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Sphaeropsis sapinea and Associated Endophytes in Scots Pine: Interactions and Effect on the Host Under Variable Water Content

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The ascomycete *Sphaeropsis sapinea* is the causal agent of the Diplodia Tip Blight disease on pines and other conifer species. This fungus has a symptomless endophytic life stage. Disease symptoms become visible when trees have been weakened by abiotic stress, usually related to warmer temperatures and drought. Currently, this disease is observed regularly in Scots pine (*Pinus sylvestris*) sites in parts of Europe, such as Germany, increasing dramatically in the last decade. Changes in climatic conditions will gradually increase the damage caused by this fungus, because it is favored by elevated temperature. Thus, host trees with reduced vitality due to climate change-related environmental stress are expected to be more susceptible to an outbreak of Diplodia Tip Blight disease. There is currently no established and effective method to control *S. sapinea*. This project aims to reveal the nature of the endophyte community of Scots pine. Utilizing the antagonistic core community of endophytes could serve as a novel tool for disease control. Results from this study provide a starting point for new solutions to improve forest health and counter *S. sapinea* disease outbreaks. We screened potential antagonistic endophytes against *S. sapinea* and infected Scots pine seedlings with the most common endophytes and *S. sapinea* alone and combination. The host was stressed by limiting access to water. The antagonism study revealed 13 possible fungi with the ability to inhibit the growth of *S. sapinea in vitro*, for example *Sydowia polyspora*. None of the tested co-infected fungi (*Desmazierella acicola, Didymellaceae* sp., *Microsphaeropsis olivacea, Sydowia polyspora, and Truncatella conorum-piceae*) showed strong necrosis development *in vivo*, even when host stress increased due to drought. However, the infection experiment demonstrated that drought conditions enhance the effect of the disease outbreak, triggering *S. sapinea* to cause more necrosis in the infected twigs.

Keywords: Diplodia tip blight, climate change, antagonism, infection, drought, *Sphaeropsis sapinea, Sydowia polyspora*

INTRODUCTION

Climate change is a potential driver of adverse effects on forest health. With climate change, there is an increase in drought-associated stress, rendering trees more susceptible to threats such as pests and pathogens which can compromise their overall health (Bußkamp, 2018; Brodde et al., 2019; Terhonen et al., 2019b). Scots pine (*Pinus sylvestris* L.) is one of the most economically important forestry tree species in Europe. Changes in the environment, especially those related to drought, will affect Scots pine pathosystems. The ascomycete fungus *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton (≡ *Diplodia sapinea* (Fr.) Fuckel, *Diplodia pinea* (Desm.) J. Kickx f.) is recognized as the most widespread necrotrophic pathogen responsible for dramatic losses of *Pinus* species across the continents, causing a disease called Diplodia tip blight (Smith et al., 1996; Fabre et al., 2011; Bußkamp, 2018; Paez and Smith, 2018; Brodde et al., 2019). The correct name of this anamorphic Botryosphaeriaceae is in discussion and it is epityped (de Wet et al., 2003; Phillips et al., 2013), but the current name after Index Fungorum is still *S. sapinea.* The preferred name after the EPPO Global Database, however, is *Diplodia sapinea*[1].

Recently, the disease Diplodia tip blight has been increasing in Germany, especially after the hot and dry years of 2018 and 2019 (Blumenstein et al., 2020). *Sphaeropsis sapinea* has a latent endophytic stage (Burgess et al., 2004; Flowers et al., 2006; CABI, 2019; Bußkamp et al., 2020; Terhonen et al., 2021) and disease symptoms become visible when trees have been weakened by stress, usually related to temperature and drought, allowing *S. sapinea* to switch its lifestyle from endophytic to pathogenic (Blodgett et al., 1997a; Stanosz et al., 2001; Blumenstein et al., 2020). The production of reactive oxygen species (e.g., hydrogen peroxide, H_2O_2) and the accumulation of free amino acids are common plant responses to drought (Sherwood et al., 2015). Because these drought-induced perturbations in metabolism also often occur during pathogenic attack, they may be partly responsible for an enhanced susceptibility to fungal pathogens (Desprez-Loustau et al., 2006; Sturrock et al., 2011; Sherwood et al., 2015) or lifestyle-switch. Nevertheless, it is not known in detail which fungal or host genetic factors are responsible in the lifestyle-switch of *S. sapinea*.

The term "endophyte" is used to describe microbes that live asymptotically inside plant tissues for the entire or at least a significant part of their life cycle without causing any clear negative harm to the host (Petrini, 1991; Saikkonen et al., 1998). Although endophytes in conifers have been studied intensively (e.g. Bußkamp, 2018; Bußkamp et al., 2020; e.g., Saikkonen et al., 1998; Sieber, 2007; Terhonen et al., 2019b), generally the interaction between different endophytes and their host remains poorly understood (Sieber and Grünig, 2013; Witzell et al., 2014; Compant et al., 2016; Terhonen et al., 2018; Witzell and Martín, 2018). Several endophytes are regarded as parasites tolerated by their host or as opportunistic or latent pathogens (Sanz-Ros et al., 2015). Endophytes can also act as primary colonizers and may remain in a physiological resting phase in their host. For example *S. sapinea* is a typical member of the fungal community of natural pruning of branches defined by Butin and Kowalski (1990). Growing inside their hosts' tissues, they can form fruiting bodies and spores (Chapela and Boddy, 1988; Griffith and Boddy, 1988; Kehr, 1998; Osono, 2006; Oses et al., 2008; Sanz-Ros et al., 2015). The question is, how do endophytes overcome a host's defenses and colonize them? This could either be due to the secretion of metabolites by the endophyte (Peters et al., 1998; Schulz et al., 2015, 2002) or by changing the phytohormone balance in the tree (Navarro-Meléndez and Heil, 2014). It is also conceivable that the endophyte defeats the metabolism of the tree by secreting lyzing enzymes (Schulz et al., 1998; Suryanarayanan et al., 2012). Sherwood et al. (2015) proposed a new model of fungal infection of plants based on interactions of the metabolisms of the free amino acid proline and H_2O_2 with drought. The authors showed that droughted Austrian pine (*Pinus nigra* Arnold) accumulated hydrogen peroxide in shoots. Hydrogen peroxide is toxic to *S. sapinea*, but the infection of the droughted Austrian pine with this pathogen led to a reduction in the H_2O_2-concentration in the host plants. *S. sapinea* is able to produce catalase and peroxidase in response to the oxidative stress by H_2O_2. Proline is a preferred nitrogen source for *S. sapinea in vitro* and it increased in the plant as well in response to drought as and in response to infection with *S. sapinea*. Sherwood et al. (2015) conclude from their results that the proline precursor, glutamate, protects *S. sapinea* from hydrogen peroxide damage.

In vitro, endophytes produce a variety of secondary metabolites such as herbicides, fungicides and antibiotics (Schulz et al., 2002; Kusari et al., 2012). The metabolites may serve the plant to maintain numerous balances: between endophytes and host, but also between other endophytic fungi and bacteria. The endophytic stage represents a balanced interaction between the fungus and its host. However, endophytic fungal species can become pathogens when this balance is disturbed or saprotrophs if the host dies (Müller and Krauss, 2005; Rodriguez et al., 2009; Bußkamp, 2018; Terhonen et al., 2016, 2019a).

Fungal endophytes are known to contribute to the health of plants, acting as growth promotors that synthesize phytohormones; in addition, they can potentially protect plants from pathogenic fungi by their anti-fungal activity (Witzell et al., 2014; Terhonen et al., 2018, 2019b). In conifer trees, it has been shown that inoculations with fungal endophytes protect the host from natural infection by *Dothistroma septosporum* (Dorogin) M. Morelet (Ridout and Newcombe, 2015). Consequently, several metabolites with antifungal properties have been isolated from foliar endophytes of *Picea rubens* Sarg. and *Picea mariana* (Mill.) Britt., E.E. Sterns & Poggenburg (McMullin et al., 2017). These results support the hypothesis that fungal endophytes may enhance the tolerance of the host tree to fungal pathogens (Sumarah et al., 2011, 2015; Richardson et al., 2014; Tanney et al., 2016; Oliva et al., 2021). Some endophytes can also be used as antagonists against potential pathogens (Arnold et al., 2003; Ganley et al., 2008; Rungjindamai et al., 2008; Martín et al., 2015). Whether an endophyte acts

[1]https://gd.eppo.int/taxon/DIPDPI

as an antagonist or competes with a particular pathogen, can be studied *in vitro* using so-called antagonism assays (Santamaría et al., 2012; Blumenstein, 2015; Romeralo et al., 2015; Bußkamp, 2018; Rigerte et al., 2019). These findings are extremely important as, in the future, the use of beneficial endophytes that can act as biocontrol agents against pathogens, in this case against *S. sapinea*, may be a valuable approach to disease control. Potential antagonistic fungi, such as *Sydowia polyspora* (Bref. & Tavel) E. Müll., *Alternaria* sp. and *Epicoccum nigrum* Link, were recently discussed in a study by Oliva et al. (2021).

