Etiology and Real-Time Polymerase Chain Reaction-Based Detection of *Gremmeniella*- and *Phomopsis*-Associated Disease in Norway Spruce Seedlings

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ABSTRACT

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In spring 2002, an unusual disease outburst was recorded on Norway spruce seedlings in southeast Norway. Extensive damage was recorded on 1- and 2-year-old Norway spruce seedlings that either had wintered in nursery cold storage or had been planted out in autumn 2001. The damage was characterized by leader shoot dieback and stem necroses on the upper or lower part of the shoot from 2001. *Gremmeniella abietina* and a *Phomopsis* sp. frequently were isolated from the diseased seedlings. Internal transcribed spacer (ITS) ribosomal (r)DNA sequence analysis and random amplified microsatellites profiling indicated that the *G. abietina* strains associated with diseased nursery seedlings belonged to the large-

tree type (LTT) ecotype of the European race of *G. abietina* var. *abietina*, and inoculation tests confirmed their pathogenicity on Norway spruce. Based on ITS rDNA sequence analysis, the *Phomopsis* strains associated with diseased seedlings did not represent any characterized *Phomopsis* spp. associated with conifers. The *Phomopsis* sp. was not pathogenic in inoculation tests, indicating that it may be a secondary colonizer. ITS-based real-time polymerase chain reaction assays were developed in order to detect and quantify *G. abietina* and *Phomopsis* in the nursery stock. We describe here the *G. abietina*-associated shoot dieback symptoms on Norway spruce seedlings and conclude that the unusual disease outburst likely was related to the *G. abietina* var. *abietina* epidemic caused by the LTT on large Scots pines in 2001.

Additional keywords: *Picea abies*.

Gremmeniella abietina (Lagerb.) M. Morelet is one of the most serious pathogens on pines in the Northern hemisphere. It causes a range of symptoms known as the Brunchorstia dieback or Scleroderris canker. *G. abietina* comprises two described varieties: var. *abietina* associated with a number of conifers and with a large geographic distribution area, and var. *balsamea* known only from Quebec, Canada, where it is associated with *Abies balsamea*, *Picea glauca*, and *P. mariana* (25). As reviewed by Hamelin and Rail (12), *G. abietina* var. *abietina*, based on morphological, serological, and genetic studies, has been divided into North American, Asian, and European races. The European race of *G. abietina* var. *abietina* consists of two ecotypes named the “small-tree type” (STT) and the “large-tree type” (LTT) (16), also previously described as biotypes A and B, respectively (30). Based on pairing tests, STT and LTT are regarded as genetically isolated (32). The LTT is most common in 15- to 40-year-old Scots pine (*Pinus sylvestris* L.) trees in southern Scandinavia and Finland (15,31), where it causes dieback of current-year shoots in the entire crown. The STT occurs on young Scots pine trees in northern Scandinavia and at higher elevations in the south, where it causes perennial cankers on the parts of the tree covered by a lasting snow layer during the winter (19).

Besides pines, *G. abietina* also may attack Norway spruce (*Picea abies* (L.) Karst.), where the infection occurs mainly on the previous year's leader shoot and results in stem girdling and top dying (20,28). During the outbreaks in the southernmost part of Norway in the early 1980s, *G. abietina* damaged Norway

spruce trees and saplings grown as the understorey below Scots pine (2,28).

The symptoms of *Gremmeniella* spp. damage on trees (here defined as having a diameter >10 cm at breast height) differ from those on saplings (>1 m tall) and seedlings (<1 m tall). On Scots pine seedlings, damage caused by *G. abietina* can be identified readily due to the characteristic umbrella-like folding of needles on the infected leader shoot (4,22). Regarding Norway spruce seedlings, there are no reports describing *Gremmeniella* infection on seedlings while still in nursery production (≤2 years old). Barklund and Hellgren (1) explained this by a long latent period after the infection and reported that shoot dieback symptoms on seedlings usually develop after they have been planted out. The symptoms at this stage are described as being similar to shoot dieback caused by *Sirococcus conigenus* (DC.) P. F. Cannon & Minter (5).

In spring 2001, a devastating epidemic of *G. abietina* occurred on large Scots pines in southeastern Norway and in adjacent parts of Sweden (29). The autumn of 2000 had been mild and wet with a sudden onset of frost periods, and the fluctuation between mild and cold periods continued throughout the whole winter. These climatic conditions are known to be conducive to *Gremmeniella* infection (7,21). The following spring (2002), a great number of 2-year-old Norway spruce seedlings, which had wintered in nursery cold storage or had been planted out in the autumn of 2001, showed wilting top shoots and symptoms of various degrees of shoot dieback. When surveying the heavily affected plant nurseries, 1-year-old seedlings showed similar symptoms, but to a lesser extent. In the commercial evaluation of 115 thousand seedlings originating from the nursery with the greatest damage, 34% of Norway spruce seedlings had to be discarded due to shoot dieback, and 13% of the seedlings eventually died after planting.

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On damaged seedlings, two distinct types of necroses were observed on the 2001 shoots, resulting in stem girdling and shoot dieback. *G. abietina* and a *Phomopsis* sp. were recovered from the necrotic tissue. The objectives of this work were to (i) describe the disease on Norway spruce seedlings, (ii) further determine the pathogenicity of the associated *G. abietina* and *Phomopsis* sp. in vivo and in vitro, (iii) identify these strains at species or ecotype level, and (iv) develop real-time polymerase chain reaction (PCR) assays that can be used for rapid diagnosis of diseased seedlings.

MATERIALS AND METHODS

Plant material and fungal isolation. In spring and summer 2002, 2-year-old Norway spruce seedlings were collected mainly from the two most affected nurseries in southeastern Norway (Prestebakke Nursery at Kornsjø in Østfold County and Telemark Nursery at Gvarv in Telemark County). In addition, Norway spruce seedlings from nurseries that did not report on similar problems were collected for inspection (Table 1). The seedlings were collected either immediately after the cold storage or from the field after having been planted out in the previous autumn. Both diseased and healthy looking plants were collected for inspection and isolation of fungi. Plants were dissected in order to trace the initial infection court, and the length of the second-year shoot (year 2001), the length of the necrosis, and the distance from the lower edge of the necrosis to the 2000–01 stem node were measured.

Fungal isolations were made from the margins of necrotic lesions by placing host tissue pieces (2 by 2 by 3 mm) on malt agar (1.25% malt and 2% agar). After an incubation period of 3 to 5 weeks at 21°C in the dark, the fungal colonies were subcultured onto fresh media.

