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SHORT COMMUNICATION

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Differences in the virulence of *Sphaeropsis sapinea* strains originating from Scots pine and non-pine hosts

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Abstract

The virulence of 15 endophytic and pathogenic *Sphaeropsis sapinea* strains was tested towards Scots pine (*Pinus sylvestris*). Two-thirds of the strains had been isolated from Scots pines with varying health status: five isolates originated from healthy tissue (endophytic fungal stage) and five from diseased tissue (pathogenic stage). One-third of the strains were isolated from symptomatic tissues of non-pine hosts: black alder (*Alnus glutinosa*), European beech (*Fagus sylvatica*), European larch (*Larix decidua*), Norway spruce (*Picea abies*) and Douglas fir (*Pseudotsuga menziesii*). The *S. sapinea* strain isolated from black alder is the first proof that this fungus can form associations with alder species. On four-year-old *P. sylvestris*, one isolate per plant was inoculated on three side-shoots of seven plants in a greenhouse (21 inoculations/strain). Differences in necrosis size caused by the isolates were measured 55 days after inoculation. The pathogenic *S. sapinea* isolates originating from diseased Scots pine and from non-pine hosts were found to cause significantly longer necroses when compared to the endophytic isolates of *S. sapinea* from symptomless pines.

KEYWORDS
Alnus glutinosa, Botryosphaeriaceae, Diplodia tip blight, pathogenicity test, *Pinus sylvestris*, *Sphaeropsis sapinea*, virulence

1 | INTRODUCTION

Diplodia tip blight, caused by the opportunistic pathogen *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton (≡*Diplodia sapinea* (Fr.) Fuckel, =*Diplodia pinea* (Desm.) J. Kickx f.), is becoming increasingly important in Europe, especially due to the dry and warm years of 2018–2020 (Blumenstein et al., 2021). For forestry and the future cultivation of *Pinus sylvestris*, it is important to understand the host–pathogen relationship. *S. sapinea* can live endophytically in symptomless pines. Fungal endophytes usually do not cause disease symptoms in their host plants *per se* and can even enhance the host immune system (Terhonen et al., 2019). Triggered by abiotic factors, *S. sapinea* can become pathogenic or saprophytic. In most cases, drought stress in pines leads to *Diplodia* tip blight outbreaks (Blumenstein et al., 2021). Research on the disease has emphasized and focused on the abiotic factors that can trigger pathogenic development (Blumenstein et al., 2021). Flowers et al. (2001) investigated the virulence of latent *S. sapinea* isolates on Black pines (*Pinus nigra*)—as well as isolates from symptomatic colonization. However, the virulence and disease potential of *S. sapinea* strains from different origins have received little attention. Previous studies have postulated that endophytic strains are as virulent as strains from diseased tissue (Flowers et al., 2001; Stanosz et al., 2007).

The aim of this study was to test for differences in the virulence of endophytic and pathogenic *S. sapinea* isolates, originating from

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Scots pine and several non-pine hosts. Strains originating from non-pine hosts may have an influence on the disease epidemics. In this study, 15 *S. sapinea* isolates of different origins (from healthy [asymptomatic] and diseased [symptomatic] tissues of Scots pine) and from different deciduous and coniferous tree species were tested for virulence on *P. sylvestris* in a controlled greenhouse experiment.

2 | MATERIALS AND METHODS

Fifteen *S. sapinea* strains originating from six different host species (Table 1) were inoculated onto 125 three-year-old *P. sylvestris* plants under greenhouse conditions. The strains were isolated from asymptomatic tissue representing endophytic strains or from symptomatic tissue representing pathogenic strains (Table 1). All *S. sapinea* strains used in this study were identified based on morphology and ITS sequences. The ITS sequences (containing ITS1, 5.8S and ITS2 regions) have been deposited in GenBank (Table 1), and all of the strains are permanently stored in the NW-FVA strain collection.

