Top shoot dieback on Norway spruce seedlings associated with *Gremmeniella* and *Phomopsis*

Isabella Børja, Halvor Solheim, Ari M. Hietala and Carl Gunnar Fossdal
Norwegian Forest Research Institute, Høgskoleveien 8, N-1432 Ås, Norway.
isabella.borja@skogforsk.no

Abstract

In spring 2002, extensive damage was recorded in southeast Norway on nursery-grown Norway spruce seedlings that had either wintered in nursery cold storage or had been planted out in autumn 2001. The damage was characterised by a top shoot dieback. Two visually distinct types of necroses were located either on the upper or lower part of the 2001-year-shoot. Isolations from the upper stem necroses rendered *Gremmeniella abietina*, while *Phomopsis* sp. was isolated mostly from the lower stem necroses. RAMS (random amplified microsatellites) profiling indicated that the *G. abietina* strains associated with diseased nursery seedlings belonged to LTT (large-tree type) ecotype, and inoculation tests confirmed their pathogenicity on Norway spruce seedlings. *Phomopsis* sp. was not pathogenic in inoculation tests, this implying it may be a secondary colonizer. We describe here the *Gremmeniella* – associated shoot dieback symptoms on Norway spruce seedlings and conclude that the unusual disease outburst was related to the *Gremmeniella* epidemic caused by the LTT ecotype on large Scots pines in 2001. The role of *Phomopsis* sp. in the tissue of diseased Norway spruce seedlings is yet unclear.

Introduction

In the spring of 2001 a devastating epidemic of *Gremmeniella abietina* (Lagerb.) M. Morelet on large Scots pines (*Pinus sylvestris* L.) occurred in the south-eastern part of Norway (Solheim 2001) and in adjacent parts of Sweden (Elna Stenström, personal comm.) which probably was the strongest outbreak recorded in these areas.

The following spring, in 2002, a frequent occurrence of diseased Norway spruce (*Picea abies* (L.) Karsten) seedlings was registered in forest nurseries in the south-eastern part of Norway. The damage was detected mostly on 2-year-old seedlings that were either planted out in the autumn 2001 or taken out from cold storage, ready to be planted out in the spring 2002. The seedlings showed various degrees of top shoot dieback. When surveying plant nurseries with heavy damage, also 1-year old seedlings were seen with similar symptoms, but to a lesser extent. At a closer examination principally two different types of stem necroses were observed. The two types of necroses yielded *Gremmeniella abietina* and *Phomopsis* sp. respectively.

Damages caused by *Gremmeniella abietina* are well documented and described on large pines as well as on Norway spruce. In Northern Europe *G. abietina* consists of two ecotypes, A and B (Uotila 1983), also described as «the small tree type» (STT) and «the large tree type» (LTT), respectively (Hellgren & Högberg 1995). The LTT is most common in 15–40 year-old Scots pine trees in southern Scandinavia and Finland (Hellgren & Barklund 1992, Uotila 1992), 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 (Karlman *et al.* 1994).

On pine seedlings it causes the typical umbrella-like folding of needles on the leader (Nef & Perrin 1999). However, to our knowledge, neither *Gremmeniella abietina* nor *Phomopsis* sp. infections have been described on Norway spruce seedlings in nursery production.

Here we report on the *Gremmeniella* and *Phomopsis* associated symptoms on Norway spruce seedlings that occurred after the epidemic *Gremmeniella*-outbreak in spring 2001. The objectives of this work were (i) to describe the disease symptoms on Norway spruce seedlings; (ii) to isolate and identify the fungi associated with this damage and further determine their pathogenicity *in vivo* and *in vitro*; (iii) to assess survival and development of the outplanted symptomatic seedlings.

Materials and methods

Plant material and fungal isolation

Norway spruce seedlings (2-year-old) were collected from affected nurseries in south-east Norway. The length and location of the necroses were measured. Tissue chips were cut out from the necrose margins, sterilized and plated on the malt (1.25 %) agar (2 %) medium, incubated at 210C in the dark for 3–5 weeks, then fungi were identified.