Pathogenicity tests. The pathogenicity test in vitro was a modification of a bioassay described by Galaaen and Venn (10). Three isolates each of *G. abietina* (2002-48/2, 2002-26/2, and 2002-47/1) and a *Phomopsis* sp. (2002-53/3, 2002-117/3, and 2002-62/1), characterized in Table 2, were tested for their ability to

cause damage on plant material. In addition, two isolates of *S. conigenus* (2000-58/2 and 2000-54/3, deposited in the fungal culture collection of the Norwegian Forest and Landscape Institute), a pathogen causing a similar shoot dieback on spruce seedlings, were included in the pathogenicity tests. Ten needles, detached from aseptically grown 5-week-old Norway spruce seedlings, were placed in front of an advancing mycelial zone growing in a petri dish with malt agar medium. Three petri dish replicates were prepared for each fungal strain tested, while needles placed on malt agar without fungus served as controls. The petri dishes were incubated in the darkness at $19 \pm 2^\circ\text{C}$. After 1, 2, 3, 4, 5, 7, and 10 days, each needle was examined under stereomicroscope and the relative tissue damage (percentage of discoloration) was estimated visually. The time required for a strain to kill 50% of the needles was determined.

To determine the pathogenicity of the isolated fungi in vivo, healthy, apparently uniform looking 1- and 2-year-old Norway spruce seedlings were inoculated with the same fungal strains as in the pathogenicity test with needles. Ten seedlings of both ages were inoculated with each strain by placing a piece of malt-agar-grown fungal mycelium ($\approx 1 \text{ mm}^3$) inside a 2-mm-long incision made with a scalpel in the bark in the middle half of the stem, and sealing the wound with parafilm. Control seedlings were treated similarly with malt agar only. Seedlings then were placed in containers and incubated in a climatic chamber, where cold storage conditions (2 to 5°C, 80% humidity, and darkness) were simulated. The extent of the necroses was measured 18 weeks later.

Outplanted symptom-bearing seedlings. In order to follow the further development of diseased seedlings, 36 diseased, 1-year-old Norway spruce seedlings from the affected Prestebakke nursery were planted out in the experimental garden of the Norwegian Forest and Landscape Institute. After outplanting in summer 2002, the seedlings were observed regularly during the following growing seasons for their condition, and for the presence of *G. abietina* and *Phomopsis* sp. fruit bodies. The experiment was ended in January 2005.

Internal transcribed spacer ribosomal DNA sequence analysis and random amplified microsatellites PCR. The *Grem-*

TABLE 1. Characteristics of Norway spruce seedlings with upper- and lower-stem necroses, and frequency of *Gremmeniella abietina* and a *Phomopsis* sp. isolated from the necroses

Isolation date ^a	Age ^b	Locality in Norway	<i>n</i> ^c	Fungi isolated (%)	
				<i>G. abietina</i>	<i>Phomopsis</i> sp.
Upper-stem necroses					
24 April 2002	A	Prestebakke, Østfold	6/6	83	0
07 May 2002	A	Prestebakke, Østfold	5/5	100	0
10 May 2002	B	Prestebakke, Østfold	5/3	66	0
23 May 2002	B	Rakkestad, Østfold	3/3	100	0
23 May 2002	B	Hokksund, Buskerud	8/4	75	0
20 June 2002	A	Granli, Hedmark	7/7	43	29
20 June 2002	A	Granli, Hedmark	5/5	20	40
20 June 2002	A	Granli, Hedmark	7/4	0	100
23 June 2002	A	Lyngdal, Vest-Agder	4/4	0	100
23 June 2002	A	Lyngdal, Vest-Agder	5/5	20	40
Lower-stem necroses					
26 April 2002	A	Biri, Oppland	4/4	0	100
30 April 2002	B	Andebu, Vestfold	5/5	0	80
08 May 2002	B	Andebu, Vestfold	14/5	0	100
23 May 2002	B	Larvik, Vestfold	10/10	0	20
21 May 2002	A	Gvarv, Telemark	3/3	0	67
31 May 2002	A	Gvarv, Telemark	5/5	0	100
23 May 2002	A	Gvarv, Telemark	5/5	0	80
29 May 2002	A	Gvarv, Telemark	22/4	0	100
29 May 2002	A	Gvarv, Telemark	24/6	0	17
03 June 2002	B	Gvarv, Telemark	3/3	0	0
10 July 2002	B	Nøss, Oppland	5/5	0	20
16 December 2002	A	Alstahaug, Nordland	17/11	0	100

^a Seedlings with upper- and lower-stem necroses.

^b A = 2-year-old seedlings from cold storage and B = 2-year-old seedlings, planted out.

^c Total number of seedlings examined/number of seedlings from which isolation was made.

meniella and *Phomopsis* isolates processed for molecular characterization are shown in Table 2. In addition, we sequenced the following four *Diaporthe* (teleomorph genus of *Phomopsis*) and *Phomopsis* strains isolated from conifers and deposited at the Centraalbureau voor Schimmelcultures (CBS): *Diaporthe conorum* (CBS186-37), *D. conorum* (CBS 199-39), *Phomopsis conorum* (CBS 587-79), and *P. juniperivora* (CBS 186-27). To obtain mycelia for DNA isolation, the fungal strains were grown on cellophane-coated malt (1.25% malt and 2% agar) and V8 juice agar (20% V8 and 2% agar) (Campbell Grocery Products LTD, Norfolk, UK), and the mycelia harvested were ground with a pestle in liquid N₂-chilled mortars. DNA isolation was performed using the Plant DNA Mini Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

For internal transcribed spacer (ITS) ribosomal (r)DNA sequence analysis, 30 cycles (annealing temperature 57°C) of PCR amplification were carried out with a 200-nM concentration of the ITS1 and ITS4 primers (33) in 50-μl reaction volumes using HotStarTaq DNA Polymerase according to the manufacturer's instructions (Qiagen). After gel electrophoresis, the PCR products were purified with QIAquick Gel Extraction Kit and QIAquick PCR purification Kit (Qiagen), ligated into a cloning vector (pDrive Cloning vector of Qiagen PCR Cloning^{plus} Kit), and transformed into Qiagen EZ Competent Cells. For sequencing, plasmid DNA was isolated and purified using QIAprep Miniprep (Qiagen). Three distinct colonies were sequenced with -21 M13 and M13 reverse primers in both directions on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) for each isolate. The alignments of complementary strands and the similarity studies within sequenced samples were determined with the Seqman software (Lasergene, DNASTAR Inc., Madison, WI). Similarities to known ITS regions were performed with the BLAST search engines of the National Center for Biotechnology Information (NCBI) GenBank (Bethesda, MD) and multiple se-

quence alignments were performed with Clustal W of Kyoto University Informatics Center, Japan.

