**Diagnostics**
**Diagnostic**

# *Gremmeniella abietina*

## Specific scope

This standard describes a diagnostic protocol for *Gremmeniella abietina*[1]

## Specific approval and amendment

Approved in 2009–09.

## Introduction

*Gremmeniella abietina* (Scleroderris-canker of conifers) is a pathogen occurring on many species of *Pinus* and several other conifers (*Picea* spp., *Abies sachalinensis*, *Abies balsamea*, *Larix* spp., *Pseudotsuga menziesii*). The pathogen attacks buds and young shoots, killing foliage and leading to dieback of twigs and branches. Whole plants may die after repeated attacks or may be girdled. On older parts bark necroses develop.

*Gremmeniella abietina* occurs almost worldwide (North America, Europe, Asia). It is widespread in Europe: it is a common disease of *Pinus nigra*, *Pinus sylvestris*, *Pinus cembra*, *Pinus mugo*, but also of *Picea abies*. Geographic distribution ranges from the Boreal to the Mediterranean region.

The species is believed to be partly of European and partly of North American origin. One race is regarded as of European origin and has spread to North America and Japan where it resulted in heavy losses in pine plantations.

Different varieties, races and biotypes have been described (see Appendix 1) but molecular data from Hamelin & Rail (1997) and Dusabenyagasani *et al.* (2002) indicate that the taxonomy of *Gremmeniella* will be undergoing major changes in the near future, resulting in raising varieties to species level. However, as the expected changes in taxonomy are still pending, the present version of this protocol refers to the actual taxonomic status of *G. abietina* with changes to be expected in the future.

## Identity

**Name:** *Gremmeniella abietina* (Lagerberg) Morelet

**Synonyms:** *Ascocalyx abietina* (Lagerberg) Schläpfer
*Crumenula abietina* Lagerberg
*Lagerbergia abietina* (Lagerberg) J.Reid
*Scleroderris abietina* (Lagerberg) Gremmen
*Scleroderris lagerbergii* Gremmen
**Anamorph:** *Brunchorstia pinea* (P. Karsten) Höhnel
**Synonyms:** *Brunchorstia destruens* Eriksson
*Brunchorstia pini* Allescher
*Excipulina pinea* P. Karsten
*Septoria pinea* P. Karsten
**Taxonomic position:** Fungi: Ascomycetes: Helotiales
**Notes on taxonomy and nomenclature:** (see introduction)
**EPPO code:** GREMAB
**Phytosanitary categorization:** EU Annex designation: II/B

## Detection

### Symptoms

Descriptions below refer to the whole species unless otherwise stated.

Initial infection by *G. abietina* occurs in developing shoots in the spring. Buds do not flush and resin droplets appear on the bark of current year's shoots, however symptoms may not be visible until the following winter when resin exudations are clearly visible. Buds die and necrosis extends from the bud into the shoot. Shoots that are one year or older may die back, and local cankers can also be formed on older stem parts. As a consequence, the previous year's needles turn red to brown (Fig. 1), beginning at their base. They can easily be stripped off the shoot. Totally brown needles soon drop. The bark of the shoot becomes shrunken and dry. Buds killed only partially may give rise to poorly developed and distorted shoots, which may die later on. Cankers near the stem base may result in girdling of the tree.

[1]Use 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.


 

**Fig. 1** Early symptoms of *G. abietina*-infection: dieback of Swiss stone pine, shoot with dead terminal bud surrounded by reddish-brown discoloured needles.

Cankers, which fail to girdle, result in shrunken, more or less callused bark patches. Dieback of the shoots often results in branching anomalies, resembling witches brooms. Inner bark layers of the cankered areas sometimes show a yellowish discoloration (not in case of the Asian race of *G. abietina* var. *abietina*).

Generally any dieback of pine or spruce's shoots could be the result of *G. abietina* infections but can also be the result of attack by a large number of other pathogenic microfungi. Therefore buds and bark of shoots and twigs should be examined carefully for fruiting structures of the anamorph *Brunchorstia pinea* or the teleomorph (Figs 2 and 3). In the case of pines the most indicative additional symptom is needles surrounding dead buds, most of them totally red to red brown, but some of them with only the basal half discoloured (see above), the outer half still green. A common dieback-fungus of several pine species is *Sphaeropsis sapinea* (Fr.) Dyko & Sutton. Symptoms may have a quite similar appearance to *Gremmeniella* – dieback, with the exception, that the characteristic symptom of needles with only the basal half discoloured surrounding dead buds is lacking.

