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Antagonistic activities of endophytic fungi isolated from pines against *Diplodia sapinea* (Fr.) Fuckel

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Abstract: *Diplodia sapinea*, an endophytic fungus belonging to the Ascomycota, is commonly found on coniferous trees. While it typically exists as an endophyte, it can transform into an opportunistic pathogen under abiotic stress factors such as drought induced by climate change. The fungus enters the host through stomata on needles or via injured tissues, causing a disease known as Diplodia tip blight. This disease affects trees in various environments, including nurseries, plantation areas, natural forests, and urban trees. The prevalence of *D. sapinea* has significantly increased in Europe in recent years, and there is currently no established and effective control method worldwide. In response to this challenge, biological control method utilizing antagonist organisms have emerged as a promising alternative to combat Diplodia tip blight. The objective of this study is to evaluate the antagonistic activities of endophytic fungi isolated from different pine tree tissues against *D. sapinea* isolates obtained from *Pinus halepensis* and *Pinus brutia* under *in vitro* conditions. Identification of the isolates was carried out using both morphological and molecular methods. Fungal inhibition tests were conducted to assess the interaction between these isolates and *D. sapinea* isolates. The results of the tests revealed that 15 fungi, including *Trichoderma* sp. and *Sydowia polyspora*, demonstrated the potential to inhibit the growth of *D. sapinea in vitro*.
Keywords: Antagonism, Biological control, Diplodia tip blight, Fungal endophyte, Türkiye

Çam ağaçlarindan izole edilen endofitik fungusların *Diplodia sapinea* (Fr.) Fuckel'e karşi antagonistik etkilerinin belirlenmesi

Diplodia sapinea, Ascomycota'ya ait endofitik bir fungustur ve genellikle iğne yapraklı ağaçlarda bulunur. Bu fungus genellikle endofit olarak varlık gösterse de, iklim değişikliğinin neden olduğu kuraklık gibi abiyotik stres faktörleri altında fırsatçı bir patojene dönüşebilmektedir. Fungus, iğne yaprakların üzerindeki stomalar veya yaralı dokular aracılığıyla konukçusuna girmekte ve Diplodia sürgün yanıklığı olarak bilinen bir hastalığa yol açmaktadır. Bu hastalık, fidanlıklar, plantasyon alanları, doğal ormanlar ve kentsel ağaçlar gibi çeşitli ortamlardaki ağaçları etkilemektedir. Son yıllarda *D. sapinea*'nın Avrupa'da yaygınlığı önemli ölçüde artmış olup, dünya genelinde halen yerleşik ve etkili bir kontrol yöntemi bulunmamaktadır. Bu soruna yanıt olarak, antagonist organizmalar kullanılarak yapılan biyolojik mücadele yöntemi, *Diplodia* sürgün kurumasına karşı umut verici bir alternatif olarak ortaya çıkmaktadır. Bu çalışmanın amacı, farklı çam ağacı dokularından izole edilen endofitik fungusların, *Pinus halepensis* ve *Pinus brutia*'dan elde edilen *D. sapinea* izolatlarına karşı *in vitro* koşullarda antagonistik aktivitelerinin değerlendirilmesidir. İzolatların teşhisleri hem morfolojik hem de moleküler yöntemler kullanılarak gerçekleştirilmiştir. Bu izolatların *D. sapinea* izolatları ile olan etkileşimlerini değerlendirmek amacıyla fungal inhibisyon testleri yapılmıştır. Test sonuçları, *Trichoderma* sp. ve *Sydowia polyspora* da dahil olmak üzere 15 fungusun in-vitro olarak *D. sapinea*'nın gelişimini inhibe etme potansiyeline sahip olduğunu ortaya koymuştur.
Anahtar Kelimeler: Antagonizm, Biyolojik mücadele, Diplodia sürgün yanıklığı, Endofitik fungus, Türkiye

1. Introduction

Plants are constantly interacting with a wide range of microorganisms. These microorganisms can be found colonising the soil and subsoil organs of the plant (root microorganisms), on the plant surface (epiphytes) and in the internal tissues of plants (endophytes). Endophytes are assumed to be organisms that colonise the living internal tissues of plants and live without causing any significant damage. These microorganisms can produce secondary metabolites that can directly inhibit insects and pathogens or stimulate the plant to activate passive resistance mechanisms. Therefore, the use of antagonistic microorganisms such as endophytes is one of the most ideal methods of plant disease control and holds great promise in the biocontrol of diseases (Trejo-Estrada et al., 1998). Considering their coordinated functions, the secondary metabolites they produce and their effective role in biological control, it is of great importance to discover new and interesting endophytic microorganisms from numerous plants living in different conditions and ecosystems. Thus, it is possible to develop new and effective strategies with measures taken with microorganisms such as endophytes in biological control. It is estimated that many important endophytes are waiting to be discovered in nature and whose effects are not yet known (Beram et al., 2016).

Diplodia sapinea (Fr.) Fuckel [syn.; *Diplodia pinea* (Desm.) J. Kickx f., *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton] is one of the most common and dangerous fungal pathogens of coniferous trees worldwide. It was identified as a saprobic fungus in Europe in the early 19th century, and its

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damage and prevalence have been increasing rapidly since the 1980s (Brodde et al., 2019; Blumenstein et al., 2020). The fungus enters the host through stomata from the needles or through injured tissues. In coniferous trees, it causes various disease symptoms such as discoloration of needles, backward death of current year shoots, crown wilt, stem cancer, root collar and root rot, damping-off of seedlings, seed rot and blue colouration of sapwood (Brookhouser and Peterson, 1971; Munck et al., 2009; Capretti et al., 2013). In addition to nearly 50 pine species, the fungus has also been detected in coniferous species such as *Pseudotsuga* spp., *Abies* spp., *Picea* spp., *Larix* spp., and *Cedrus* spp. (Kaya et al., 2014; Zlatković et al., 2017; Oskay et al., 2018).

There are different stages in the life cycle of *D. sapinea*. While the fungus can exist as an asymptomatic endophyte on the host tree, it can also transform from a latent pathogen to an opportunistic pathogen and/or a saprotroph fungus (Swart and Wingfield 1991; Diekmann et al., 2002). With stress factors such as drought, hail, extreme temperatures or mechanical injury (Parnell, 1957; Chou, 1987), it can rapidly become pathogenic and cause sudden disease outbreaks (Stanosz et al., 2001; Langer et al., 2011; Blumenstein et al., 2021). The fungus can increase in nurseries, plantation areas and natural forests under hot climatic conditions and becomes more aggressive due to stress factors such as drought (Blumenstein et al., 2021). Due to the endophytic stage of *D. sapinea*, disease outbreaks caused by this pathogen in the host can progress undetected while still small in scale (Brodde et al., 2019).

The presence of *D. sapinea* in Türkiye was first reported by Ünligil and Ertaş (1993) on shoots of *P. pinaster* Aiton. and *P. pinea* L. near Kemerburgaz, northwest of Istanbul. In another study by Sümer (2000), the presence of the fungus was mentioned on *P. nigra* Arnold, *P. brutia* var. *eldarica* and *P. brutia* stands in Kahramanmaraş region. So far, this fungal agent has been reported in many species such as *P. nigra*, *P. sylvestris* L., *P. brutia* Ten., *P. pinea*, *P. halepensis* Mill, *Cedrus libani* A. Rich. and *Pseudotsuga menziesii* (Mirb.) Franco. in Türkiye (Ünligil and Ertaş 1993; Sümer, 2000; Soylu et al., 2001; Yeltekin, 2015; Oskay et al., 2018; Kaya et al., 2014; Kaya et al., 2019). The damage caused by this fungus has increased dramatically in recent years in Europe and in Türkiye. There is not yet an established and effective control method used in the world for the control of this disease agent.