The random amplified microsatellite (RAMS) technique was used to further characterize the *Gremmeniella* and *Phomopsis* isolates. The PCR reactions were carried out in the reaction conditions recommended by the manufacturer of the HotStarTaq DNA Polymerase using 2 μM concentrations of the degenerate ACA and CCA primers described by Hantula and Müller (14). The PCR cycling parameters also were as described in that study. Amplification products were separated by gel electrophoresis in 1.5% agarose gels using Tris-acetate-EDTA running buffer and visualized under UV light after ethidium bromide staining.

Quantification of host and pathogen by real-time PCR. To allow rapid and high throughput detection of *G. abietina* and *Phomopsis* sp. in diseased seedlings, we developed real-time PCR assays for detecting and quantifying their DNA. The assays were used for analyzing diseased Norway spruce seedlings sent from the forest nurseries during the summers of 2003 and 2004. Stem sections (5 mm long) were taken from the middle and the margin of necrotic stem lesions and from the neighboring healthy tissue. DNA isolation was performed using Plant DNA Mini Isolation Kit (Qiagen) according to the manufacturer's instructions, and using a 50-μl elution volume.

The real-time PCR primers used for monitoring *G. abietina* and *Phomopsis* sp. colonization in infected seedlings were designed with the Primer Express software 1.5a provided with Applied Biosystems real-time quantitative PCR systems (Applied Biosystems) by employing conserved and species-specific sequence areas in the ITS rDNA gene cluster. The forward primer 5'-CACCGTGCCCTATATCTCTGTTG-3', reverse primer 5'-TATAGTACTCAGACGGTAAATAATTCATAGGTT-3', and a VIC-labeled probe 5'-TTCCCGGGCCTCAACCCCC-3' were selected for real-time PCR quantification of *G. abietina*. For quantification of the *Phomopsis* sp., the forward primer 5'-GCACCCAG-

TABLE 2. *Gremmeniella abietina* and *Phomopsis* strains processed for molecular characterization

Species	Strain ^a	Accession no. ^b	Host	Age ^c	Locality in Norway	Note ^d
<i>G. abietina</i>	1974-46/1	Ns	<i>Pinus sylvestris</i>	Sapling	Malmmyra, Rendalen, Hedmark	STT
<i>G. abietina</i>	1975-32/1	DQ445885	<i>P. sylvestris</i>	Sapling	Folldal verk, Folldal, Hedmark	STT
<i>G. abietina</i>	2002-20/4	Ns	<i>P. sylvestris</i>	Sapling	Øyerberget, Kongsvinger, Hedmark	LTT
<i>G. abietina</i>	2002-47/1	DQ116556	<i>P. sylvestris</i>	Tree	Kongsvinger, Hedmark	LTT
<i>G. abietina</i>	1985-393/16/1	DQ445884	<i>P. contorta</i>	Sapling	Matrand, Eidskog, Hedmark	LTT
<i>G. abietina</i>	1985-393/19/1	Ns	<i>P. contorta</i>	Sapling	Matrand, Eidskog, Hedmark	LTT
<i>G. abietina</i>	1988-306/1	Ns	<i>P. contorta</i>	Sapling	Malmmyra, Rendalen, Hedmark	STT
<i>G. abietina</i>	1988-307/3	DQ445883	<i>P. contorta</i>	Sapling	Malmmyra, Rendalen, Hedmark	STT
<i>G. abietina</i>	1966-163/2	Ns	<i>P. abies</i>	Sapling	Holmegil, Aremark, Østfold	LTT
<i>G. abietina</i>	1985-111/6	Ns	<i>P. abies</i>	Sapling	Viblemo, Audnedal, Vest-Agder	LTT
<i>G. abietina</i>	2002-107/2	DQ445882	<i>P. abies</i>	Seedling	Granli nursery, Granli, Hedmark	LTT
<i>G. abietina</i>	2002-109/4	DQ445881	<i>P. abies</i>	Seedling	Granli nursery, Granli, Hedmark	LTT
<i>G. abietina</i>	2002-124/1	Ns	<i>P. abies</i>	Seedling	Lyngdal nursery, Lyngdal, Vest-Agder	LTT
<i>G. abietina</i>	2002-26/2	DQ116557	<i>P. abies</i>	Seedling	Prestebakke nursery, Kornsjø, Østfold	LTT
<i>G. abietina</i>	2002-29/3	DQ116559	<i>P. abies</i>	Seedling	Prestebakke nursery, Kornsjø, Østfold	LTT
<i>G. abietina</i>	2002-4/4	DQ445880	<i>P. abies</i>	Seedling	Prestebakke nursery, Kornsjø, Østfold	LTT
<i>G. abietina</i>	2002-48/2	DQ116558	<i>P. abies</i>	Seedling	Rakkestad, Østfold	LTT
<i>G. abietina</i>	2002-79/2	Ns	<i>P. abies</i>	Seedling	Vestfold	LTT
<i>Phomopsis</i> sp.	2002-18/1	DQ445887	<i>P. abies</i>	Seedling	Andebu, Vestfold	...
<i>Phomopsis</i> sp.	2002-34/3	Ns	<i>P. abies</i>	Seedling	Andebu, Vestfold	...
<i>Phomopsis</i> sp.	2002-51/3	Ns	<i>P. abies</i>	Seedling	Brunlanes, Larvik, Vestfold	...
<i>Phomopsis</i> sp.	2002-53/3	DQ445888	<i>P. abies</i>	Seedling	Brunlanes, Larvik, Vestfold	...
<i>Phomopsis</i> sp.	2002-62/1	DQ116550	<i>P. abies</i>	Seedling	Telemark nursery, Gvarv, Telemark	...
<i>Phomopsis</i> sp.	2002-73/3	Ns	<i>P. abies</i>	Seedling	Telemark nursery, Gvarv, Telemark	...
<i>Phomopsis</i> sp.	2002-105/4	Ns	<i>P. abies</i>	Seedling	Granli nursery, Granli, Hedmark	...
<i>Phomopsis</i> sp.	2002-113/2	DQ445886	<i>P. abies</i>	Seedling	Granli nursery, Granli, Hedmark	...
<i>Phomopsis</i> sp.	2002-117/3	DQ445890	<i>P. abies</i>	Seedling	Granli nursery, Granli, Hedmark	...
<i>Phomopsis</i> sp.	2002-120/1	DQ445889	<i>P. abies</i>	Seedling	Lyngdal nursery, Lyngdal, Vest-Agder	...
<i>Phomopsis</i> sp.	2002-131/2	Ns	<i>P. abies</i>	Seedling	Næss, Nordre Land, Oppland	...
<i>Phomopsis</i> sp.	2002-132/3	Ns	<i>P. abies</i>	Seedling	Næss, Nordre Land, Oppland	...

