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Sphaeropsis sapinea found as symptomless endophyte in Finland

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Highlights

- *Sphaeropsis sapinea* was found for the first time as an endophyte in healthy Scots pine in Finland.
- This finding confirms that *S. sapinea* can proliferate in a symptomless stage in Scots pine in Finland.

Abstract

The aim of this study was to determine if the ascomycete fungus *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton (syn. *Diplodia sapinea* (Fr.) Fuckel) could be cultured from surface sterilized Scots pine twigs presenting the endophytic stage of this fungus. This fungus causes the disease called Diplodia tip blight in conifers. Symptoms become visible when trees have been weakened by abiotic stressors related to temperature, drought and hailstorms. The disease is rapidly increasing and is observed regularly in Scots pine (*Pinus sylvestris* L.) forests in Europe. Changes in climatic conditions will gradually increase the damage of this pathogen, because it is favored by elevated temperatures and additionally the host trees will be more susceptible due to related environmental stress. Diplodia tip blight is emerging towards Northern latitudes, thus, actions to monitor the spread of *S. sapinea* in pine-dominated forests should be undertaken in Finland. Our aim was to search for *S. sapinea* in Scots pine along a transect in Finland. Branch samples were collected from healthy Scots pine, fungal endophytes were isolated and morphologically identified. Sixteen *S. sapinea* strains were found from four Scots pine trees from two locations. This finding confirms that *S. sapinea* is found as an endophyte in healthy Scots pine in Finland.

Keywords *Diplodia sapinea*; *Pinus sylvestris*; Diplodia tip blight; Scots pine

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1 Introduction

Scots pine (*Pinus sylvestris* L.) is the main source of forest tree products for the forest sector in Finland contributing several billion EUR net yearly (Finnish Forest Statistics 2019). The major threats to the sustainable supply of forest tree products are adverse climate, pests and diseases. Damages due to fungal pathogens are predicted to increase under climate change scenarios that include increasing temperatures and longer periods of drought (Seidl et al. 2017). The forest pathosystems' behavior can be unpredictable in the future due to changes in environment that favor fungal pathogens rather than the host (Linnakoski et al. 2017; Terhonen et al. 2019; Stewart et al. 2020). When the local environment changes and an increase in drought/temperature-associated stress may be observed, Scots pines become more susceptible to pathogens (Stanosz et al. 2001; Bußkamp 2018). Southern Finland is a high-risk drought area (Veijalainen et al. 2019), where a dry growing season may result in a 30–50% probability of detecting physiological changes such as defoliation, decreased carbon and nutrient assimilation, and breakdown of the photosynthetic process through loss of hydraulic conductivity in forest trees (Muukkonen et al. 2015).

Endophytes are described as microbes that live asymptotically in their host-plant tissues for the entire or at least a significant part of their life cycle, without causing any visible negative symptoms to the host (Petrini 1991; Saikkonen et al. 1998). In practical applications (e.g. when isolating endophytes), the definition of endophytes is stated by Hallmann et al. (1997) as "...those that can be isolated from surface-disinfested plant tissue or extracted from within the plant, and that do not visibly harm the plant." Plant pathogens and saprophytes can be endophytically present in host tissues and when the conditions become favourable for them (e.g. dead needle tissue is generated) they change from endophyte to pathogenic/saprophytic (Müller et al. 2001; Parfitt et al. 2010; Álvarez-Loayza et al. 2011). The endophytic stage is the typical strategy for shoot pathogens that exploit periods of abiotic stressors in the host (Slippers and Wingfield 2007; Blumenstein et al. 2020; Oliva et al. 2020). *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton (syn. *Diplodia sapinea* (Fr.) Fuckel) is an ascomycete fungus with different trophic stages (Smith et al. 1996). It can live asymptotically as an endophyte in its host tree (Langer et al. 2011; Luchi et al. 2014; Blumenstein et al. 2020; Bußkamp et al. 2020), while being a latent and opportunistic pathogen (Brodde et al. 2019; Blumenstein et al. 2020; Bußkamp et al. 2020) or/and saprophyte (Müller et al. 2019; Oliva et al. 2020). The abundance of *S. sapinea* is currently increasing in European forests as an endophyte (Bußkamp 2018; Blumenstein et al. 2020; Bußkamp et al. 2020; Oliva et al. 2020) and it is emerging in Northern latitudes (Hanso and Drenkhan 2007; Adamson et al. 2015; Brodde et al. 2019; Müller et al. 2019). *Sphaeropsis sapinea* causes the disease called Diplodia (syn. Sphaeropsis) tip blight and the outbreaks in Europe threaten the ecologically important and commercially valuable Scots pine forests (Fabre et al. 2011; Langer et al. 2011; Brodde et al. 2019; Blumenstein et al. 2020; Oliva et al. 2020).