Climate change could transform *S. sapinea* into a global threat to forest health, as the growth rate will be favored by climate warming (Fabre et al., 2011). Indirect effects (temperature, drought) are important because susceptibility of pines to *S. sapinea* is strongly enhanced by water stress (Blodgett et al., 1997a,b; Stanosz et al., 2001; Desprez-Loustau et al., 2006; Bußkamp, 2018). Hence, understanding how the lifestyle of fungi switches due to environmental stress is critical for deciphering the evolution of host–microbe interactions (Kuo et al., 2014). Fungal lifestyles are not in that sense stable but dynamic, and are likely to be influenced by the genetics of the fungal species, host factors and changing environments (Kuo et al., 2014). Endophytes may have evolved to switch their lifestyles to adapt different environmental conditions (Kuo et al., 2014). *S. sapinea* can be considered a very good model to test the direct effects of environmental changes on a fungal pathogen (lifestyle-switch), as well as indirect effects on the host susceptibility (stress). The high levels of Diplodia tip blight symptoms in forest stands are not due to a high abundance but rather depend on environmental conditions (Feci et al., 2002; Bußkamp, 2018; Blumenstein et al., 2020). The impact of climate change could shift the endophyte communities and will probably also affect the nature of fungal pathogens. Increased temperature may mean that host resistance to disease may be overcome more quickly as a result of rapid disease cycles. For *S. sapinea*, warmer temperatures will be suitable for accelerated growth and reproduction. To mitigate the impacts of climate change, understanding the factors that trigger development of forest tree disease epidemics will be essential.

The aims of this study on the pathosystem of *S. sapinea* and *P. sylvestris* were (1) to identify fungal Scots pine endophytes which may have the ability to antagonize the pathogen *S. sapinea in vitro*, (2) to test how the strength of drought-induced stress due to different levels of water availability effects potential disease symptoms, measured by necrosis length, in inoculation tests performed with *S. sapinea* and selected Scots pine endophytes *in planta*, and (3) to investigate whether there are potential remote influences between Scots pine endophytes and *S. sapinea* in an *in vivo* experiment. Aims 2 and 3 were tested in a greenhouse experiment. We infected Scots pine saplings with *S. sapinea* and selected endophytes from pine that are either reported to be latent pine pathogens or putative antagonistic endophytes. The host plants were inoculated with a single test strain or combined with different fungal strains on a separate twigs.

MATERIALS AND METHODS

Test for Antagonistic Endophytes Against *S. sapinea*

Fungal Material: Isolates From Scots Pine Tips

In this study, 30 fungal isolates (two *Sphaeropsis sapinea* strains and 28 endophytes) were used for either the antagonism assay or inoculations (**Table 1**). The fungal strains chosen are regularly isolated from Scots pine tips in German forests according to Bußkamp et al. (2020). Origin and locations of the strains are described in Blumenstein et al. (2020). One strain (*Didymellaceae* sp., NW-FVA ID 5756) originated from a sample of the nursery pine plants (see section "Pre-experiment Detection of Endophytes Including *Sphaeropsis sapinea* in Plant Material") that were used in this study for the antagonism assay.

In vitro Study: Antagonism Assay

In order to identify endophytic fungal isolates that may have the ability to antagonize the pathogen *S. sapinea* inside the pine trees' tissue (*in planta)*, the interactions of 27 isolated pine endophytes (Blumenstein et al., 2020) against two different *S. sapinea* strains were investigated by means of a paired-growth assay (antagonism assay *in vitro*) as described in Rigerte et al. (2019). The ability of an endophyte to antagonize the pathogen was determined based on the inhibition level (defined as pathogen growth with and without the endophyte) over a given period of time. This was achieved by assessing and measuring the concurrent growth of the pathogens on 1.5% Malt-Yeast-Peptone (MYP-agar, after Langer (1994)) nutrient medium (pH = 6) and the assessment by eye of the growth behavior of the paired fungal strains. The latter observations were categorized as follows: (1) Inhibition of *S. sapinea* growth (**Figure 1A**), (2) endophyte superior = the endophyte has overgrown and inhibited the mycelium of *S. sapinea* (**Figure 1B**), (3) Equal growth capability = the tested endophyte and *S. sapinea* did not overgrow or obviously inhibit each other (**Figure 1C**), and (4) *S. sapinea* superior = *S. sapinea* inhibited and may also have overgrown the tested endophyte strain (**Figure 1D**). The endophyte and pathogen were placed opposite each other on the surface of MYP agar plates within 2.1 cm of the perimeter of the petri dish (**Figure 2**; Rigerte et al., 2019). Such pairings were prepared in triplicate. Control plates containing only the pathogen (located identically on the plate as in the paired setup) were also prepared. All these plates were then incubated under the same conditions: room temperature (ca. 22°C) and diffuse daylight. Growth was assessed 3, 7, and 10 days after inoculation. Measurements were performed with a ruler for both the endophytes and the pathogens in the antagonism test plate. For analysis of the data and to determine whether the presence of an endophyte had an effect to the pathogen, a *t*-test to compare the pathogens' growth (alpha direction, **Figure 2**) after 10 days with the growth of the control was performed with SPSS version 26.0 (IBM Corporation, New York, United States).

Greenhouse-Study

Four endophytes, *Desmazierella acicola* Lib., *Microsphaeropsis olivacea* (Bonord.) Höhn., *Sydowia polyspora*, *Truncatella*

TABLE 1 | All isolates used in antagonist study or/and inoculation study, their accession numbers and observations against *S. sapinea*.

			Antagonist test results		
Isolate ID (NW-FVA)	**Gene Bank Accession No.**	**Species name**	**Visual observation**	**Paired *t*-test based on measured growth along two axes**	
5756	MW365344	*Didymellaceae* sp.	NA	Endophyte versus *S. sapinea*	
4739	MT790326	*Sphaeropsis sapinea*	NA	*S. sapinea*, strain 4739	*S. sapinea* strain 4740
4740	MT790327	*Sphaeropsis sapinea*	NA	*p*-Value	*p*-Value
4741	MT790316	*Diaporthe sp.* 1	Equal growth capability	0.008	0.004
4742	MT790328	*Sydowia polyspora*	Inhibition of pathogen growth	0.010	0.005
4743	MT790320	*Microsphaeropsis olivacea*	Equal growth capability	0.014	0.002
4744	MT790317	*Epicoccum nigrum*	Equal growth capability	0.011	0.005
4745	MT790329	*Truncatella conorum-piceae*	Pathogen superior	0.018	0.000
4746	MT790311	*Alternaria alternata*	Inhibition of pathogen growth	0.019	0.006
4747	MT790325	*Rosellinia* sp.	Endophyte superior	0.017	0.003
4748	MT821234	*Xylaria polymorpha*	Endophyte superior	0.016	0.005
4749	MT821235	*Fusarium* sp.	Equal growth capability	0.015	0.005
4750	MT821236	*Nemania diffusa*	Inhibition of pathogen growth	0.019	0.007
4751	MT790315	*Desmazierella acicola*	Equal growth capability	0.016	0.005
4753	MT821237	*Diaporthe* sp. 2	Inhibition of pathogen growth	0.013	0.004
4754	MT790312	*Biscogniauxia mediterranea*	Endophyte superior	0.016	0.008
4755	MT790318	*Hypoxylon fragiforme*	Equal growth capability	0.021	0.005
4756	MT790313	*Biscogniauxia nummularia*	Equal growth capability	0.036	0.008
4757	MT790324	*Pyronema domesticum*	Endophyte superior	0.011	0.004
4758	MT790314	*Botrytis cinerea*	Equal growth capability	0.013	0.004
4759	MT790323	*Pseudocamarosporium brabeji*	Equal growth capability	0.025	0.003
4760	MT790321	*Nemania serpens*	Pathogen superior	0.029	0.006
4761	MW365343	*Pezicula eucrita*	Inhibition of pathogen growth	0.024	0.005
4762	MT790322	*Preussia funiculata*	Inhibition of pathogen growth	0.032	0.009
4763	MT821238	*Mollisia sp.*	Pathogen superior	0.037	0.006
4764	MT821239	*Preussia sp.*	Inhibition of pathogen growth	0.011	0.002
4765	MT790319	*Jugulospora rotula*	Endophyte superior	0.010	0.003
4766	MT821240	*Daldinia sp.*	Endophyte superior	0.011	0.003
4767	MT790330	*Therrya fuckelii (*strain 6)	Pathogen superior	0.019	0.006
4768	MT821241	*Therrya fuckelii* (strain 7)	Pathogen superior	0.126	0.005