^a All strains are deposited at the culture collection of Norwegian Forest Research Institute and are available at request.

^b National Center for Biotechnology Information GenBank Sequence Database accession numbers; Ns = not sequenced.

^c Seedlings <1 m tall, saplings >1 m tall, and tree >10 cm in diameter at breast height.

^d STT = small-tree-type reference and LTT = large-tree-type reference.

AAACCCTTTGTG-3', reverse primer 5'-AAGAGTTGACTTG-GCCGCC-3', and a VIC-labeled probe 5'-CGGTAACGAGGA-GCAGCCCGC-3' were selected.

Co-precipitation of compounds that inhibit PCR confounds molecular analyses of environmental samples by producing false negative results (34). To eliminate false negatives, Norway spruce DNA was used as an endogenous reference in analyzed samples from infected nursery seedlings, and estimated by using the polyubiquitin primer/probe set previously described (17). To investigate the specificity of the primer/probe sets used, the following ascomycetes, related to *G. abietina* or the *Phomopsis* sp. and commonly associated with shoots of Norway spruce, *S. conigenus* (strains 2000-54/3 and 2000-58/2), *Botrytis cinerea* (strain 1983-145/1), *Herpotrichia juniperi* (strain 1973-53), *Rhizosphaera kalkhoffii* (strain 1997-1/6), *Epicoccum nigrum* (strain 1959-1898/1/1), *Nectria fuckeliana* (strain 197-74), and *Tiarosporrella parca* (strain 1991-727/28) were processed as described above for the *Gremmeniella* and *Phomopsis* strains. In addition, detection of *D. conorum* (CBS186-37), *D. conorum* (CBS 199-39), *P. conorum* (CBS 587-79), and *P. juniperivora* (CBS 186-27) DNA by the *Phomopsis* primer/probe set was examined. The real-time PCR detection of DNA of Norway spruce, *G. abietina*, and the *Phomopsis* sp. was performed with TaqMan Universal PCR Master Mix (P/N 4304437; Applied Biosystems). Primer and probe concentrations of 150 and 330 nM, respectively, were used for detecting DNA of Norway spruce (17). To optimize primer/probe concentrations for quantitative detection of *G. abietina* and the *Phomopsis* sp., several primer and probe concentrations were tested by using the corresponding standard curve samples (described below) as templates. For both fungi, a primer concentration of 300 nM and a probe concentration of 400 nM were chosen because they showed a stable amplification efficiency over the concentration range used in the corresponding standard curve. All PCR reactions were performed under singleplex conditions.

To construct standard curves for quantification of Norway spruce and pathogen DNA, DNA isolated from a healthy control seedling and from pure cultures of *G. abietina* and the *Phomopsis* sp. were quantified by using a Versafluor fluorometer (Bio-Rad, Hercules, CA) and a PicoGreen DNA quantification kit (Molecular Probes, Eugene, OR), and then pooled together in known concentrations. The host DNA standard curve samples (100,000, 10,000, 1,000, and 100 pg of Norway spruce DNA) all were spiked with 1,000 pg of *G. abietina* and 1,000 pg of the *Phomopsis* sp. DNA. The *Gremmeniella* DNA standard curve samples (1,000, 100, 10, 1, and 0.1 pg of *G. abietina* DNA) all were spiked with 1,000 pg of Norway spruce and 1,000 pg of *Phomopsis* sp. DNA. The *Phomopsis* DNA standard curve samples (1,000, 100, 10, 1, and 0.1 pg of *Phomopsis* sp. DNA) all were spiked with 1,000 pg of Norway spruce and 1,000 pg of *G. abietina* DNA. To ensure that the signals from the experimental samples fell within the standard curves, and to investigate the presence of compounds inhibitory to PCR, 4-log dilution series were prepared for experimental samples. As a template, 3 µl of the DNA solution for both experimental and standard curve samples were used for each 25-µl PCR reaction. Each reaction was repeated twice. PCR cycling parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence emissions were detected with an ABI Prism 7700 (Applied Biosystems). Data acquisition and analysis were performed with the Sequence Detection System software package (1.7a; Applied Biosystems). The amounts of Norway spruce and fungal DNA in the samples were calculated from the respective standard curves by using critical threshold (Ct)-based calculation procedures. Genomic DNA from healthy Norway spruce and from included reference fungi was used as a negative control for the fungal primer/probe sets, while fungal DNA served as negative controls for the host primer/probe set. To further test the function of the *Gremmeniella* and

Phomopsis primer/probe sets, we spiked DNA obtained from selected seedlings from which *Gremmeniella* or *Phomopsis* spp. had been isolated or detected by real-time PCR. The former was spiked with 500 pg of the *Phomopsis* sp. DNA, whereas the latter was spiked with 500 pg of *G. abietina* DNA, and the ability of the primer/probe sets to detect and quantify the added DNA was determined.

Statistical analysis. The data for necrosis length on 1- and 2-year-old Norway spruce seedlings in the *in vivo* pathogenicity test were subjected to analysis of variance (ANOVA) by using one-way ANOVA (JMP; SAS institute, Cary, NC). For comparison of all pairs the Tukey-Kramer highly significant difference test was applied ($P < 0.0002$).

RESULTS

Description of disease symptoms. The symptoms on Norway spruce seedlings became visible during the spring of 2002, 1 year after the *Gremmeniella* epidemic on Scots pine trees. Both 1- and 2-year-old plants showed symptoms of desiccated leader shoots and had necrotic lesions on the stems of shoots from 2001 (Fig. 1A). Often, the seedlings appeared healthy and the necroses became visible only after the stem bark had been peeled off (Fig. 1B and C). The first visible signs of a stem lesion were a local indentation in the bark and grayish-green foliage on the lesion area. Later, the foliage and branches distal to the lesion area became yellow and brown. Some lesions were located only on one side of the stem, whereas others ringed the whole stem, causing dieback of the shoot. Occasionally, there were two to three separate necroses on one stem. Generally, two types of necroses, upper- and lower-stem necroses, could be distinguished.

Upper-stem necroses associated with *G. abietina*. Mean 2001 shoot length on 2-year-old seedlings with this type of necrosis was 25 cm. Necroses on the upper stem were located a mean distance of 14.9 cm above the 2000–01 stem node and their average length was 4.3 cm. The necrotic, dark-brown-colored bark was profusely impregnated with resin (Fig. 1A and C). In this area, the stem usually was girdled, the nearby needles were brown at the base, and the shoots above the necrosis were dead or dying. The edges of the necroses were sharp and distinct. In most cases, *G. abietina* was isolated from the advancing edge of the necrotic tissue (Table 1). *G. abietina* alone was isolated predominantly from seedlings sampled during April and May. In isolations performed later (June and later), a *Phomopsis* sp. occasionally was recovered from this type of necrosis (Table 1). These two fungi were isolated from the same batch of plants and the same type of necrosis, but never together from the same plant or the same necrosis. No other potentially pathogenic fungi were isolated from the upper-stem necroses. Most of the seedlings with upper-stem necroses yielding *G. abietina* originated from Prestebakke Nursery, where Scots pine trees were in the close vicinity of the nursery area.

