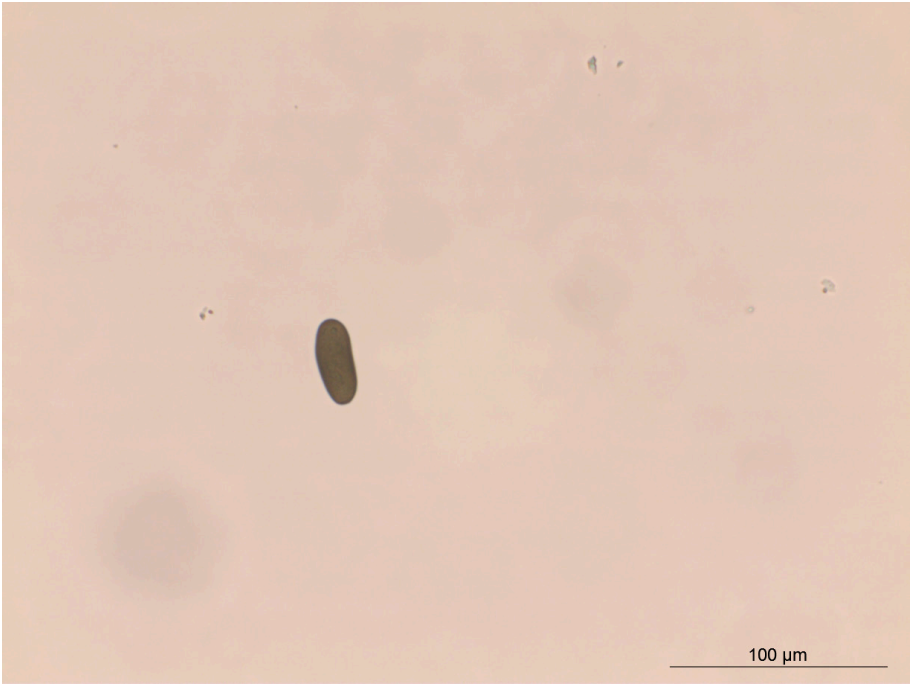
Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2023:43
Faculty of Forest Sciences

Diplodia tip blight affecting Scots pine

Factors determining infection and spread in Swedish forests

Laura Brodde



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SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

DOCTORAL THESIS
Uppsala 2023

Acta Universitatis Agriculturae Sueciae
2023:43

Cover: Conidium of *Diplodia sapinea* obtained from culture. Isolate collected at the first detected outbreak of Diplodia tip blight in Sweden, 2016.
(photo: L. Brodde)

ISSN 1652-6880
ISBN (print version) 978-91-8046-138-2
ISBN (electronic version) 978-91-8046-139-9
https://doi.org/10.54612/49nu5u98e1
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Print: SLU Grafisk service, Uppsala 2023

Diplodia tip blight affecting Scots pine. Factors determining infection and spread in Swedish forests

Abstract

Diplodia sapinea (syn. *Sphaeropsis sapinea*) is a globally distributed pathogen of conifers. Symptoms of Diplodia tip blight can develop when its host is affected by stressors such as drought, heat or mechanical wounding. This thesis builds on the first detected outbreak of Diplodia tip blight on Scots pine (*Pinus sylvestris* L.) in Sweden, which marked the northernmost record of such an outbreak of this disease at that time. The aim of this thesis was to generate knowledge about the emergence of Diplodia tip blight. The work showed that the fungus has no apparent restrictions to cause damages on Scots pine in the Nordic climate. Climate, and variation in drought adaptation of pines were potential drivers of the first detected outbreak discovered in 2016. Further, the work showed that *D. sapinea* is not a recently introduced pathogen in Sweden and is genetically similar to the general European population. Large areas of Scots pine showing severe crown dieback were discovered after the drought of 2018 on Gotland. *Diplodia sapinea* was associated with the observed symptoms, though not the driver of the dieback. Mortality was highest in severely damaged pines, but pines could recover when the drought stress lessened. *Diplodia sapinea* was found to be present in healthy-looking stands, trees, and twigs. However, high abundances of *D. sapinea* were only found in symptomatic twigs of trees showing crown dieback. Endophytic fungal communities depended on health status and these differences were apparent across scales, from tissues to stands. Four species were associated with healthy tree tissue, indicating potential for antagonistic fungi in the endophytic community of Scots pine. A standardized *in vitro* sporulation method was developed to facilitate future research on the pathosystem *D. sapinea* – Scots pine. The findings in this thesis contribute knowledge about the range expansion of Diplodia tip blight to the North, and underline the importance of managing stress-related forest pathogens to maintain forest health in the ongoing climate change.

Keywords: Diplodia tip blight, *Diplodia sapinea*, Scots pine, emerging disease,endophyte, drought, fungal community, in vitro sporulation, inoculation assay

Diplodiasjuka på tall. Faktorer som påverkar infektion och spridning i svenska skogar

Sammanfattning

Diplodia sapinea (syn. *Sphaeropsis sapinea*) är en global patogen som infekterar barrträd och symtom på Diplodiasjukan kan utvecklas när värdträdet utsätts för stress, t.ex. torka, värme eller mekanisk skada. Arbetet som denna avhandling bygger på startade med det första utbrottet av Diplodia på tall (*Pinus sylvestris* L.) i Sverige, vilket då var den nordligaste kända storskaliga utbrottet av sjukdomen. Syftet med avhandlingen var att skapa kunskap om sjukdomen om Diplodia orsakar i tall. Arbetet visade att svampen inte har några uppenbara begränsningar för att orsaka skador på tall i det nordiska klimatet. Vidare visade arbetet att *D. sapinea* inte är en nyligen introducerad patogen i Sverige, utan den genetiska variationen liknar den generella europeiska populationen. Klimatet och variationer i tallarnas anpassning till torka var möjliga drivkrafter bakom det första utbrottet som hittades 2016.

