots). After surface sterilization, needles are cut into segments 4–6 mm 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 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 slow growth
in culture and the presence of endophytic fungi in pine needles, isolation 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.

**Method for surface sterilization of pine needles**

Needles are immersed in 70% ethanol for 30 s, or NaOCl (commercial bleach, 2% of active chlorine) for 60 s, or 96% ethanol for 10 s, then rinsed in sterile water.

**Identification**

**Identification on the basis of morphological features**

A presumptive indication of the presence of *M. dearnessii* in pine needles is the appearance of yellow, occasionally resin-soaked spots which later become brown in the centre, contrasting with the prominent yellowish border (Web Fig. 1a,b). Final confirmation can only be obtained by microscopic identification of the conidial stage when the typical conidia are produced (Web Fig. 6). Ascostromata are produced irregularly on fallen needles and are not particularly useful for identification.

Mature conidiomata split the epidermis by one or two longitudinal slits, raising the epidermal and hypodermal tissue, and producing a slimy olive spore mass under moist conditions. This turns to a blackish-green, not shining tuft under dry conditions (Web Fig. 5, hand lens 10×). Sporulation can be induced by placing needles with mature conidiomata in a moist chamber for 1–2 days.

**Comparison with similar species**

The macroscopic symptoms and morphological features can easily be confused with red band needle blight (*Mycosphaerella pini*, anamorph: *Dothistroma septospora*) especially at the beginning of the disease and even later, if typical red bands are not produced (Evans, 1984; Pehl & Wulf, 2001). Some symptoms and features of *Mycosphaerella gibsonii* (anamorph: *Pseudocercospora pini-densiflorae*) are also very close to *M. dearnessii*. The teleomorphs of these three species (if available for examination) are very similar morphologically, and can hardly be distinguished in the absence of some other characteristic information, such as the profuse reddish tint of the necrotic needle tissue characteristic by attack by *M. pini*.
The anamorphs, however, are consistently different and provide the best characters to separate the species. The conidiomata known as Lecanosticta and Dothistroma vary in form between acervuli and pseudopycnidia, according to host and climate, but differ clearly from the fruit bodies of Pseudocercospora, which are sporodochia. The most important and consistent character to distinguish *M. dearnessii* from *M. pini* is the nature of the spore wall of the conidium. Conidia of *M. dearnessii* (of *Lecanosticta* type) have melanin granules in their outer wall. Viewed under the microscope, the spores are pigmented, thick-walled with a verrucous surface. The best view is obtained using differential interference contrast optics at an magnification over ×400. In contrast, the conidia of *M. pini* (of *Dothistroma* type) are hyaline, thin walled and smooth. However, the conidia of *M. dearnessii* do not differ distinctly in size from those of *M. pini*.

The three fungi can also be distinguished by cultural characteristics. On MEA, colonies of *M. pini* are slow-growing, grey-brown-black, stromatic and produce a whitish conidal slime. A reddish-brown diffusate is present in the agar. *M. dearnessii* on the same media grows significantly larger and show green-black stromatic colonies with an olive-green conidial slime. Typically a yellow diffusate is visible. *M. gibsonii* is the fastest-growing species and forms woolly mycelial grey-coloured colonies.

**Identification by molecular biological methods**

Pehl *et al.* (2004) have used a PCR-based ITS-RFLP technique to differentiate *M. pini* from *M. dearnessii* and 10 other fungi frequently occurring in Europe on pine needles. rDNA is obtained from 0.5–1 mg freeze-dried mycelium of fungi grown on liquid culture media or directly from 1 or 2 infected needle segments (1–3 mm length, 1.5–3 mg) showing immature fruit bodies or stroma. Mycelium or fresh needle segments are homogenized using a glass micro mortar (25–100 bodies or stroma. Mycelium or fresh needle segments are homogenized using a glass micro mortar (25–100 bodies or stroma. Mycelium or fresh needle segments are homogenized using a glass micro mortar (25–100 μL). Extraction buffer from the Qiagen DNeasy Plant Mini Kit. rDNA is isolated by using the Qiagen DNeasy Plant Mini Kit following the manufacturer’s instructions. Because of the small quantity of material tested, the amounts listed in the protocol (e.g. buffers, RNase) were reduced to 1/4 of the prescribed values. rDNA concentration is determined fluorimetrically using fluorescent dye Hoe 33258 and a DyNa Quant 200 fluorimeter (e.g. Pharmacia).

PCR is carried out employing 50 μL reaction volume and a Biometra Tpersonal 48 thermocycler. The reaction mixture contains 3 units Taq DNA polymerase (e.g. Stratagene), 5 μL 10x reaction buffer (e.g. Stratagene), 0.5 mM MgCl₂, 0.1 mM dNTPs (e.g. Boehringer Mannheim), 0.6 μM forward ITS-4 primer 5′-TCTTCGGCTATTTGATGC-3′ and 0.6 μM reverse ITS-5 primer 5′-GGAAGTAAAAGTCGTAACAAGG-3′ according to White *et al.* (1990) and 2 ng template rDNA. The PCR programme consists of an initial denaturation for 150 s at 94°C, 35 cycles with 60 s denaturation at 94°C, 60 s annealing at 55°C and 120 s extension at 72°C, and a final extension for 5 min at 70°C. After completion of the PCR, aliquot samples are separated electrophoretically using a 2% agarose gel, and amplified rDNA is stained with 1 μg mL⁻¹ ethidium bromide and visualized using an UV-transilluminator.

