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Diplodia sapinea in Swedish forest nurseries

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Abstract: *Diplodia sapinea* is a common forest pathogen on *Pinus* spp. in a large part of the world. In 2013, disease caused by this pathogen on Scots pine (*Pinus sylvestris*) trees in Sweden was reported for the first time. In this study, we report the first detection of *D. sapinea* on diseased seedlings of *P. sylvestris* from two Swedish forest nurseries. Infected seedlings were collected July–November 2019. *Diplodia sapinea* was identified by morphological characteristics of fungal structures on plant tissues and from culture grown on Hagem agar media, followed by sequencing of fungal ITS rDNA. The result emphasizes the susceptibility of *P. sylvestris* seedlings. More research is needed to better understand the risk for disease spreading within forest nurseries and into the forest through infected plant material.

Keywords: fungal disease; ITS rDNA; pathogen; *Pinus sylvestris*; pine seedlings

In Sweden, ca. 370 million forest tree seedlings are produced annually and almost half of these constitute of *P. sylvestris*, which are primarily cultivated using a container system (www.skogsstyrelsen.se). In this system, intensive management practices (e.g. extensive monocultures, dense cultivation, chemical and mechanical weed and pest control, and shortage of beneficial organisms) may stress seedlings, thereby creating favourable conditions for the establishment and outbreak of fungal diseases.

Diplodia sapinea (Fr.) Fuckel [syn. *Diplodia pinea* (Desm.) Kickx., *Sphaeropsis sapinea* (Fr.) Dyko & Sutton] is a common fungal pathogen on *Pinus* spp. found in all continents (Phillips et al. 2013). Recent observations suggest that distribution of *D. sapinea* has expanded northwards and in 2013 it was reported for the first time in Sweden (Oliva et al. 2013). In 2016, an outbreak was observed in central Sweden, affecting a plantation of *P. sylvestris* the size of ca. 15 ha (Brodde et al. 2019).

Favoured conditions for *D. sapinea* are those that induce host stress, such as drought. Under such conditions, *D. sapinea* is known to cause severe damage on red pine (*Pinus resinosa*) and jack pine (*Pinus banksiana*) seedlings in forest nurseries in North America (Stanosz et al. 2007 and references therein). However, *D. sapinea* has to our knowledge never been reported causing disease on *P. sylvestris* seedlings. In Europe, *P. sylvestris* is one of the principal tree species that is mainly produced in forest nurseries for replantation of harvested forest stands. The lack of knowledge on both *D. sapinea* spread in forest nurseries and effective control measures poses a risk for production of healthy *P. sylvestris* stock. This study reports the first disease incidence by *D. sapinea* in Swedish forest nurseries, and highlights the need for further research in order to understand the potential risk of disease development and spread.

MATERIAL AND METHODS

Infected seedlings of *P. sylvestris* were detected from two forest nurseries in Sweden. Seeds were sown in March 2019 and seedlings were cultivated using an open container system with plastic trays elevated from the ground. During vegetation season, seedlings were regularly monitored for disease infections.

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The first two infected seedlings were collected 9th of July and 6th of August 2019, respectively, from a nursery in central Sweden (59°37.9181' N, 17°30.8624' E). No early stages of disease were observed until confirmed infected seedlings were detected. Surrounding area consisted of a mixed *P. sylvestris* and *Picea abies* forest stand, but also some broadleaved species were found there. Three more infected seedlings were later collected on the 6th of November 2019 from a nursery in southern Sweden (56°42.0931' N, 16°7.4479' E), where no early stages of disease had been observed until confirmed infected seedlings were detected as well. The area around this nursery consisted of mixed broadleaved and coniferous tree species, i.e. *P. sylvestris*, *P. abies*, and *Larix sibirica*. In addition, 20 *P. sylvestris* cones, 10 shoots from mature *P. sylvestris* trees, and 72 asymptomatic *P. sylvestris* seedlings were collected from the nursery in central Sweden and tested for the presence of *D. sapinea*. Similarly, 10 *P. sylvestris* cones, 10 shoots from mature *P. sylvestris* trees and 10 asymptomatic *P. sylvestris* seedlings were tested from the forest nursery in southern Sweden.