The Scots pine plants (single provenance: HKG 85103 Heide and Altmark, Germany, tree nursery Reinke, Rellingen, Germany) were observed to be in very good health at the beginning of the experiment, and no symptoms of Diplodia tip blight were visible. The inoculation experiment was performed at the University of Goettingen, Germany (51°33′28.4″ N 9°57′30.5″ E), from November 2020 until January 2021 (55 days). Altogether, 130 plants were randomly assigned to three benches and maintained at 14°C mean air temperature (min 5°C and max 24°C). As *S. sapinea* can infect pines without causing symptoms in the endophytic stage, we chose five trees randomly (leaving 125 for inoculations) and examined the branches, stems and needles before the inoculation experiment for possible colonization by *S. sapinea*. Therefore, plant material was surface-sterilized (1 min in 70% EtOH/5 min 4% NaOCl/1 min 70% EtOH) and cut into pieces (0.5 cm). These pieces were plated on a Petri dish of malt yeast peptone (MYP) agar, three units per Petri dish. After 7 and 14 days, dishes were checked for the presence of *S. sapinea* and other outgrowing fungi.

The plants were watered regularly. For each *S. sapinea* isolate, three side branches on seven trees were inoculated. Only one isolate was used for the inoculation per plant, resulting in 21 replicates per inoculated *S. sapinea* strain. For this purpose, the first centimetre of the shoot tip was cut off from the terminal tip side of the shoot, and an agar plug with *S. sapinea* (fungus-side-down) was placed on the freshly cut shoot and wrapped with Parafilm®. The *S. sapinea* strains had been cultured on 1.5% MYP (malt yeast peptone) agar for one week prior to the inoculations. For a more detailed protocol, see Blumenstein et al. (2021). Ten pines were treated identically but with pure MYP agar as a control. Ten pines were left untreated.

TABLE 1 Isolates of *S. sapinea* used in this study: original host, Northwest German Forest Research Institute (NW-FVA) strain identification and collection number, sampling locality, year of isolation, NCBI accession number and necrosis length

Original host	NW-FVA No.	Locality	Isolation year	NCBI accession number	Necrosis length, cm (arithmetic mean)
Pine host					
Pinus sylvestris (asymptomatic, endophytic)	2364	Hesse	2014	MW529089	3.9
	2697	Saxony-Anhalt	2015	MW529090	1.8
	2702	Bavaria	2015	MW529091	1.5
	2715	Baden-Wuerttemberg	2015	MW529092	4.6
	5697*	Lower Saxony	2020	MW529100	5.6
Pinus sylvestris (symptomatic, pathogenic)	5305	Saxony-Anhalt	2019	MW529094	8
	5306	Saxony-Anhalt	2019	MW529095	6.9
	5329	Lower Saxony	2019	MW529096	3.6
	5747	Saxony-Anhalt	2020	MW529101	2.9
	5772	Saxony-Anhalt	2020	MW529102	4.7
Non-pine host (symptomatic)					
Alnus glutinosa	5654	Mecklenburg-Western Pomerania	2020	MW529099	5.4
Fagus sylvatica	4932	Hesse	2019	MN698984	3.1
Larix decidua	3626	Lower Saxony	2016	MW529093	7.5
Picea abies	5355	Saxony-Anhalt	2020	MW529098	7.7
Pseudotsuga menziesii	5346	Hesse	2019	MW529097	5.3
Agar control	MYP	-	-	-	0.2
Untreated control	-	-	-	-	0

Note: Strains were isolated from woody tissue, except number 5697 which was isolated from a needle*.

At the end of the experiment, lesion lengths were measured with a ruler in the vertical direction to an accuracy of 1 mm. The bark was gently peeled back to expose any necrosis in the phloem. After measurements, three seedlings were selected at random from each fungal or control treatment. To confirm infection and that Koch's postulates were fulfilled, pieces from the interface between necrotic and healthy tissue were surface-sterilized (as described above) and plated on MYP agar. The fungi used in the inoculations had been identified by ITS sequencing.

Differences in length of the necrosis caused by different strains and categories (control, strains isolated as endophytic or pathogenic, from *P. sylvestris* or other non-pine trees) were investigated using ANOVA and Tukey's HSD post hoc test. All data analyses were conducted in R, version 3.6.2. Differences were considered significant if the *p*-value was below 0.01.