Sphaeropsis sapinea spreads through conidial dispersion, transmitted by wind or water droplets (Brookhouser and Peterson 1971; Swart et al. 1987; Brodde et al. 2019). Seedlings can also transmit *S. sapinea* as asymptomatic nursery stock when the fungus is present as an endophyte (Stanosz et al. 2007). As for most tree endophytes, *S. sapinea* is transmitted horizontally (Rodriguez et al. 2009; Bihon et al. 2011). Fruiting bodies (pycnidia) are produced on dead twigs, needles or cones (Fig. 1A–B) and the asexual spores are released into the air from spring to fall (Brodde et al. 2019). Conidia are oval with a size of 30–55 × 11–18 µm (Sutton 1980; Sutton and Dyko 1989; Fig. 1C). Unwounded healthy tissue may become infected in spring/early summer during bud burst and tissue elongation (Brookhouser and Peterson 1971; Chou 1976, 1978). Similarly, *S. sapinea* also enters unwounded twig tissues through needle stomata (Brookhouser and Peterson 1971; Chou 1976; Flowers et al. 2006; Schlößler 2020). In addition, *S. sapinea* enters pine trees

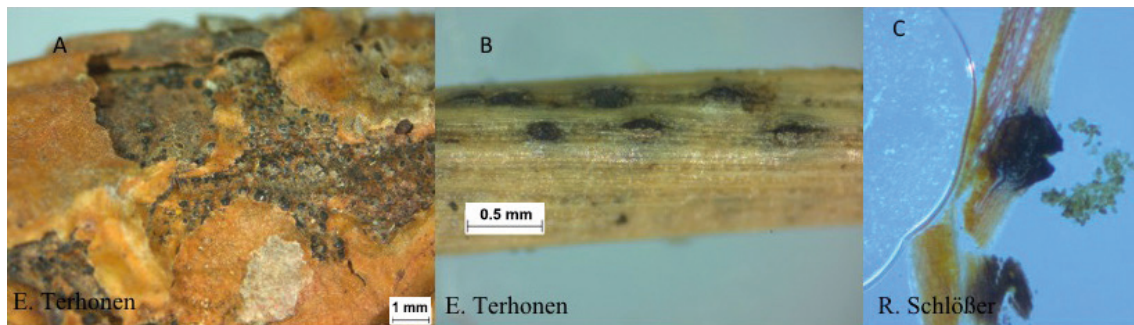


Fig. 1. Pycnidia of *Sphaeropsis sapinea* on (A) a Scots pine twig and (B) a needle. (C) Conidia of *Diplodia sapinea* released from a pycnidium (\varnothing 250 μ m, modified from Schlöber 2020).

via injured tissue (Munck et al. 2009; Oliva et al. 2020), grow and persist for long time periods in young and older twig tissues without causing the disease (Bihon et al. 2011). When the environment changes towards conditions more adverse for the host tree (e.g. drought) and more favorable for pathogenic fungi (e.g. elevated temperature), *Sphaeropsis sapinea* switches from the endophytic stage to pathogenic (Fabre et al. 2011; Bosso et al. 2017; Bußkamp et al. 2020). After this lifestyle change, *Sphaeropsis sapinea* kills the twigs, and eventually it can kill even mature trees during one growth season (Blumenstein et al. 2020; Bußkamp et al. 2020; Oliva et al. 2020). Diplodia tip blight disease symptoms include tip blight, stem canker, dieback of current year shoots, and blue staining of the sapwood (Luchi et al. 2014; Bußkamp et al. 2020; Oliva et al. 2020). The lifestyle change can lead to fast developing epidemics in Scots pine-dominated forests (Bußkamp 2018; Brodde et al. 2019; Blumenstein et al. 2020). The disease, Diplodia tip blight, has increased rapidly but still the invasion and epidemiology studies are lacking (Flowers et al. 2006; Bußkamp 2018; Brodde et al. 2019; CABI 2019).