Pycnidia are generally more common than apothecia in the case of attack by *G. abietina*. They are formed on the needles (often near the base) and on twigs around the base of killed buds, emerging from the abscission scar of detached needles or from cankered areas. Pycnidia can be found isolated or in groups, sometimes closely packed, reaching about one mm in size. On *Pinus sylvestris, Pinus resinosa* and *Pinus banksiana*, 'cryptopycnidia'6 are formed, which can be detected only by removing the outer bark layers of twigs.

Apothecia emerge from the bark and needle bases to become superficial. Under moist conditions they show a cream to light grey coloured hymenium. Apothecia are irregularly dispersed on dead shoots or dead needle tissue (Fig. 2).

Sometimes fruiting structures are difficult to find or absent. This may be a common phenomenon for instance on *Pinus cembra*, where they may be found only during a short period in spring, often developing under the snow cover. In this case, the pathogen can be isolated from infected tissues. Isolation from infected tissues is time consuming and may be unsuccessful during summer (Skilling & Kienzler, 1983), consequently it is recommended to look carefully for fruiting structures.

**Fig. 2** Ascomata of *G. abietina* (Photograph R. Engesser, WSL).

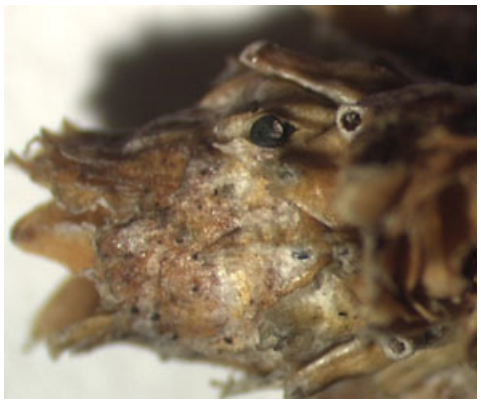

**Fig. 3** Conidioma of *B. pinea*, emerging from a terminal bud of Norway spruce (*Picea abies*).

### Similar symptoms caused by other factors

Another fungus, *Cenangium ferruginosum* Fr. (usually a saprophytic species) can be found on dead twigs of many pines. Trees weakened by various stress agents may show dieback phenomena with *C. ferruginosum* involved. It produces large numbers of