Stora områden med tall som uppvisade allvarlig kronutglesning upptäcktes efter torkan 2018 på Gotland. *Diplodia sapinea* kunde förknippas med de observerade symptomen, även om den inte var drivkraften bakom kronutglesning. Dödligheten var störst i de allvarligast skadade tallarna, men även svårt skadade tallar kunde återhämta sig när torkstressen minskade. *Diplodia sapinea* hittades även i friska bestånd, träd och kvistar. Stora mängder av *D. sapinea* förekom dock endast i symptomatiska kvistar på träd som visade kronförlust. De endofytiska svampsamhällena skiljde sig åt beroende på hälsostatus - från växtplats till vävnadsnivå. Fyra arter var förknippade med frisk tallvävnad, vilket tyder på att det potentiellt finns svampantagonister mot *D. sapinea* i det endofytiska samhället hos tall. En standardiserad metod för att producera infektiösa *D. sapinea* sporer *in vitro* utvecklades inom ramen för detta arbete för att underlätta framtida forskning om patosystemet *D. sapinea* - tall. Resultaten i denna avhandling bidrar med kunskap om faktorer av utvidgningen av Diplodiasjukan i norr och understryker vikten av att hantera stressrelaterade skogspatogener för att upprätthålla skogens hälsa i den pågående klimatförändringen.

Nyckelord: Diplodiasjuka, *Diplodia sapinea*, tall, framskridande sjukdom, endophyt, torka, svampsammhället, *in vitro* sporulation, inoculationsmethod

Diplodia-Triebsterben der Waldkiefer. Einflussfaktoren der Infektion und Verbreitung in den Wäldern Schwedens

Zusammenfassung

Diplodia sapinea (syn. *Sphaeropsis sapinea*) ist ein weltweit verbreiteter pilzlicher Schaderreger der Nadelbäume befällt. Symptome von *D. sapinea* können auftreten, sobald die Wirtspflanze durch Stress wie Trockenheit, Hitze oder mechanische Schäden beeinträchtigt wird. Diese Doktorarbeit begann mit dem ersten in Schweden entdeckten Ausbruch vom Diplodia-Triebsterben an der gemeinen Waldkiefer (*Pinus sylvestris* L.). Dies ist der bisher nördlichste Nachweis eines großflächigen Ausbruchs dieser Krankheit. Ziel dieser Arbeit war es, Erkenntnisse über das Auftreten des Diplodia-Triebsterbens in Schweden zu gewinnen. Es wurde gezeigt, dass der Schaderreger keine offensichtlichen Beschränkungen hat, im nordischen Klima Schäden an Kiefern zu verursachen. Außerdem zeigt diese Arbeit, dass *D. sapinea* kein erst kürzlich in Schweden eingebrachter Pilz ist und genetisch der allgemeinen europäischen Population ähnelt. Mögliche Ursachen für den ersten Ausbruch 2016 waren das Klima und die unterschiedlichen Anpassungen der Kiefern an Trockenheit.

In Folge des Dürrejahres 2018 wurden auf Gotland große Flächen mit Kiefern entdeckt, welche ein starkes Kronensterben aufwiesen. Die Sterblichkeit war bei stark geschädigten Kiefern am höchsten. Überlebende Kiefern konnten sich aber erholen, als der Trockenstress nachließ. *Diplodia sapinea* wurde mit den beobachteten Symptomen in Verbindung gebracht, war aber nicht der Auslöser für das Absterben. *Diplodia sapinea* wurde auch in gesunden Beständen, Bäumen und Zweigen gefunden. Große Mengen des Erregers wurden jedoch nur in erkrankten Zweigen gefunden. Die endophytischen Pilzgemeinschaften in den Kiefernzweigen unterschieden sich je nach Gesundheitszustand, vom Holzgewebe bis zum Standort. Vier Arten waren mit gesundem Holzgewebe assoziiert, was auf ein Potenzial für antagonistische Pilze in der endophytischen Pilzgemeinschaft der Kiefer hindeuteten könnte. Es wurde eine

standardisierte *in vitro*-Sporenbildungsmethode entwickelt, um künftige Forschungen über das Pathosystem *D. sapinea* - Kiefer zu erleichtern. Die Ergebnisse dieser Arbeit tragen zum Wissen über die Ausbreitung von Diplodia-Triebsterben in nördlichere Breiten bei. Außerdem wird die Bedeutung der Kontrolle von stressbedingten Waldpathogenen unterstichen, die für die Erhaltung der Waldgesundheit im Zuge des Klimawandels von essentieller Bedeutung ist.

Stichworte: Diplodia-Triebfäule, *Diplodia sapinea*, Kiefer, Endophyt, Trockenheit, Pilzgemeinschaft, *in vitro* Sporenbildung, Inokulationstest

Dedication

To my mother and her infinite support.

An meine Mutter und ihre unendliche Unterstützung.

We rest; a dream has power to poison sleep.
We rise; one wand'ring thought pollutes the day.
We feel, conceive, or reason; laugh or weep,
Embrace fond woe, or cast our cares away;
It is the same: for, be it joy or sorrow,
The path of its departure still is free.
Man's yesterday may ne'er be like his morrow;
Nought may endure but mutability!

Mary Wollstonecraft Shelley, *Frankenstein*, 111

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Brodde, L.**, Adamson, K., Julio Camarero, J., Castaño, C., Drenkhan, R., Lehtijärvi, A., Luchi, N., Migliorini, D., Sánchez-Miranda, Á., Stenlid, J., Özdağ, Ş., & Oliva, J. (2019). Diplodia Tip Blight on Its Way to the North: Drivers of Disease Emergence in Northern Europe. Frontiers in Plant Science, 9 (January). https://doi.org/10.3389/fpls.2018.01818 (*published*)

II. **Brodde, L.**, Stein Åslund, M., Elfstrand, M., Wågstörm, K., Oliva, J., and Stenlid, J. (2022). *Diplodia sapinea* as a contributing factor in the crown dieback of Scots pine (*Pinus sylvestris*) after a severe drought. (*manuscript draft*)

III. **Brodde, L.**, Elfstrand, M., Redondo, M.A., Oliva, J., and Stenlid, J. 2023, The endophytic mycobiome of Scots pine (*Pinus sylvestris*) is affected by Diplodia tip blight (*Diplodia sapinea*) under drought. (*manuscript draft*)

IV. Oostlander, A.*, **Brodde, L.***, Bargen, M., Leiterholt, M., Trautmann, D., Enderle, R., Elfstrand, M., Stenlid, J., and Fleißner, A. (2023). A reliable and simple method for the production of viable pycnidiospores of the pine pathogen Diplodia sapinea and a spore-based infection assay on Scots pine. (* *shared first authorship; accepted in* Plant Disease APS Journals*)*

Papers I and IV are reproduced with the permission of the publishers.