Desprez-Loustau et al. (2006) classify *S. sapinea* as cryptogenic. Cryptogenic species are defined as species with an uncertain origin, but that are suspected to be exotic (invasive). Similarly, in the “Handbook of alien species in Europe”, *S. sapinea* is classified as cryptogenic (DAISIE 2009). Although the origin of *S. sapinea* is still unknown, it can be introduced to new regions in host material such as cones, seeds, and diseased seedlings (Stanosz et al. 2007; Brodde et al. 2019; Cleary et al. 2019). It has been considered that *S. sapinea* could be an invasive species in Europe, or that it has changed its behavior in the last decades from an asymptomatic endophyte to a pathogen due to the change in environmental conditions (Stanosz et al. 2001; Bußkamp 2018; Brodde et al. 2019; Müller et al. 2019; Blumenstein et al. 2020). However, the invasive nature of *S. sapinea* in Northern Europe has not been confirmed. *Sphaeropsis sapinea* was recorded to cause disease symptoms for the first time in Europe in the early 1980s (van Dam and de Kam 1984) and in the mid-1990s (Heydeck and Dahms 2012), respectively. Other reports from Central-Europe indicate emergence of this opportunistic pathogen (Bußkamp 2018; Brodde et al. 2019; Bußkamp et al. 2020; Blumenstein et al. 2020; Oliva et al. 2020). *Sphaeropsis sapinea* was observed in Estonia in 2007 (Hanso and Drenkhan 2009) and in Sweden in 2013 (Oliva et al. 2013). In southern Finland it was found on cones (living as a saprophyte) in 2015 and in 2016 (Müller et al. 2019). The disease Diplodia tip blight caused by *S. sapinea* is emerging in the northern parts of Europe and poses a serious threat to pine-based silviculture in the Nordic and Baltic countries. Our aim was to determine if *S. sapinea* could be found as an endophyte (as defined by Petrini 1991) in symptomless Scots pine trees in Finland.

2 Materials and methods

2.1 Study sites and trees

Tips of branches from 80 Scots pine trees were randomly collected at fourteen sites in various parts of Finland during the summer of 2019 (Table 1). The site number three (3) is described in Müller et al. (2019). The collected branches included annual shoots of 2017, 2018, and 2019. Shoots were sampled at various heights of the trees of various ages (Table 1). Samples from Kivalo (site 13) were collected from several heights altogether from six trees (tree 1: 17 m, 19 m and 21 m; tree 2: 11 m, 13 m, 15 m, 17 m and 19 m; tree 3: 9 m, 11 m and 15 m; tree 4: 4 m and 8 m; tree 5: 8 m, 10 m and 12 m; tree 6: 11 m and 17 m).

2.2 Fungal isolation

Altogether one branch of each tree, presenting altogether 240 twigs, were sampled (240 twigs from 80 trees; each twig either 2017, 2018 or 2019 growth). The needles were removed from each sample, and 3 cm sections cut from the tip of each annual shoot were sterilized. The sections were dipped in 70% ethanol for 1 min, followed by surface sterilization in 2% NaOCl for 1 min, they were rinsed four times with autoclaved deionized water. The sections were then cut in 0.5 cm long pieces and individually placed on Petri plates containing 1.5 % malt extract agar (MEA). The plates were incubated at room temperature for 3 to 4 weeks to reveal the slow growing endophytes. The plates were sub-cultured until pure colonies were obtained. The plates were examined and the colonies with *S. sapinea* morphology (Fig. 2) were selected for molecular identification.