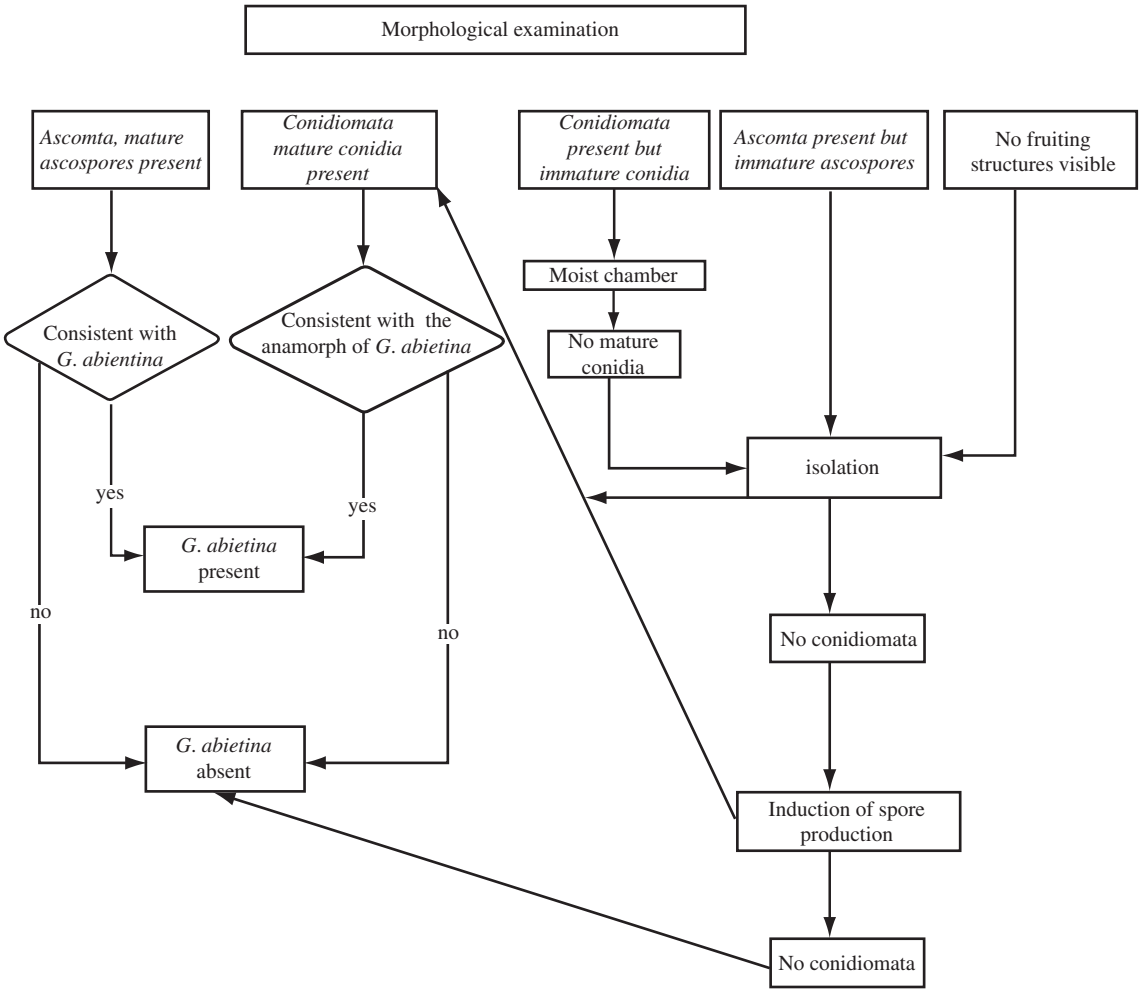


**Fig. 4** Flow diagram for the detection and identification of *G. abietina*.

blackish apothecia with yellowish discs on twigs, branches and stems.

Cankers of variable size are also typical for *Crumenulopsis sororia* (P. Karsten) Groves, which attacks European pines as well. On stems a bluish discoloration of the wood is often observed.

On larch, *G. laricina* (Ettlinger) Schläpfer-Bernhard causes similar symptoms.

Finally similar symptoms may also result from abiotic factors.

## Identification

The procedures for detection and identification described in this protocol, and the flow-diagram in Fig. 4, should be followed. If mature fruiting structures are present, *Gremmeniella abietina* can easily be identified *in vivo*. In the absence of visible fruiting structures, the symptomatic samples should be incubated in a damp chamber to induce sporulation. This is only successful if there are already immature conidiomata present. The most distinguishing feature is the presence of pycnostromata and the conidia of the anamorph as well as the ascomata and ascospores of the teleomorph. Culturing may also reveal the fruiting structures and is then followed by classical morphological examination.

### Morphological characteristics *in vivo*

*Anamorph*

Pycnidia are dark-brown to black, stromatic, with one to several locules incompletely separated from each other, without an ostiole and spindle shaped, pulvinate, globose or irregular. They may remain inserted deeply in the bark, but more often they emerge through a slit finally appearing nearly superficial (Fig. 3).

Walls and base are thick, composed of outer thick-walled colourless or dark brown textura angularis and an inner region of thinner walled paler pseudoparenchymatic cells, 200–600 × 800–1400 (2000) µm.

When cut with a scalpel, they are quite soft, the content is white and sometimes pinkish shades can be seen (hand lens). They extrude slimy spore masses often covering the bark surface as a white to pinkish coating.

