Silva Fennica vol. 56 no. 4 article id 22008
Category: research note
https://doi.org/10.14214/sf.22008

SILVA FENNICA

http://www.silvafennica.fi
ISSN-L 0037-5330 | ISSN 2242-4075 (Online)
The Finnish Society of Forest Science

Eeva Terhonen

# First report of Diplodia tip blight on Scots pine in Finland

**Terhonen E.** (2023). First report of Diplodia tip blight on Scots pine in Finland. Silva Fennica vol. 56 no. 4 article id 22008. 7 p. https://doi.org/10.14214/sf.22008

## Highlights

- Diplodia tip blight is a new disease on Scots pine in Finland.
- *Diplodia sapinea* can be identified reliably with the presented pipeline.

## Abstract

*Diplodia sapinea* (Fr.) Fuckel causes shoot blight on Scots pine (*Pinus sylvestris* L.). This fungus has been discovered in Finland as a saprophyte in 2015 on Scots pine cones. The endophytic mode of this fungus was later discovered in healthy Scots pine twigs. In 2021 the disease, Diplodia tip blight was observed on Scots pine in Finland. Currently, the disease symptoms are poorly identified so the role of *D. sapinea* in disease outbreaks in Finland are easily overlooked. The identification of the fungi is challenging in field conditions and requires targeted identification in laboratory. In this research note I report the first Diplodia tip blight outbreaks observed in Finland, the typical disease symptoms, and methodology for the species identification. Samples were collected from symptomatic trees based on observations made by the citizens. *Diplodia sapinea* was isolated from defoliated and surface sterilized twigs. The species identification by morphological characters was further confirmed with sequencing of ITS region of rDNA and with species-specific primers. A pathogenicity test confirmed that *D. sapinea* was the disease agent causing shoot blight. This is the first report of Diplodia tip blight on Scots pine in Finland.

**Keywords** *Diplodia sapinea*; *Pinus sylvestris*; *Sphaeropsis sapinea*; drought; emerging fungal disease
**Address** Natural Resources Institute Finland (Luke), Forest health and biodiversity, Latokartanonkaari 9, FI-00790 Helsinki, Finland
**E-mail** eeva.terhonen@luke.fi
**Received** 30 September 2022 **Revised** 10 January 2023 **Accepted** 11 January 2023

## 1 Introduction

Diplodia tip blight is a shoot blight disease of Scots pine (*Pinus sylvestris* L.), caused by ascomycete *Diplodia sapinea* (Fr.) Fuckel. *Diplodia sapinea* is present in its host as dormant saprotroph (Müller et al. 2019) or asymptomatic endophyte (Terhonen et al. 2021). Due to additional biotic stressors (e.g. drought) that the host encounters (Blumenstein et al. 2021a, 2022), *D. sapinea* can switch its lifestyle to pathogenic, leading to unexpected disease (Diplodia tip blight) outbreaks on Scots pine in the field and nurseries (Brodde et al. 2019; Blumenstein et al. 2021b; Larsson et al. 2021; Oliva et al. 2021). Scots pine is the most common forestry species in Finland with a volume of 1250 million cubic meters, corresponding to 50% of the growing stock (Mäkisara et al. 2022). As conifers are long living organisms, they might not be able to locally adapt to the rapid changes in climate and environment. This may make Scots pine more prone to drought stress that can lead to higher disease outbreaks due to *D. sapinea* (Brodde et al. 2019).

*Diplodia sapinea* was first recorded on cones in southern Finland in 2015 (Müller et al. 2019). In 2019 it was found as endophyte from healthy Scots pine twigs collected in southern Finland (Terhonen et al. 2021). In autumn 2021 and spring 2022, first blighted shoots of Scots pine were observed in several locations in coastal Finland. The symptoms resembled *D. sapinea* causing the disease Diplodia tip blight. *Diplodia sapinea* can be assumed to be a new pathogen on Scots pine in Finland. Previously, it has been recorded only as a harmless endophyte or dormant saphrophyte in Finland, but various stressors of the host tree might promote the launch to pathogenic mode. The aim of this research note is to show the disease symptoms in the field, raise awareness and develop pipeline to correctly identify *D. sapinea* in the laboratory.