Lower-stem necroses associated with a *Phomopsis* sp. Mean 2001 shoot length on 2-year-old plants with this type of necrosis was 21 cm. The mean distance from the lower edge of the necroses to the 2000–01 stem node was 3.9 cm. These necroses often were located at the base of the 2001 shoot or partly at the end of the 2000 shoot. Necroses on the lower stem were lighter in color compared with the upper-stem necroses, and had a characteristic water-soaked appearance without any resin flow (Fig. 1D). The edges of the necroses were diffuse and nondistinct. Occasionally, such necroses also were found on the upper part of the 2001 shoot. The most frequently isolated fungus from these lesions was a *Phomopsis* sp., which was recovered in the period from April to December (Table 1). Apart from two cases where a *Botrytis* sp. was recovered, no other potentially pathogenic fungi were isolated from these necroses. Fruit bodies of the *Phomopsis* sp. developed readily on plants after storage at +4°C. Seedlings

with lower-stem necroses originated mostly from Telemark Nursery, where there were no Scots pine trees in the immediate vicinity.

Pathogenicity tests. In the pathogenicity test in vitro with needles (Fig. 2), *G. abietina* strains killed 50% of the needle tissue within 4 to 6 days, strain 2002-48/2 being the most aggressive. One of the two included isolates of *S. conigenus* (strain 2000-58/2) caused 50% needle damage after 7 days of incubation, whereas the other *S. conigenus* (strain 2000-54/3) caused the same extent of damage after 9 days. Two *Phomopsis* strains

(2002-62/1 and 2002-117/3) showed 50% damage after 8 days. The *Phomopsis* strain 2002-53/3 showed no signs of pathogenicity 10 days after the incubation, when the experiment was ended. In contrast to the necrotic spots and shrivelling of the needles caused by *G. abietina*, needles overgrown by the *Phomopsis* sp. showed a uniform discoloration, but remained turgid, suggesting a different mode of interaction with needles than that by *G. abietina*.

In the pathogenicity test in vivo, seedlings were stored in climatic chambers for 18 weeks in the period from mid-November to the end of March. In 1-year-old seedlings, *G. abietina* strains

Fig. 1. Symptoms of top dieback in Norway spruce seedlings associated with *Gremmeniella abietina* and a *Phomopsis* sp. **A**, Typical symptom of dry leader on 2-year-old seedlings after planting out; *G. abietina* was isolated from the resinous necrosis (**A**, detail below). **B**, Seedling appeared to be healthy but, when bark was peeled off, extensive necrosis in the leader was revealed. **C**, Note also the browning of needles from the bases (arrows). **D**, Light-colored and water-soaked necroses from which the *Phomopsis* sp. was isolated usually were located close to the stem node.

Fig. 2. Pathogenicity test in vitro. Discoloration of aseptic spruce needles incubated together with cultures of *Gremmeniella abietina* (G1–G3), a *Phomopsis* sp. (P1–P3), and *Sirococcus conigenus* (S1–S2), all isolated from Norway spruce seedlings with top dieback symptoms. Pathogenicity of fungal isolates was estimated as the time needed for the fungus to kill 50% of the needles.

2002-48/2 and 2002-26/2 caused significantly longer necroses than the other strains (Fig. 3). The necroses produced by the other strains were not significantly different from the control. In 2-year-old seedlings, the longest necroses were caused by *G. abietina* strains 2002-26/2 and 2002-48/2, but only *G. abietina* strain 2002-26/2 differed significantly from the control (Fig. 3).

Symptom-bearing seedlings in outplanted plots. In spring 2003, at the time of the first assessment, 23% of the seedlings (8 of 36 seedlings) had a tendency to develop a double shoot (i.e., two side shoots were competing for dominance). At this time, four dead shoots had pycnidia of *Brunchorstia pinea* (P. Karst.) Höhn., the anamorph stage of *G. abietina*, with conidia still present. In January 2005, at the time of final harvesting, 64% of the seedlings (9 of 14 seedlings) had developed a double stem (unfortunately, 22 seedlings were destroyed by accident before the last evaluation; thus, only the 14 remaining seedlings were inspected at the end of the experiment). The seedlings were still alive, and showed good growth. The originally diseased leader shoots had been taken over by a new leader. Of the 14 dead shoots collected at the last inspection, 4 had old, empty pycnidia still present, whereas 10 had only visible scars after pycnidia. No apothecia were observed in any seedling.

Molecular characterization of isolates of *G. abietina* and the *Phomopsis* sp. The ITS rDNA sequences obtained from *G. abietina* isolates from Norway spruce seedlings, deposited as GenBank accession nos. DQ116556 to DQ116558 and DQ445881 to DQ445885, were identical to each other (not shown). In BLAST analysis against the NCBI GenBank Sequence Databases, these sequences showed 100% sequence identity to the European race of *G. abietina*. The ITS rDNA sequences of the included reference strains of LTT and STT were indistinguishable from each other and from those obtained from Norway spruce seedlings, indicating that the ITS rDNA sequence cannot be used to distinguish between the ecotypes. The RAMS-ACA and RAMS-

CCA banding patterns were identical among the *G. abietina* isolates from Norway spruce seedlings, whereas the included reference strains of LTT and STT showed type-specific banding patterns (Fig. 4A and B). The assay confirmed that all the *G. abietina* isolates from Norway spruce seedlings belonged to the LTT, because their banding patterns were identical to those from the reference strains of the type. For example, with the ACA primer, the STT reference strains produced a band of approximately 0.7 kb, which was absent in the LTT reference strains and in the strains from the seedlings. With the CCA primer, only the LTT reference strains and the strains from the seedlings had a 1.5-kb band.