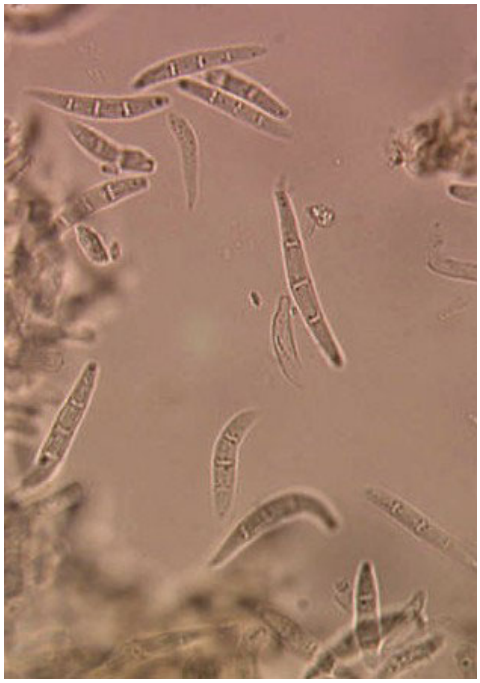

**Fig. 5** Conidia of *G. abietina*.

**Fig. 6** Asci and ascospores of *G. abietina* (Photograph R. Engesser, WSL).

The conidia (Fig. 5) are hyaline, 25–40 × 3–3.5 µm, with 0–8 (mostly 3 to 7) septate, curved, fusiform, smooth, thin-walled and eguttulate, developing from irregularly branched, cylindrical conidiogenous structures (Sutton, 1980; Heiniger, 1988).

A diagnostic method, in the absence of superficial fruiting structures of *G. abietina* visible on twigs of *Pinus* spp., is described by Cauchon & Lachance (1980). The pathogen can be detected at an early stage, where only the cambial necroses are present or the first needles have become red. Pycnidia can be observed, which appear as small locules or pockets varying in size from 200 µm to 2 mm. They are completely hidden in the bark of the twigs and shoots and resemble resin-pockets, since they reflect light like crystallized resin or wax. These locules are conidiomata of the anamorph of *G. abietina* containing the characteristic conidia. The colour of the conidial masses is white to whitish pink. Unlike the typical fruiting bodies, the pycnidia are not enclosed by dark wall layers. There is no characteristic mode of spore release (no typical cracks or fissures), although in the case of small shoots or twigs the conidia seem to be expelled through microscopical cracks between the peridermal ribs. Normally these structures are larger and contain a larger quantity of spores than typical conidiomata of *G. abietina*. These structures can be seen using a stereomicroscope with a strong illumination. The pycnidia may be located randomly along a shoot or twig, but they are often situated close to the detachment scars of needles. In the case of *P. sylvestris* they are arranged in a characteristic circle around the needle scars and are not as deeply immersed as in the case of *P. resinosa* and *P. banksiana*. On larger branches or stems, which have been infected for a long time, the pycnidia may be found occasionally in cankers, even in old and partly decayed bark, but they vary considerably in number. They may or may not occur mixed with the typical conidiomata or ascomata. Even if the majority of the locules are empty, there are commonly some conidia remaining, which enable the diagnosis. In general, the conidia show a higher viability: isolation of the fungus from those conidia is as easy as from the typical form, and aseptic removal of the conidia using a sharp needle or scalpel immediately after the bark has been opened is easier than from superficial conidiomata (Cauchon & Lachance, 1980).

Comparison with conidia of similar species:

A common dieback-fungus of several pine species is *Sphaeropsis sapinea* but its conidia are very large (30–45 × 10–16 µm, Sutton, 1980) and dark brown. The anamorph of *Crumenulopsis sororia* (P.Karsten) Groves (*Digitosporium pinophilum* Gremmen) is characterized by digitate conidia. The blackish ascomata are bordered by distinct, dark hairs. The conidia of *Brunchorstia laricina* Ettl. resemble *B. pinea*, but they are shorter (15–23 × 3–4 µm) and one to three-septate.

*Teleomorph*

Ascomata are brown to black-brown, disc-shaped, folded when dry and short stiped (pseudothecium – ascolocular), composed of pseudoparenchymatic, thick-walled cells and measure 400–1200 × 120–250 µm.