The contribution of Laura Brodde to the papers included in this thesis was as follows:

I. Participated in designing the experiment; performed field work and parts of laboratory work; performed parts of the analysis, contributed to writing the manuscript and correspondence with the journal.

II. Contributed to conceptualisation; contributed to the experimental design; coordinated and performed field work, laboratory work and analysis; was the main writer of the manuscript.

III. Contributed to conceptualization; designed the experiment; coordinated and performed field and laboratory work; coordinated and contributed to analysis; was part of writing the manuscript.

IV. Conceptualized the project; took part in designing the experiment; contributed to coordinate laboratory work and greenhouse experimenters; performed analysis; was part of the main writing team of the manuscript; contributed to correspondence with the journal.

List of figures

Abbreviations

CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
NSC	Non-structural carbohydrate
ITS	Internal transcribed spacer
OTU	Operational taxonomic units
ha	Hectare
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
SSR	Simple sequence repeats
IndVal	Indicator Value
CD	Cazpek-Dox medium
LNA	Low nutrient agar medium
OA	Oat meal medium
PDA	Potato dextrose agar medium
VMM	Vogel´s minimal medium

1. Introduction

More than one third (38%) of the global habitable land area is covered by forests (Ritchie et al., 2021). They play a key-role for not only the environment, but also to the human population and economy. Providing protection from soil erosion, water protection, and playing a critical role in the global carbon cycle are only a few of the plenty ecosystem services that forests supply with high impact on the globe, and on our human lives.

Forest health can be defined as conditions that are derived from human needs, and lead to sustainable ecological environments by resilience, recovery, persistence, and biophysical processes (www.fs.usda.gov). Forest health can be impaired by single or interacting abiotic (e.g. drought, storm, fire) and biotic (e.g. browsing, insect pests, pathogens) disturbances, while the classification of a disturbance as "natural disturbance" *vs.* "forest damage" depends on your point of view.

One of the biggest challenges of the recent, as well as coming centuries is dealing with consequences of the human-driven change of climate, where changes manifest quicker than ecosystems can adapt. A general increase in frequency and intensity of drought, caused by high temperatures, as well as changes in precipitation patterns are predicted to affect forest ecosystems by challenging tree and forest health (IPCC, 2014; Anderegg et al., 2022). Coping with disturbances affects not only tree growth, but also tree defence. Consequently, opportunistic pests and pathogens are given the chance to develop disease, which are expected to increase in impact in the scope of climate change (Ghosh et al., 2022).

An opportunistic, stress related pathogen that is expected to further increase in impact on forest health is *Diplodia sapinea* causing Diplodia tip blight on conifers (Roy et al., 2022). Most susceptible trees belong to the

genus of Pines (*Pinus* spp.), which is distributed almost all over the world. With a world-wide colocalisation of *D. sapinea* with its host, this opportunistic pathogen has potential to impact forest health substantially.

Formerly, damages by Diplodia tip blight have been more common in the southern hemisphere. The relationship of forest disturbances, as drought or hail, and increased disease severity of Diplodia tip blight is well described in those areas.

An increase of damages in more northern latitudes of Europe has been observed during the recent century. First detections of Diplodia tip blight occurred in Estonia (2007), then in Sweden (2013) and Finland (2015) on single, infected pines has brought attention to a potential threat to forest health of the formerly unnoticed disease also in the Northern countries. The dominating forest type of these countries is the boreal forest, which is a globally important carbon sink. Impacts on boreal forests have direct impact on the atmosphere, influencing the preservation of healthy ecosystems in the future (Bradshaw et al., 2015).

In this thesis, I demonstrated the ability of *D. sapinea* to cause damages on Scots pine in boreal conditions with the first detected outbreak of Diplodia tip blight in a forest plantation in Sweden, 2016.

Most of Sweden's land is covered by forests (70 %), and the majority of these are productive forest lands (83%) (Skogsdata, Nilsson et al., 2022). The Nordic European countries, together with Sweden, host 1.6% of the world's commercial forest areas, but provide approximately 15% of forest products (saw timber, pulp and paper) on the global market which reflects the economic importance of forests in Fennoscandia. Furthermore, societal aspects, such as cultural or recreational value of the forests, are directly impaired by forest damages.

With Scots pine (*Pinus sylvestris* L.), one of *D. sapinea*'s most susceptible hosts, covering 40% of the production forests, Diplodia tip blight has a great potential to impact forest landscapes in Sweden.

In this thesis, I provide new knowledge about Diplodia tip blight affecting Scots pine in the North with insights ranging from stand-level damages to spore infection methodology.

2. Background

2.1 Diplodia tip blight

2.1.1 Overview

Diplodia sapinea (Fr.) Fuckel (syn. *Diplodia pinea* (Desm.) Kickx., *Sphaeropsis sapinea* (Fr.: Fr.) Dyko & Sutton) is one of the most widely distributed pathogens of conifers worldwide (Whitehill et al., 2007). Substantial losses for the forest industry are caused by the fungus, including apical shoot dieback (Diplodia tip blight), canker, blue stain and root disease (Chou, 1976; Nicholls & Ostry, 1990; Peterson, 1977; Wingfield & Knox-Davies, 1980).