In recent decades, there has been a growing interest in screening and testing endophytic fungi for their potential to act as antagonists against pathogenic fungi (Rodriguez et al., 2009; Bamisile et al., 2018; Silva et al., 2019). Currently, the use of endophytes as biocontrol agents in forestry is a developing area of research (Witzell et al., 2014; Terhonen et al., 2018, Terhonen et al., 2019; Prospero et al., 2021). Exploring and testing endophytic fungi for their antagonistic potential against forest pathogens have shown promise in developing novel biocontrol agents for managing such pathogens (Tellenbach et al., 2013; Raghavendra and Newcombe, 2013; Terhonen et al., 2018; Costa et al., 2020; Kowalski and Bilański, 2021; Oliva et al., 2021; Blumenstein et al., 2021).

Diplodia shoot blight, caused by *D. sapinea*, is an emerging disease throughout Türkiye, posing a significant and increasing threat to pine forests in the country. Therefore, it is of utmost importance to develop effective management strategies to mitigate its impact. Previous research has suggested that fungal endophytes in pine trees could play a pivotal role in the biological control of this pathogen. This study aims to evaluate the antagonistic activities of endophytic fungi against *D. sapinea* under controlled *in vitro* conditions, utilizing fungal inhibition tests in dual cultures. The fungal endophytes and the *D. sapinea* isolates were obtained from various tissues including seeds, needles, and shoots of five different pine species (*Pinus* spp.) from Türkiye and identified using morphological and molecular methods. By assessing the biological control potential of these endophytic isolates, we aim to advance our understanding of sustainable strategies for managing Diplodia shoot blight. This research has the potential to significantly contribute to the development of innovative and environmentally friendly approaches for combating Diplodia shoot blight in Turkish pine forests.

2. Material and method

2.1. Fungal isolation

2.1.1. Isolation of the pathogenic fungi

D. sapinea isolates used in the experiment were obtained from symptomatic *P. halepensis* and *P. brutia* shoots (Table 1). After conducting macroscopic and microscopic examinations, isolations were carried out from symptomatic shoots and cones. The spores were plated onto 2% Potato Dextrose Agar (PDA; Merck, Germany) supplemented with 0.5 mg/ml streptomycin sulfate in 90 mm diameter Petri dishes. These cultures were then incubated for seven days at 25 ± 1°C. Hyphal tips of fungi emerging from tissue pieces were transferred to fresh PDA, and ultimately, single-spore cultures were grown on PDA at 25 ± 1°C. Indicator isolates were selected from different haplotype groups of *D. sapinea* isolates.

2.1.2. Isolation of the endophytic fungi

Endophytic fungi were obtained from various pine tree tissues, including seeds, needles, shoots, and cones (Table 2). After surface sterilizations, the tissues were plated onto 2% PDA supplemented with 0.5 mg/ml streptomycin sulfate in 90 mm diameter Petri dishes. These cultures were then incubated for seven days at 25 ± 1°C. Hyphal tips of fungi emerging from tissue pieces were carefully transferred to fresh PDA. Single-spore cultures were cultivated on PDA at 25 ± 1°C.

2.2. Identification of fungi

2.2.1. Morphological identification of fungi

The identification of fungi on the basis of their macroscopic and microscopic characteristics has been carried out using various sources related to the subject (Ryvarden, 1978; Bernicchia, 2005). Macroscopic characteristics of fungal spores were determined by using culture colour and shape, and microscopic characteristics were determined by examining the colour, shape, wall characteristics and spore size of the spores. Conidia were mounted in water and dimensions were measured at 100× using an Olympus compound microscope and the program Olympus DP-Soft.

Table 1. *Diplodia sapinea* isolates used in the study and their characteristics

Code	Host	Location	Tissue/substrate	Habitat
DS80	*P. halepensis*	İzmir	Symptomatic shoot	Natural *P. halepensis* forest stand
DS85	*P. halepensis*	İzmir	Symptomatic shoot	Natural *P. halepensis* forest stand
DS99	*P. brutia*	Muğla	Old cone from forest floor	Natural *P. brutia* forest stand
DS110	*P. brutia*	Antalya	Old cone from forest floor	Natural *P. brutia* forest stand

Table 2. Source of endophytic fungal isolates used in the study

Code	Host	Location	Tissue/Substrat	Habitat
E1CF	*P. pinea*	İzmir/Bergama	Healthy looking bud	Natural *P. pinea* forest stand
E2CS	*P. sylvestris*	Çankırı/Eldivan	Old needle from forest floor	Natural *P. sylvestris* forest stand
E3CS	*P. sylvestris*	Çankırı/Eldivan	Wood from dead tree	Natural *P. sylvestris* forest stand
E4CS	*P. sylvestris*	Çankırı/Eldivan	Wood from dead tree	Natural *P. sylvestris* forest stand
E5CF	*P. pinea*	İzmir/Bergama	Healthy looking bud	Natural *P. pinea* forest stand
E6CZ	*P. brutia*	Denizli/Bozkurt	Symptomatic shoot	*P. brutia* plantation site
E7CF	*P. pinea*	İzmir/Bergama	Healthy looking cone	Natural *P. pinea* forest stand
E8CZ	*P. brutia*	Denizli/Bozkurt	Healthy looking shoot from symptomatic tree	*P. brutia* plantation site
E9CZ	*P. brutia*	Denizli/Bozkurt	Healthy looking shoot from symptomatic tree	*P. brutia* plantation site
E10CZ	*P. brutia*	Denizli/Bozkurt	Symptomatic shoot from symptomatic tree	*P. brutia* plantation site
E11CK	*P. nigra* subsp. *pallasiana*	Denizli/Acıpayam	Healthy looking shoot from symptomatic tree	Natural *P. nigra* forest stand
E12CK	*P. nigra* subsp. *pallasiana*	Denizli/Acıpayam	Healthy looking shoot from symptomatic tree	Natural *P. nigra* forest stand
E13CH	*P. halepensis*	İzmir/Urla	Healthy looking shoot from symptomatic tree	Natural *P. halepensis* forest stand
E14CT	*P. thunbergii*	İstanbul/Sarıyer	Dead needle from symptomatic tree	Arboretum
E15CF	*P. pinea*	Adana	Healthy looking cone from healthy tree	Natural *P. pinea* forest stand
E16CK	*P. nigra* subsp. *pallasiana*	Çankırı/Eldivan	Healthy looking shoot from healthy tree	Natural *P. nigra* forest stand
E17CK	*P. nigra* subsp. *pallasiana*	Isparta/Keçiborlu	Healthy looking seed from healthy tree	Natural *P. nigra* forest stand
E18CF	*P. pinea*	İzmir/Bergama	Healthy looking shoot from healthy tree	Natural *P. pinea* forest stand
E19CZ	*P. brutia*	Antalya	Dead cone from healthy tree	Natural *P. brutia* forest stand
E20CF	*P. pinea*	Bursa	Healthy looking cone from healthy tree	Natural *P. pinea* forest stand
E21CZ	*P. brutia*	Isparta/Eğirdir	Symptomatic stem from symptomatic seedling	Forest nursery

2.2.2. Molecular identification of fungi

To confirm the morphological identification, the analysis of the internal transcribed spacer sequence of ribosomal DNA (ITS rDNA) region was conducted for five representative isolates, each exhibiting distinct morphotypes. Genomic DNA was extracted from fresh mycelium using the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The isolated DNAs were measured using a microplate reader (Epoch, Biotek.) to determine their quality and concentration. DNAs of sufficient concentration and quality were stored under appropriate conditions for use in PCR amplifications.