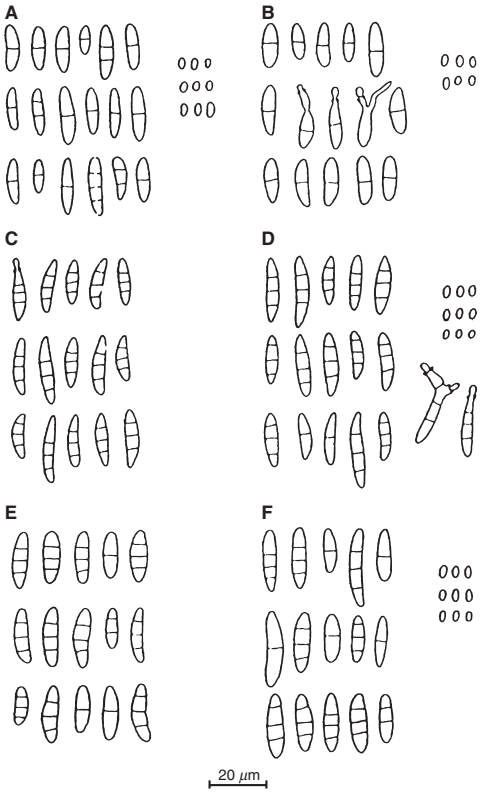


**Fig. 7** Ascospores and conidia of *Gremmeniella* spp. (Petrini *et al.*, 1989). (A,B) Ascospores and primary microconidia of *G. laricina.* (C,D) Ascospores and primary microconidia of *G. abietina* var. *balsamea.* (E,F) Ascospores and primary microconidia of *G. abietina* var. *abietina.*

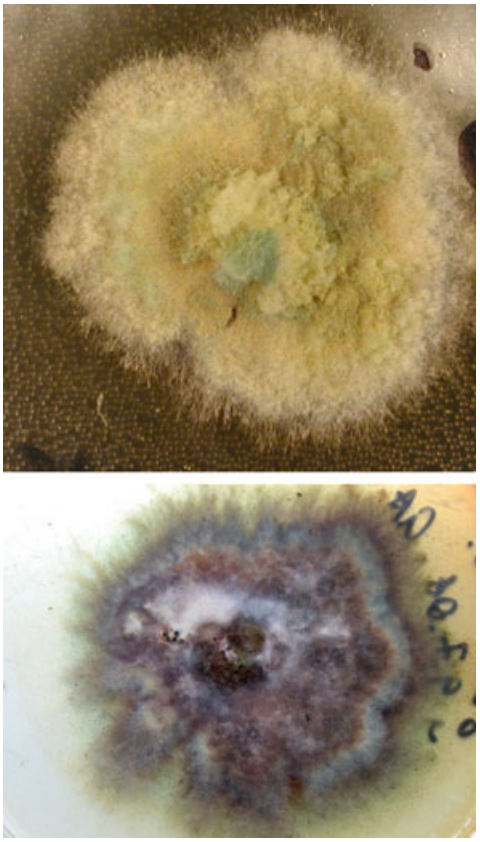

**Fig. 8** Cultural characteristics of *G. abietina* (medium Benhamou *et al.*, 1984; originally isolated from *Pinus cembra*, Austria, Carinthia, 2007).

Asci are saccate to cylindrical, bitunicate, 8-spored, hyaline, inoperculate with a rounded apex (not amyloid), and measure 100–120 × 6.5–10.5 µm (Fig. 6).

Ascospores are fusiform, 3-septate, hyaline, typically 4-guttulate, 15–22 × 3–5 µm.

Paraphyses are hyaline, filiform and septate.

Comparison with ascospores of similar species:

Another fungus, *Cenangium ferruginosum* Fr., can be found on dead twigs of many pines but the ascospores are non-septate and oval. The ascospores of *G. laricina* are one-septate and about the same size as those of *G. abietina* (Fig. 7).

### Morphological identification *in vitro*

*Isolation*

In the absence of mature fruiting structures, isolation is required. Identification of *G. abietina* from cultures may take between 2 weeks and 1 month. Culture media are described in Appendix 2.