The isolates of the *Phomopsis* sp. originating from diseased Norway spruce seedlings showed RAMS-ACA and RAMS-CCA banding patterns that were identical among the isolates (data not shown), and sequencing of the strains (DQ116550 and DQ445886 to DQ445890) revealed 99 to 100% sequence similarity to each other in the ITS1 and ITS2 regions. The sequenced isolates of the *Phomopsis* sp. from nursery-grown Norway spruce seedlings and the included reference strains of *P. conorum*, *P. juniperivora*, and *Diaporthe conorum* (anamorph *P. occulta*) differed in the ITS1 and ITS2 regions. The two strains of *D. conorum* (DQ116551 and DQ116552) from the CBS culture collection showed 90% sequence similarity to each other and 91% sequence similarity to

Fig. 3. Length of necroses in 1- and 2-year-old Norway spruce seedlings 18 weeks after inoculation with different isolates of *Gremmeniella abietina* (G1–G3), a *Phomopsis* sp. (P1–P3), *Sirococcus conigenus* (S1–S2), and the control (C). Different letters indicate significant differences between treatments (Tukey's highly significant difference test, $P < 0.0001$ and 0.0002 for 1- and 2-year-old seedlings, respectively; $n = 180$).

Fig. 4. Random amplified microsatellite (RAMS) patterns obtained from small-tree-type (STT) and large-tree-type (LTT) *Gremmeniella abietina* using the A, ACA and B, CCA primer sets. Obtained RAMS patterns of three STT (1988-306/1, 1988-307/3, and 1974-46/1) *G. abietina* reference strains, five LTT (2002-20/4, 2002-47/1, 1985-111/6, 1985-393/16/1, and 1966-163/2) *G. abietina* reference strains, and four isolates (2002-4/4, 2002-79/2, 2002-107/2, and 2002-124/1) obtained from diseased Norway spruce seedlings from Norwegian nurseries (NS) with *Gremmeniella* problems. Lane M: DNA size marker (GeneRuler DNA ladder mix).

the isolates of the *Phomopsis* sp. from Norway spruce seedlings. *P. conorum* (DQ116553) and *P. juniperivora* (DQ116555) showed 92 and 70% ITS sequence similarity, respectively, to our isolates of the *Phomopsis* sp. from Norway spruce seedlings over the region sequenced. In BLAST against the NCBI GenBank Sequence Databases, a previously sequenced *D. phaseolorum* (anamorph *P. phaseoli*), GenBank accession no. AF001027, isolated from diseased soybean, showed the highest ITS sequence similarity (95%) to the isolates of the *Phomopsis* sp. recovered from Norway spruce seedlings.

Real-time PCR. The primer set developed for monitoring Norway spruce did not detect DNA of *G. abietina* and the *Phomopsis* sp. and the primer/probe sets used for detecting DNA of these fungi did not detect DNA of Norway spruce. In addition, these two sets did not detect DNA of *S. conigenus*, *B. cinerea*, *H. juniperi*, *R. kalkhoffii*, *E. nigrum*, *N. fockeliana*, and *T. parca* when DNA (1,000 pg) from these references was used as the template. Excluding *P. juniperivora*, the reference strains of *D. conorum* and *P. conorum* could be amplified in a SYBR Green assay with the primers for detecting the *Phomopsis* sp. However, none of these were detected when the probe-based real-time PCR assay we are reporting here was used. In the standard curve samples constructed for Norway spruce (Norway spruce DNA spiked with *G. abietina* and *Phomopsis* sp. DNA), there was a linear relationship between the Ct values and the logarithm of the amount of spruce DNA in the range of 100,000 to 100 pg of host DNA. In the standard curve for samples constructed for *G. abietina* (*Gremmeniella* DNA spiked with Norway spruce and *Phomopsis* DNA) and the *Phomopsis* sp. (*Phomopsis* DNA spiked with Norway spruce and *Gremmeniella* DNA), there was a linear relationship between the Ct values and the logarithm of the amount of DNA in the range of 1,000 to 0.1 pg of pathogen DNA, showing that the assays can be used for quantitative monitoring of the fungi in a broad range of colonization. The standard curves were constructed based on the relationship of Ct values (x) and the amount of template (y) generated from known host and pathogen DNA concentrations: Norway spruce, *G. abietina*, and the *Phomopsis* sp. DNA amount standard curves were $\log y = 11.45 - 0.280x$ ($R^2 = 0.999$, $P < 0.0001$), $\log y = 8.74 - 0.280x$ ($R^2 = 0.999$, $P < 0.0001$), and $\log y = 8.47 - 0.272x$ ($R^2 = 0.999$, $P < 0.0001$), respectively (Fig. 5).

Approximately 100 Norway spruce seedlings with necrotic stem lesions, collected in the years 2003 and 2004 from the nurseries in southeastern Norway with disease problems in 2002, were screened for the presence of *G. abietina* and a *Phomopsis* sp. The differences in the host and pathogen Ct values between 10- and 100-fold diluted DNA samples from the seedlings were in the ranges (3.3 to 3.6) observed in the corresponding log-diluted standard curves as well (data not shown). This indicates that the diluted DNA samples from seedlings did not contain any substances inhibitory to PCR amplification, and that the assays were not saturated with the template levels present in the diluted DNA samples from seedlings. Thus, the Ct values from 10-fold-diluted DNA samples were used to calculate the amount of host and pathogen DNA in the seedlings. More than 80% of the seedlings with necrotic stem lesions hosted a *Phomopsis* sp., whereas DNA of *G. abietina* was detected in only one seedling. In both cases, the host DNA yield was low in the samples taken from the middle and from the margin of a necrotic lesion, whereas samples taken outside the lesions had host DNA yields comparable with the healthy control stems (Table 3). For the *Phomopsis* sp., the pathogen/host DNA amount ratio showed a gradient within the lesion, the highest levels of the pathogen colonization being in the middle of the lesion. We also spiked DNA obtained from selected infected and healthy seedlings to further test the function of the assays. Close to 100% of the added *G. abietina* or *Phomopsis* sp. DNA was detected in all samples that were diluted similarly (10-fold) to the experimental samples (Table 3).

DISCUSSION

Most of the literature on nurseries reports *G. abietina* exclusively as a pathogen on pine seedlings and, if associated with Norway spruce, *G. abietina* is mentioned as a pathogen on sap-

Fig. 5. Standard curves for quantifying the DNA amount of Norway spruce, *Gremmeniella abietina*, and a *Phomopsis* sp. Cycle threshold values were plotted against log₁₀-transformed DNA amounts, and linear regression equations were calculated for the quantification of DNA pools by interpolation in unknown samples. **A**, The host DNA standard samples (100,000, 10,000, 1,000, and 100 pg of Norway spruce DNA) all were spiked with 1,000 pg of *G. abietina* and 1,000 pg of the *Phomopsis* sp. DNA. **B**, The *G. abietina* DNA standard curve samples (1,000, 100, 10, 1, and 0.1 pg of *G. abietina* DNA) all were spiked with 1,000 pg of Norway spruce and 1,000 pg of *Phomopsis* sp. DNA. **C**, The *Phomopsis* DNA standard curve samples (1,000, 100, 10, 1, and 0.1 pg of *Phomopsis* sp. DNA) all were spiked with 1,000 pg of Norway spruce and 1,000 pg of *G. abietina* DNA. Three polymerase chain reaction replicates were run on each sample.

lings (18) and on larger seedlings in plantations (27). In pine seedlings, the disease is easily recognized by the characteristic umbrella-like folding of needles on the leader shoot (4,22), whereas the symptoms of *G. abietina* infection in Norway spruce seedlings (necroses and shoot dieback) are rather nonspecific and may resemble symptoms caused by other pathogens (such as *S. conigenus*) as well as abiotic stresses, such as frost, drought, or cold storage. Because multiple factors can cause these symptoms in Norway spruce seedlings, the incidents of *G. abietina* infection easily may be misidentified, especially during nonepidemic periods when the infection origin is less obvious.