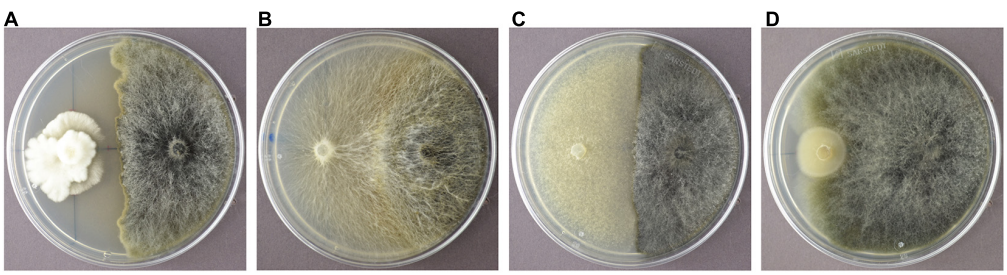

FIGURE 1 | Examples of the different reactions of the endophyte (white mycelium in examples A-D) during the antagonism assays growing in dual culture with *S. sapinea* (brownish-gray morphology). **(A)** *Nemania diffusa* (4750) vs. *S. sapinea*: inhibition of *S. sapinea* growth; **(B)** *Biscogniauxia mediterranea* (4754) vs. *S. sapinea*: endophyte superior; **(C)** *Botrytis cinerea* (4758) vs. *S. sapinea*: equal growth; **(D)** *Mollisia* sp. (4763) vs. *S. sapinea*: *S. sapinea* superior.

conorum-piceae (Tubeuf) Steyaert, and *S. sapinea* were chosen for the greenhouse inoculation experiment. We compared the infection capacity of these fungi under variable water content on infected pine trees both alone and in combination. Combined infections were set up so that different twigs of the plants were infected with different fungi, to test for possible remote synergistic effects of the endophytic fungi on *S. sapinea*. Additionally, one endophyte (*Didymellaceae* sp.) was included in the study that was isolated with the highest frequency in pre-experiment detection of endophytes (section "Pre-experiment

Detection of *Endophytes Includ*ing Sphaeropsis sapinea in Plant Material").

Plant Material

The plant material consisted of 1008 2-year-old, apparently healthy and vital, Scots pine (*P. sylvestris*) seedlings purchased from Niedersächsische Landesforsten Services GmbH (seeds originated from the collin Black Forest area, Germany) and LIECO GmbH & Co KG. Seedlings were potted into plastic containers arranged in trays with six containers / tray. These were filled with fertilized peat (Flora gard, TKS®2 Instant Plus and PERLIGRAN® Extra 2-6mm, Hermann Meyer KG, Rellingen, Germany). Containers were placed on tables covered with plastic sheets where excess water could accumulate and be absorbed later. The seedlings were acclimatized to the greenhouse conditions for 30-days prior to receiving water according to their treatment group (section "Experimental Design of the Green-House Study"), during the initial period they received tap water as required to maintain moist soil. No additional fertilization was given during the experiment.

Pre-experiment Detection of Endophytes Including *Sphaeropsis sapinea* in Plant Material

Six seedlings were examined for pre-colonization of the internal woody tissues by *S. sapinea* and to reveal the endophyte community. The methodology followed the procedures described in Bußkamp et al. (2020). The complete stem and all shoots of each plant were investigated by culture-based isolation of endophytes. Needles were removed and the tips und stem were surface sterilized (1 min in 70% ethanol/5 min 4% sodium hypochlorite/1 min 70% ethanol) and cut into segments (0.5 cm). Three segments were plated on a petri dish filled with 1.5% MYP-agar. In total, 457 woody plant segments were plated (Ø 76 segments, min. 50, max. 122). Additionally, discolored or dead needles that were found occasionally were checked for infection with *S. sapinea*. Discolored needles (n = 6 needles, =62 plated tissue segments) were treated according to the same procedure as the woody plant part, except with a shorter 1 min sterilization in 4% sodium hypochlorite. Dead needles were incubated in a moist chamber for potential formation of pycnidia by *S. sapinea*. After 7 and 14 days all petri dishes were checked for the presence of *S. sapinea* and other outgrowing endophytes.

DNA Extraction, PCR, and Sequencing

Before inoculations, the DNA was extracted from 150 mg of the homogenized mycelium sample as described in Keriö et al. (2020). 1000 µl of PVP extraction buffer (1 M NaCl, 100 mM Tris-Hcl, 10 mM EDTA, 2% PVP (w/v)) was added to a 1.5 ml Eppendorf tube with 0.3 g of ground mycelium sample. After incubation at 65°C for 15 min, the sample was centrifuged 5000 rpm for 10 min. The supernatant was transferred into a 1.5 ml tube (ca. 500 µl). One volume of SDS (1% SDS (w/v), 0.5 M KCl) was added. The sample was vortexed for 20 s and centrifuged at 15 000 rpm for 10 min. The supernatant (ca.700 µl) was transferred into a 1.5 ml tube. 0.85 volume of isopropanol was added and mixed by inversion for 20 s, followed by centrifuging 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 centrifuging 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).

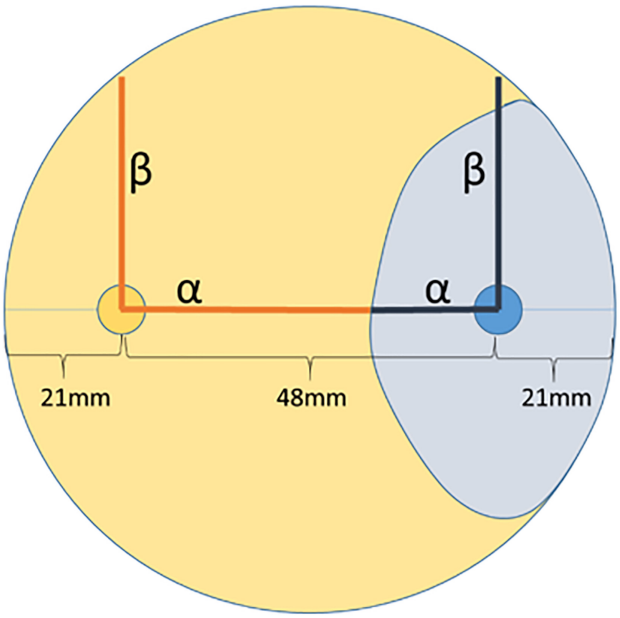

FIGURE 2 | Arrangement of the two-paired culture assay of one fungus (e.g., endophyte) in orange and the other fungus (e.g., pathogen) in blue. Measurement was along the alpha and beta axes. The light orange and blue areas suggest possible growth areas of the fungi and how their growth could be measured.

Taq DNA polymerase (Qiagen) was used for PCR amplification of ITS regions with the primer pair ITS1-F and ITS4 (White et al., 1990; Gardes and Bruns, 1993). Briefly, the PCR protocol was as follows: 1X PCR Buffer, 200 µM dNTP, 0.5 µM primer 1, 0.5 µM primer 2, 100 ng template DNA, 0.2 U/µL DNA polymerase; the reaction was adjusted to 25 µL with autoclaved MQ H_2O. The 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. Possible contaminants were determined with a negative control using sterile water as a template in both PCR protocols. StainIN RED™ Nucleic Acid Stain was used to confirm DNA amplicons on a 1.5% agarose gel and the visual detection was undertaken with ultraviolet transillumination. ITS region PCR products were purified and sequenced using the ITS4 primer at Microsynth SEQLAB (Göttingen, Germany).

The ITS sequences were extracted with the open source software ITSx to separate 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. The sequences with ≥98% similarity and a query coverage ≥97% were set to constrict species level (Arnold and Lutzoni, 2007; Koukol et al., 2012) and confirmed the morphological identification (**Table 1**).

Experimental Design of the Green-House Study

The experiment was conducted at the University of Göttingen, Germany (51°33′28.4″ N 9°57′30.5″ E) from mid-March until August 2019. The 1008 seedlings were randomly block-assigned to waterproof tables in four water treatment groups: the optimal water content (100%) and addition of water equivalent to 75%, 50%, and 25% of the optimal amount (Linnakoski et al., 2019; Terhonen et al., 2019b). The optimal amount of water was considered to be sufficient to maintain moist soil (not soaked), which was measured regularly with a soil moisture meter. Fungal isolates were plated on 2% Malt Extract Agar (MEA) and grown at 21°C for 2 weeks prior to the experimental inoculations. Three months after the planting and two months after the start of water availability treatments, the trees were inoculated (**Table 2**): 768 trees were infected with one species (= one tip per tree, I): *S. sapinea* (25 per water treatment group), *T. conorum-piceae* (25 per treatment group), *M. olivacea* (25 per treatment group), *Didymellaceae* sp. (25 per treatment group), *Sy. polyspora* (25 per treatment group), *D. acicola* (25 per treatment group), mock-inoculated controls with an agar plug (20 per treatment group), and 22 per water treatment group were left entirely untreated. A total of 240 trees were inoculated with four fungi, one on each tip (**Table 2**):

- treatment group II; mock-control, *S. sapinea*, *T. conorum-piceae*, *S. polyspora* (20 per treatment group);
- treatment group III; mock-control, *S. sapinea*, *M. olivacea*, *D. acicola* (20 per treatment group) and
- treatment group IV; mock-control, *Didymellaceae* sp., *T. conorum-piceae*, *Sy. polyspora* (20 per treatment group).