Pathogenicity test in vitro *and* in vivo

The fungi isolated from the diseased seedlings were tested for their ability to induce dieback on fresh living tissue *in vitro* and *in vivo*. For both tests, three isolates of *Gremmeniella abietina* (2002–48/2, 2002–26/2, 2002–47/1), and *Phomopsis* sp. (2002–53/3, 2002–117/3, 2002–62/1) were chosen. The *in vitro* test compared the ability of the fungi to kill the tissue of freshly detached, aseptic spruce needles. Needles from aseptically grown spruce seedlings (about 5 weeks old) were detached, placed in a petri plate containing malt agar medium, together with the actively growing culture of the fungus. The needles were positioned in front of the advancing mycelium. Needles on malt agar without any fungal culture were used as controls. The petri plates were incubated in the darkness at room temperature. The visual inspection of all needles was done

once per day. The relative amount of discoloration on each needle was recorded and the percentage of damage for each needle was registered. There were three replicates for each fungal culture with 10 needles in each petri dish. The pathogenicity for each fungal culture was estimated as a time necessary for the fungus to kill 50 % of the needles.

To determine the pathogenicity of the isolated fungi *in vivo*, healthy looking seedlings were inoculated with the same fungi as in the pathogenicity test *in vitro*. Both 1-and 2-year-old seedlings of Norway spruce, were delivered from the nursery production in November 2003. Ten seedlings of each kind were inoculated with 3 isolates of *Gremmeniella*, 3 isolates of *Phomopsis* sp., respectively. A scalpel incision (2 mm) was made in the middle of the stem and a piece of fungal mycelium (about 1 mm^3) on agar medium was placed inside. The wound was sealed with parafilm. Control seedlings were mock inoculated with agar only. Seedlings were then placed in containers and moved over to a climatic chamber where cold storage conditions (2–50 C, 80 % humidity and darkness) were simulated. Eighteen weeks later extend of the necroses and the shoot lengths were measured.

Outplanted symptomatic seedlings

In order to investigate and follow the further development of diseased seedlings, an outdoor outplanting experiment was set up. One year old Norway spruce seedlings, originating from the nursery with large amount of typical *Gremmeniella*-diseased seedlings, were selected for outplanting. Thirty-six seedlings with the same symptoms were taken to Hoxmark, the experimental garden of Norwegian Forest Research Institute, and outplanted during the summer 2002. All seedlings had dead top shoots. Total shoot length, the length of the diseased shoot and the extent of the necrotic part of the shoot were measured, in spring 2003. All outplanted seedlings showed a tendency of the side shoot taking over the dead leader. In 8 cases out of 36, there was a tendency to develop a double leader (double stem). The seedlings were regularly observed during the following growing seasons, and development of fungal fruitbodies was monitored. In January 2005 all seedlings were cut off , their health condition, shoot length and fungal fruitbody development was evaluated.

RAMS-PCR-assay of Gremmeniella *isolates*

Random amplified microsatellite (RAMS) technique was used to further characterize the *Gremmeniella* – isolates and determine which biotype they represented. The *Gremmeniella* – isolates were grown on cellophane-coated malt and V8 juice agar, and the mycelia harvested were ground with a pestle in liquid N_2 chilled mortars. DNA isolation was performed by using Plant DNA Mini Isolation Kit (Qiagen) according to the manufacturer's instructions. The PCR reactions were carried out in the reaction conditions recommended by the manufacturer of the HotStarTaq™ DNA Polymerase by using 2 µM concentration of the degenerate CCA primers described by Hantula and Müller (1997). The PCR cycling parameters were also as described in that study. Amplification products were separated by gel electrophoresis in 1.5 % agarose gels using TAE running buffer and visualized under UV-light after ethidium bromide staining.

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 by using Oneway ANOVA (JMP, SAS institute)

Results and discussion

The symptoms on Norway spruce seedlings became visible during the spring of 2002, one year after the *Gremmeniella* epidemic on large Scots pines. Both 1- and 2-year-old plants showed symptoms of desiccated leader shoot (Fig. 1) and had necrotic stem lesions on the 2001-year shoot. The first visible signs of a stem lesion were a local indentation in the bark, and greyish 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, while others ringed the whole stem, causing top dying of the shoot. Occasionally there were 2–3 separate necroses on one stem. Generally, two types of necroses, «upper stem necroses» and «lower stem necroses», could be distinguished (Fig. 2).