Table 1. Investigated Scots pine stands and number of collected samples.

Site No.	Location	Latitude, Longitude	No. of trees	No. of branches	No. of tips	Avg. age of trees	Sampling month	Sampling height, m
1	Vantaa	60°25'N, 25°07'E	10	10	30	40	July	2
2	Mäntsälä	60°68'N, 25°19'E	1	1	3	20	July	10
3	Lohja	60°29'N, 23°55'E	10	10	30	20	July	2
4	Lapinjärvi	60°64'N, 26°15'E	6	6	18	60	July	10
5	Akaa	61°16'N, 23°90'E	11	11	33	40	July	2
6	Urjala	61°10'N, 23°52'E	4	4	12	40	July	2
7	Hyytiälä	61°80'N, 24°30'E	6	6	18	40	July	4
8	Route 66	61°80'N, 24°30'E	3	3	9	20	July	4
9	Setälänkangas	61°80'N, 24°20'E	3	3	9	20	July	2
10	Setälänkangas	61°80'N, 24°20'E	4	4	12	40	July	4
11	Siikakangas	61°90'N, 24°20'E	5	5	15	40	July	4
12	Skaftung	62°15'N, 21°33'E	8	8	24	40	August	2
13	Kivalo	66°00'N, 25°50'E	6	6	18	80 and 40	July	several
14	Lapajärvi	66°70'N, 28°40'E	3	3	9	35	August	10
SUM:			80	80	240			



Fig. 2. Three examples of *Sphaeropsis sapinea* morphology on 1.5% MEA plate. The hyphae color can vary from light grey to intense black.

2.3 DNA extraction and PCR

Seven *S. sapinea* strains provided by Dr. Michael Müller (Müller et al. 2019) were included in DNA analysis in this study to confirm the taxonomy of new strains. Fungal DNA was extracted for the purpose of molecular identification following the protocol of Keriö et al. (2020). Briefly, 1000 µl of PVP extraction buffer (1M NaCl, 100 mM TrisHCl, 10 mM EDTA, 2% PVP (w/v)) was added to a 1.5 ml Eppendorf tube with 0.3 g of grounded mycelium sample. After incubation at 65 °C for 15 minutes, the sample was centrifuged at 5000 rpm for 10 min. The supernatant was transferred into a 1.5 ml tube (ca 500 µl). One volume of SDS buffer (1% SDS (w/v), 0.5 M KCl) was added. The sample was vortexed for 20 seconds and centrifuged at 15 000 rpm for 10 min. The supernatant was transferred into a 1.5 ml tube (ca 700 µl). Isopropanol (0.85 volumes) was added and mixed by inversion for 20 seconds, followed by centrifugation for 10 min at 15 000 rpm. The supernatant was removed by pouring it away, and the pellet was washed with 200 µl of cold 70% ethanol. After centrifugation at 15 000 rpm for 5 min, ethanol was removed, and the pellet dried for 15 min at 65 °C. The pellet was re-suspended in nuclease-free water (50 µl).

Taq DNA polymerase (VWR) was used for PCR amplification of ITS regions with primer pair ITS1-F (White et al. 1990) and ITS4 (Gardes and Bruns 1993), for Large Sub Unit (LSU) region with primers nu-LSU-287-5'-mpnF (Nelsen et al. 2011) and LR 6R (Vilgalys and Hester 1990). Briefly, the PCR protocol was as follows: 1X PCR Buffer, 200 µM dNTP, 0.5 µM primer 1, 0.5 µM primer 2, 1.5M MgCl₂, 100ng template DNA, 0.2 U/µl DNA polymerase; the reaction was adjusted to 25 µl with autoclaved MQ H₂O. The PCR conditions used for ITS region were 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and 72 °C for 10 min. For LSU region, the only difference was the annealing temperature, 48 °C.

Possible contaminations were determined with a negative control using sterile water as template in both PCR protocols. RedStain was used to confirm DNA amplicons on a 1.5% agarose gel, and the visual detection was made by ultraviolet transillumination. ITS and LSU region PCR products were purified and sequenced using the ITS4 and LR-6R primers at Microsynth SEQLAB (Germany). The DNA extraction, PCR and sequencing were successful for 10 strains found in this study and for all seven strains from Müller et al. (2019).