The ITS rDNA region was amplified using the universal primer pair ITS1 and ITS4 (White et al., 1990). PCR reactions were performed with Xpert Fast Hotstart Mastermix (Grisp, Portugal), following the company’s protocol instructions. Amplification was carried out in a 25 µL reaction mix containing 1 µL of each mentioned primer at 10 pmol/µL, 12.5 µL of Xpert Fast Hotstart Mastermix (2X), 7.5 µL PCR-grade water, and 3 µL template DNA. Amplification reactions were conducted in a PCR thermocycler (Kyratec, SuperCycler Thermal Cycler, Australia) with the following conditions: an initial cycle of 3 min at 95 °C (enzyme activation, denaturation of template DNA), followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 15 s, with a final elongation at 72 °C for 3 min. PCR products were sequenced at BMLabosis (ANKARA). The DNA sequences were initially edited and subsequently compared with other sequences in the NCBI (National Center for Biotechnology Information) GenBank using the nucleotide BLAST algorithm. BioEdit version 7.2.6 software was employed to align the most similar sequences, as well as selected outgroup sequences obtained from the NCBI database.

2.3. In vitro determination of antagonistic interactions

In this study, 21 different endophytic fungal (antagonist) isolates were used against 4 different pathogen (*D. sapinea*) isolates in fungal inhibition tests. The dual culture method "Fungal Disc Technique", which is based on the principle of simultaneous cultivation of pathogen and potential antagonists on PDA medium, was used to determine the interactions between the isolates. Inhibition between the tested isolates was determined by simultaneously placing two fungal discs in a Petri dish (Parkinson, 1994).

The pathogen and antagonist isolates to be tested were grown in Petri dishes containing 2% PDA in an incubator set at 25±1°C in the dark. After 7 days of incubation, 5 mm diameter discs were taken from the growing colonies of antagonist and pathogen isolates using a cork borer. Two discs; one from an antagonist and the other from a pathogen was placed onto a Petri dish containing PDA with 5 cm space between them. The dual cultures for each antagonist-pathogen combination (84 pairing in total) were replicated 5 times.

In order to evaluate the growth of pathogenic fungi in antagonist-free medium, 5 mm diameter discs were taken from each fungus and placed onto Petri dishes containing PDA in 5 replicates. The process was positioned in the same way on the Petri dish as in the pairings. All the above-mentioned procedures were carried out in a microbiological safety cabinet. Cork borers used to remove fungal discs were sterilised by dipping in alcohol and passing through a flame before each use. The inoculated Petri dishes were covered with parafilm and placed in the incubator. All Petri dishes were then incubated under the same conditions in the dark in an incubator set at 25±1°C.

2.3.1. Data analyses of antagonistic interactions

The inhibition of the growth of the pathogen fungus (percentage of fungal inhibition) was calculated by the following formula (1) (Grondana et al., 1997). Measurements were made using a ruler and values were recorded in mm.

$$RI = 100 \times \frac{(R_2 - R_1)}{R_2} \quad (1)$$

R1 represents the growth of the pathogen fungus inoculum and the colony formed by it measured in the direction of the antagonist inoculum; R_2 represents the growth of the pathogen fungus measured in the direction of maximum radius development. In the study, the R_2 value given in the formula for fungal inhibition values was determined by measuring the radius of the indicator fungus growing in the antagonist-free medium, not by measuring the growth of the pathogen fungus in the direction of maximum radius development. Each test was repeated 5 times and the percentage inhibition (R_I) value was calculated by taking the average of five repetitions.

The evaluation of the findings obtained in the study was carried out in MiniTab 16 statistical programme. Firstly, simple variance analysis (Anova Test) was performed. In case of statistical differences as a result of Anova test, Duncan test was used to determine the different groups.

3. Results

Experiments were carried out to determine the antagonistic activities of 21 endophytic fungi isolated from five different pine species (*P. brutia*, *P. halepensis*, *P. nigra*, *P. pinea* and *P. sylvestris*), against *D. sapinea* isolates from *P. halepensis* and *P. brutia* under *in vitro* conditions.

The identification of the fungal isolates obtained was carried out by classical methods using morphological characteristics and molecular methods using DNA sequence information. Morphological identification procedures were carried out based on macroscopic and microscopic features of the fungi. The macroscopic characteristics of the spores of the fungi were determined by using culture colour and shape and the microscopic characteristics were determined by examining the colour, shape, characteristics of the walls and spore size. For morphological identification of *D. sapinea* isolates, fragments taken from the colony grown on 2% PDA medium at 20 °C were transferred to 2% water agar medium and sterilised *P. brutia* needles were placed in the same Petri dish for pycnidia formation (Figure 1) and incubated.

Out of 21 endophyte isolates, E10CZ could not be morphologically and molecularly assigned at the genus level (*Ascomycota* sp.). The morphological and molecular characterization results of antagonist fungi and pathogenic fungi are given in Table 3.

Fungal inhibition tests were performed by assessing and measuring the simultaneous growth of pathogens on the medium and evaluating the growth behaviour by measurements and zone formation. The final observations made on the paired fungal species were categorised as follows: (1) inhibition of *D. sapinea* growth (Figure 2A), (2) endophytic dominance (Figure 2B), (3) equal growth ability, no inhibition (Figure 2C) and (4) *D. sapinea* dominance (Figure 2D).

In fungal inhibition tests, 21 different endophytic fungal isolates were used against 4 different pathogenic fungal isolates (DS88, DS85, DS99, DS110). The ability of an endophyte to antagonise the pathogen was determined based on the level of inhibition (defined as pathogen growth with and without endophyte) over a given period of time. The maximum growth of *D. sapinea* isolates, the rate of inhibition by antagonist isolates (%) and the day of maximum growth are given in Table 4.

When Table 4 is analysed; the highest inhibition rate is 72% between E5 and DS85. This rate was followed by the inhibition rate between E5 and DS80, and E5 and DS99. When the control groups were analysed, DS80, DS85, DS99 and DS110 isolates reached maximum growth on days 11, 11, 9 and 9, respectively. Compared to the control groups, DS80 was inhibited by E2, E10 and E11 endophytes, DS85 by E2 and E21 endophytes, DS99 by E1, E2, E20 and E21 endophytes, DS110 by E1, E2, E19, E20 and E21 endophytes. Against endophytes, DS80, DS85, DS99 and DS110 isolates reached maximum growth on average in 9, 8, 8 and 8 days, respectively. From this point of view, DS85 was the most inhibited fungus in terms of total inhibition. This isolate was followed by DS80, DS110 and DS99, respectively. E3 and E5 coded antagonist isolates were observed to inhibit the growth of the pathogen on day 4. Antagonists coded E4, E6, E7, E12 and E16, which had different inhibition rates, inhibited the growth of pathogenic fungi on the 7th, 9th, 9th, 8th and 8th days, respectively. The control of the arithmetic means of the maximum growth amount, inhibition rate percentage and maximum growth day values of the antagonist isolates applied against *Diplodia* isolates was performed by simple analysis of variance (Anova test). Duncan test was applied as a result of the difference in arithmetic means as a result of Anova test (Table 5).