Fig. 1. Top shoot dieback caused by G. abietina *on 2-year-old Norway spruce seedling. Photo: H. Solheim.*

Fig. 2. Characteristic location and appearance of the necroses on stems of the 2-year-old Norway spruce seedlings. Typical upper stem-necrosis (photo on the left), with brown, resinous tissue, where G. abietina *was isolated. Lower stem necrosis (photo on the right), with light brown and waterlogged tissue, were often located close to the stem node.* Phomopsis *sp. was frequently isolated here. Photos: H. Solheim.*

Upper stem necroses: associated with Gremmeniella

Mean 2001-shoot length on 2-year-old seedlings with this type of necroses was 25 cm. Necroses on the upper stem were located 14.9 cm (mean distance) above the 2000–2001 stem node and their average length was 4.3 cm. The necrotic, dark brown coloured bark was profusely impregnated with resin (Fig. 2). In this area, the stem was usually 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. *G. abietina* alone was isolated predominantly from seedlings sampled in April-May period. In isolations performed later (June and later), also *Phomopsis* was occasionally recovered from this type of necroses. No other potentially pathogenic fungi were isolated from the upper stem necroses. Most of the seedlings with upper stem necroses yielding *Gremmeniella* originated from a nursery, where large pine trees were in close vicinity to the nursery area.

Lower stem necroses: associated with Phomopsis

Mean 2001-shoot length on 2-year-old plants with this type of necroses was 21 cm. The mean distance from the lower edge of the necroses to the 2000–2001 stem node was 3.9 cm. These necroses were often located at the base of the 2001-shoot or partially at the end of the 2000-shoot. Necroses on lower stem were lighter in colour compared to the upper stem necroses, and had a characteristic water-soaked appearance without any resin flow (Fig. 2). The edges of necroses were diffuse, non-distinct. Occasionally, such necroses were found also on the upper part of the 2001-shoot. The most frequently isolated fungus from these lesions was *Phomopsis* sp., which was recovered in the period from April to December. Apart from two cases where *Botrytis* sp. was recovered, no other potentially pathogenic fungi were isolated from these necroses. Fruitbodies of *Phomopsis* sp. developed readily on plants after storage at + 4×C. Seedlings with lower stem necroses originated mostly from nurseries, where there were no pine trees in the immediate vicinity.

The stem necroses may have originated from the bark fissures, cracks in the bark associated with rapid growth, usual for plants in nurseries. The damage above the necroses first became visible in 2002. The seedlings were probably infected during spring or summer 2001 and the disease was already latent during their moving to cold storage or outplanting, in autumn 2001. Presumably, the seedlings at this point had no visible symptoms, which would explain why infected plants were not discarded.

Pathogenicity tests

In the pathogenicity test *in vitro* with needles (Fig. 3), *G. abietina* strains killed 50 % of the needle tissue within 4–6 days, strain 2002–48/2 (G3) being the most aggressive. The *Phomopsis* strains (P1 and P3) caused 50 % damage on needle tissue after 9 days, while P2 showed no signs of pathogenicity at 10 days after the inoculation, when the experiment was ended.

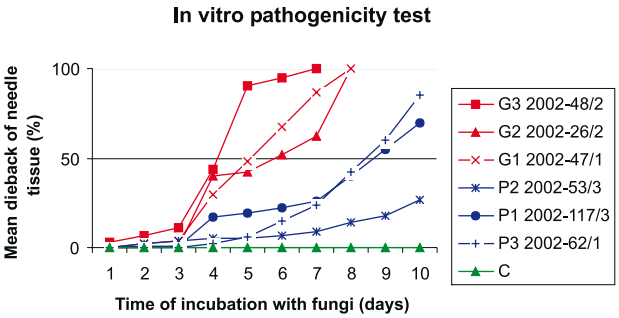

Fig. 3. Pathogenicity test in vitro. Dieback of aseptic spruce needles inoculated with three isolates of G. abietina *(G1-G3) and* Phomopsis *sp. (P1-P3) compared to non-inoculated control needles (C). All fungi were isolated from Norway spruce seedlings with top dieback symptoms.*