The inoculation tests performed with the strains examined here resulted in necroses and confirmed the pathogenicity of the fungus on Norway spruce seedlings. By simulating the bark fissure infection in the pathogenicity tests carried out with seedlings, we reproduced bark necroses by *G. abietina* inoculation. The symptoms of shoot dieback were not reproduced due to the short duration of our in vivo experiment, caused by a technical problem. In the Norway spruce nurseries affected by *G. abietina*, stem necroses became visible after wintering or planting out in 2002, a year after the infection. The timing of symptom appearance agrees with observations of Barklund and Unestam (3), who reported that, in artificially inoculated Norway spruce seedlings, a latent stage occurs in summer and fall, during which the fungus colonizes only the outer stem tissues. During the winter, the fungus actively colonizes the inner stem tissues and the symptoms develop during the following spring or summer (3). Due to this long latent stage, seedlings infected by *G. abietina* can be planted out before the symptoms develop in the nursery. Our conclusion is that the incidence of *G. abietina* on nursery-grown Norway spruce seedlings probably is not new but simply has been overlooked due to the long latent development, nonspecific symptoms, and sparse occurrence during nonepidemic periods.

Hamelin and Rail (12) analyzed the ITS sequence of a number of *G. abietina* var. *abietina* strains obtained from different pine

species and showed that the ITS sequences were identical for all members of the European race, and that the European race differs from the North American race of *G. abietina* var. *abietina* at two nucleotides in this region. In the present study, ITS rDNA sequence analysis of the *G. abietina* strains isolated from necroses formed on nursery-grown Norway spruce seedlings indicated that the strains belonged to the European race of this *G. abietina* variety, because their ITS sequences were identical to those of the European-race strains reported by Hamelin and Rail (12).

To further characterize the *G. abietina* strains associated with Norway spruce seedlings, we employed RAMS analysis, a technique that provides ecotype-specific banding patterns (14). The RAMS banding patterns obtained from *G. abietina* strains isolated from Norway spruce seedlings were identical to each other and to those from the included reference strains representing the LTT, but differed from those of the STT. These data confirmed that the strains associated with nursery-grown Norway spruce seedlings belonged to the LTT of the European race. The presently studied nursery samples were collected from the geographical area in southeastern Norway where a devastating epidemic of *G. abietina* had occurred on Scots pine trees in the previous year. This epidemic was a typical LTT outbreak, characterized by dieback of shoots in the entire crown (29). All of the isolates of *G. abietina* from diseased nursery seedlings of Norway spruce were grouped in the LTT, suggesting that the unusual disease outburst on Norway spruce seedlings in 2002 likely was related to the previous year's epidemic on Scots pines. Apparently, similar damage in Norway spruce seedlings after the pine epidemic also was observed in Sweden (E. Stenström, *personal communication*) and in Finland (24).

G. abietina attack on spruce seedlings can be difficult to identify (no fruit bodies present, nonspecific disease symptoms, and causal pathogen often may be dead or inactive at the time of identification); therefore, we developed real-time PCR assays for detecting its presence and for monitoring the development of the

TABLE 3. Amount of Norway spruce, *Gremmeniella abietina*, and a *Phomopsis* sp. DNA in representative diseased (D) and healthy (H) seedlings sampled from *Gremmeniella*-affected nurseries during the years 2003 to 2004^a

Seedling no.	Position ^b	DNA yield (ng/μl)	DNA amount ratio (%)		Recovery of DNA added (%)		Condition	Nursery
			<i>Gremmeniella</i> /host	<i>Phomopsis</i> /host	<i>G. abietina</i> ^c	<i>Phomopsis</i> sp. ^c		
6	Middle	4	0.29	0	No DNA added	Missing data	D	Gvarv
	Margin	8	2	0	No DNA added	99.7	...	...
	Outside	130	0	0	No DNA added	104.5	...	...
38	Middle	0.5	0	0.53	99.9	No DNA added	D	Gvarv
	Margin	22	0	0.41	100.8	No DNA added	...	...
	Outside	160	0	0	95.2	No DNA added	...	...
39	Middle	4	0	0.40	105.2	No DNA added	D	Gvarv
	Margin	26	0	0.14	99.9	No DNA added	...	...
	Outside	125	0	0	98.3	No DNA added	...	...
55 ^d	Middle	9	0	0.47	125.5	No DNA added	D	Prestebakke
	Margin	21	0	0.13	105.5	No DNA added	...	...
	Outside	120	0	0	98.0	No DNA added	...	...
57 ^d	Middle	20	0	0.34	99.2	No DNA added	D	Prestebakke
	Margin	25	0	0.1	96.4	No DNA added	...	...
	Outside	190	0	0	92.7	No DNA added	...	...
53	Middle	125	0	0	95.8	106.4	H	Prestebakke
	Margin	97	0	0	94.2	112.7	...	...
	Outside	153	0	0	94.2	104.9	...	...
43	Middle	123	0	0	95.8	100.4	H	Gvarv
	Margin	148	0	0	93.3	102.3	...	...
	Outside	70	0	0	96.7	106.4	...	...

^a For real-time polymerase chain reaction (PCR), 10-fold diluted DNA samples were used. To further test the functionality of the real-time PCR assays, a fixed amount of *G. abietina* or *Phomopsis* sp. DNA was added to selected samples.

^b Position within lesion: 5-mm-long samples from diseased seedlings were taken from the middle, margin, and outside of a necrotic stem lesion. For healthy seedlings, the 5-mm-long samples were taken next to each other.

^c *G. abietina* or *Phomopsis* sp. DNA (500 pg in a volume of 1 μl) of was added to a 3-μl volume of undiluted DNA stock solution from the seedlings. The amount of the added DNA was quantified by real-time PCR in 10-fold diluted samples by using a 4-μl-volume template. The recovery was estimated from the obtained critical threshold values by using the standard curve equation generated for the corresponding pathogen. A value of 100% indicates that 50 pg of the added DNA was detected in the 10-fold diluted sample.