Inoculations were on a first-year shoot. A sterile Ø 3 mm cork-borer was used to punch through hyphae (fungi or control, 2% MEA) in order to get a round-shaped plug. One 1 cm tip of a side shoot per seedling or four tips of four side-shoots (combination-infections), were cut off with a scissor. The agar plug was placed onto the exposed surface, with the mycelium facing the cut, and sealed with Parafilm® (**Figure 3**).

The inoculation experiment ran for 66 days. Since seedling water intake varied with the ambient temperature, we continuously monitored the amounts of water applied, in order to maintain the essential level in the 100% watering treatment group, considered to be the optimal amount. The moisture for each treatment was measured with a soil moisture meter for the same seedlings throughout the experiment. The water amounts needed to be adjusted to the increasing temperatures during the growing season in 2019. At the beginning of the experiment, each seedling in the optimum (100%) water treatment group was given 60 ml of water three times per week, and each seedling in the drought stress water treatment group (25%) was given 15 ml of water three times per week. After 2 weeks, the watering regimes were modified to 120 ml for the 100% water treatment group, and 30 ml × 3 for the 25% water treatment groups. After 5 weeks, the water level was modified again to 180 ml × 3 and 45 ml × 3, respectively. The 75% and 50% water treatment groups received the corresponding amounts per seedling relative to the optimal. Water quantities were increased in July and maintained at that level until August.

Data Collection and Post-experiment Detection of Fungal Species

The lesion lengths (nearest 0.001 mm) were measured with a stereomicroscope (Stemi 508, Carl Zeiss Microscopy GmbH, Jena, Germany) with an attached camera (Axiocam ERc5s, Carl Zeiss Microscopy GmbH, Jena, Germany) using the freely available software Labscope (Carl Zeiss Microscopy GmbH, Jena, Germany). First, the bark was gently peeled to expose the necrosis in the phloem and then measured. The lesion length was measured only in the vertical direction. Dead twigs were removed from necrosis length analysis and analyzed separately. After measurements, five randomly chosen seedlings (in total 35 seedlings) were selected from each fungal or control treatment, to confirm the infection and to guarantee the fulfillment of Koch's postulates. Pieces from the interface of necrosis and healthy tissue were surface sterilized (as described above) and plated onto a 1.5% MYP plate. The fungi were first identified by morphology, followed by DNA extraction and ITS region sequencing to confirm identity (as described above). The sequences obtained were aligned with BLASTN (Zhang et al., 2000) to confirm that the isolate was the same as used in infections (**Table 3**).

Data Analysis

Data (water content and necrosis length) were analyzed using SPSS version 26.0 (IBM Corporation, New York, United States). A generalized linear model (GLM) was constructed to evaluate the fixed effects of inoculation method (control/fungal species) and combination (single or combined infection) under different water treatments (25%, 50%, 75%, and 100%) on necrosis length in tips. Initial fixed explanatory variables in the necrosis length model included inoculation method (categorical value), water treatment (categorical value), and combination (categorical value). Tray (categorical) was set as random factor in the model. The necrosis lengths in statistically different treatments were further assessed by Tukey HSD test for two-samples assuming equal variances. Differences were considered statistically significant if the p-value was below the threshold of 0.01. Similarly, the water content during different time points (each week) between water treatments was assessed by ONE-WAY-ANOVA and Tukey HSD test for two-samples.

The generalized linear model (glm) in R version 3.5.1 (R Core Team, 2019) was run for twig mortality as with the results of the water treatment / fungal inoculation. Further one-way-ANOVA (aov) was conducted and Tukey's HSD test was used to examine the differences between groups (in water/fungal treatment).

RESULTS

In vitro Study: Antagonism Assay

Four different kinds of interaction between the Scots pine endophytes and *S. sapinea* strains were observed (**Figure 1**

TABLE 2 | Total amount of seedlings and the number of the infections of Scots pine seedlings in different water treatments.

Treatment group (running No. of the treatment)	Method	Fungal species	Strain No.	25% water	50% water	75% water	100% water	SUM (total number of seedlings)
			NW-FVA	Low water (number of seedlings)		High water (number of seedlings)		
I (1)	Single infected	Mock-inoculated control	-	20	20	20	20	80
I (2)		*Sphaeropsis sapinea*	4740	25	25	25	25	100
I (3)		*Sydowia polyspora*	4742	25	25	25	25	100
I (4)		*Truncatella conorum-piceae*	4745	25	25	25	25	100
I (5)		*Microsphaeropsis olivacea*	4743	25	25	25	25	100
I (6)		*Desmazierella acicola*	4751	25	25	25	25	100
I (7)		*Didymellaceae* sp.	5756	25	25	25	25	100
I (8)		Untreated	-	22	22	22	22	88
II (1, 2, 3, 4)	Combination infected	Mock-control, *S. sapinea*, *S. polyspora*, *T. conorum-piceae*	x, 4740, 4745, 4742	20	20	20	20	80
III (1, 2, 5, 6)		Mock-control, *S. sapinea*, *M. olivacea*, *D. acicola*	x, 4740, 4743, 4751	20	20	20	20	80
IV (1, 3, 4, 7)		Mock-control, *S. polyspora, T. conorum-piceae, Didymellaceae sp.*	x, 4745, 4742, 5756	20	20	20	20	80
Total amount of seedlings								1008

and **Table 1**). About a quarter of the tested endophytes (26%, *Alternaria* alternata (Fr.) Keissl., *Diaporthe* sp. 2, *Nemania diffusa* (Sowerby) Gray, *Pezicula eucrita* (P. Karst.) P. Karst., *Preussia* sp., *Preussia funiculate* (Preuss) Fuckel, and *S. polyspora*, **Figure 1A**) inhibited the growth of *S. sapinea.* Twenty-two percent of all tested endophytes were superior in growth *v*ersus *S. sapinea* (**Figure 1B**, *Biscogniauxia mediterranea* (De Not.) Kuntze, *Jugulospora rotula* (Cooke) N. Lundq., *Daldinia* sp. *Pyronema domesticum* (Sowerby) Sacc., *Rosellinia* sp., and *Xylaria polymorpha* (Pers.) Grev.). A third of the tested strains (33%, *Biscogniauxia nummularia* (Bull.) Kuntz*, Botrytis cinerea* Pers.*, D. acicola, Diaporthe* sp. 1*, E. nigrum, Fusarium* sp., *Hypoxylon fragiforme* (Pers.) J. Kickx f., and *M. olivacea*) showed equal growth capability (**Figure 1C** and **Table 1**). Nineteen percent of endophytes tested against *S. sapinea* were inferior (= *S. sapinea* was superior; **Figure 1D**, *Mollisia* sp., *Nemania serpens* (Pers.) Gray, *T. conorum-piceae*, and *Therrya fuckelii* (Rehm) Kujala). All of the endophytes had statistical effect with respect at least to one of the *S. sapinea* strains after ten days (**Table 1**).

Sphaeropsis sapinea, strain 4740, had higher *p*-Values (presence of endophytes had stronger effect). If stricter rules for the *p*-value had been applied and only lower *p*-values accepted ($p < 0.01$), only two endophytes would have exhibited statistically significant inhibition (*Sydowia polyspora* and *Diaporthe* sp. 2). *Sy. polyspora* (**Figure 4A**), *M. olivacea* (**Figure 4B**), *T. conorum-piceae* (**Figure 4C**), and *D. acicola* (**Figure 4D**) were chosen for inoculation studies because of their frequent endophytic occurrence in *P. sylvestris*.

In planta Study: Greenhouse Inoculations

Pre-experiment Detection of *Sphaeropsis sapinea* and the Fungal Isolates

Eighteen different endophytic fungal species were isolated from stems and shoots of the six studied nursery pine trees (**Table 4**); these included *Alternaria* spp., *Diaporthe* spp., *E. nigrum, M. olivacea, Sy. polyspora,* and *T. conorum-piceae*. The most frequently isolated species was identified as *Didymellaceae* sp. (NW-FVA ID 5756) and it occurred in all tested trees and 30.9% of all analyzed shoot segments. *S. sapinea* was not isolated from asymptomatic woody tissues of those pines nor detected in any discolored or dead needles that were attached to the plants.