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 one-year-old seedlings, *G. abietina* strains 2002–48/2 (G3) and 2002–26/2 (G2) caused significantly longer necroses than the other strains (Fig. 4). The necroses produced by the other strains were not significantly different from the control. In two-year-old seedlings, the longest necroses were caused by *G. abietina* strains 2002–26/2 (G2) and 2002–48/2 (G3), but only *G. abietina* strain 2002–26/2 (G2) differed significantly from the control. (Fig. 4).

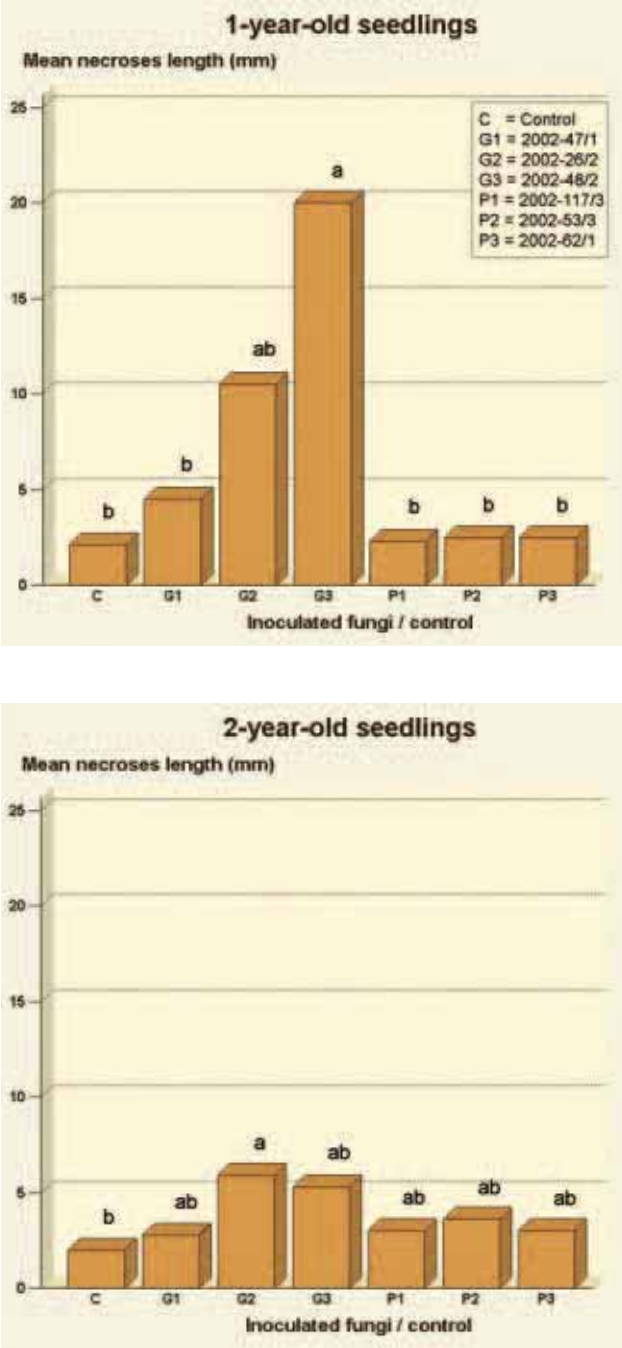

Fig. 4. In vivo *pathogenicity test. Length of necroses in one- and two-year-old Norway spruce seedlings 18 weeks after inoculation with different isolates of* G. abietina *(G1-G3),* Phomopsis *sp. (P1-P3) and mock inoculated control (C).*