D. sapinea can infect various conifers, with *Pinus* as the main host genus. Infections of species of other genera have been reported, e.g., blue spruce (*Picea pungens*), white fir (*Abies concolor*) (Luley et al., 1988), and common juniper (*Juniper communis*) (Terhonen et al., 2023). Even infection of deciduous trees (*Fagus sylvatica*) has been reported (Zlatković et al., 2017). Nevertheless, highest aggressiveness is shown infecting *Pinus* spp., including Scots pine (*Pinus sylvestris*) (Peterson, 1981).

Diplodia sapinea was first described by Elias Fries as *Sphaeria sapinea* in 1823. The exact geographic origin of the primary type specimen is not clear, but likely from Småland in southern Sweden.

Diplodia tip blight was first recorded as a disease in plantations on non-native pine species in South Africa in 1909 (Lundquist, 1987). It is

assumed that *D. sapinea* was introduced to South Africa during the 1800s, with initial pine introductions, such as *P. pinea* and *P. sylvestris*, by European colonialists (Burgess et al., 2001b; Burgess et al., 2004a). Since then, the highest economical impact of Diplodia tip blight has been found in South Africa, New Zealand, and many other parts of the southern hemisphere (Swart et al., 1991; Burgess et al., 2004b). During the last decades, impact of Diplodia tip blight has increased further in the northern hemisphere. Severe damages caused by *D. sapinea* in central Europe were recorded for the first time in the Netherlands in 1982 (Swart et al., 1991) with increasing severity in France and Germany since early 2000 (e.g., Blaschke et al., 2007; Fabre et al., 2011). In Northern Europe, *D. sapinea* was first detected in Estonia, 2007 (Hanso et al., 2009), followed by Sweden in 2016 (Oliva et al., 2013), and Finland in 2019 (Müller et al., 2019). Adamson et al. (2021) suggested that *D. sapinea* was introduced from central Europe, Netherlands and Germany with the trade of seeds and saplings to Northern Europe. Nevertheless, is not known if increasing symptoms of Diplodia tip blight in Northern Europe were caused by an introduction of *D. sapinea*, or if changed environmental conditions has led to more frequent symptomatic infections.

Diplodia sapinea spreads through asexual pycnidiospores (spores), which are released from pycnidia that form at the base of necrotic branches, shoots and needles, on leaf sheaths, and on cone scales of the host plant (Phillips et al., 2013). Development of pycnidia, and spore release have been shown to be associated with precipitation (Brookhouser et al., 1971), as well as temperature conditions after high precipitation periods (Swart et al., 1987; Kuntzmann et al., 2009).

Spores of *D. sapinea* have been found on various insects, but mainly on bark beetles (Coleoptera: Curculionidae), e.g., *Ips acuminatus* Gyll., *Tomicus piniperda* L. or *Hylurgops palliatus* Gyll. (e.g., Goldazarena et al., 2012; Davydenko et al., 2017). Many bark beetles are mainly secondary pests which affect stressed trees, which are also susceptible to Diplodia tip blight. It is plausible that insects play a role in transmitting *D. sapinea*'s conidia over longer distances, though it has not been shown to which extent they act as vectors of *D. sapinea*.

It has been suggested that transmission of *D. sapinea* happens mostly horizonally, from mature trees via spores or mycelium to young trees. Vertical transmission, from mature trees via seeds to their offspring, seems

to be unlikely (Bihon et al., 2011b; Decourcelle et al., 2015). Diplodia tip blight infected seedlings have been found in nurseries, where infected mature pines surrounding the nurseries most likely acted as inoculum sources (e.g., Stanosz et al., 2005; Larsson et al., 2021). Nevertheless, *D. sapinea* has also been found in seed batches (Decourcelle et al., 2015; Cleary et al., 2019). These might contribute to the spread of the disease; infection from seed to seedling has been observed, but with varying and mostly little infection success (e.g., Rees et al., 1988; Decourcelle et al., 2015). Still, even a very low probability of transmission by seeds could contribute to a significant spread when large quantities of seeds are handled. More research in the form of e.g. infection studies is needed to investigate the role of seeds in the spread of Diplodia tip blight.

Diplodia sapinea has been suggested to be obligately asexual (Burgess et al., 2004b), though its reproduction remains cryptic since signs for sexual reproduction have been found (e.g., Bihon et al., 2012b; Lopes et al., 2018). Though sexual structures have never been observed, *D. sapinea* has been suggested to be heterothallic (self-sterile), indicated by the presence of MAT loci (MAT1-1-1 and MAT1-2-1) (Nagel et al., 2021). Further evidence for sexual reproduction was found in linkage disequilibrium (e.g. Bihon et al., 2012a; Adamson et al., 2021).

The population structure of *D. sapinea* has been investigated by several studies and varies in genetic diversity between geographic locations. Low diversity has been reported in Australia, intermediate in New Zealand, and very high in South Africa (Burgess et al., 2001a). High genetic diversity in South Africa (mainly on *P. radiata*) suggested that each genotype is a single introduction, reflecting the number of introductions and history of planting exotic pine species to plantations (Burgess et al., 2001b). In European populations the domination of a single genotype and high genetic similarity between regions has been found (Adamson et al., 2021, Paper I). Very high genetic diversity was also found in North America, which was proposed as a potential center of origin (Adamson et al., 2021). The reason of differences in genetic diversity between the geographic regions is not fully understood.

Another factor that was shown to influence genetic diversity is the type of tissue which was sampled; isolations obtained from cones seem to show higher genetic diversity compared to isolates from symptomatic shoots, the stem or roots (Aragonés et al., 2021).