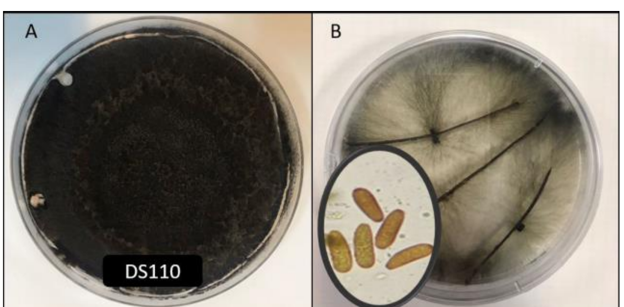

Figure 1. Morphological identification of pathogen isolates based on macroscopic and microscopic characteristics A) 20-day-old colony of *Diplodia sapinea* (DS110) incubated in 2% PDA at 20 °C in the dark B) Incubation of *Pinus brutia* needles in a 2% water agar inoculated with *Diplodia sapinea* for the induction of pycnidia formation

Table 3. Morphological and molecular characterisation results of pathogenic and endophytic fungi

Code	Morp. Ident.	Molec. Ident.	Most close GenBank matches	
			Query cover	Accession number
DS80	*Diplodia sapinea*	*Diplodia sapinea*	99%	*MH183336.1*
DS85	*Diplodia sapinea*	*Diplodia sapinea*	99%	*MH183342.1*
DS99	*Diplodia sapinea*	*Diplodia sapinea*	100%	*MN698985.1*
DS110	*Diplodia sapinea*	*Diplodia sapinea*	100%	*MT587369.1*
E1CF	*Fusarium* sp1	-	-	-
E2CS	*Fusarium* sp2	*Fusarium* sp2	98%	*KX618492.1*
E3CS	*Trichoderma* sp1	-		
E4CS	*Trichoderma* sp2	*Trichoderma* sp2	98%	*KC576692.1*
E5CF	*Trichoderma* sp3	*Trichoderma* sp3	-	-
E6CZ	*Trichoderma* sp4	*Trichoderma* sp4	-	-
E7CF	*Aspergillus* sp.	*Aspergillus ochraceus*	100%	*MH856959.1*
E8CZ	*Oxyporus corticola*	*Oxyporus corticola*	100%	*KC176669.1*
E9CZ	*Epicoccum* sp.	-	-	-
E10CZ	Unknown isolate	-	-	-
E11CK	*Alternaria* sp1	*Alternaria* sp1	100%	*MT448892.1*
E12CK	*Fusarium* sp3	*Fusarium* sp3	100%	*MG274297.1*
E13CH	*Pseudocamasparium sp.*	*Pseudocamasparium brabeji*	100%	*MN833937.1*
E14CT	*Coniothyrium sp.*	*Coniothyrium juniperi*	100%	*MH860594.1*
E15CF	*Akanthomyces* sp.	*Akanthomyces* sp.	99%	*NR_111096*
E16CK	*Sydowia polyspora*	*Sydowia polyspora*	99%	*MT556703.1*
E17CK	*Alterneria alternata*	-	-	-
E18CF	*Alternaria* sp2	-	-	-
E19CZ	*Akanthomyces attenuatus*	*Akanthomyces attenuatus*	100%	*MT889904.1*
E20CZ	*Trihothecium* sp.	-		
E21CF	*Fusarium* sp4	*Fusarium avenaceum*	99%	*MT357238.1*

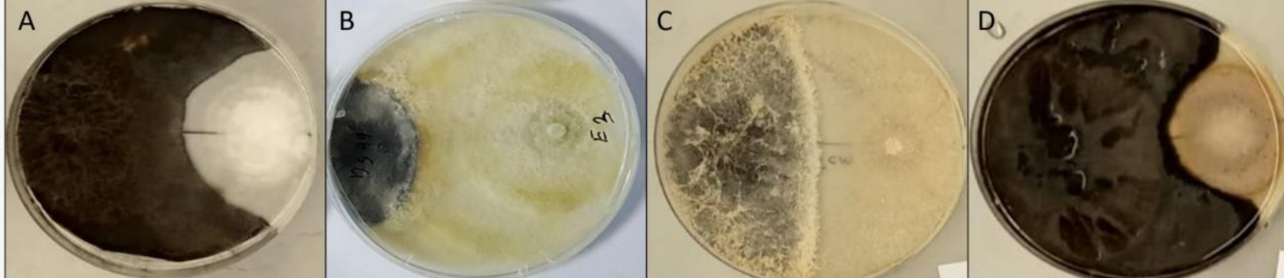

Figure 2. Examples of endophyte fungi showing different reactions with *Diplodia sapinea* (brown-grey morphology) isolate in dual cultures during antagonism trials. (A) E15 and D. sapinea; (B) E3 and *D. sapinea*; (C) E20 and *D. sapinea*; (D) E8 and *D. sapinea*.

Table 4. Maximum growth of *D. sapinea* isolates, inhibition rates caused by the endophytic isolates on the pathogen (%) and maximum growth days

Antagonist	Maximum growt amount (r=cm)				Inhibition rate values (%)				Maximum growth days			
	DS80	DS85	DS99	DS110	DS80	DS85	DS99	DS110	DS80	DS85	DS99	DS110
E1CF	4.2	4.2	4.2	4.2	2.33	2.33	2.33	2.33	10	9	11	11
E2CS	4.2	4.2	4.2	4.1	2.33	2.33	2.33	4.65	11	11	11	10
E3CS	2.1	2.6	2.5	2.4	51.16	39.53	41.86	44.19	3	3	5	4
E4CS	2.4	4.1	3.2	4.1	44.19	4.65	25.58	4.65	7	7	5	6
E5CF	1.3	1.2	1.5	2	69.77	72.09	65.12	53.49	4	3	3	3
E6CZ	1.7	1.7	1.9	1.9	60.47	60.47	55.81	55.81	9	8	9	7
E7CF	2.1	4.2	4.2	4.2	51.16	2.33	2.33	2.33	9	9	7	9
E8CZ	4.2	4.2	4.2	4.2	2.33	2.33	2.33	2.33	7	7	6	7
E9CZ	4.2	4.2	4.2	4.2	2.33	2.33	2.33	2.33	9	5	5	6
E10CZ	4.2	4.2	4.2	4.2	2.33	2.33	2.33	2.33	11	8	8	7
E11CK	4.2	4.2	4.2	4.2	2.33	2.33	2.33	2.33	11	10	9	7
E12CK	3.7	3.6	3.8	3.5	13.95	16.28	11.63	18.60	8	10	9	4
E13CH	4.2	4.2	4.2	4.2	2.33	2.33	2.33	2.33	9	7	5	6
E14CT	4.2	4.2	4.2	4.2	2.33	2.33	2.33	2.33	7	6	5	6
E15CF	4.2	4.2	4.2	4.2	2.33	2.33	2.33	2.33	9	6	5	6
E16CK	3.9	2.1	2.2	2.1	9.30	51.16	48.84	51.16	9	6	5	7
E17CK	4.2	4.2	4.2	4.2	2.33	2.33	2.33	2.33	8	7	5	8
E18CF	4.2	4.2	4.2	4.2	2.33	2.33	2.33	2.33	6	7	5	5
E19CZ	4.2	4.2	4.2	4.2	2.33	2.33	2.33	2.33	7	6	8	10
E20CZ	4.2	4	4.2	4.2	2.33	6.98	2.33	2.33	10	8	11	11
E21CF	4.2	4.2	4.2	4.2	2.33	2.33	2.33	2.33	9	11	11	11
control	4.3	4.3	4.3	4.3	-	-	-	-	11	11	9	9