## 2 Material and methods

Declining shoots (7) were collected from either seedlings (Fig. 1A), saplings (15-year-old trees) (Fig. 1B, C) or mature trees (Fig. 2A, B) in five different Finnish locations (Table 1). The branches

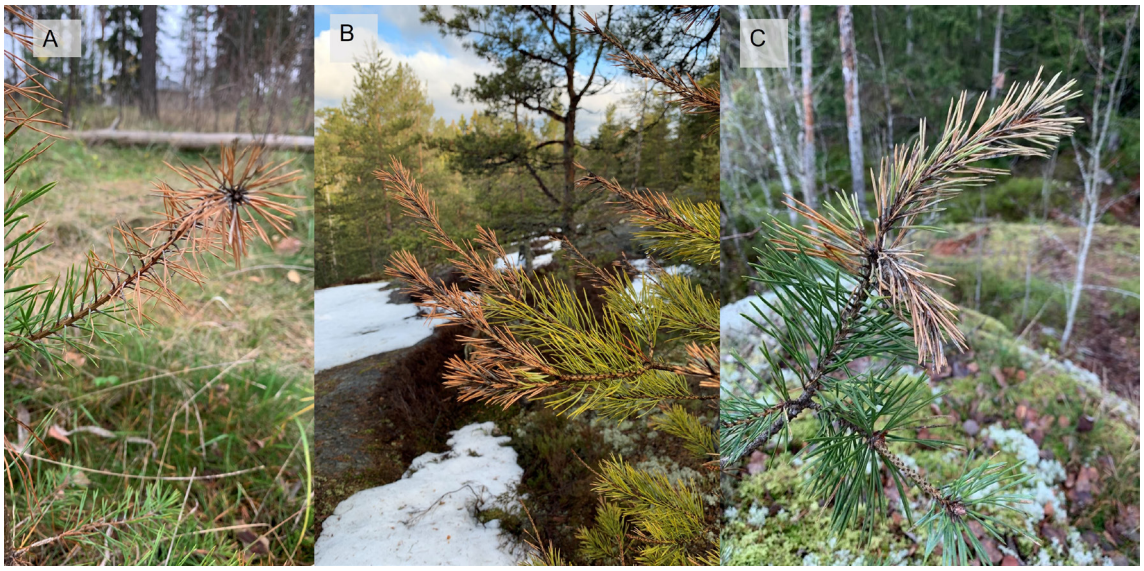


**Fig. 1.** A) Symptoms of Diplodia tip blight observed in the field on Scots pine seedling in August 2021, three-year-old seedling died due to the disease; B) Symptoms of Diplodia tip blight could be seen easily still in April 2022 in the field in young Scots pine (~15 years old); C) Symptoms of Diplodia tip blight recorded in November 2021 in 15 years old Scots pine.


**Fig. 2.** A) Mature Scots pine with Diplodia tip blight symptoms in the field, April 2022. B) The shoots were brown and dead needles were still attached to the tips.

were defoliated, sprayed with 70% EtOH, air dried and surface sterilized 1 min in 2.4% NaOCl. Thereafter, twigs were gently scraped to show the borderline of healthy and necrotic tissue and cut into pieces (~5 mm segments). The segments (containing bark, phloem, and sapwood) were plated on modified malt yeast peptone agar (MYP) (Langer 1994; Bußkamp et al. 2020). Maximum five twig segments were placed on MYP medium and incubated at room temperature and daylight. Emerging mycelia could be easily morphologically identified as *D. sapinea*. The hyphae are downy, turning from white to greenish/grey/black in few days. Representative *D. sapinea* strains are stored on MYP slants at 4 °C at Natural Resources Institute Finland (Luke).

The DNA were isolated from presentative strains (Table 1) by using PrepMan™ Ultra Sample preparation reagent (Applied Biosystems, Foster City, CA, USA) (Linnakoski et al. 2016). The ITS1-5.8S-ITS2 region of rDNA was amplified using primer pair ITS1-F (White et al. 1990) and ITS4 (Gardes and Bruns 1993). Briefly, PCR protocol was as follows: Dream Tag green mix (2X) (Thermo Scientific™), 200 µM dNTP, 0.5 µM primer 1, 0.5 µM primer 2, 1 µl of crude template DNA; the reaction was adjusted to 15 µl with autoclaved MQ $H_2O$. PCR conditions used 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.