Soil Water Content

At week 3, the soil moisture in the 25% water content treatment was statistically lower than in the 50% ($p = 0.008$) and 100% ($p = 0.010$) treatments (**Figure 5**). After 4 weeks the soil moisture in the 100% treatment was statistically higher than in the 25%, 50%, and 75% treatments. Similarly, the soil moisture in the 25% treatment was lower than the soil moisture of the other

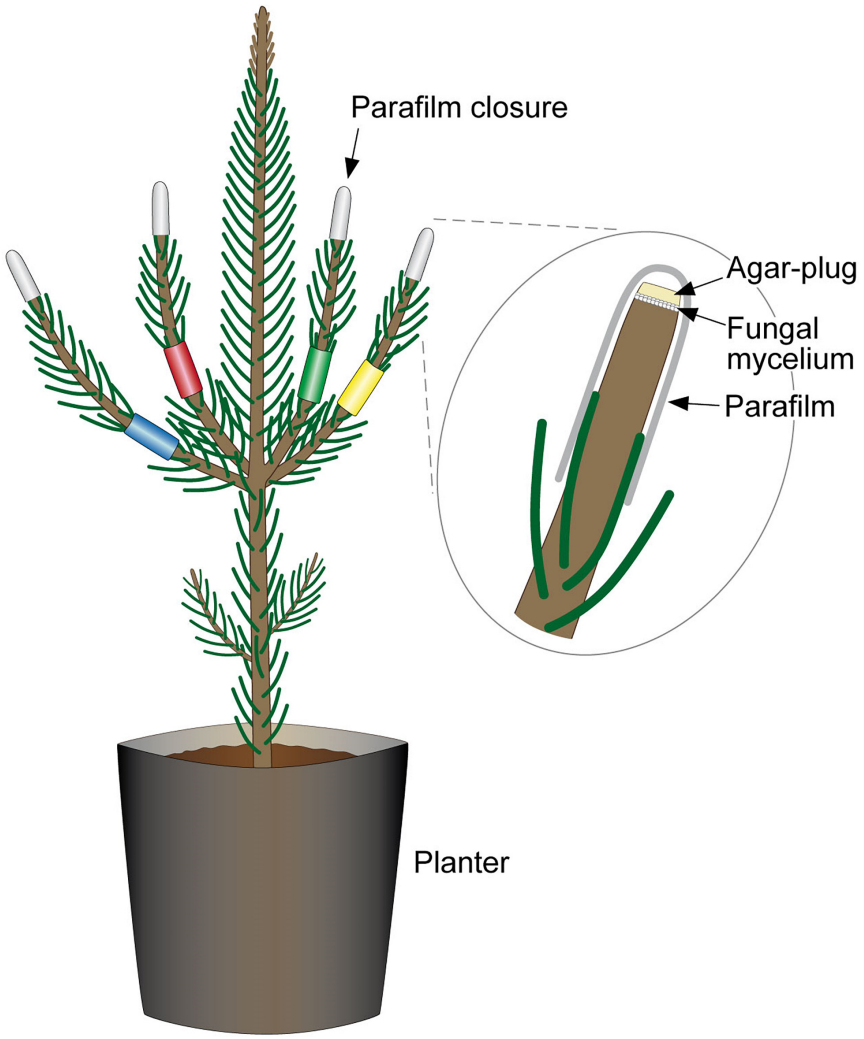

FIGURE 3 | Combined-infection of pine seedlings. Each of the side-shoots is color-labeled to indicate the different fungal strains with which pine was infected. The labeling allowed differentiation during necrosis measurements and re-isolations. Excised tips were replaced with a mycelium-covered agar plug and were sealed with Parafilm®.

treatment groups (all p-Values 0.00, except for 100% versus 50% $p = 0.003$). The soil moisture in the 50% and 75% treatments was, overall, the same throughout the study. In the middle of the experiment (week 5), the soil moisture in the 25% treatment was statistically lower than in the 100% ($p = 0.00$), 75% ($p = 0.003$) and 50% ($p = 0.035$) treatments. The same results were obtained for week 6. At week 7, the soil moisture in the 25% treatment was statistically lower than in the 100% $p = 0.00$ and 75% ($p = 0.001$) treatments and the soil moisture in the 75% treatment was statistically lower than in the 100% treatment ($p = 0.013$). Similarly, the soil moisture in the 50% treatment was lower than in the 100% treatment ($p = 0.035$). At week 8, the soil moisture in the 75% treatment was still statistically lower than in the 100% treatment ($p = 0.019$), and the soil moisture in the 25% treatment was lower than in all other groups: 100% $p = 0.000$, 75% $p = 0.04$, 50% $p = 0.028$. At week 9 the soil moisture in the 25% group was again lower than in the other groups (100% $p = 0.000$, 75% $p = 0.009$, 50% $p = 0.024$), the soil moisture in the 50% ($p = 0.017$) and 75% ($p = 0.011$) treatments was lower than in the 100% treatment. At the end of the study (week 10), the soil moisture in the 25% treatment was statistically lower ($p = 0.042$, 0.001, and 0.00) than the 50%, 75%, and 100% treatments.

Necrosis Length and Dead Twigs

The untreated control plants stayed healthy during the experiment and no dead twigs were observed. In the mock-inoculated control 7.1% of the twigs died. The inoculation with *S. sapinea* led to the highest shoot mortality of inoculated Scots pine twigs in the greenhouse study (**Figure 6**; **Table 3**). Over all treatment groups these necroses caused complete die-off of 18% of all inoculated twigs ($n = 260$) compared to 1.2-8.3% for individual inoculated endophyte and 7.1% for twigs inoculated with the mock community. *S. sapinea* killed 31.9% of inoculated

TABLE 3 | Greenhouse-experiment: Mean necrosis length in mm, percentage of dead twigs, and results of re-isolation.

No.	Treatment method*	Water treatment	I				II				III				IV				All treatments				Percentage of dead twigs in the treated twigs	Re-isolation of inoculated strains	Re-isolation of non-inoculated *S. sapinea*
			25%	50%	75%	100%	25%	50%	75%	100%	25%	50%	75%	100%	25%	50%	75%	100%	25%	50%	75%	100%			
1	Mock-inoculated control		2.0	1.1	1.8	0.6	0.9	0.8	0.9	0.5	1.4	0.9	0.9	0.9	1.1	0.8	0.8	0.7	1.3	0.9	0.9	0.7	7.1	No	No
2	*Sphaeropsis sapinea*		7.6	6.0	8.8	5.1	5.8	6.8	7.4	6.0	4.3	4.7	9.3	6.2	0	0	0	0	5.8	6.0	8.3	5.7	18.1	Yes	NA
3	*Sydowia polyspora*		1.2	1.1	2.0	1.3	0.7	1.1	0.7	0.6	0	0	0	0	1.4	1.5	1.4	0.7	1.0	1.1	1.1	0.8	4.2	Yes	No
4	*Truncatella conorum-piceae*		2.3	1.2	1.7	0.9	0.8	0.7	1.1	0.9	0	0	0	0	1.0	0.6	2.3	0.4	0.9	0.7	1.5	0.6	7.3	Yes	No
5	*Microsphaeropsis olivacea*		2.4	1.6	1.7	1.8	00	0	0	0	2.0	1.5	2.1	1.2	0	0	0	0	1.8	1.4	1.8	1.4	8.3	Yes	No
6	*Desmazierella acicola*		1.3	1.9	1.5	0.9	0	0	0	0	1.6	1.4	2.1	1.2	1.5	2	1.8	1.8	1.4	1.5	1.5	1.2	7.8	Yes	No
7	*Didymellaceae* sp.		2.2	1.6	2.3	2.0	0	0	0	0	0	0	0	0	0	0	0	0	1.7	1.7	1.8	1.9	2.2	Yes	No

**I = single infection of fungi/control; II = combination of No. 1, 2, 3, 4; III = combination of No. 1, 2, 5, 6; IV = combination of No. 1, 3, 4, 7.*

twigs in the 25% water treatment, 27.7% in both the 50% and the 75% water treatments and only 13% in the 100% water treatment (**Supplementary Table 1**). After *M. olivacea* inoculation, 8.3% of the twigs were dead, followed by *D. acicola* (7.8%), *T. conorum-piceae* (7.3%), mock-inoculated control (7.1%), *Sy. polyspora* (4.2%), and *Didymellaceae sp.* (2.2%).

The necrosis length model for endophytes and pathogen (GLM) showed that necrosis size (length) was statistically affected by treatment (fungal species) and water availability but not by the combination used (single infection or several in one plant) (**Table 5**). Statistical differences are listed only in **Table 6** and omitted from **Figure 7**. Statistical differences were found between *S. sapinea* (**Figure 7** and **Table 6**) and all other inoculation treatments in all water treatments. Similarly, necrosis caused by *Didymellaceae* sp. was statistically higher than the mock-control and *T. conorum-piceae* in the 100% water treatment. Necrosis length was statistically higher (mock-control) in the 25% water treatment than in the 100% treatment (**Figure 7** and **Table 6**). The necrosis was greatest statistically in the 75% water treatment compared to the 100% (for *S. sapinea* and *T. conorum-piceae*) (**Figure 7** and **Table 6**). Similarly, for *S. sapinea* inoculation, the necrosis was statistically higher in the 75% compared to the 25% water treatment (**Figure 7** and **Table 6**).

The generalized linear model showed that the number of dead twigs was affected by fungal inoculation due to *S. sapinea* (p = 0.0079, **Supplementary Figure 1A**). The water treatment also affected twig mortality (p = 0.05). The Tukey HSD test showed that the mortality of the twigs was statistically higher in the 25% (p = 0.03) and 50% (p = 0.03) water treatments compared to the 100% water treatment (**Supplementary Figure 1B**).