The taxonomy and thereby nomenclature of fungi based on morphological and later molecular features has been frequently rearranged. Belonging to the Ascomycota, *D. sapinea* is classified as an anamorph in the family of *Botryosphaeriaceae* (Jacobs et al., 1998). The most recent classification changed the genus to *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton 1980. Denman et al. (2000) and Phillips et al. (2013) provide arguments for keeping the species within the genus of *Diplodia*. Phillips et al. (2013) argues that conidia of *S. sapinea*, Desm. are smaller (25 - 32 x 12 - 15µm) than reported by Dyko & Sutton (1989) (30 - 55 x 11-18 µm) and are rather matching the range of *S. sapinea* (33-39 x 11.5 - 13 µm) (Palmer et al., 1987; Swart et al., 1991). Therefore, they conclude *S. sapinea* Desm. and *Diplodia sapinea* Fr. to be two different species and the original lectotype to be *Diplodia*. In my opinion, conidial dimensions of both suggested genera are too variable and overlapping, and therefore questionable to consider as a clear indicator for distinction between them.

Denman et al. (2000) pointed out that the distinction between *Diplodia* and *Sphaeropsis* has never been straight forward. Spore morphology has been a key feature in the differentiation of the two genera. Denman et al. (2000) argues that these features are not clearly distinguishable between the two genera and therefore questionable in value for differentiation between *Diplodia* and *Sphaeropsis*. Furthermore, Phillips et al. (2013) refers to paraphyses (sterile filaments between sporogenous organs) as a characteristic to distinguish the two genera. Paraphyses are present in *Sphaeropsis*, while they are absent in the genus *Diplodia*.

The latest suggestion of nomenclature by Dyko & Sutton can be used synonymously to the previous one, *Diplodia sapinea* (Fr.) Fuckel 1870, which is the recommended name for the fungus causing Diplodia tip blight in Sweden (https://namnochslaktskap.artfakta.se/taxa/, TaxonID: 6011671) and is therefore used in this thesis.

2.1.2 Infection biology

Diplodia sapinea can enter its host through the epidermis, stomata and injured tissues. Elongating needles and shoots, as well as wounds have been suggested to be entry points for new infections. (e.g., Brookhouser et al., 1971; Chou, 1978; Li et al., 2019)

Pycnidia develop near the needle base and on leaf sheaths, while attached cones also become infected and show pycnidia on their scales (Waterman, 1943; Peterson et al., 1968). As a latent pathogen, *D. sapinea* can be present in asymptomatic trees (Smith et al., 1996). Latent infections seem to be locally and discontinuous, with a more frequent occurrence in the phloem and bark compared to the xylem and pith of asymptomatic shoots (e.g., Flowers et al., 2003; Bußkamp, 2018). Trees affected by Diplodia tip blight also showed infections by *D. sapinea* in the xylem and pith of symptomatic shoots (Flowers et al., 2001).

An outbreak of Diplodia tip blight symptoms is induced by stress factors, such as drought (e.g., Bachi et al., 1985; Stanosz et al., 2001b) or mechanical wounding as caused by hail (e.g., Chou, 1987; Zwolinski et al., 1990; Oliva et al., 2021; Caballol et al., 2022). Induction of fast tree growth caused by exposure to overly fertile soil has also been shown to increase susceptibility of Scots pine to Diplodia tip blight (Blodgett et al., 2005).

Hyphal growth within shoots is suggested to be signalised beforehand with necrosis of the tissues, possibly indicating the importance of phytotoxins in symptomatic infections (Flowers, 2006). Furthermore, *D. sapinea* infections cause changes to the phytohormone profiles on a transcriptomic level in needles of infected conifers, similar to stress response patterns to drought (Hu et al., 2022). However, the exact mechanisms behind the infection, and especially the switch in lifestyle from endophytic to pathogenic are not known.

2.2 Scots pine (*Pinus sylvestris*)

Scots pine is a long-lived conifer. As one of the widest distributed pine species, it is native to northern Eurasia, present from southern Spain in the west to the far east of Siberia, and from northern Turkey in the south to well inside the arctic circle in the north of Fennoscandia (Critchfield et al., 1966) (**Figure 1**). Economically, it is one of the most important tree species, particularly in northern Europe. The wide distribution of Scots pine reflects

Figure 1 Distribution map of *Pinus sylvestris* L. (Caudullo et al., 2017). Native range shown in **green**, introduced and naturalised areas shown in **yellow**. Isolated populations outside the main domain indicated as **dots**.

its capacity to adapt to a range of habitats. As a pioneer and light-demanding species, it can colonise low-nutrient soils (e.g., Picon-Cochard et al., 2006; Vacek et al., 2021). A relatively sensitive control of transpiration makes Scots pine capable to respond to moderate drought by reducing stomatal conductance (e.g., Irvine et al., 1998). Nevertheless, more frequent and severe drought can induce a decline in Scots pine, where carbon starvation and hydraulic failure impair tree health (e.g., Sánchez-Salguero et al., 2012; Sevanto et al., 2014). Drought stress can be caused by reduced water availability, an increase of evapotranspiration, or a combination of both. If water loss from transpiration is higher than water uptake by the roots, a high xylem water tension can result in cavitation and conductivity loss in the xylem, interrupting water transportation (e.g., Sperry et al., 1998; McDowell et al., 2008). Reducing the loss of water through transpiration is achieved by closing the stomata. Consequently, CO_2 supply needed for photosynthesis becomes limited. If drought conditions persist for long enough, a depletion of non-structural carbohydrates (NSCs), and possibly tree death caused by carbon starvation, can follow (e.g., McDowell, 2011).

To withstand abiotic and biotic stressors, trees have evolved various constitutive or inducible defence responses which can be for instance, mechanical and chemical. Examples of mechanical defence responses are the physical barriers we can observe in Scots pine, such as thick cuticle layers

on needles or bark on the trunk (e.g., Łaźniewska et al., 2012). The production of secondary metabolites (e.g. phenolics or terpenoids), both constitutively or induced after attacks, are often effective defence mechanisms (Bennett et al., 1994). Variation in these traits can lead to varying susceptibility to diseases within and among pine species (Sampedro, 2014; Hurel et al., 2021).