3 | RESULTS AND DISCUSSION

Sphaeropsis sapinea is known to cause necrosis mainly in species of Pinaceae and Cupressaceae. According to CABI, our own studies and Smahi et al. (2017) the causal agent of *Diplodia* tip blight can be isolated endophytically or from symptomatic tissues of Betulaceae, Viscaceae and Fagaceae including *Fagus sylvatica* and *Quercus suber*. This study is the first report of *S. sapinea* associated with diseased tissue of *Alnus glutinosa* (Betulaceae). The infected alder host tree, located in north-eastern Germany, had poor vigour due a complex disease pattern induced mainly by heat and drought.

3.1 | Pre-experiment detection of *Sphaeropsis sapinea*

No *S. sapinea* was isolated from the Scots pine nursery plants used in the inoculations, although 8 fungal morphotypes were identified in pre-isolations. All isolates were identified based on morphology. Almost half of the tissue samples analysed (n = 140 units) were colonized by *Phoma* species (47%), followed by *Alternaria* spp. (30%), *Microsphaeropsis olivacea* (21%), *Epicoccum* sp. (5%), *Fusarium* sp. (2%) *Sordaria* sp. (1%), *Hypoxylon fragiforme* (1%) and *Diaporthe* sp. (>1%).

3.2 | Re-isolation of *Sphaeropsis sapinea*

Sphaeropsis sapinea was re-isolated from all inoculated shoots after completion of the experiment. At the end of the experiment, no *S. sapinea* was detected in the controls that were treated with MYP or left untreated.

3.3 | Necroses lengths

All shoots inoculated with *S. sapinea* showed symptoms of infection: browning of the shoots and death of the needles. All control plants remained healthy and symptomless. Some of the shoots treated with MYP showed minor discoloration of the phloem (0.2 cm arithmetic mean length) at the position where the shoot tip was excised. The necrosis lengths caused by the endophytic strains of *S. sapinea* were significantly smaller (3.5 cm arithmetic mean length) than those caused by strains isolated from symptomatic *P. sylvestris* tissues (5.2 cm arithmetic mean length) and from non-pine hosts (5.8 cm arithmetic mean length).

The endophytic strains of *S. sapinea* caused necrosis and clearly could cause the Diplodia tip blight symptoms. Significant differences in necrosis size were also evident within the categories (Figure 1). There was variation between necrosis caused by the endophytic strains. The isolate obtained from a needle caused significantly longer necroses compared to endophytic strains from twigs (NW-FVA 2702 and NW-FVA 2697) (Figure 1). However, no differences in necrosis length were observed between endophytic strains isolated from woody tissues (Figure 1). The largest necrosis (by median) was caused by the isolate from *Larix decidua* (NW-FVA 3626), followed by the one from *Picea abies* (NW-FVA 5355, Figure 1) and by pathogenic strains from *P. sylvestris* (NW-FVA 5305). Within the tested group of non-pine host strains, *S. sapinea* isolated from beech woody tissues exhibited the lowest virulence.

Necrosis length could be influenced by culture age and thus the vitality of the inoculated strains, due to loss of virulence of *S. sapinea* during storage in artificial conditions. Other influencing factor can be isolate origins: all tested endophytic strains were isolated from woody tissue, with the exception of the one from needles (NW-FVA 5697). The needle endophyte caused the longest necroses within the endophytic category (Table 1 and Figure 1). This strain had not established an endophytic interaction with the host in the woody tissue. Pathogenic strains from symptomatic woody tissues were isolated more recently (in 2019 and 2020), except for the isolate from larch which was obtained in 2016 and caused the second longest necroses (Table 1 and Figure 1). A further aspect to consider is that the temperature during the experiment (mean temperature 14°C) was not optimal for growth of *S. sapinea*, which is between 25 and 30°C *in vitro* (Bußkamp, 2018).