The water treatment (drought) affected the mortality of the twigs in the *S. sapinea* treatment (**Supplementary Figure 2**). The number of dead twigs was higher in 25% compared to the 100% water treatment (p = 0.04, **Supplementary Figure 2**). Drought, caused by the reduced water treatment, was not found to have any impact on the number of dead twigs in other fungal/control treatments.

Re-isolations of Inoculated Fungi

Sphaeropsis sapinea did not grow out from the surface sterilized samples of the mock-controls. From all inoculated samples, the respective fungus was isolated 66 days after inoculation and identified as the original inoculated species (**Table 3**).

DISCUSSION

The forest pathosystems' behavior can be unpredictable in the future due to changes in the environment that favor fungal pathogens rather than the hosts' vitality. Similarly, this might have unknown effects on host trees core fungal endophytes. We found that abiotic stress, here defined as drought, increased the aggressiveness of *S. sapinea* (no. of dead twigs, necrosis length) but not the other tested endophytes. In case of *S. sapinea*, the negative impact can be expected to increase. Similarly, we observed different modes of competition between other endophytes against *S. sapinea*. Overall these results indicate that

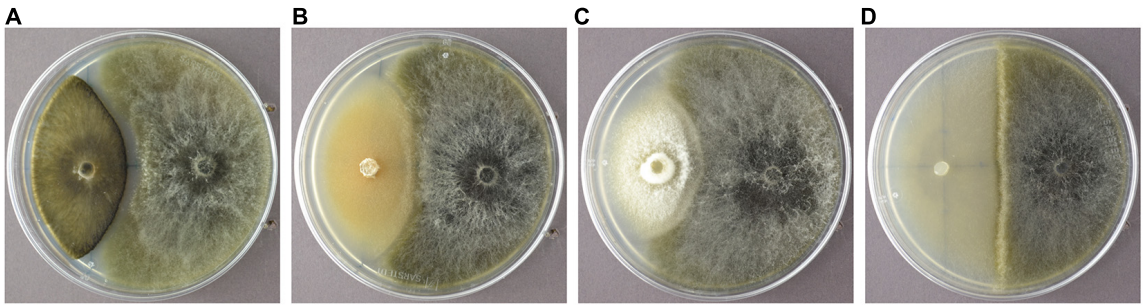

FIGURE 4 | *Sydowia polyspora* **(A)**, *Microsphaeropsis olivacea* **(B)**, *Truncatella conorum-piceae* **(C)**, and *Desmazierella acicola* **(D)** were the endophytes chosen for the infection experiment.

TABLE 4 | Frequency as percent of twig-inhabiting fungal endophytes isolated from two-year-old *Pinus sylvestris*.

Pine plant	1	2	3	4	5	6	Total
Stem including shoots of the growth years	2018-2019	2018-2019	2018-2019	2018-2019	2018-2019	2018-2019	
Incubated tissue segments	122	87	88	54	50	56	457
no outgrowth	23	10.3	44.3	7.4	0	3.6	17.9
outgrowth of yeasts	2.5	0	3.4	3.7	6	7.1	3.3
Alternaria alternata	20.5	2.3	5.7	11.1	24	23.2	13.8
Alternaria spp.	11.5	0	0	7.4	0	0	3.9
Didymellaceae sp.	26.2	24.1	15.9	22.2	68	50	30.9
Ascomycete sp. 2	4.9	34.5	12.5	18.5	10	0	13.6
Ascomycete sp. 3	1.6	0	0	35.2	2	16.1	6.8
Ascomycete sp. 4	19.7	28.7	2.3	0	0	0	11.2
Ascomycete sp. 5	0	3.5	6.8	0	0	0	2
Diaporthe sp. 1	10.7	4.6	13.6	14.8	12	12.5	10.9
Diaporthe sp. 2	0	0	8	0	0	0	1.5
Diaporthe sp. 3	0	0	0	7.4	0	1.8	1.1
Epicoccum nigrum	13.9	20.7	18.2	7.4	26	35.7	19.3
Fusarium sp.	0	0	0	11.1	0	1.8	1.5
Microsphaeropsis olivacea	0	0	0	0	0	10.7	1.3
Paraphaeosphaeria sp.	0	1.2	0	0	0	0	0.2
Sordaria fimicola	0	0	0	0	2	0	0.2
Sydowia polyspora	0	3.5	2.3	0	0	0	1.1
Trichoderma spp.	0	0	0	0	2	0	0.2
Truncatella conorum-piceae	0	2.3	0	0	0	0	0.4
Fungus spp.	1.6	2.3	0	0	0	0	0.9
Total No. of identified filamentous species	8	10	9	9	8	8	18

other endophytes in Scots pine tissues (especially in twigs) can play a role in the disease development of "Diplodia Tip Blight." Hypothetically, favoring certain tree microbiome in tree health would produce effective, durable, and environmentally friendly control method against severe disease outbreaks.

Antagonism Studies

Scots pine endophytes and *S. sapinea* interacted with each other in various ways as demonstrated by the *in vitro* antagonism assays. Four interaction categories were found: about half of the tested strains were able to either inhibit *S. sapinea in vitro* or were superior in their growth towards *S. sapinea*. Similar results were presented by Bußkamp (2018). In our study, thirteen species could be considered potential antagonists against *S. sapinea in vitro,* because they had either a faster growth than *S. sapinea* or inhibited the pathogen's development. This partly corresponds with the results of Bußkamp (2018), who found 22% of the tested 89 endophytic strains inhibited the growth of *S. sapinea*. A contactless inhibition of *S. sapinea* was observed for 26% of the endophytes in our study (e.g., *A. alternata* and *Pe. eucrita*). Chemical antagonism can be assumed when a fungus reacts to the presence of an opponent fungus with an inhibition zone between the two fungal colonies. One fungus might excrete secondary metabolites that inhibit the opponent fungus (Schulz et al., 2002).

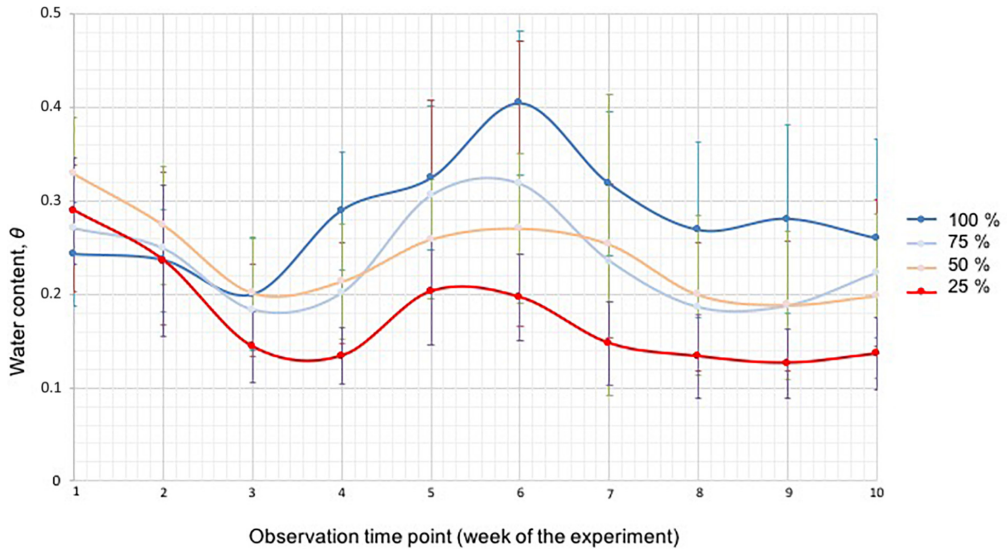

FIGURE 5 | The soil moisture of the four different water treatment groups (100% blue, 75% light blue, 50% green, and 25% red) measured with a soil moisture meter, shown as water content ϴ for each week during the duration of the experiment. The bars represent standard deviation.

Secondary metabolites should be extracted and tested to see whether the same reaction towards the pathogen can be observed, in order to determine whether a certain metabolite can inhibit growth (Tellenbach et al., 2013). *S. sapinea*, could not penetrate the barrier surrounding one of those endophytes.

Several of the tested endophytes in this study over-grew the *S. sapinea* mycelium. This ability might indicate a stronger capacity to metabolize nutrients. The faster growth and better ability to utilize nutrients are two strategies with clear advantages during competition (Mgbeahuruike et al., 2011). The fungi *Sy. polyspora*, *Xylaria* sp. and *Diaporthe* sp. are typical endophytes in Scots pine (Bußkamp et al., 2020). If their growth or infection in the host tissues could be promoted, in theory they could provide strong competition against pathogens in nature (Terhonen et al., 2019a). A co-occurrence analysis of shoot-inhabiting endophytes in mature pines by Oliva et al. (2021) identified a cluster of species that was negatively correlated with *S. sapinea* and could be potential antagonists. The most negatively correlated was an *Alternaria* species, accompanied by *E. nigrum* and *Sy. polyspora*. Similarly, we show that *Sy. polyspora* had antagonistic capability against *S. sapinea*. Oliva et al. (2021) provided some evidence that the competition between *S. sapinea* and other endophytes for stress-related metabolites could prevent the growth of *S. sapinea* and development of Diplodia tip blight symptoms. Our results support this conclusion as the tested endophytes (e.g., *Sy. polyspora*) sharing the same niche (in this case MYP plates) with *S. sapinea* could inhibit the fungus' growth *in vitro*. The co-infection of the studied Scots pines with different endophytes together with *S. sapinea* on different twigs did not cause a visible increase in the host plant's immune system *in planta*. In our study, we did not aim at directly inhibiting *S. sapinea* growth but rather testing for an influence (lower necrosis caused by *S. sapinea*) on the host plant as a consequence of the combined infections (e.g., induced systemic resistance due to the presence of an endophyte).