Fungal pycnidia on infected seedlings and conidia were analysed and photographed using the Leica dissection microscope (Leica M165 FC, Wetzlar, Germany) and Leica light microscope (Leica DM5500 B, Wetzlar, Germany), respectively. Infected needles were surface sterilized in 70% ethanol for 30 s, placed in 2% sodium hypochlorite for 5 min, and washed three times in sterile distilled water before being placed on Hagem-agar media (Stenlid 1985) in Petri dishes sealed with Parafilm (Bemis Company Inc., USA) in order to isolate fungal cultures. The petri dishes with needles were kept at ca. 21 °C in darkness and inspected daily for the outgrowth of fungal mycelia that was usually observed within three days of incubation. The outgrowing mycelia was subcultured to new Petri dishes and one isolate per seedling was used for species identification by ITS rDNA sequencing (Menkis et al. 2005). Needle and shoot samples from mature trees and seedlings of *P. sylvestris* as well as cone samples were subjected to DNA isolation and tested for the presence of *D. sapinea* using species-specific PCR assay (Smith & Stanosz 2006).

RESULT AND DISCUSSION

Morphological assessment showed that infected *P. sylvestris* seedlings had symptoms characteristic of *D. sapinea* i.e. round and pointy pycnidia on needles and stems (Figure 1A and B). The size of pycnidia was 0.3 ± 0.08 mm in diam. (average ± S.D. of 27 pycnidia). Conidia had an oval shape, were dark brown in colour, were characteristically pigmented and were 30.9 ± 2.1 × 11.5 ± 0.8 µm in size (average ± S.D. of 29 conidia; Figure 1C). Fungal mycelia on Hagem agar media was white at the beginning, but after 7–10 days became dark grey (Figure 1D) and agar media became complete black (Figure 1E). DNA analysis confirmed that all isolates were of *D. sapinea.* Sequences are available from Genbank under accession No. MT457611–M457614.

D. sapinea-infected seedlings from two geographically separated forest nurseries were found in this study. An early study from 1961 reports a *D. sapinea* finding in roots of nursery-grown *P. sylvestris* in Sweden, where Molin et al. (1961) referred to *D. sapinea* as a parasitic root fungus. However, their findings does not correspond to the current view. Furthermore, they did not provide any characterisation of the symptoms or fungal isolates. This suggest that the fungus reported by Molin et al. (1961) was probably another species. This study provides the first evidence that *D. sapinea* can cause disease on *P. sylvestris* seedlings in Swedish forest nurseries. Since no disease symptoms were detected during regular observations, seedlings were most likely infected about three to four weeks before detection. Moreover, *D. sapinea* has an incubation period of about three weeks until pycnidia are formed and maturate, which was visible on infected seedlings (Figure 1B). Diseased seedlings were noticeably smaller than the surrounding uninfected seedlings. This may suggest that seedlings infected by *D. sapinea* might have already been stressed and therefore became more susceptible to infection. In addition, these seedlings had not been exposed to any chemical treatment against fungal diseases which could explain why infection of *D. sapinea* were detected.

A species-specific PCR assay has showed the presence of *D. sapinea* on several cones and shoots of mature *P. sylvestris* trees growing in a radius of ca. 150 m distance from infected seedlings in each forest nursery, indicating that these may be the source of fungal inoculum. Among the 82 asymptomatic seedlings from both forest nurseries, a weak PCR band for *D. sapinea* was detected for a single seedling (1.2% of all seedlings tested) from the forest nursery in southern Sweden, suggesting an early stage of infection or the presence of conidia on