^d The presence of a *Phomopsis* sp. within the lesion also was confirmed by fungal isolation.

disease in the affected nurseries after the problematic year 2002. Specificity of assays used for quantifying a pathogen under field conditions is crucial, so that coexisting microbes can be excluded. The primer/probe sets used here for monitoring *G. abietina* and a *Phomopsis* sp. did not detect the included reference ascomycetes, species that can be commonly associated with diseased shoots of Norway spruce seedlings. The different species and varieties of *Gremmeniella* are very similar but not identical in the ITS regions. In the annealing sites of the forward primer, probe, and reverse primer used here for monitoring *G. abietina*, var. *abietina* differs from var. *balsamea* in one, one, and seven nucleotides, respectively (data not shown). *G. abietina* var. *abietina* is the only *Gremmeniella* sp. or *G. abietina* variety occurring on spruce in Europe (26). However, should the developed primer/probe set be used for monitoring *G. abietina* var. *abietina* in hosts or areas where several varieties or species of *Gremmeniella* can coexist, further specificity tests need to be performed. For example, both *G. abietina* var. *abietina* and *G. laricina* can infect *Larix* spp., whereas *G. abietina* var. *balsamea*, the variety known only from Quebec, Canada, can infect *Picea glauca* and *P. mariana* in addition to *A. balsamea* (25). The high specificity of the *Phomopsis* primer/probe used was confirmed, because the assay did not detect *D. conorum* (anamorph *Phomopsis occulta*) and *P. conorum*, species closely related to the investigated *Phomopsis* sp. but also commonly present in diseased shoots of Norway spruce (6,8).

Although a *Phomopsis* sp. commonly was detected in necrotic lesions of seedlings collected during the years 2003 and 2004 (2 and 3 years after the *G. abietina* epidemic, respectively), *G. abietina* was detected in only 1 of the 100 seedlings examined. The good recovery of the added *G. abietina* and *Phomopsis* DNA from DNA obtained from infected seedlings in the spiking test indicates that the monitoring data obtained from the sampled seedlings were reliable. Therefore, the absence of *G. abietina* in the real-time PCR screening indicates a rapid decline in the *G. abietina* population in the nurseries after the epidemic outbreak in 2001, which is in agreement with the absence of similar epidemics in the nearby forests during the two monitored years. That Scots pine forests near the nurseries were the most possible source of inoculum, a fact reflected in the observation that *G. abietina* was recovered most often from seedlings originating from the nursery having large pines in the vicinity, whereas isolations from seedlings originating from nurseries with no pines nearby resulted in no *G. abietina* being found.

In addition to *G. abietina*, a *Phomopsis* sp. frequently was associated with the shoot dieback or stem necrosis symptoms in the Norway spruce seedlings examined. However, whereas *G. abietina* strains were recovered from cankers formed on the upper part of the stem, *Phomopsis* sp. was obtained primarily from cankers formed on the lower part of the stem. Compatible with our observations, Hansen and Hamm (13) report on *Phomopsis* spp. associated with top-kill symptoms of Douglas fir seedlings, where necroses were formed at the base of new shoots. They suggested that the infection takes place during the summer, possibly through the bud scales. In addition to location, the appearance of necroses associated with *G. abietina* and the *Phomopsis* sp. also differed. Resin flow, a characteristic conifer response upon pathogen attack, commonly was observed in necroses hosting *G. abietina*, whereas *Phomopsis* sp.-associated necroses were water soaked and without any resin flow. Moreover, necrotic spots and shriveling of needle tissue was characteristic on needles infected by *G. abietina* in the needle bioassay, whereas needles overgrown by the *Phomopsis* sp. showed a uniform discoloration, with needles remaining turgid. These data suggest that the fungi differ in their mode of interaction with the host tissue.

Based on the ITS rDNA sequence analysis performed, the *Phomopsis* isolates did not represent any previously characterized *Phomopsis* sp. associated with conifers, because the included reference strains of *D. conorum* (anamorph *P. occulta*), *P. conorum*,

and *P. juniperivora* showed relatively low sequence similarities to the isolates of the *Phomopsis* sp. from Norway spruce seedlings. In the deposits of the NCBI GenBank Sequence Database, our isolates of the *Phomopsis* sp. had the highest sequence similarity (95%) with *D. phaseolorum*, a pathogen causing stem cankers near the stem node on soybean (9). The ITS sequence similarity of the isolates of the *Phomopsis* sp. from Norway spruce seedlings to deposits at the NCBI GenBank Sequence Database also was relatively low ($\leq 95\%$); therefore, it is likely that the *Phomopsis* sp. strains here studied represent an as yet uncharacterized species. This complicates comparison with other studies. Bearing this warning in mind, *P. occulta* has been associated with stem cankers (8,11), while *P. conorum* has been observed in shoot dieback of young spruce trees in Austria (6,8). Cech (T. Cech, personal communication) confirms the occurrence of *Phomopsis* spp. on spruce, but has the opinion that the genus *Phomopsis* is a secondary fungus, infecting after (for example) *Sirococcus* or *Gremmeniella* spp. Similarly, Perny et al. (23) also described *Phomopsis* spp. as merely weak parasites of spruce that are favored only in cases of adverse climatic conditions, wrong provenance, or localization. Our data are consistent with the latter two cases because, in the included pathogenicity tests, the *Phomopsis* sp. strains showed low pathogenicity. Our current hypothesis is that, in order to become pathogenic, the presently examined *Phomopsis* sp. strains need specific host-predisposing conditions, such as infection by other pathogens or abiotic stress.

In conclusion, disease symptoms caused by *G. abietina* in nursery-grown Norway spruce seedlings are reported here for the first time. The incidence of the disease on Norway spruce seedlings was observed 1 year after a severe *G. abietina* var. *abietina* epidemic on Scots pine trees and likely was related to this. The absence of the pathogen in Norway spruce seedlings in the affected nurseries during the two monitored years after the epidemic indicates that, as in pine seedlings, the disease is not a problem in Norway spruce seedlings during nonepidemic years. Removal of the Scots pine trees, a source of *G. abietina* inoculum, from the immediate vicinity of the nursery may diminish the damage to seedlings. The developed real-time PCR assay makes it possible to monitor the presence of *G. abietina* var. *abietina* in nursery seedlings during the early, latent asymptomatic stage of this disease, but this tool also can be employed to monitor *G. abietina* in the nearby forests. In years with high infection pressure of *G. abietina*, selective chemical treatment of Scots pine and also Norway spruce seedlings seems warranted. We report here on a *Phomopsis* sp. associated with lower-stem necroses in Norway spruce seedlings; however, the pathogenicity potential and function of this fungus is still unclear.

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