In Sweden, Scots pine is one of the dominant trees species, representing around 40% of the standing forest volume (Nilsson et al., 2022). Over the last 80 years the dominant management strategy has been clear cutting, combined with active regeneration by planting (Jonsson et al., 2019). Lately damages on Scots pine have been given more attention. Biotic factors, such as browsing by mammals, pathogens and insects cause substantial damages in Scots pine forests. Especially browsing by ungulates causes large losses in particular in young Scots pine stands (Nilsson et al., 2022). Fungal diseases also lead to noticeable losses. Common fungal diseases in Northern pine forests are Scots pine blister rust (*Cronartium pini* (Willd.) Jørst) (Samils et al., 2022), Lophodermium needle cast (*Lophodermium seditiosum* Minter, Staley & Millar) (Stenström et al., 2005), twisting rust (*Melampsora pinitorqua* Rostr.) (Martinsson, 1985), shoot disease caused by *Gremmeniella abietina* Lagerb. (Hellgren et al., 1992), and *Heterobasidion annosum* s.l. (Woodward et al., 1998). In central Europe, *D. sapinea* is one of the most common fungi inhabiting Scots pine (Bußkamp et al., 2020), while records of Diplodia tip blight have increased since the 1990s (Heydeck et al., 2012). Damages caused by *D. sapinea* are more frequent in southern Europe with several affected pine species e.g., *Pinus pinea* L., *P. nigra* J.F.Arnold, *P. radiata* D. Don, and *P. halepensis*, Mill. (e.g., Botella et al., 2010; Fabre et al., 2011; Luchi et al., 2014). In common garden experiments, Scots pine has been shown to be intermediately to highly susceptible to Diplodia tip blight (Iturritxa et al., 2013; Caballol et al., 2022).

2.3 (*Un*)balanced interactions of fungi with their hosts

Fungi are heterotrophic organisms, and as such being dependent on an external source for energy. They have evolved several strategies to use carbon sources, from dead organic material to various living hosts and tissues. The different ways of how, and from which tissue fungi obtain carbon of their hosts, can be classified as different lifestyles (Watkinson et al., 2015):

Saprotrophs feed through decomposition of dead organic matter, being key regulators of nutrient cycling in ecosystems. Necrotrophic pathogens gain energy by killing living host tissue before degrading them, while biotrophic pathogens depend on living plant tissue to parasitise and obtain nutrition.

Disease establishment is dependent on the host's and pathogen's genotypes, as well as the environment (biotic and abiotic) (**Figure 2**). Time can be added as a fourth factor to account for complexity and evolution of the interactions that lead to disease in the disease framework.

Biotrophs form close contact with, and are dependent on living tissue of a host plant, to feed on their products of photosynthesis. This interaction can be mutualistic or parasitic, depending on the benefits both partners gain.

Mutualistic connections are formed by mycorrhizal fungi, establishing close connections, and exchanging micro- and macronutrients with plant roots.

Fungi can colonise their host on the surface (epiphytes) or grow within the living tissues (endophytes).

The literal definition of endophytes means "in the plant" (*gr*. *endon* = within, *phyton* = plant). Usage of this term is diverse as many organisms might be classified as endophytes. Most commonly, they are defined as metabolically active organisms that enter the healthy tissues of their hosts without detectable symptoms of infection (Stone et al., 2000; Schulz et al., 2005). They have various lifestyles, growing systematically or locally inside their hosts, and are localised intra- or intercellular (Rodriguez et al., 2009). To be able to colonise their hosts asymptomatically, endophytes have to balance and maintain antagonisms with their hosts, but also with other microbial inhabitants (Schulz et al., 2015). Keeping this balance is determined by the plasticity of the host's, as well as the endophyte's phenotype. Their momentary status is shaped by environmental factors and

their tolerance to those factors, forming a continuum of the endophytic interaction (Schulz et al., 2005). Within these interactions, endophytes have been shown to affect plant disease, by either antagonising or facilitating pathogens (Busby et al., 2016) .

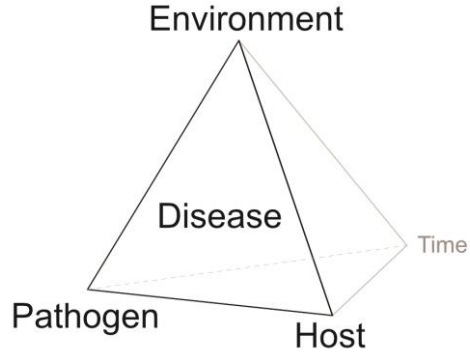

Figure 2 The classic disease triangle (black) with modification to add a fourth factor (grey) as e.g. time (after Stevens (1960) & Agrios (2005)).

The lifestyles of fungi are not necessarily fixed and as clearly defined as the classifications suggest; some pathogens can initiate infection as biotrophs and then switch to a necrotrophic mode later during infection (hemi-biotrophs). Other pathogens can have an "endophytic" phase, inhabiting plant tissue without causing symptoms prior disease, questioning the definition used for "endophytes".

Opportunistic pathogens, as *D. sapinea*, fall within the interaction framework of an endophyte as well as that of a pathogen at different life stages. Asymptomatic colonisation of their host can be followed by a pathogenic phase when conditions are suitable. Inhabiting a host plant comes along with benefits of a protected environment, followed by an aggrandised stage of dispersal when pycnidia can develop on diseased, necrotic tissues – and escaping plant defence once again when conditions change in favour of the host.

3. Objectives and Aims

This study began with the first detected outbreak of Diplodia tip blight on Scots pine in Sweden, which was the northernmost record of a large-scale infection of a pine stand with this disease up to that date. The main aim of this thesis was to generate knowledge about the emergence of Diplodia tip blight in Sweden. I studied the disease from a landscape – perspective, down to stand- , tree- , twig- , and tissue level, and finally transferred the disease complex to the laboratory to improve the methodology of infection assays. The specific objectives where:

- Describing the first detected outbreak and understanding drivers of Diplodia tip blight emergence in Sweden (**Paper I**).

Hypotheses

- Observed damages are the result of an introduction of a new, aggressive genotype of *D. sapinea*.
- The outbreak is an isolated event.
- Weather conditions played a role in the development of the observed outbreak.
- Scots pine phenotypes with lower water-use efficiency are more susceptible to *D. sapinea*.
- *D. sapinea* is dispersed across short distance.