Table 5. Duncan test results of maximum growth amount of *D. sapinea* isolates, inhibition rate values (%) of the pathogen by antagonist isolates and maximum growth day

Antagonist	Maximum growth amount (r=cm)	Antagonist	Inhibition rate values (%)	Antagonist	Maximum growth day
5	1.5 (0.01)[1] a[2]	1	2.33 (0.02) a	5	4 (0.03) a
6	1.8 (0.02) a	8	2.33 (0.02) a	3	4 (0.04) a
3	2.4 (0.03) b	9	2.33 (0.02) a	18	6 (0.06) b
16	2.6 (0.02) b	10	2.33 (0.02) a	14	6 (0.07) b
4	3.3 (0.03) c	11	2.33 (0.03) a	4	7 (0.06) bc
12	3.6 (0.03) cd	13	2.33 (0.03) a	9	7 (0.08) bc
7	3.7 (0.04) cd	14	2.33 (0.03) a	15	7 (0.08) bcd
20	4.2 (0.04) de	15	2.33 (0.45) a	8	7 (0.08) bcd
2	4.2 (0.04) de	17	2.33 (0.02) a	13	7 (0.07) bcd
1	4.2 (0.04) de	18	2.33 (0.03) a	16	8 (0.08) bcde
8	4.2 (0.04) de	19	2.33 (0.02) a	17	8 (0.09) bcde
9	4.2 (0.03) de	20	3.49 (0.02) a	12	8 (0.09) bcde
10	4.2 (0.04) de	21	2.33 (0.02) a	19	8 (0.09) bcde
11	4.2 (0.04) de	2	2.91 (0.02) a	6	9 (0.08) cdef
13	4.2 (0.04) de	7	14.53 (0.16) ab	7	9 (0.10) defg
14	4.2 (0.04) de	12	15.12 (0.15) ab	10	9 (0.10) defg
15	4.2 (0.03) de	4	19.77 (0.21) b	11	10 (0.11) efgh
17	4.2 (0.03) de	16	40.12 (0.02) c	20	10 (0.11) fgh
18	4.2 (0.04) de	3	44.19 (0.48) c	1	11 (0.12) fgh
19	4.2 (0.04) de	6	58.14 (0.60) d	21	11 (0.12) gh
21	4.2 (0.04) de	5	65.12 (0.64) d	2	11 (0.11) h
control	4.3 (0.04)	control	-	control	10 (0.11)

1: Standard deviation, 2: Homogeneous groups formed according to Duncan's test are indicated by letters in each column. F=23.046, F=23.197 and F=9.561 and $p<0.001$ for maximum growth amount, inhibition rate percentages and maximum growth days, respectively.

When Table 5 is analysed, the inhibition rates of E1, E8, E9, E10, E11, E13, E14, E15, E17, E18, E19, E20 and E21 coded endophytes were 2.3%. Visual analyses showed that endophytes coded E2, E9, E10, E13, E14, E15, E17, E19 formed an inhibition zone with Diplodia isolates. Isolates coded E1, E8, E21 did not form any inhibition zone and Diplodia isolates showed superior growth. Isolates E11, E18 and E20 showed equal growth characteristics with Diplodia isolates. Among the isolates tested, the fungus with the highest antagonistic effect on average was isolate E5 (65.12%), followed by isolate E6 (58.14%). These isolates were followed by E3 (44.19%), E16 (40.16%), E4 (19.77%), E12 (15.12%) and E7 (14.53%). According to the visual inspection results, 4 different types of interactions were observed between the tested endophytes and *D. sapinea* isolates. In the study, 71% of the tested strains were able to inhibit *D. sapinea in vitro* or provide superiority over *D. sapinea* in their growth. As a result of the tests, a total of 15 strains can be considered as potential antagonists against *D. sapinea in vitro* because they showed faster growth than *D. sapinea* or inhibited the growth of the pathogen.

In the study, 33% of the endophytes (E3, E4, E5, E6, E7, E12, E16) showed faster growth than *D. sapinea*. 14% (E11, E18, E20) of the endophytes tested in the study showed equal growth with *D. sapinea* isolates without inhibition. *D. sapinea* isolates tested in our study were unable to cross the endophyte barrier in 38% of the antagonist isolates tested and non-contact inhibition was observed. These isolates (E2, E9, E10, E13, E14, E15, E17, E19) react to the presence of the competitor fungus, forming an inhibition zone. In this study, 14% of the endophytes tested (E1, E8, E21) caused superior growth of mycelium of *D. sapinea* isolates without showing inhibition and were observed as deficient.

4. Discussion and Conclusion

The ability of many fungi to antagonise various microorganisms, especially bacteria and other fungi, has been known and studied by researchers for many years (Baker, 1987; Harman, 2006; Thambugala et al., 2020). Furthermore, numerous fungi have been assessed for their potential as biological control agents against plant diseases, with many already being successfully utilized in agriculture and forestry as commercial-scale biological control agents (Thambugala et al., 2020; Guzmán-Guzmán et al., 2023). To effectively implement biological control strategies against plant diseases, it is required to evaluate the interaction between a pathogen and its antagonist in a controlled laboratory setting using in vitro assays as the initial step (Bosmans et al., 2016; Köhl et al., 2019). These assays not only describe antagonistic interactions, but also offer valuable insights for selecting and refining biocontrol agents, which ultimately establishes the foundation for successful biological control strategies in the field.

The dynamics of forest pathosystems may become unpredictable in the future due to environmental changes that favor fungal pathogens over the viability of hosts. Similarly, these changes may have unknown impacts on the essential fungal endophytes of host trees. It is anticipated that abiotic stress, specifically drought, may enhance the aggressiveness of *D. sapinea* in the future (Blumenstein et al., 2021). This study observed various forms of competition among different endophytes against *D. sapinea*. In general, these findings suggest that other endophytes in pine tissues, particularly on branches, might contribute to the development of "Diplodia shoot blight" disease. Hypothetically, promoting a specific tree microbiome in tree health could lead to an effective, long-lasting, and environmentally friendly control method against severe disease outbreaks.

In this study, pine endophytes and *D. sapinea* isolates interacted with each other in various ways, as demonstrated by in vitro antagonism assays. Overall, four interaction categories were identified in all tests. Similar results were presented by Bußkamp (2018) and Blumenstein (2021). In our study, 71% of the strains tested were able to inhibit *D. sapinea in vitro* or outcompete *D. sapinea* in growth. Generally, 15 strains can be considered as potential antagonists against *D. sapinea in vitro*, because they either

showed a faster growth than *D. sapinea* or inhibited the growth of the pathogen. This partly agrees with the results of Bußkamp (2018), who found that 22% of the 89 endophytic strains tested inhibited the growth of *D. sapinea*.