**Table 1.** Site locations in Finland, sequence ID in GeneBank and necrosis length of the representative *Diplodia sapinea* strains.

| Location | Tree age status | Sequence ID in NCBI | Inoculation experiment, arithmetic necrosis length (cm) |
|---|---|---|---|
| Helsinki | mature | OP103745 | 1.03 |
| Borderline Helsinki-Sipoo | mature | OP103747 | 4.03 |
| Borderline Helsinki-Sipoo | sapling* | OP103746 | 1.57 |
| Vantaa | seedling | OP103744 | 1.60 |
| Uusikaupunki | mature | OP103748 | 2.00 |
| Naantali | mature | OP103743 | 2.03 |
| Naantali | seedling | OP103742 | 3.20 |

* 15-year-old Scots pines

Amplifications were confirmed on a 1.5% agarose gel (Ethidium Bromide staining), and the visual detection was made by ultraviolet transillumination. Samples were purified using the EXO-SAP (Exonuclease I–Shrimp Alkaline Phosphatase, Thermo Fisher Scientific, Waltham, MA, USA) protocol (Linnakoski et al. 2016) and sequenced using the ITS1 primer at Microsynth SEQLAB (Germany).

The *D. sapinea* ITS sequences are deposited in GenBank with accession numbers OP103742-OP103748 (Table 1). The species-specific primers DiSapi-F (3′- CCCTTATATATCAAACTATGCTTTGT-5′) (Adamson et al. 2021) and Diplo-R (3′-TTACATAGAGGATTGCCTTCG-5′) (Adamson et al. 2021) confirmed the species identification. PCR protocol was 0.2 U/µl DNA polymerase (DreamTaq DNA Polymerase, Thermo Scientific™), 1X PCR Buffer, 200 µM dNTP, 0.5 µM primer 1, 0.5 µM primer 2, 10 µl of crude DNA (diluted to 1:50 from the original extracted concentration), and finally the reaction was adjusted to 25 µl with MQ $H_2O$. PCR conditions were 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and 72 °C for 10 min (Adamson et al. 2021). Sterile MQ $H_2O$ were used as negative control. The correct species (*D. sapinea*) was determined by the visual detection (the formed band with a size of 500 bp) made by ultraviolet transillumination of DNA amplicons on a 1.5% agarose gel (1 hour, 120V) (Adamson et al. 2021).

In June 2022 one-year-old Scots pine seedlings were inoculated with seven *D. sapinea* strains (Table 1) as described in Blumenstein et al. (2021a, see Fig. 3). The terminal shoot growth length had ended, and terminal bud had developed. Briefly, mycelial plugs (5 mm diameter) were taken from a seven-day-old culture growing on malt yeast peptone (MYP) plates. The terminal bud of each seedling (three seedlings per strain) was cut (~0.2 cm) with scissors and the mycelial plug placed on the exposed area. The inoculated shoots were then wrapped with Parafilm® for seven days. Seedlings were kept outside for seven weeks and watered when needed. The observed symptoms were similar to those seen in the field (Fig. 3a). Each shoot inoculated with *D. sapinea* showed symptoms of the disease: browning of the shoots and death of the needles. Necrosis in phloem


**Fig. 3.** A) Disease symptoms of Scots pine seedling inoculated with *Diplodia sapinea*; B) Mock-control seedlings remained asymptomatic.

and sapwood was measured and *D. sapinea* was reisolated from infected parts as described above. The three control shoots, inoculated with MYP plugs, remained healthy (Fig. 3b) and attempts to isolate *D. sapinea* were made.

Data were analysed using the R, version 3.5.1. (R Core Team, 2019). A generalized linear model was constructed to evaluate the fixed effects of strain, seedling height and number of side shoots on the length of the necrosis. Differences were considered significant if $p$-value ≤ 0.05.