*Isolation techniques*

• Isolation from needles (Barklund & Rowe, 1981):

Needles are separated from the shoot, washed in tap water with a small droplet of detergent. They are surface sterilised for 30 s in sodium hypochlorite (8–12% active chlorine). Subsequently, the needles are washed three times in sterile water, dried with sterile filter paper, and cut into small pieces and placed on agar Petri dishes (Skilling & Kienzler, 1983).

• Isolation from shoots, branches and stems

Two methods are recommended

Barklund & Rowe, 1981:

Sections from shoots, branches or stems are washed in tap water with a small droplet of detergent. Then, samples are surface sterilised for 30 s in sodium hypochlorite (8–12% active chlorine). Subsequently, samples are washed three times in sterile water, and dried with sterile filter paper. The outer bark layers are then removed under sterile conditions, and pieces of tissue are cut out and plated onto agar medium.

Skilling & Kienzler, 1983:

Branches with the characteristic yellowish stain should be selected and wood chips removed, these are surface sterilized for 1 minute in 10% (8–12% active chlorine) hypochlorite solution. These wood chips are then placed onto V-8 medium.

*Incubation*

Inoculated agar plates are incubated in the dark at 5 to 10°C for three weeks (Barklund & Rowe, 1981). After a pure mycelial culture is obtained from the original transfer, it should be incubated at 15–17°C for optimal mycelial growth (Skilling & Kienzler, 1983).

*Induction of spore production (anamorph)*

There is a high variation among races of *G. abietina* considering the development of conidiomata in culture. While many isolates produce conidiomata in abundance within several weeks, others fail or remain sterile for months.

For optimal spore production cultures should be kept under artificial light for at least 16 hours per day (Marosy *et al.*, 1989).

If there is no development of conidiomata, cultures should be stored under near UV light for one week at 20°C (Barklund & Rowe, 1981) or for 2–3 months at room temperature (Dorworth & Krywienczyk, 1975).

Alternatively, cultures are transferred onto wheat substrate (see Appendix 2). They should be cultured at 15°C for 2–3 months. Masses of pink conidia are produced (Bazzigher *et al.*, 1986).

Further information on sporulation-media for *G. abietina* is given by Hudler *et al.* (1984).

*Cultural characteristics*

Description of cultural characteristics of colonies have been made on V8 (Campbell's) agar (Dorworth, 1971). *Gremmeniella abietina* is a slow growing fungus producing a white, greenish or greyish-green, sometimes yellow aerial mycelium in Petri-dishes (Fig. 8). Experience with other rich agar media (e.g. malt extract agar and other vegetable juice agar) show a similar range of cultural characteristics (Cech, pers. comm. 2009).

Minimum temperature for growth is −5°C, maximum is 30°C and the optimal temperature between 13°C and 20°C (Donaubauer & Stephan, 1988).

After about one month on 2% Malt Extract Agar at 18°C (Schlaepfer-Bernhard, 1968) or two weeks on a media stimulating sporulation (Hudler *et al.*, 1984) pink droplets containing conidia (Schlaepfer-Bernhard, 1968; Skilling, 1968) develop usually in concentric rings (Dorworth & Krywienczyk, 1975).

An overview on the variation of *G. abietina* in culture morphology is given by Dorworth & Krywienczyk (1975).

### Molecular methods

Some tests have been developed for the identification of *G. abietina* on infected plant material or pure cultures (Hellgren & Högberg, 1995; Hamelin *et al.*, 1996, 2000; Hantula & Müller, 1997; Zeng *et al.*, 2005; Børja *et al.*, 2006). Some of the tests allow the differentiation of biotypes within the European race of *G. abietina* var. *abietina.* Nevertheless none of them allow the identification of all varieties races and biotypes of *G. abietina.* Given the uncertainty about the taxonomic position of the varieties of *G. abietina*, none of these tests are recommended.

### Differentiation of varieties and races

Tests have been described in the literature for the differentiation of races and are described in Appendix 1, but such differentiation is not required for quarantine purposes.

## Reference material

Numerous herbaria and culture collections provide specimens and cultures of *G. abietina*.