Several studies indicate that fungal endophytes may increase their host plant's immune system *in vivo* (e.g., Witzell and Martín, 2018). Ganley et al. (2008) demonstrated that *Pinus monticola* Douglas gained resistance against *Cronartium ribicola* J.C. Fisch. as a result of prior infection with certain endophytes. Mejía et al. (2008) were able to enhance *Theobroma cacao* L. plants' defense against herbivore and pathogen attacks, by inoculating the leaves with the endophyte *Colletotrichum tropicale* E.I. Rojas, S.A. Rehner & Samuels. The host plant's defense was enhanced due to a priming effect caused by the endophytes, increasing the expression of suites of host genes involved in in plant defense pathways or e.g., cell wall development (Mejía et al., 2008). Similar results were found by Raghavendra and Newcombe (2013); Martínez-Arias et al. (2019) with endophytic fungi on *Populus* species. Enhancement of resistance to Dutch elm disease by elm endophytes has been recently described by Martínez-Arias et al. (2021). The combination infections did not impact the necrosis length, indicating no activation of systematic resistance. The inoculation method in our study was very aggressive (mycelium) and perhaps, infecting *S. sapinea* later than the endophytes in the host would have produced different results. Similarly, mimicking more natural infection methods through conidia infections would give more legitimate results.

The remaining tested strains in the antagonism assays showed either neutral (33% of strains with equal growth capability) interaction or were inferior to *S. sapinea*. Therefore, they do not appear to be suitable potential antagonists against *S. sapinea*. Pairing with equal growth capability may indicate that the endophytes' and *S. sapinea*s' growth was not disturbed by each other, meaning no antagonism took place. In the host,

TABLE 5 | The General Linear Model values.

Variable	Fixed Effects	Std. Error	*F*	Sig.
Necrosis length	Water treatment	0.141	7.692	0.000
	Inoculation method	0.188	245.600	0.000
	Combination	0.152	0.460	0.710
	Random Effects			
Necrosis length	Tray	0.045		0.108

this might mean the fungi can grow together and neither specifically reacts to the presence of the other. *S. sapinea* has a comparatively high growth rate (Bußkamp, 2018) and therefore, it can be assumed that it has a fast metabolism to use the available nutrients. Is not surprising that *S. sapinea* was found to be superior and occupied a larger area of the plate more rapidly than several more slow growing endophytic test partners, such as *T. conorum-piceae*, *N. serpens*, *Mollisia* sp. and two *Th. fuckelii* strains. When the Scots pine host is weakened due to drought stress for example, the common endophytes inside the tree could be disadvantaged while *S. sapinea* can become more established as secondary pathogen and occupy more tissues faster in the host, thus outcompeting these inferior endophytes. This could be one reason why *S. sapinea* is present in higher numbers in diseased sites (Bußkamp et al., 2020).

Pre-colonization of the Trees With Fungal Community

The pre-examination of the nursery Scots pines indicated a relatively small fungal community. No *S. sapinea* could be detected from the tested seedlings prior to the experiments, whereas the other fungal strains used for the greenhouse-infections were found to be present. We concluded that these species belong to the naturally observed Scots pine endophyte community (Blumenstein et al., 2020; Bußkamp et al., 2020). This corresponds to results of Bihon et al. (2011) who found that *S. sapinea* was not an endophyte of healthy seedlings collected from greenhouses and nurseries. The composition of endophytes isolated from two-year-old Scots pine plants in this study was dominated by *A. alternata* and *E. nigrum*, and was typical of young pines from tree nurseries (own unpublished data). In contrast to intensive studies on the fungal endophytic community of Scots pine shoots of mature trees (Blumenstein et al., 2020; Bußkamp et al., 2020; Oliva et al., 2021), the endophyte α-diversity of the tested two-year old Scots pines was lower. In total, only 18 filamentous, ascomycetous endophytes (8 – 10 species per seedling) were isolated in this study, whereas 103 different species (5 – 22 isolated per tree) were found in mature pine shoots in a study by Bußkamp et al. (2020). Foliar fungal endophyte assemblages have been shown to vary between the developmental stage of the host (mature vs. seedling (Helander et al., 2011; Koukol et al., 2012; Skaltsas et al., 2019). The core foliar endophytes seem to be the same in seedlings and mature Scots pine (Blumenstein et al., 2020; Bußkamp et al., 2020) but community compositions and species differences are probably related to the age of the host (Taudière et al., 2018).

The genesis of the Scots pine endophytic community is poorly known. There are hints that composition of the endophytic community is mainly determined by the host species and organ type (Peršoh, 2013), health condition, composition of the forest trees, and vegetation which is growing in the surroundings of the host tree (Peršoh et al., 2010; Nguyen et al., 2016; Bußkamp, 2018). The latter factor may explain why plants could be species-poorer in a nursery field than in a mixed forest. Horizontal transmission from mature trees in forest stands is probably the main source for young trees to obtain endophyte inocula, as shown for *Pinus patula* Schltdl. & Cham. And *S. sapinea* (Bihon et al., 2011). Additionally, the composition of endophytes depends on the age of the tissue (Fröhlich et al., 2000; Arnold and Herre, 2003; Terhonen et al., 2011,

TABLE 6 | Statistical differences of necrosis length between different groups ($p < 0.01$).

		Water treatment			
Group 1	**Group 2**	**100%**	**75%**	**50%**	**25%**
Mock-inoculated control	*Sphaeropsis sapinea*	0.000	0.00	0.00	0.00
Mock-inoculated control	*Didymellaceae* sp.	0.001	NA	NA	NA
Sphaeropsis sapinea	*Sydowia polyspora*	0.000	0.000	0.000	0.000
Sphaeropsis sapinea	*Truncatella conorum-piceae*	0.000	0.000	0.000	0.000
Sphaeropsis sapinea	*Microsphaeropsis olivacea*	0.000	0.000	0.000	0.000
Sphaeropsis sapinea	*Desmazierella acicola*	0.000	0.000	0.000	0.000
Sphaeropsis sapinea	*Didymellaceae* sp.	0.000	0.000	0.000	0.000
Truncatella conorum-piceae	*Didymellaceae* sp.	0.001	NA	NA	NA
		Inoculation treatment			
Group 1	**Group 2**	**Mock-inoculated control**	***Sphaeropsis sapinea***	***Truncatella conorum-piceae***	
100%	25%	0.001	NA	NA	
100%	75%	NA	0.003	0.002	
75%	25%	NA	0.007	NA	

FIGURE 6 | Pine shoot with combined infections at the end of the experiment. The red labeled side-shoot was infected with *S. sapinea* and shows full necrosis.

2019a; Bußkamp, 2018), which determines whether an endophyte community can accumulate over years or if it represents only annual accumulation, such as in leaves. Peršoh (2013) found that one- and three-year-old pine stem sections had different assemblies of endophytic fungi, supporting the theory that forest trees mainly accumulate their endophytic community as the infection rate tends to increase with age (Guo et al., 2008; Deckert and Peterson, 2011).

Interaction of Endophytes With *S. sapinea* Infection

Necrosis of the Scots pine was marginal when infected with *D. acicola, Didymellaceae* sp., *M. olivacea, Sy. polyspora*, and *T. conorum-piceae*. These fungi (excluding *S. sapinea*) were most likely already endophytically present in the Scots pine plants before inoculation (based on pre-examination). The fact that *D. acicola* (anamorph *Verticicladium trifidum* Preuss) did not cause higher necrosis compared to the control is not surprising, as it is a typical twig endophyte on *Pinus* spp. in Europe (Petrini and Fisher, 1988; Kowalski and Kehr, 1992; Kowalski and Zych, 2002; Bußkamp, 2018) and lives endophytically in pine needles (Kendrick, 1962). This saprotrophic species also colonizes needles after fall (Ponge, 1991) and ascocarps can be found on dead blackened leaves. No pathogenic behavior has ever been described and it is one of the most common fungi growing in pine leaf litter all over the world (Martinoviæ et al., 2016). The tested *Didymellaceae* sp. was not identified to the species level based on the ITS analysis. The *Didymellaceae* is a species rich, cosmopolitan family, assigned to the Pleosporales and includes genera such as *Didymella, Leptosphaerulina, Macroventuria, Monascostroma, Platychora* and moreover some species of *Phoma* and *Ascochyta* (Zhang et al., 2009). Most species of the *Didymellaceae* are associated with dicotyledon plants and mostly hemibiotrophic and saprobic (Zhang et al., 2009) or pathogens, causing leaf and stem lesions (Chen et al., 2017). *M. olivacea* (basionym: *Coniothyrium olivaceum* Bonord.) has been described from *Pinus* plants in very few publications (Petrini and Fisher, 1988; Kowalski and Kehr, 1992; Bußkamp et al., 2020). Petrini and Fisher (1988) described *M. olivacea* as a typical endophytic colonizer of conifers. Bußkamp et al. (2020) were able to confirm this observation as it was the third most common fungus isolated from pine twigs and they found it in nearly all studied stands in Germany. In contrast, Kowalski and Kehr (1992), isolated the fungus endophytically only from very few pine branches. *M. olivacea* is known to be plurivorous and was found on twigs and branches of other tree species (e.g., Hormazabal et al., 2005), causing brown spine rot of Camelthorn (*Alhagi maurorum* Medik.) (Razaghi and Zafari, 2016).