In work by Flowers et al. (2001), 66 *Pinus nigra* plants were inoculated with *S. sapinea* isolates of different origin (latent from *P. nigra*, from diseased tissue of *P. nigra* and latent from *P. sylvestris*). Similar to the results in our study, Flowers et al. (2001) concluded that latent *S. sapinea* isolates are as virulent as those from diseased tissue. Stanosz et al. (2007) also examined the virulence of *S. sapinea* isolated endophytically, finding that these strains cause symptoms on red pine (*Pinus resinosa*) and Jack pine (*Pinus banksiana*). Our study also shows that endophytic strains caused necrosis in Scots pine; however, the necrosis caused by pathogenic strains was clearly more severe (Figure 1).

The results of this study could indicate that it is not only the vitality of the host tree that is important in the development of disease, but also the virulence of the *S. sapinea* strain. One explanation for the lower virulence of the endophytic strains could be that the fungus is in a different 'mode' when living endophytically inside host tissues.

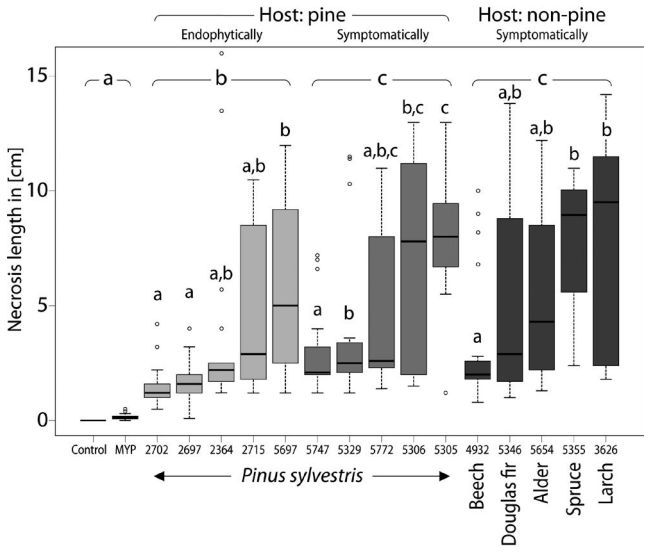


FIGURE 1 Necrosis caused by the 15 *S. sapinea* strains isolated from healthy tissues (endophytically) or diseased tissues (symptomatically) from Scots pine and diseased (symptomatically) non-pine host (different categories: from *Pinus sylvestris* endophytic or symptomatic and from other non-pine trees), 55 days after inoculation. Boxplot of necrosis length describing the necrosis measured from $n = 21$ branches per strain, and control (non-treated) and MYP (agar plug) =30 branches analysed. Different letters (a-c) indicate significant differences at $p < .01$ for grouped data, between four categories: control group (MYP and untreated control) – strains from *Pinus sylvestris*: endophytic and symptomatic and from non-pine host. Significant differences within the categories are also indicated with different letters (a-c) at $p < .01$

However, as it can cause necrosis in its host, *S. sapinea* can switch lifestyle to pathogenic most probably triggered by abiotic disturbances (Blumenstein et al., 2021). Perhaps the fungus only releases compounds toxic to the host when the tree is stressed, or it is possible that this fungus shows variable virulence between strains. The endophytic life strategy may help *S. sapinea* proliferate in new areas (Terhonen et al., 2021) and similarly in new hosts (in this study: alder).

Our research indicates that a large proportion of *S. sapinea* strains can colonize Scots pine and other alternative hosts. The isolates from alternative hosts caused severe symptoms in Scots pine. The potentially wide host range, high aggressiveness of the isolates and increasing disease severity under drought stress (Blumenstein et al., 2021) make *S. sapinea* a high-risk pine pathogen. However, it is not known whether the latent strains are as virulent on hosts under drought stress as the strains from diseased tissue. As this was the pilot study, in the future we will repeat this experiment with newly isolated endophytic strains combined with different water availabilities to induce host stress (Blumenstein et al., 2021). We aim to test whether *S. sapinea* can switch its lifestyle from endophytic and become more pathogenic.

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PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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