Infected host material and pure cultures can be obtained from the following research centres:

The Finnish Forest Research Institute, Unioninkatu 40 A, FIN-00170 Helsinki (FI)

Swedish University of Agricultural Sciences, Department of Forest Mycology and Pathology Box 7026, S-750 07 Uppsala (SE)

Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre, P.O. Box 3800, 1055 du PEPS, Sainte-Foy, Québec G1V 4C7 (CA)

Federal Research and Training Centre for Forests, Natural Hazards and Landscape, Seckendorff-Gudent-Weg 8 A-1131 Vienna (AT).

## Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM7/77 (OEPP/EPPO, 2006).

## Further information

Further information on this organism can be obtained from: Cech T, Austrian Federal Office and Research Centre for Forests, Seckendorff-Gudent-Weg 8, A-1131 Vienna (AT).

Karjalainen R, University of Kuopio, 31600 Jokioinen, Finland.

## Acknowledgements

This protocol was originally drafted by: Cech T, Federal Forest Office and Federal Research and Training Centre for Forests, Natural Hazards and Landscape, Vienna (AT).

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## Appendix 1

### Differentiation of varieties and races

Differentiation of varieties and races is not required for quarantine purposes.

At present, the pathogen is divided into varieties, races and biotypes but identification to the species level is sufficient for quarantine purposes. It should be noted that among the varieties and races, as well as within the races, there are further different types corresponding to different disease pattern and aggressiveness.

Varieties are:

- *abietina* (*typica*), which is the most abundant, occurring in Europe, North America and Asia mainly on *Pinus* spp. and *Picea* spp.
- *balsamea*, which occurs in a restricted area in Eastern North America, mainly on *Abies balsamea* and *Picea* spp.

Var. *abietina* is further divided into a North American race, occurring on *Pinus banksiana* and *P. contorta*, a European race (on *Pinus* spp., *Larix* spp. and *Picea* spp.), and an Asian race (on *Abies sacchalinsensis,* Japan).

Within the European race of *G. abietina* four biotypes have been identified:

- A or European biotype (=Large tree type mainly of *Pinus sylvestris* and *Picea abies*, affecting large trees as well as seedlings). Biotype A has been introduced into North America causing increasing damage among conifers.
- B or Fennoscandian biotype (=Small tree type associated with *Pinus sylvestris* and *P. contorta*, affecting seedlings and shoots related to long snow cover in winter).
- A third biotype, known as Alpine biotype, was described as *G. abietina* var. *cembrae.*
- In Spain, isolates of *G. abietina*, clearly differing from the two Northern and the Alpine biotypes have been characterized in 2005, indicating the existence of a forth biotype within the European race (Santamaria *et al.*, 2005).

Biotypes A and B are able to hybridize, but the progeny shows a low fitness.

According to Tuomivirta *et al.*, 2002 several isolates of biotype A of the European-race of *G. abietina* var. *abietina* contain double-stranded RNA (dsRNA), which may be of viral origin. Pathogenicity tests, however, did not reveal any differences in aggressiveness between isolates with and without dsRNA.

Molecular data from Hamelin & Rail (1997) and Dusabenyagasani *et al.* (2002) indicate that the taxonomy of *Gremmeniella* will be undergoing major changes in the near future, resulting in

raising varieties to species level. The following conclusions can be drawn:

1. The Asian race of *G. abietina* var. *abietina* is closely related to var. *balsamea*
2. *Gremmeniella abietina* var. *balsamea* is more related to *G. laricina* than to var. *abietina*.
3. The divergence between *G. abietina* var. *abietina* and *G. laricina* is about the same as between *G. abietina* var. *abietina* and var. *balsamea*.

Therefore, it can be expected, that the two varieties of *G. abietina* will become new distinct species and the Asian race might belong to *balsamea*. However, as the expected changes in taxonomy are still pending, the present version of this protocol refers to the actual taxonomic status of *G. abietina* with changes to be expected in the future.

### Differentiation of varieties

*Morphology*

*Gremmeniella abietina* var. *balsamea* differs morphologically from var. *abietina* by the following morphological features:

Ascospores are slightly larger (12.7–34 × 2.8–5.7 µm). Furthermore, they are acuminate, but this also occurs in *G. abietina* var. *abietina* and thus cannot be considered as a distinctive feature (Uotila *et al.*, 2006). Conidia are also larger in var. *balsamea* (21.7–53.7 × 1.8–4.2 µm (Petrini *et al.*, 1989).