Aliquot samples containing the amplified rDNA should be digested with each of the restriction endonucleases Hae, Hha I, Hpa II, Hinf I and Nci I (e.g. Gibco), following the manufacturer’s instructions. The DNA restriction fragments obtained are separated electrophoretically using a 2.5% agarose gel and visualized as described above. Fragment sizes are estimated by comparison with a DNA size marker (100 bp ladder, e.g. Gibco). *M. dearnessii* is identified on the basis of the species-specific DNA restriction fragments obtained by carrying out the ITS-RFLP analysis as described above. An example of species differentiation by ITS-RFLP restriction fragment patterns of *M. dearnessii* and *M. pini* isolates is shown in Web Fig. 9.

**Conclusion**

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 one molecular method (PCR-based ITS-RFLP). All are equivalent and each method is adequate by itself. At least one of these methods should have been positive for a positive diagnosis. The essential distinguishing features of *M. dearnessii* are the conidia of its anamorph *L. acicola* produced on mature conidiomata on infected needles and in culture. Because of the limitations of classical identification methods, the use of PCR-based ITS-RFLP allows fast and reliable identification of *M. dearnessii*.

Symptoms on pines infected by *M. dearnessii* and adequate identification methods to be undertaken are shown in Web Fig. 10.

**Reference culture**

M 275.

**Reporting and documentation**

Guidance on reporting and documentation is given in EPPO Standard PM7/– (in preparation).

**Further information**

Further information on this organism can be obtained from:

Dr L. Pehl, Federal Biological Research Centre for Agriculture and Forestry, Institute for Plant Protection in Forests, Messweg 11/12, D-38104 Braunschweig (Germany)

Dr T. L. Cech, Bundesamt und Forschungszentrum für Wald, Institut für Forstschutz, Abteilung für Phytopathologie, Seekendorff-Gudent-Weg 8, A-1131 Wien (Austria)

Prof Dr Ottmar Holderrieder, ETH-Zentrum, Department Wald- und Holzforschung, Rämistr. 101, 8092 Zürich (Switzerland).
Acknowledgements

This protocol was originally drafted by Dr L. Pehl, BBA, Braunschweig (DE).

References


Web Figs. 1a-c: Brown spots and necrotic bands (1c: resin-soaked spots) on needles of *Pinus mugo* caused by *Mycosphaerella dearnessii* (*Lecanosticta acicola*).

Web Figs. 2a, b: Attack of *Mycosphaerella dearnessii* (*Lecanosticta acicola*) on *Pinus mugo*. 
Web Fig. 3: Spots of black stroma of *Mycosphaerella dearnessii* (*Lecanosticta acicola*) developing under the needle epidermis (*Pinus mugo*).

Web Fig. 4a, b: Fructifications of *Mycosphaerella dearnessii* (*Lecanosticta acicola*) rupturing needle epidermis of *Pinus mugo*.
Web Fig. 5: Slimy-dark green spore mass of *Lecanosticta acicola* produced from conidiomata under moist conditions.

Web Fig. 6: Conidia of *Lecanosticta acicola*.
Web Fig. 7: Cross section through an conidioma of *Lecanosticta acicola* (staining: thionine).

Web Fig. 8: Sporulating culture of *Lecanosticta acicola* on malt extract agar.
Web Fig. 9: ITS-RFLP patterns of a *Dothistroma septospora* isolate compared with a *Lecanosticta acicola* isolate. M) 100 bp marker, 1) ITS-Amplicon, 2) *Hinf* I, 3) *Hae* III, 4) *Hha* I, 5) *Nci* I, 6) *Hpa* II.
Web Fig. 10 Symptoms on pines infected by *M. dearnessii* (*Lecanosticta acicola*)

**Suspicious symptoms**

- Pine trees with strong needle cast. Twigs looking often like paint-brushes.

**Identification scheme**

- Needles with necroses, stroma or fruit bodies especially in the litter. Further studies (microscope or PCR / RFLP) are necessary.

- Needles with typical yellow bordered brown spots or bands especially on current-years-old needles in spring and mid summer. Strong indication for *M. dearnessii*. Further research is necessary by isolation of the pathogen on culture media. Comparison with reference culture or PCR / RFLP.

- Needles with brown necrotic tissue, showing black spots of stroma developing under the epidermis. Further research is necessary by isolation of the pathogen on culture media or direct evidence by PCR / RFLP procedure.

- Needles with brown necrotic tissue, showing fruit bodies breaking through the epidermis by longitudinal slits, raising a flap of epidermis. Microscopically examination for conidia or ascospores are necessary. If no spores are present, sporulation can be induced by placing the needles in a moist chamber for 1 or 2 days. Also direct evidence by PCR / RFLP procedure is possible.