Both pathogenicity tests confirmed the virulence of the *Gremmeniella abietina* isolates on Norway spruce seedlings. Most of the literature on nurseries reports *Gremmeniella* exclusively as a pathogen on pine seedlings, and if associated to Norway spruce, *G. abietina* is mentioned as a pathogen on saplings (Kaitera *et al.* 2000) and on larger seedlings in plantations (Roll-Hansen 1967). In pine seedlings, the disease is easily recognized by the characteristic umbrella-like folding of needles on the leader shoot (Björkman, 1959, Nef & Perrin 1999), whereas the symptoms of *Gremmeniella* infection on Norway spruce seedlings, necroses and shoot dieback, are rather non-specific and can be caused by several pathogens as well as by abiotic stresses, such as frost, drought or cold storage. Since multiple factors can cause these symptoms in Norway spruce seedlings, incidents of *Gremmeniella*-infection may be misidentified.

Symptomatic seedlings in outplanted plots

In spring 2003, at the time of the first assessment, 23 % of the seedlings (8 seedlings out of 36) had a tendency to develop a double shoot, i.e. two sideshoots were competing for the 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 % (9 seedlings out of 14) of the seedlings had developed a double stem (unfortunately, 22 seedlings were destroyed by accident before the last evaluation, and thus only 14 remaining seedlings were inspected at the end of the experiment). The seedlings were alive, and showed good growth (Fig. 5). The originally diseased leader shoots had been taken over by a new leader. Out of the 14 dead shoots collected at the last inspection, four had old, empty pycnidia still present, while ten had only visible scars after pycnidia. No apothecia were observed in any seedling.

The outplanting experiment confirmed that the infected Norway spruce seedlings survive the damage. Even if the part above the stem necrosis dies, in young plants usually the side shoot takes over the dead leader. Some of the seedlings develop double leaders after the *Gremmeniella* infection.

Fig. 5. Long term field performance of damaged 1-year-old Norway spruce seedlings. Left: Seedlings were 1-year-old (in 2002) when top shoot damage occurred (arrow). One year later (in 2003) the dead shoot was taken over by side-shoots. Right: The same seedling in 2005. Photos: H. Solheim

The RAMS-PCR assay

The RAMS-CCA banding patterns were identical among the *Gremmeniella* isolates from Norway spruce seedlings, while the included reference strains of LTT and STT ecotypes showed type specific banding patterns (Fig. 6). The assay confirmed that the *Gremmeniella* isolates from Norway spruce seedlings belonged to the LTT ecotype, as their banding patterns were identical to those from the reference strains of the type and differed from the STT reference strains. With the CCA primer, only the LTT reference strains and the strains from the seedlings had a 1500-bp band.

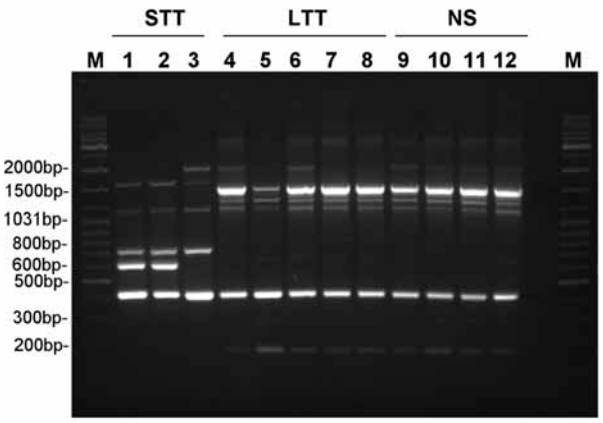

Fig. 6. RAMS patterns (CCA primer) of three small-tree type (STT) (1988–306/1, 1988–307/3 and 1974–46/1, respectively) and five large-tree type (LTT) (2002–20/4, 2002–47/1, 1985–111/6, 1985–393/16/1 and 1966–163/2, respectively) G. abietina *reference strains, and four isolates (2002–4/4, 2002–79/2,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

These data confirmed that the strains associated to nursery-grown Norway spruce seedlings belonged to the LTT ecotype of *G. abietina*. Our nursery samples were collected from the geographical area in south-eastern Norway, where a devastating epidemic of *G. abietina* had occurred on large pines the previous year. This epidemic was a typical LTT outbreak characterised by dieback of shoots in the entire crown (Solheim 2001). As the *Gremmeniella* strains from diseased nursery seedlings of Norway spruce grouped to the LTT, we conclude that the unusual disease outburst on Norway spruce seedlings in 2002 was related to the previous year's epidemic on Scots pines. Apparently similar damages in Norway spruce seedlings after the pine epidemic were observed in Sweden (Stenström, pers. comm.) and in Finland (Petäistö 2003) as well.