# 3 Results

## 3.1 Symptoms observed in the field

The symptoms of Diplodia tip blight were seen from July onwards (Fig. 1–3). Brown shoots (current-year growth) were declining towards the late summer (July–August), and they looked like drought symptoms. Similarly, needles turned brown from the base as the phloem died, and needles either shed or stayed attached to the shoot until spring (Fig. 1, 2). The blighted shoots could be observed until the following spring (Fig. 1, 2). The disease development in one shoot can easily be followed during the summer (browning of the needles and shoot, Fig. 1A).

## 3.2 Identification of the disease agent

*Diplodia sapinea* was isolated on the borderline of the living and dead phloem and sapwood. The inoculation of presentative strains to Scots pine seedlings lead to typical symptoms of Diplodia tip blight (Fig. 3). TheKoch's Postulates 2–4 were fulfilled for the putative causal agent *D. sapinea* because: (2) *D. sapinea* was the most commonly isolated fungi from the diseased Scots pine; (3) Inoculation of a healthy Scots pine with *D. sapinea* caused the disease; (4) *D. sapinea* was re-isolated from the inoculated, diseased Scots pine.

The blighted shoots observed in the field (Fig. 1, 2) were dying due to *D. sapinea*. No significant difference in the necrosis in the shoot was observed, based on the strain ($p = 0.0912$), seedling height ($p = 0.819$), or number of side shoot ($p = 0.283$). Necrosis varied between the strains (Table 1) and were much higher than in control plants (arithmetic length 0.1 cm). However, due to the high variation and low sample number no statistical differences were observed in the data.

# 4 Discussion and conclusion

This is the first report of Diplodia tip blight on Scots pine in Finland. The first symptoms were observed in autumn 2021 and after release of the bulletin by Luke (Luke, 15.11.2021) the information reached several forest owners. The number of the contacts from forest owners concerning the disease symptoms after March 2021 showed that this disease might be more common (E. Terhonen, personal observation) than previously thought. Here I report only the very first findings. The disease agent, *D. sapinea*, has been earlier found in Finland from cones (Müller et al. 2019) and from healthy twigs as an endophyte (Terhonen et al. 2021). *Diplodia sapinea* can turn from harmless to pathogenic causing Diplodia tip blight in stressed trees. The stress can be consequence from changes in environment (drought, temperature) (Oliva et al. 2021; Blumenstein et al. 2022) or related to biotic damage, e.g. *Heterobasidion annosum* s.s. (Fr.) Bref. disturbing the water supply (Dr. Gitta Langer, NW-FVA, pers. obs.). *Diplodia sapinea* is a previously unknown, most likely opportunistic, Scots pine pathogen in Finland, and the disease Diplodia tip blight has potential to

increase in the near future (Brodde et al. 2019). Symptoms can be identified in late summer, however, the confirmation for the fungal species still requires DNA based identification. The protocol presented in this research note can be shortened, e.g. DNA can be extracted from the phloem (preferably including also sapwood), followed by the species-specific primer identification (Adamson et al. 2021). The culture-based method using MYP was adapted from Bußkamp et al. (2020), used routinely to isolate *D. sapinea* in Germany. For the isolation studies, I recommend using MYP media over the commonly used 1.5% MEA (Malt Extract Agar) or PDA (Potato Dextrose Agar). The texture, growth rate and colour of this fungi differ between these medias, and MYP results easiest morphological identification. Our aim in Luke is to collect *D. sapinea* strains from all the areas where the typical disease has been observed. For this Luke continues to collect observations and samples from citizens of Finland.

## Declaration of openness of research materials

The research material is available from the author on reasonable request.

## Funding

This study was funded by Alfred Kordelin Foundation.

## Acknowledgements

The author would like to thank Katri Leino, Tuija Hytönen and Dr. Suvi Sutela for their excellent assistance in laboratory and in the field. Professor Jarkko Hantula is acknowledged for his support.