The quality of all the obtained FASTA files were checked before analysis. The ITS sequences were extracted with an open source software ITSx to extract the ITS1 and ITS2 subregions from the fungal ITS sequences (Bengtsson-Palme et al. 2013). The ITS1 and ITS2 sequences were used for BLASTN (Zhang et al. 2000) searches against GenBank/NCBI (Sayers et al. 2011) to provide taxonomic identification. Similarly, the sequences were blasted against ITS1, ITS2 and

LSU regions of the *S. sapinea* strains found in Müller et al. (2019). The sequences obtained in this study and the ones from Müller et al. (2019) were aligned with MUSCLE (Edgar 2004), and a phylogenetic tree was generated in MEGA 5.01 (Tamura et al. 2011) using the Neighbour-joining (NJ) analysis with 1000 bootstrap replicates. The sequences of the isolates found in this study were deposited in GenBank with the following accession numbers: MT763348–MT763357 for ITS, and MT763358–MT763367 for LSU.

3 Results

Sphaeropsis sapinea was isolated from sixteen annual shoots harvested from four healthy symptomless Scots pine trees located in two different sites (Fig. 3, Table 2). They were considered as endophytic isolates as the hosts were healthy and the bark was intact. This means that the mycelium of the endophyte was already well established inside the host (under the bark) and it originated from surface sterilized plant tissue.



Fig. 3. Locations of the investigated Scots pine sites in this study as dots. Reprinted and modified from Free Vector Maps. Red dots indicate sites where *Sphaeropsis sapinea* was found in this study; number one (Vantaa) and five (Akaa).

Table 2. Number and origin of *Sphaeropsis sapinea* isolates from healthy Scots pine shoots.

No. of site	Location	No. of trees infected	Age of trees	No. of <i>S. sapinea</i> isolates in annual shoots of Scots pine		
				2019	2018	2017
1	Vantaa	2	40	5	4	3
5	Akaa	2	40	0	0	4

Morphological identification was performed for all 16 isolates (Fig. 2). Identification of *S. sapinea* was verified by ITS and LSU sequences for 10 isolates originating from the stands 1 (7 strains) and 5 (3 strains). ITS1 and ITS2 sequences were identical between strains and identical to numerous sequences in GenBank assigned to *D. sapinea*, or *S. sapinea*. They were also identical to those strains obtained recently from cones in Finland (Müller et al. 2019). LSU regions (for strains found in this study and those found in Müller et al. 2019) were identical (Query coverage 99%–100%; Per. Identity 98%–100%) to TYPE material of *D. sapinea* (gene bank ID: NG_069010). Scots pine stands infested by *S. sapinea* were close to the southwestern coast of Finland (Fig. 3). None of the fourteen investigated stands showed typical disease symptoms caused by *S. sapinea* at the time of sampling.

4 Discussion

Sphaeropsis sapinea is present as a symptomless endophyte in Scots pine twigs (Langer et al. 2011; Fabre et al. 2011; Luchi et al. 2014; Blumenstein et al. 2020; Bußkamp et al. 2020; Oliva et al. 2020). Similarly, we found *S. sapinea* as a symptomless endophytic fungus in healthy Scots pine trees (as defined Petrini 1991 and Hallmann et al. 1997) in Finland. This confirms that *S. sapinea* is emerging horizontally and has an endophytic trophic level in the northern limits of its known distribution area. *Sphaeropsis sapinea* was found on Austrian pine (*Pinus nigra* J.F. Arnold) in 2007 (Hanso and Drenkhan 2009) and on Scots pine in 2012 (Adamson et al. 2015) in Estonia. In 2013 the first observations of the pathogen were found in Sweden and in northwestern Russia (~110 km from the Finnish border) (Oliva et al. 2013; Adamson et al. 2015). Similarly, *Sphaeropsis sapinea* was observed as a saprophyte on the cones in 2015 in South Finland (Müller et al. 2019). It is notable that *S. sapinea* was not observed in Finland when Müller et al. (2019) did preliminary inventories in 2004 (Müller et al. 2019). The information describing how and where *S. sapinea* spread to Finland is still missing. The endophytic stage of *S. sapinea* can be detrimental for its Scots pine host, as *S. sapinea* can switch to pathogenic stage when triggered by host stress, and kill the occupied twigs leading eventually to the death of mature trees (Blumenstein et al. 2020; Fig. 4).