The *D. sapinea* isolates tested in our study failed to cross the endophyte barrier in 38% of the antagonist isolates tested and non-contact inhibition was observed. These isolates (E2, E9, E10, E13, E14, E15, E17, E19) react to the presence of the competitor fungus, forming an inhibition zone. Chemical antagonism can be assumed when a fungus reacts to the presence of a competitor fungus with an inhibition zone between two fungal colonies. A fungus can secrete secondary metabolites that inhibit the competitor fungus (Schulz et al., 2002). To determine whether a particular metabolite can inhibit growth, secondary metabolites must be extracted and tested to see if the same reaction can be observed against the pathogen (Tellenbach et al., 2013). Blumenstein et al. (2021) observed a non-contact zone of inhibition between *A. alternata* isolates and *D. sapinea* isolates. The *A. alternata* isolate coded E17 and the *Alternaria* isolate coded E18, which we used in our study, formed a non-contact zone of inhibition with the pathogen.

In the analyses carried out, E1, E2, E8, E9, E10, E11, E13, E14, E15, E17, E18, E19, E20 and E21 coded endophytes were found to inhibit the the pathogen at an average rate of 2.3%. This does not mean that all of these endophytes failed to inhibit the fungus. Visual analyses showed that endophytes E2, E9, E10, E13, E14, E15, E17, E19 formed a non-contact zone of inhibition with *Diplodia* isolates. These isolates form an inhibition zone by reacting to the presence of the competitor fungus. Fungal inhibition tests are carried out by evaluating and measuring the simultaneous growth of pathogens on the medium and evaluating the growth behaviour according to measurements and zone formation.

In this study, 14% of the endophytes tested (E1, E8, E21) caused superior growth of mycelium of *D. sapinea* isolates without showing inhibition. Among the isolates used in the antagonism tests, the antagonist isolates with neutral interactions and lower growth than *D. sapinea* do not appear to be suitable potential antagonists. These isolates did not form any inhibition zone and at the same time showed lower growth than the pathogen. This growth ability of *Diplodia* isolates may indicate a stronger capacity to metabolise nutrients than other endophytes (Bußkamp, 2018). Faster growth and better utilisation of nutrients are two strategies with clear advantages during competition (Mgbeahuruike et al., 2011). Some typical endophytes from host tree species can in theory provide strong competition against pathogens in nature if they can show sufficient distribution in host tissues (Terhonen et al., 2019; Bußkamp et al., 2020). When a pine species is weakened by drought stress, the common endophytes within the tree may be at a disadvantage. This creates an opportunity for *D. sapinea*, a secondary pathogen, to establish itself more readily, invade more host tissue, and outcompete other endophytes. This shift in the microbial community could explain why *D. sapinea* is more prevalent in areas experiencing tree disease (Bußkamp et al., 2020; Blumenstein et al., 2021). This observation suggests that environmental stressors like drought may create conditions that favor certain pathogens over the typical endophytes, leading to increased disease occurrence and spread.

In the study, 14% of the endophytes tested (E11, E18, E20) showed equal growth with *D. sapinea* isolates without showing inhibition. In this case, it can be said that the isolates were not affected by each other and antagonism did not occur. It may mean that these fungi can grow together in the host and neither of them reacts specifically to the presence of the other.

In addition, 33% of the endophytes, E5, E6, E3, E16, E16, E4, E12, E7 coded isolates showed superiority against the pathogen and these isolates have the potential to be used as biological control agents. Isolates coded E3, E4, E5 and E6 were identified as fungi belonging to the genus *Trichoderma* as a result of morphological and molecular identification. *Trichoderma* isolates, sourced from various hosts in the study, demonstrated diverse mechanisms in inhibiting the growth of indicator fungi. Fungi belonging to the genus *Trichoderma* have been used for biological control against plant pathogens since 1920s. These species accelerate plant growth, increase plant defence mechanisms and make plants more resistant to pathogens. In addition, the various broad s pectrum of antibiotic compounds they produce provide an effective control against pathogens (Dennis and Webster, 1971; Küçük and Kıvanç, 2003; Oskay and Şimşek 2017; Guzmán-Guzmán et al., 2023).

Another isolate determined in the study to have the potential to be used as a biological control agent is the isolate coded E12. As a result of morphological and molecular diagnosis, this isolate was determined to be *Fusarium* sp3. It is known that non-virulent fungi belonging to the *Fusarium* genus are commercially produced due to their adaptation to the ecosystem in which they are found and their positive effects on plant development. E7-coded *Aspergillus* sp. is among the successful isolates. The *Aspergillus* genus is a large group of fungi known for its biological diversity and metabolic flexibility. Many species in this genus are used in various industrial and biotechnological applications, often capable of producing valuable chemical compounds (Wilson et al., 2002; Keller and Turner, 2012).

Another fungus with high biological control potential is *S. polyspora* isolate coded E16. *S. polyspora* is a typical endophyte of pines that is widespread throughout the world (Muñoz-Adalia et al., 2017; Pan et al., 2018). As an endophyte, it has a high consistency and frequency in pine branches (Sanz-Ros et al., 2015; Blumenstein et al. 2020; Bußkamp et al. 2020). In a study conducted by Bußkamp (2018), *S. polyspora* isolates did not exhibit antagonistic behavior in double culture with *D. sapinea*. The recent reports by Oliva et al. (2021) and Blumenstein et al. (2021) highlighted the biological control potential of *S. polyspora*, indicating its antagonistic effects against *D. sapinea*. Oliva et al. (2021) identified a cluster of potential antagonistic species by analyzing the relationships between the *D. sapinea* pathogen and endophytes present in the shoots of adult pine trees. In their study, *S. polyspora* was identified as one of the species with antagonistic ability against *D. sapinea*, along with an *Alternaria* species accompanied by *E. nigrum*. The dynamic nature of these interactions underscores the importance of further research in understanding the variability of antagonistic behavior among endophytic species and their potential role in disease control strategies.

The general purpose of this study is not to directly inhibit the growth of *D. sapinea*, but to determine how much *D. sapinea* growth is inhibited in the presence of endophytes. In future studies, effective fungal endophytes should be tested *in vivo* on plants. Some show that fungal endophytes can

enhance the host plant's immune system *in vivo*. (Ganley, 2008; Mejia et al., 2008; Witzell and Martín, 2018).

In this study, fungal antagonists that can be used as biological control agents, especially *Trichoderma* sp., which were found to be very effective in *in vitro* tests. The potential of the isolates to be used against the *D. sapinea*, an important pathogen in forest trees, has emerged. Endophyte fungi have a very important place among potential biological control agents. One of the important issues in the selection of biological agents is that potential antagonists are not only successful in vitro against the tested organisms, but also that they remain stable in natural environments and have the ability to reach a population level that can suppress disease agents in field conditions. In addition, it is a very important factor that potential antagonists can be effective without causing any harm to nature or disturbing the natural balance. Although *in vitro* tests are useful in determining the enzymatic and antibiotic activities of biological control agents, they cannot determine how and how effective these mechanisms are while maintaining the interactions of organisms in their natural environment (Whipp, 1987). In this study, isolates were planted opposite each other in petri dishes at the same time. Inoculating *D. sapinea* later than endophytes may lead to different results in the study.