- Studying development of crown dieback in Scots pine after extreme drought conditions in combination with Diplodia tip blight occurrence (**Paper II**).

Hypotheses

- Severely damaged trees (>70%) show higher mortality rates
- Mildly damaged trees (<30%) show higher probability of recovery.
- *D. sapinea* is equally abundant in symptomatic and asymptomatic trees on affected and healthy sites.
- Spore dispersal of *D. sapinea* is associated with occurrence of dieback of Scots pine at the sample site.

- Investigating the endophytic community of Scots pine infected with *D. sapinea* (**Paper III**).

Hypotheses

The endophytic community of Scots pine differs between:

- **sites** with and without obvious Diplodia tip blight symptoms.
- symptomatic and asymptomatic **trees** at sites with obvious Diplodia tip blight.
- symptomatic and asymptomatic **twigs** in trees with obvious Diplodia tip blight.
- healthy and necrotic **tissue** in twigs with Diplodia tip blight.

- Developing methods for more effective and standardised pathogenicity tests of *D. sapinea* infecting Scots pine at the greenhouse (**Paper IV**).

Hypotheses

- spores produced *in vitro* are more effective at causing infection in Scots pine seedlings compared to mycelium-based methods.

4. Finding *Diplodia sapinea* – from the forest to the lab

4.1 Damage estimations and sampling in the field

In the scope of this thesis, I used different techniques to detect and study *Diplodia sapinea* in the field.

The typical symptom of Diplodia tip blight is shoot dieback, which can easily be confused with other pests and pathogens or abiotic factors as an underlying cause. If a fungal disease is suspected, localisation and morphology of potential fruiting bodies on diseased tissues can give information regarding the species. Together with microscopically examining spore morphology, many known fungi can be identified (Agrios, 2005). In the case of Diplodia tip blight, characteristic pycnidia containing pycnidiospores can be found on cones, and symptomatic twigs, and needles of infected pines. With a size of $40.8 \pm 4.9 \times 15.5 \pm 2.1$ µm in combination with a dark brown colour and presence of none, or one to two septa, the pycnidiospores of *Diplodia* are relatively easy to recognise (Cheng-guo et al., 1985; Phillips et al., 2013). If pycnidia are absent, culturing diseased, surface-sterilised tissues can provide evidence in the form of culture morphology. If induction of sporulation in culture is possible, one can further verify the species. Fungal DNA can be extracted from the culture or directly from infected tissues. Amplification with species-specific primers or sequencing of the internal transcribed spacer (ITS) regions of the ribosome encoding genes can give certainty about the identity of the fungus. *Diplodia scrobiculata* is a closely related species to *D. sapinea*. Since differentiation between those two species by spore morphology is

challenging (Phillips et al., 2013), the usage of species specific primers for certain identification is recommended (Smith et al., 2006).

A genetic marker for general species identification is the ITS region, a popular phylogenetic marker (Schoch et al., 2012). Publicly available databases e.g., NCBI (http://www.ncbi.nlm.nih.gov) or UNITE (https://unite.ut.ee/) contain a multitude of reference sequences for identification by matching the obtained sequence with previously published sequences from identified fungi.

At the studied outbreak (**Paper I**), *D. sapinea* was identified as the cause of the observed shoot dieback by obtaining cultures from 40 trees in the stand. DNA from one isolate per tree, matching morphology of *D. sapinea* in culture, was extracted and verified with specific *D. sapinea* primers (DpF/BotR, Smith et al. (2006)). A subset of the cultures was sequenced at the ITS 1 and 2 region (ITS1f/ITS4, Ihrmark et al. (2012)), which was then blasted in Genbank for further species verification. Once the identification of *D. sapinea* was established at the outbreak, we examined the population of the fungus by studying allelic variation in 10 simple sequence repeat (SSR) markers (Burgess et al., 2001c; Bihon et al., 2011a). The population of the outbreak was compared to isolates obtained earlier from Sweden (2013) and other countries in Europe and Asia Minor (Turkey) to investigate the potential appearance of a novel genotype in this outbreak.

At the drought-triggered dieback of Scots pine on Gotland (**Paper II**), evidence of the presence of *D. sapinea* was first obtained by examining cones, and necrotic shoots and needles for pycnidiospores on four affected sites. DNA was extracted and quantified (qPCR, Luchi et al. (2005a)) for diseased and healthy tissue within the affected region to give further insights into the presence and abundance of *D. sapinea*. Twig samples were sampled in 2018 from symptomatic and asymptomatic trees at affected and healthy sites (**Figure 3**) within the affected region, which gave further insights into the presence, and abundance of *D. sapinea*. The endophytic fungal community was studied with the same samples, but including an additional sample level of symptomatic and asymptomatic tissues within symptomatic twigs (**Paper III**). Metabarcoding of the fungal community was carried out by high-throughput sequencing the ITS2 region (fITS7/ ITS4, Ihrmark et al. (2012)), using an inhouse protocol(Clemmensen et al., 2016). The PacBio Sequel platform by SciLifeLab NGI (Uppsala, Sweden) was used for sequencing. The sequence reads were quality controlled, clustered and

demultiplexed using the in-house SCATA pipeline (https://scata.mykopat.slu.se/). Sequences were clustered into operational taxonomic units (OTUs) based on 98% similarity to differentiate species, but not intra-species variation (Lindahl et al., 2013). The most common sequence in each OTU was used for taxonomic examination utilising the Protax software (Somervuo et al., 2016; Abarenkov et al., 2018) implemented in PlutoF (https://plutof.ut.ee). Relative abundance of the OTUs were compared between healthy and affected sites, symptomatic and asymptomatic trees, and asymptomatic and symptomatic twigs and tissues in symptomatic trees. Hellinger transformation of the relative abundancies were carried out to account for differences in magnitude of sequence and species counts, and to control for different total reads between sample groups (Laporte et al., 2021). Furthermore, analysis was carried out to identify indicator species characterising healthy or symptomatic sites, trees, twigs, and tissues. The Indicator Value (IndVal) measures the association between a species and a site or sample group, followed by a permutation test to test the statistical significance of this relationship (Dufrêne et al., 1997).