*Isozymes*

*Gremmeniella abietina* var. *balsamea* differs from var. *abietina* by its pectic enzyme pattern (polygalacturonase-bands, Lecours *et al.*, 1994) and a length polymorphism in the small subunit of the ribosomal DNA (Bernier *et al.*, 1994).

## Differentiation of races of *Gremmeniella abietina* var. *abietina*

*Isozymes*

The North American race of *G. abietina* var. *abietina* shows a characteristic constant polygalacturonase-band in its pectinase isozyme pattern in contrast to the European and Asian races (Lecours *et al.*, 1994). It can also be distinguished from the other races by its water-soluble protein pattern revealing a characteristic band (band 6 in Petrini *et al.*, 1989).

The Asian race can be differentiated by the water-soluble protein pattern and by immunoelectrophoresis, but not sufficiently by pectic enzyme banding patterns (Dorworth & Krywienczyk, 1975; Benhamou *et al.*, 1984; Petrini *et al.*, 1989; Lecours *et al.*, 1994). Morphologically, this race cannot be distinguished from the others (Dorworth & Krywienczyk, 1975).

*Polyacrylamide gel electrophoresis of soluble proteins*

Benhamou *et al.* (1984) developed a method for characterizing isolates of *G. abietina*.

A method developed by Petäistö *et al.* (1996) allows a differentiation between the European and the North American race of *G. abietina* var. *abietina* (SDS-PAGE protein pattern) as well as between the biotypes of the European race (Western blot).

*SDS-PAGE protein pattern*

A test has been described by Petäistö *et al.* (1994).

There are minor differences in the banding patterns of the three races. The North American race can be distinguished from the European race by the presence of a 2.5 kD protein band, but within the European race no differences can be observed (Petäistö *et al.* (1996).

*Immunoblotting*

A test has been described by Petäistö *et al.* (1994). The affinity purified antibody gives a clear double band with B-type (or small tree type = STT) isolates in western blots. No double band can be observed with A-type isolates or isolates of the North American race (Petäistö *et al.*, 1996).

*Other immunological detection methods*

Dorworth & Krywienczyk (1975) developed a method to identify different strains of *G. abietina*. This method was adapted and refined by Skilling & Kienzler (1983), who give a detailed description of the procedure.

## Appendix 2

Culture media for *G. abietina*

Numerous media have been tested for isolating *G. abietina*. The following are most common.

Gremmeniella-*medium*:

25 mL Campbell's V8 juice, 15 g Bacto-Agar, 7.5 g malt extract and 475 mL distilled water, dispensed at 25 mL per Petri dish (Benhamou *et al.*, 1984).

Experience with other rich agar media (e.g. malt extract agar and other vegetable juice agar) show a similar range of cultural characteristics (Cech pers. comm. 2009).

*Skilling & Kienzler, 1983*:

V-8-medium: 45 g Malt Agar, 200 mL Campbell's V-8 juice (unfiltered), 800 mL distilled water.

*Medium, especially for measuring growth rates*:

Dorworth & Krywienczyk, 1975:

200 mL filtered Campbell's V-8 juice, 20 g Difco Agar, 800 mL distilled water, dispensed at 25 mL per Petri dish.

*Medium, especially for stimulating conidia production*:

Wheat substrate

10 g wheat and 25 mL distilled water in 100 mL Erlenmeyer.

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# Addendum – PM 7/92 (1) *Gremmeniella abietina*

In the EPPO Diagnostic protocol PM 7/92 *Gremmeniella abietina*, approved and published in 2009, a reference is made in the introduction to the fact that "molecular data from Hamelin & Rail (1997) and Dusabenyagasani *et al*. (2002) indicate that the taxonomy of *Gremmeniella* will be undergoing major changes in the near future, resulting in raising varieties to species level".

The reader of the protocol is warned that for various reasons this taxonomic revision never happened.

## Reference

EPPO (2009) PM 7/92 (1) *Gremmeniella abietina*. *EPPO Bulletin* **39**, 310–317.

 