This study demonstrated that certain tested endophytes, such as *Trichoderma* sp., *Alternaria* sp., *Sydowia polyspora*, sharing a similar habitat with *D. sapinea*, effectively inhibited the *in vitro* growth of *D. sapinea* isolates. The findings of this study suggested that competition between *D. sapinea* and other endophytes might impede the growth of *D. sapinea* and the manifestation of Diplodia shoot blight symptoms. Pine endophytes exhibiting antagonistic properties against the *D. sapinea* pathogen without harming the host tree could serve as biological control agents, aiding in the protection of the host tree. Future research can be designed to explore innovative and efficient approaches for harnessing the beneficial tree microbiome amidst upcoming challenges. Notably, *D. sapinea* is known to intensify disease severity under drought stress, indicating that Diplodia shoot blight may lead to more widespread outbreaks in the future due to climate change-induced severe and prolonged drought conditions. Consequently, preventive measures against the disease agent should be implemented promptly, and in-depth studies on combat strategies should be continued.

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References

Baker, K.F., 1987. Evolving concepts of biological control of plant pathogens. Annual review of phytopathology, 25: 67–85.

Bamisile, B.S., Dash, C.K., Akutse, K.S., Keppanan, R., Wang, L., 2018. Fungal endophytes: beyond herbivore management. Frontiers in Microbiology, 9: 544.

Beram, R.C., Beram, A., Lehtıjarvi, H.T.D., 2016. Fungal endofitler ve etkileşimleri. Mehmet Akif Ersoy Üniversitesi Fen Bilimleri Enstitüsü Dergisi, 7(2):161-166.

Bernicchia A., 2005. *Polyporaceae* s.l. Fungi Europaei 10: 808 p. Ed. Candusso, A.

Blumenstein, K., Bubkamp, J., Langer, G.J., Schlöber, R., Rojas, N.M.P., Terhonen, E., 2021. *Sphaeropsis sapinea* and associated endophytes in scots pine: interactions and effect on the host under variable water content. Frontiers in Forests and Global Change, 4: 655769.

Blumenstein, K., Langer, G., Bubkamp, J., Langer, E., Terhonen, E., 2020. The opportunistic pathogen *Sphaeropsis sapinea* is found to be one of the most abundant fungi in symptomless and diseased Scots pine in Central-Europe. Research Square, 3:48366.

Bosmans, L., Bruijn, I., Mot, R., Rediers, H., Lievens, B., 2016. Agar composition affects in vitro screening of biocontrol activity of antagonistic microorganisms. Journal of Microbiological Methods, 7-9:127.

Brodde, L., Adamson, K., Camarero, J.J., Castaño, C., Drenkhan, R., Lehtijärvi, A., Luchi, N., Miglliorini, D., Miranda, A.S., Stenlid, J., Özdağ, Ş., Oliva, J., 2019. Diplodia tip blight on its way to the north: drivers of disease emergence in northern Europe. Frontiers in Plant Science, 9:1818.

Brookhouser, L.W., Peterson, G.W., 1971. Infection of Austrian, Scots, and ponderosa pines by *Diplodia pinea*. Phytopathology, 61(4): 409-414.

Bußkamp, J., 2018. Schadenserhebung, Kartierung und Charakterisierung des Diplodia-Triebsterbens "der Kiefer, insbesondere des endophytischen Vorkommens in den klimasensiblen Räumen und Identifikation von den in Kiefer (*Pinus sylvestris*) vorkommenden Endophyten.PhD Dissertation, Universität Kassel, Fachbereich 10 Mathematik und Naturwissenschaften Fachgebiet Ökologie, Germany.

Bußkamp, J., Langer, G.J., Langer, E.J., 2020. *Sphaeropsis sapinea* and fungal endophyte diversity in twigs of Scots pine (*Pinus sylvestris*) in Germany. Mycological Progress, 19(9):985-999.

Capretti, P., Santini, A., Solheim, H., 2013. Pathogens, Significance and Distribution. In Infectious forest diseases, Ed. Gonthier, P., Nicolotti, G., 21 Branch and Tip Blights, Italy pp. 420-435.

Chou, I. M., 1987. Phase relations in the system NaCl-KCl-H_2O. III: Solubilities of halite in vapor-saturated liquids above 445° C and redetermination of phase equilibrium properties in the system NaCl-H_2O to 1000° C and 1500 bars. Geochimica et Cosmochimica Acta, 51(7):1965-1975.

Costa, D., Tavares, R.M., Baptista, P., Lino-Neto, T., 2020. Cork oak endophytic fungi as potential biocontrol agents against *Biscogniauxia mediterranea* and *Diplodia corticola*. Journal of Fungi, 6(4):287.

Dennis, C., Websterm, J., 1971. Antagonistic properties of species-groups of *Trichoderma*: I. Production of non-volatile antibiotics. Transactions of the British Mycological Society, 57(1):25-39.

Diekmann, M., Sutherland, J.R., Nowell, D.C., Morales, F.J., Allard, G., 2002. *Pinus* spp. Food and Agriculture Organization of the United Nations, Technical Guidelines for the Safe Movement of Germplasm, Rome.

Ganley, R., 2008. Density and diversity of fungal endophytes isolated from needles of *Pinus radiata*. Client Report, N 2925.

Grondona, I., Hermosa, R., Tejada, M., Gomıs, M. D., Mateos, P. F. Brıdge, P. D., Monte, E., Garcıa-Acha, I., 1997. Physiological and Biochemical Characterization of Trichoderma Harzianum, A Biological Control Agent Against Soilborne Fungal Plant Pathogens. Applied And Envıronmental Microbiology, 63 (8):3189–3198.

Guzmán-Guzmán, P., Kumar, A., Santos-Villalobos, S., Parra-Cota, F.I, Orozco-Mosqueda, M., Fadiji, A.E., Hyder, S., Babalola, O.O., Santoyo, G., 2023. *Trichoderma* Species: Our best fungal allies in the biocontrol of plant diseases - A Review. Plants, 12(3): 432

Harman G. E., 2006. Overview of mechanisms and uses of *Trichoderma* spp. Phytopathology,96 (2):190–194.

Kaya, A.G.A., Lehtijärvi, A., Kaya, Ö., Doğmuş-Lehtijärvi, T., 2014. First report of *Diplodia pinea* on *Pseudotsuga menziesii* in Turkey. Plant disease, 98(5): 689-689.

Kaya, A.G.A., Yeltekin, Ş., Lehtijärvi, T.D., L., Lehtijärvi, A., Woodward, S., 2019. Severity of Diplodia shoot blight (caused by *Diplodia sapinea*) was greatest on *Pinus sylvestris* and *Pinus nigra* in a plantation containing five pine species. Phytopathologia Mediterranea, 58(2):249-259.

Keller, N. P., Turner, G., 2012. Fungal secondary metabolism: regulation and function. Current Opinion in Microbiology, 15(5):508-515.

Kowalski, T., Bilański, P., 2021. Fungi detected in the previous year's leaf petioles of *Fraxinus excelsior* and their antagonistic potential against *Hymenoscyphus fraxineus*. Forests, 12(10):1412.

Köhl, J., Kolnaar, R., Ravensberg, W., 2019. Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. Frontiers in Plant Science, 10:845.

Küçük, Ç., Kivanç, M., 2003. Isolation of *Trichoderma spp*. and determination of their antifungal, biochemical and physiological features. Turkish Journal of Biology, 27(4):247-253.

Langer, G., Bressem, U., Habermann, M., 2011. Diplodia-Triebsterben der Kiefer und endophytischer Nachweis des Erregers *Sphaeropsis sapinea*. AFZ-Der Wald, 11: 28-31.