The endophytic stage represents a balanced interaction between the fungus and its host tree. When conifer trees are stressed due to changes in the environment such as drought, temperature increases or hailstorms, *Sphaeropsis sapinea* transforms from an asymptomatic to pathogenic fungus (Stanosz et al. 2007; Bußkamp 2018; Blumenstein et al. 2020; Oliva et al. 2020). However, the mechanisms underlying the appearance of *S. sapinea* as first an asymptomatic fungal colonizer that turns pathogenic, after stimulation by host stress, remains unknown. Two possible scenarios related to temperature and drought could affect this transition. *Sphaeropsis sapinea* spores germinate best at 25 °C (Jiangyan et al. 1999). Similarly, the optimum of hyphae growth of *S. sapinea* is at 25 °C, while for other Scots pine endophytes the optimum growth is at 20 °C (Bußkamp 2018). In Finland, the annual mean temperature in the years 1979–2018 increased in Southern (+1.9 °C) and Northern (+2.7 °C) areas in Finland (Räisänen 2019). The increase in temperature is expected



Fig. 4. Example of mature Scots pine stand that died during growth season of 2019, due disease caused by *Sphaeropsis sapinea*. Scots pine trees were stressed due to high temperatures and drought during 2010 and 2018 that lead to Diplodia tip blight disease epidemic, Germany 2019 (A). Blue stain develops fast in dead trees that can be seen after felling, partly this blue stain is due to *S. sapinea*, Germany 2019 (B).

to be between 2.5 °C and 5 °C by the year 2100 (Mikkonen et al. 2015). Drought is an important abiotic disturbance factor, as the susceptibility of pine trees to *S. sapinea* is strongly enhanced by water stress (Stanosz et al. 2001; Boland et al. 2004; Deprez-Loustau et al. 2006; Blaschke and Cech 2007; Sturrock et al. 2011; Bußkamp 2018). The average annual precipitation sums were observed to vary from 450 mm in Northern Lapland to 750 mm in Southern and Eastern Finland for 1981–2010 (Pirinen et al. 2012). Veijalainen et al. (2019) analyzed that drought risk due to climate change can increase in Southern and Central Finland. Especially the drought risk during summer and early autumn can escalate due to an increase in temperature and longer summer periods (Veijalainen et al. 2019). Additional stress in Scots pines due to higher temperatures and drought in most vulnerable areas in Finland could lead to epidemics caused by *S. sapinea*. Brodde et al. (2019) showed that the epidemics observed in Sweden started to culminate already 10 years beforehand (*S. sapinea* emerging as an endophyte). Similarly, Blumenstein et al. (2020) showed that *S. sapinea* is the most common endophyte in healthy and diseased Scots pine and the diseased trees can die fast in one dry summer season (Blumenstein et al. 2020). This scenario can happen in near future in Finland, and it could be possible that changes in local environment causing stress to Scots pines can trigger these epidemics.

To mitigate the impacts of environmental changes, it is essential to understand the factors that trigger the development of forest tree disease epidemics and what increase host susceptibility. To design effective, durable and environmentally friendly disease prevention we need to understand the detailed epidemiology of this emerging fungi as well as the co-evolution history with its host. At the moment we don't know all the factors participating to the epidemiology of this fungi, e.g. what is needed for the activation of the pathogenic stage and how *S. sapinea* can emerge to new areas. This information can ultimately be used to improve forest health via resistance breeding, to discover biocontrol methods, and to develop diagnostic methods to limit/detect the spread of the endophytic *S. sapinea*. These facts highlight the urgent need to restrict the influence of *S. sapinea* and to develop new management protocols to secure the health of pine-dominated forests in Finland.

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