## References

Adamson K, Laas M, Blumenstein K, Busskamp J, Langer GJ, Klavina D, Kaur A, Maaten T, Mullett MS, Müller MM, Ondrušková E, Padari A, Pilt E, Riit T, Solheim H, Soonvald L, Tedersoo L, Terhonen E, Drenkhan R (2021) Highly clonal structure and abundance of one haplotype characterise the *Diplodia sapinea* populations in Europe and Western Asia. J Fungi 7, article id 634. https://doi.org/10.3390/jof7080634.

Blumenstein K, Bußkamp J, Langer GJ, Schlößer R, Parra Rojas NM, Terhonen E (2021a) *Sphaeropsis sapinea* and associated endophytes in Scots pine: interactions and effect on the host under variable water content. Front For Glob Change 4, article id 655769. https://doi.org/10.3389/ffgc.2021.655769.

Blumenstein K, Bußkamp J, Langer G, Langer E, Terhonen E (2021b) The Diplodia tip blight pathogen *Sphaeropsis sapinea* is the most common fungus in Scots pines' mycobiome irrespective of health status – a case study from Germany. J Fungi 7, article id 607. https://doi.org/10.3390/jof7080607.

Blumenstein K, Bußkamp J, Langer G, Terhonen E (2022) Diplodia tip blight pathogen's virulence empowered through host switch. Front Fungal Bio 3, article id 939007. https://doi.org/10.3389/ffunb.2022.939007.

Brodde L, Adamson K, Julio Camarero J, Castaño C, Drenkhan R, Lehtijärvi A, Luchi N, Miglio-

rini D, Sánchez-Miranda Á, Stenlid J, Özdağ Ş, Oliva J (2019) Diplodia tip blight on its way to the North: drivers of disease emergence in Northern Europe. Front Plant Sci 9, article id 1818. https://doi.org/10.3389/fpls.2018.01818.

Bußkamp J, Langer GJ, Langer EJ (2020) *Sphaeropsis sapinea* and fungal endophyte diversity in twigs of Scots pine (*Pinus sylvestris*) in Germany. Mycol Progress 19: 985–999. https://doi.org/10.1007/s11557-020-01617-0.

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for higher fungi and basidiomycetes: application to identification of mycorrhizae and rusts. Mol Ecol 2: 113–118. https://doi.org/10.1111/j.1365-294X.1993.tb00005.x.

Langer G (1994) Die Gattung Botryobasidium Donk (Corticiaceae, Basidiomycetes). Bibliotheca Mycologica 158. J Cramer, Berlin.

Larsson R, Menkis A, Olson Å (2021) *Diplodia sapinea* in Swedish forest nurseries. Plant Protect Sci 57: 66–69. https://doi.org/10.17221/68/2020-PPS.

Linnakoski R, Mahilainen S, Harrington A, Vanhanen H, Eriksson M, Mehtätalo L, Pappinen A, Wingfield M (2016) Seasonal succession of fungi associated with *Ips typographus* beetles and their phoretic mites in an outbreak region of Finland. PLoS ONE 11, article id e0155622. https://doi.org/10.1371/journal.pone.0155622.

Mäkisara K, Katila M, Peräsaari J (2022) The Multi-Source national forest inventory of Finland – methods and results 2017 and 2019. Natural resources and bioeconomy studies 90/2022. Natural Resources Institute Finland, Helsinki. http://urn.fi/URN:ISBN:978-952-380-538-5.

Müller MM, Hantula J, Wingfield M, Drenkhan R (2019) *Diplodia sapinea* found on Scots pine in Finland. For Pathol 49, article id e12483. https://doi.org/:10.1111/efp.12483.

Oliva J, Ridley M, Redondo MA, Caballol M (2021) Competitive exclusion amongst endophytes determines shoot blight severity on pine. Funct Ecol 35: 239–254. https://doi.org/10.1111/1365-2435.13692.

Terhonen E, Babalola J, Kasanen R, Jalkanen R, Blumenstein K (2021) *Sphaeropsis sapinea* found as symptomless endophyte in Finland. Silva Fenn 55, article id 10420. https://doi.org/10.14214/sf.10420.

White T, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal-RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, pp 315–322. https://doi.org/10.1016/b978-0-12-372180-8.50042-1.

*Total of 15 references.*