Mejía, L.C., Rojas, E.I., Maynard, Z., Van Bael, S., Arnold, A.E., Hebbar, P., Samuels, G., Robbins, N., Herre, E.A., 2008. Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. Biological control, 46(1):4-14.

Mgbeahuruike, A.C., Sun, H., Fransson, P., Kasanen, R., Daniel, G., Karlsson, M., Asiegbu, F.O., 2011. Screening of *Phlebiopsis gigantea* isolates for traits associated with biocontrol of the conifer pathogen *Heterobasidion annosum*. Biological Control, 57(2):118-129.

Munck, I.A., Smith, D.R., Sickley, T., Stanosz, G.R., 2009. Site-related influences on cone-borne inoculum and asymptomatic persistence of Diplodia shoot blight fungi on or in mature red pines. Forest Ecology and Management, 257(3):812-819.

Muñoz-Adalia, E.J., Sanz-Ros, A.V., Flores-Pacheco, J.A., Hantula, J., Diez, J.J., Vainio, E.J., Fernández, M., 2017. *Sydowia polyspora* dominates fungal communities carried by two *Tomicus* species in pine plantations threatened by *Fusarium circinatum*. Forests, 8(4):127.

Oliva, J., Ridley, M., Redondo, M.A., Caballol, M., 2021. Competitive exclusion amongst endophytes determines shoot blight severity on pine. Functional Ecology, 35(1):239-254.

Oskay, F., Lehtijärvi, A., Lehtijärvi, H.T.D., Woodward, S., 2018. First report of *Diplodia sapinea* on *Cedrus libani* in Turkey. New Disease Reports, 38: 13.

Oskay, F., Şimşek, Z., 2017. Çankırı (Eldivan) Karaçam Orman topraklarında saptanan bazı mikrofungusların in vitro koşullarda antagonistik etkileşimlerinin belirlenmesi. Anadolu Orman Araştırmaları Dergisi, 3(2):130-138.

Pan, Y., Ye, H., Lu, J., Chen, P., Zhou, X.D., Qiao, M., Yu, Z.F., 2018. Isolation and identification of *Sydowia polyspora* and its pathogenicity on *Pinus yunnanensis* in Southwestern China. Journal of Phytopathology, 166(6):386-395.

Parkinson, D., 1994. Filamentous fungi. Methods of Soil Analysis: Microbiological and Biochemical Properties, America.

Parnell, I.W., 1957. Some notes from Australia. Scottish Agriculture, 37(2): 84-87.

Prospero, S., Botella, L., Santini, A., Robin, C., 2021. Biological control of emerging forest diseases: How can we move from dreams to reality? Forest Ecology and Management, 496:119377.

Raghavendra, A.K., Newcombe, G., 2013. The contribution of foliar endophytes to quantitative resistance to *Melampsora rust*. New Phytologist, 197(3):909-918.

Rodriguez, R.J., White, J.F., Arnold, A.E., Redman, R.S., 2009. Fungal endophytes: diversity and functional roles. New Phytologist, 182(2):314-330.

Ryvarden, L., 1978. The Polyporaceae of North Europe. Inonotus-Tyromyces, 2: 219-507.

Sanz-Ros, A.V., Müller, M.M., San Martín, R., Diez, J.J., 2015. Fungal endophytic communities on twigs of fast and slow growing Scots pine (*Pinus sylvestris* L.) in northern Spain. Fungal Biology, 119(10):870-883.

Schulz, B., Boyle, C., Draeger, S., Römmert, A. K., Krohn, K., 2002. Endophytic fungi: a source of novel biologically active secondary metabolites. Mycological Research, 106(9):996-1004.

Silva, N.I.S., Brooks, S., Lumyong, S., Hyde, K.D., 2019. Use of endophytes as biocontrol agents. Fungal Biology Reviews, 33:133-148.

Soylu, S., Kurt, Ş., Soylu, E.M., 2001. Determination of important fungal disease agents on pine trees in the Kahramanmaraş regional forests. Journal of Turkish Phytopathology, 30: 79.

Stanosz, G.R., Blodgett, J.T., Smith, D.R., Kruger, E.L., 2001. Water stress and *Sphaeropsis sapinea* as a latent pathogen of *P. brutia* seedlings. New Phytologist, 149(3): 531-538.

Sümer, S., 2000. Shoot Blight Disease Caused by *Sphaeropsis sapinea* in Pine Stands at the South-Eastern Region, Turkey. (OGM research report)

Swart, W.J., Wingfield, M.J., 1991. Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. Plant Disease, 75(8):761-766.

Tellenbach, C., Sumarah, M.W., Grünig, C.R., Miller, J.D., 2013. Inhibition of *Phytophthora* species by secondary metabolites produced by the dark septate endophyte *Phialocephala europaea*. Fungal Ecology, 6(1): 12-18.

Terhonen, E., Blumenstein, K., Kovalchuk, A., Asiegbu, F. O., 2019. Forest tree microbiomes and associated fungal endophytes: Functional roles and impact on forest health. Forests, 10(1): 42.

Terhonen, E., Kovalchuk, A., Zarsav, A., Asiegbu, F.O., 2018. Biocontrol Potential of Forest Tree Endophytes. In: Forestry Sciences (Ed: Pirttilä, A.M., Frank, C.), Endophytes of Forest Trees, Finland, pp. 283-318.

Thambugala, K., Daranagama, D., Phillips, A., Kannangara, S., Promputtha, I., 2020. Fungi vs. fungi in biocontrol: an overview of fungal antagonists applied against fungal plant pathogens. Frontiers in Cellular and Infection Microbiology, 10: 604923.

Trejo-Estrada, S.R., Sepulveda, I.R., Crawford, D.L., 1998. In vitro and in vivo antagonism of Streptomyces violaceusniger YCED9 against fungal pathogens of turfgrass. World Journal of Microbiology and Biotechnology, 14: 865-872.

Ünligil, H., Ertaş, A., 1993. İstanbul yakınlarındaki çam ağaçlarında *Sphaeropsis sapinea* (Fr.) Dylco & Sutton mantar hastalığı. Journal of the Faculty of Forestry Istanbul University, 43(1): 131-138.

Whipp, S. C., 1987. Protease degradation of Escherichia coli heat-stable, mouse-negative, pig-positive enterotoxin. Infection and immunity, 55(9), 2057-2060.

White, T.J., Bruns, T., Lee, S.J.W.T., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications, 18(1): 315-322.

Wilson, D. M., Mubatanhema, W., Jurjevic, Z., 2002. Biology and ecology of mycotoxigenic Aspergillus species as related to economic and health concerns. Mycotoxins and Food Safety, 504:3-17.

Witzell, J., Martín, J.A., 2018. Endophytes and forest health. Endophytes of Forest Trees: Biology and Applications, 86: 261-282.

Witzell, J., Martín, J.A., Blumenstein, K., 2014. Ecological aspects of endophyte-based biocontrol of forest diseases. Advances in Endophytic Research, 321-333.

Yeltekin, Ş., 2015. Kerpe araştırma ormanı konifer türlerinde *Diplodia* spp.'den kaynaklanan kozalak ve sürgün enfeksiyonlarının belirlenmesi. Yüksek lisans Tezi, Süleyman Demirel Üniversitesi. Fen Bilimleri Enstitüsü, Isparta.

Zlatković, M., Keča, N., Wingfield, M.J., Jami, F., Slippers, B., 2017. New and unexpected host associations for *Diplodia sapinea* in the Western Balkans. Forest Pathology, 47(3): e12328.