During the periods of high inoculum density, the pathogen can also infect the Norway spruce seedlings in the neighbouring nurseries. In order to avoid infection from the pines, it is important to keep the pines away from the forest nurseries and Christmas tree plantations.

Besides *Gremmeniella*, a *Phomopsis* species was frequently associated with the shoot dieback-stem necrosis symptoms in the Norway spruce seedlings now examined. Compatible with our observations on *Phomopsis*, Hansen & Hamm (1988) report on *Phomopsis* 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, also the appearance of necroses associated with *Gremmeniella* and *Phomopsis* differed. Resin flow, a characteristic conifer response upon pathogen attack, was commonly observed in necroses hosting *Gremmeniella*, whereas *Phomopsis*-associated necroses were water soaked and without any resin flow.

Based on the ITS rDNA sequence analysis performed, the *Phomopsis* isolates do not represent any previously characterized *Phomopsis* species associated to conifers (Børja *et al.*, submitted). Since the ITS sequence similarity of the *Phomopsis* strains from Norway spruce seedlings to deposits at the NCBI GenBank Sequence Database was also relatively low (£ 95 %), it is likely that these *Phomopsis* strains now studied represent an yet uncharacterized species on Norway spruce. This complicates comparison to other studies. Bearing this caution in mind, *P. occulta* (Sacc.) Traverso has been associated with stem cankers (Donaubauer 1995, Hahn 1943), while *P. conorum* (Sacc.) Died has been observed in correlation with shoot dieback of young spruce trees in Austria (Donaubauer 1995, Cech & Perny 1995). In British Columbia, P. *occulta* is considered as a pathogen on spruce seedlings in nurseries (Thompson *et al.* 2002). Cech (pers. comm.) confirms the occurrence of *Phomopsis* spp. on spruce, but has the opinion that *Phomopsis* is a secondary fungus, infecting after e.g. *Sirococcus* or *Gremmeniella*. Consistently, Perny *et al.* (2002) described also *Phomopsis* species as merely a weak parasite of spruce that is favoured only in cases of adverse climatic conditions, wrong provenance or localization. Our own data are consistent with the latter two cases as in the included pathogenicity tests the *Phomopsis* strains were non-pathogenic. Our current hypothesis is that in order to become pathogenic, the now examined *Phomopsis* strains need specific host-predisposing conditions, such as infection by other pathogens and/or abiotic stress.

The occurrence of the disease is not new, but overlooked. The unique event of *Gremmeniella* epidemics on large pines, which occurred in 2001, allowed us to follow and describe the *Gremmeniella*-disease development on Norway spruce seedlings in nurseries.

Conclusions

In conclusion, the massive *Gremmeniella* infection in nursery-grown Norway spruce seedlings is reported here for the first time. The incidence of the disease is correlated with the serious *Gremmeniella* epidemic on large Scots pine and Norway spruce trees the previous season. The

resulting extreme infection pressure combined with predisposing weather conditions, cold and high rainfall periods in the summer followed by mild winter account for the atypical outbreak of *Gremmeniella* on nursery-grown Norway spruce seedlings. Removal of the large Scots pines, a source of *G. abietina*-inoculum, from the immediate vicinity of the nursery, may diminish the damage on seedlings. In years with high infection pressure of *G. abietina*, selective chemical treatment of Scots pine but also Norway spruce seedlings seems warranted. We report here on *Phomopsis* sp., associated with lower stem necroses in Norway spruce seedlings, yet the pathogenicity potential and function of this fungus is unclear.

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