Sydowia polyspora and *T. conorum-piceae*, which can be conifer pathogens (Sutton and Waterston, 1970; Heydeck, 1991; Talgø et al., 2010; Pan et al., 2018), did not cause strong necrosis on the twigs. The reason may be that they are specialized true endophytes of Scots pine (Bußkamp et al., 2020) and the host-fungi continuum is balanced. *Sy. polyspora* is widely distributed all over the world (Muñoz-Adalia et al., 2017; Pan et al., 2018). As an endophyte it has a high consistency and frequency in Scots pine twigs (Sanz-Ros et al., 2015; Blumenstein et al., 2020; Bußkamp et al., 2020). Predominately, *Sy. polyspora* lives saprophytically on dead plant material but also occurs as a secondary pathogen on previously damaged needles and twigs (Heydeck, 1991), as wound pathogen and blue stain-fungus (Sutton and Waterston, 1970), or as causal agent of current season needle necrosis (CSNN) on true fir (*Abies* spp.) (Talgø et al., 2010). Ascomata and pycnidia appear on dead pine branches and needles (Gremmen, 1977). Cleary et al. (2019) suggest that this fungus could benefit from climate change as additional stresses are added to the host so that *Sy. polyspora* can become a more commonly opportunistic pathogen. Here we show that the necrosis caused by *Sy. polyspora* in Scots pine is not affected by drought. In a study by Bußkamp (2018), *Sy. polyspora* did not show antagonistic behavior in dual culture

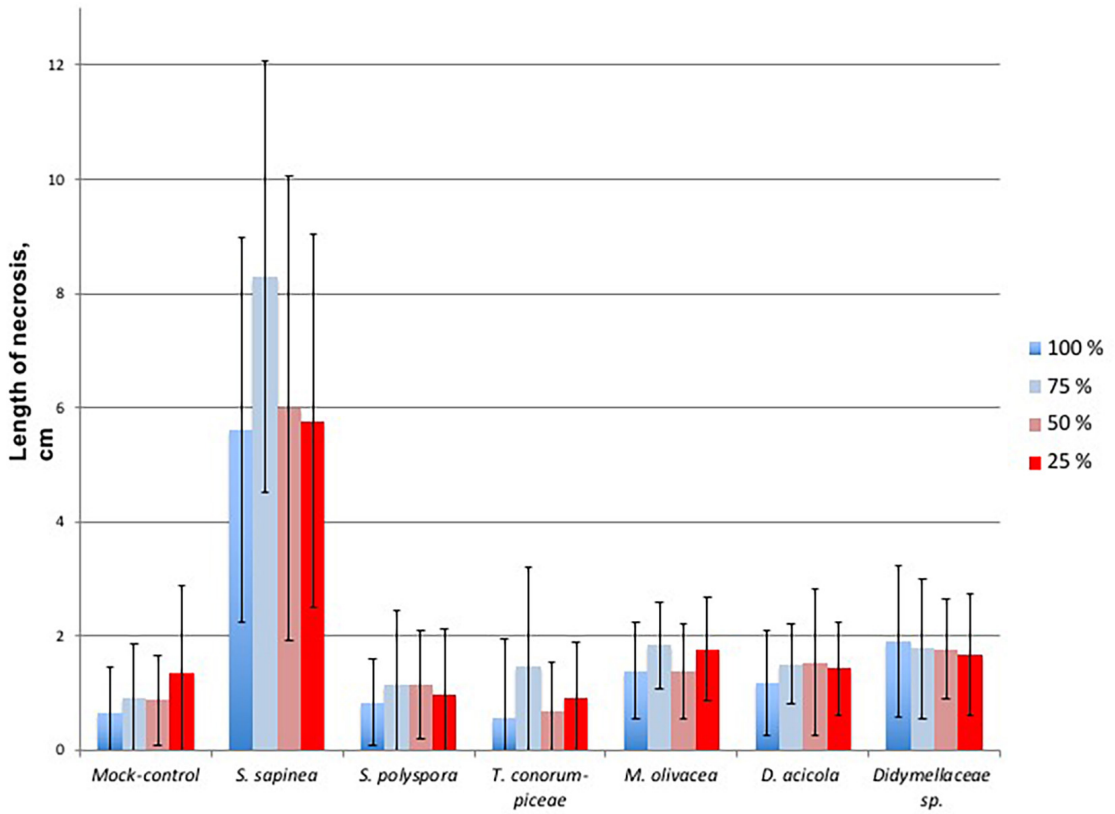

FIGURE 7 | Necrosis length of the single infected shoots per fungal isolate, each in relation to the water treatment groups, where blue is 100% water treatment, light blue 75%, light red 50%, and red 25%. The bars represent standard deviation.

with *S. sapinea* so the observed antagonism in this study might be strain related. *T. conorum-piceae* (≡ *Pestalotia conorum-piceae* Tubeuf) is also a frequently occurring twig endophyte of Scots spine with high consistency in Germany (Bußkamp et al., 2020). Mainly, it is a decomposer of pre-damaged needles of pine and is saprotrophic. However, *T. conorum-piceae* is also known as a secondary pathogen of pine and spruce needles and cones (Landeskompetenzzentrum Forst Eberswalde (LFE) 2015). The fungus is hardly described in the literature, but due to the fact that it is very abundant (Blumenstein et al., 2020; Bußkamp et al., 2020), it is important to gain more knowledge about this species in future studies. Other allied *Amphisphaeriaceae* such as *Pestalotiopsis* species can be primary causal agents of spots, blights and diebacks of various host plants. For example, the very common *Pestalotiopsis funerea* (Desm.) Steyaert, with a worldwide distribution, causes damping off, root and collar rots of seedlings, needle blight, tip-blight, twig dieback, and stem canker of conifers, especially Cupressaceae (Sinclair and Lyon, 2005).

The twig mortality was highest in the 25% water group and different statistically from the 100% treatment. This aspect is important with regard to the disease development in mature trees, as the twig mortality and thus the tree health, increases due to drought. Other studies have shown that water availability can affect the growth of pathogens in conifer trees during water stress. *Heterobasidion* species were able to increase necrosis under water stress (Terhonen et al., 2019b). Similarly, the blue stain fungus *Endoconidiophora polonica* (Siemaszko) Z.W. de Beer, T.A. Duong & M.J. Wingf. caused greater necrosis and mortality in *Pi. abies* seedlings with low water availability compared to those with high water availability (Linnakoski et al., 2017). Even a 25% decrease in water was able to trigger the activity of *S. sapinea*. The necrosis caused by *S. sapinea* was statistically higher in all water treatments compared to other fungi, indicating that host stress can particularly benefit this opportunistic pathogen.

Overall, the findings of our greenhouse-study demonstrate how severe the infection of *S. sapinea* is in drought stressed Scots pines, evidenced by high twig mortality and necrosis. The other fungi with the potential to become pathogens (as described above) did not benefit from host stress. In conclusion, pine endophytes showing antagonism against *S. sapinea* and with no detrimental effect to the host could be applied as inoculants to facilitate host protection. We will plan new research to find innovative and effective ways of utilizing the beneficial tree mycobiome in the context of future challenges. *Sphaeropsis sapinea* is a high-risk Scots pine pathogen because the disease severity (mortality of twigs, necrosis length) is increasing under

drought stress. Climate change, leading to more severe and prolonged drought, will therefore lead to more outbreaks of the disease "Diplodia Tip Blight" in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KB planned and performed the greenhouse experiment. JB and GL performed the isolation of the endophytes and the dual-culture study. RS and NPR contributed to the greenhouse experiment, the measurements of the necrosis, and the re-isolation of the fungi. ET contributed to the study design and analyzed the data. KB wrote the first draft. KB, JB, GL, and ET contributed to the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2021.655769/full#supplementary-material

Supplementary Figure 1 | Boxplot representing the mortality of the twigs due to fungal **(A)** and water **(B)** treatment.

Supplementary Figure 2 | Boxplot presenting number of dead twigs after *Sphaeropsis sapinea* treatment in different water treatments.

Supplementary Table 1 | Number of dead twigs in the end of the greenhouse-experiment.

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