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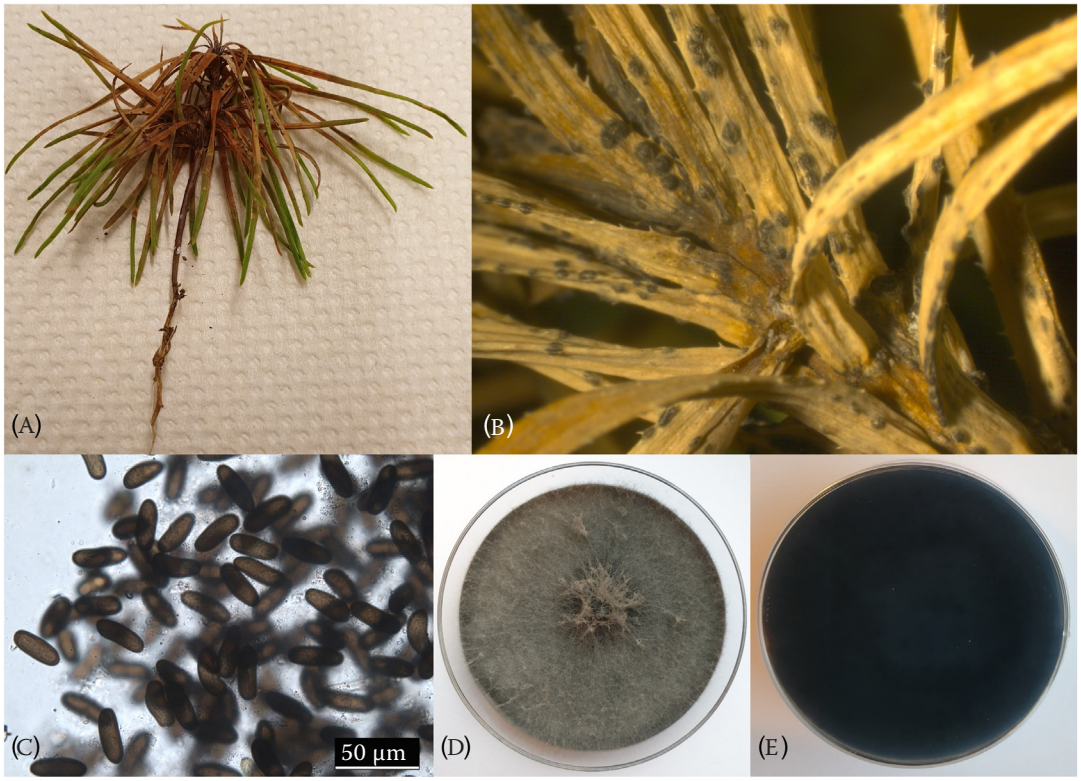

Figure 1. (A) One year-old *Pinus sylvestris* seedling infected by *Diplodia sapinea*, (B) characteristic pycnidia on needles and stem, (C) conidia of *D. sapinea*, 400 × magnification, (D) *D. sapinea* culture on Hagem medium with dark-grey mycelium on the surface and (E) black agar media on the reverse side

the surface. In case this is a latent infection, the rate of such infection would be much lower compared to earlier studies, since up to 20% of asymptomatic *P. banksiana* seedlings were shown to be infected by *D. sapinea* in forest nurseries in North America (Stanosz et al. 2007).

Since chemical pest control is commonly used within forest seedling production, this could explain the low level of latent infections. However, higher rates of latent infections in Swedish forest nurseries could have an impact on the spread of the disease. Changed conditions in the seedling production could pose a potential risk of increasing infection rate of *D. sapinea*, owing to for example drier and/or warmer weather conditions due to climate change or restrictions of the use of chemicals for disease control. Conditions that could stress seedlings, and thus predispose *D. sapinea* infections, should be considered in further studies.

Indeed, studies from North America have demonstrated that infected nursery stock can be responsible for disease outbreak in young pine plantations (Stanosz et al. 2007 and references therein). The result of this study emphasizes that more research is needed to understand the potential risk for disease outbreaks in nursery-grown *P. sylvestris*, the risk of latent infections and control strategies effective against *D. sapinea*.

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