In **Paper I** and **II**, we also investigated spore dispersal of *D. sapinea* after detecting symptomatic pines. At the outbreak (**Paper I**), simple spore traps, consisting of one horizontally fixed filter paper, and one microscopy slide covered with strips of tape coated with permanent adhesive on both sides. Traps were placed directly below affected trees, and sampled during one week with low, and one week with high precipitation. On Gotland (**Paper II**), spore traps with only filter paper were used, which were placed in the centre of each site. Here, two consecutive weeks for each season were sampled, in total 16 weeks within two years (January 2019 – October 2020).

Estimation of crown dieback of Scots pine caused by Diplodia tip blight were done visually in both field studies (**Paper I & II**). The level of crown dieback was estimated in 10% steps as the proportion of dead twigs in the upper third of the living crown in relation to a completely healthy tree in the same population. In **Paper I**, tree ring analysis was carried out for each estimated tree to reconstruct radial growth. Furthermore, quantification of non-structural carbohydrates (NSCs), as well as carbon isotope analysis was conducted to test phenotypical variation in water use efficiency of a selection of severely affected vs unaffected Scots pines.

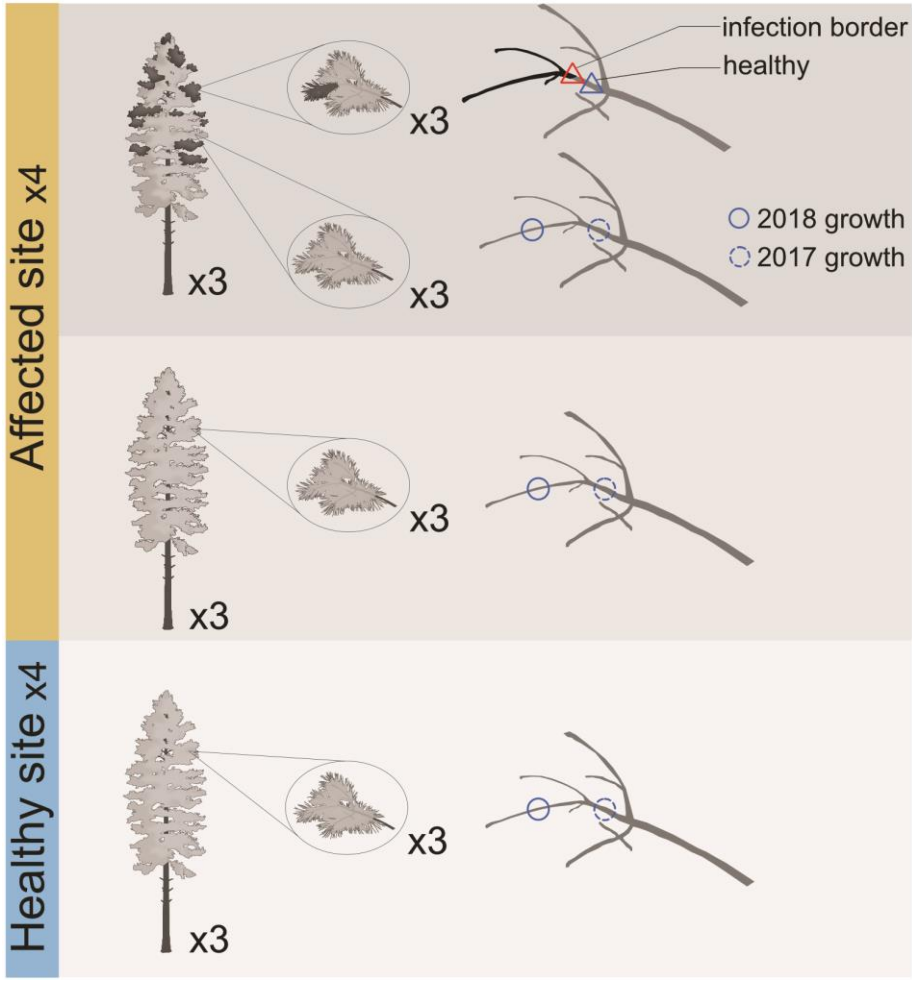

Figure 3 Overview of the sampling scheme for the study of endophytic fungi in Scots pine twigs. Four affected and four healthy-looking sites were selected. On all sites, three twigs were sampled from three asymptomatic trees. Of these, growth years from 2018 and 2017 were sampled. On affected sites, additionally three symptomatic trees were sampled for three symptomatic, and also three asymptomatic twigs. Asymptomatic twigs were sampled as described before. Symptomatic twigs were sampled at the infection border, and just below for healthy- looking tissue. (**Paper III**)

4.2 Infection studies in the laboratory and greenhouse (**Paper IV**)

Studying the pathosystem of Diplodia tip blight affecting Scots pine *in natura* comes close to investigating the true interaction of the host, pathogen, and their environment. On the downside, a non-laboratory environment goes along with an unknown number of varying factors impossible to control, influencing observations and thereby introducing uncertainty in the interpretation of the findings. Controlled studies in the laboratory or greenhouse provide more control of the interacting partners e.g., the genetic background and physiological stage of the host and pathogen, source of inoculum, application of inoculum, as well as environmental factors, such as temperature and irrigation prior, during and post infection. Simplifying the experimental set up in an imitation of the infection process reduces the number of unknown factors with impact on the observations. The challenges with an artificial set up are to represent the natural process as good as possible, and to minimise biases introduced by the chosen method, which might also limit the ability to draw conclusions from the experiment carried out.

Diplodia tip blight has been thoroughly studied in the greenhouse including investigations of the impact of drought or wounding on symptom development. Infections have typically been carried out by wounding the host followed by fixing a piece of agar covered with mycelium of *D. sapinea* grown in culture onto the tissue lesion (e.g., Paoletti et al., 2001; Luchi et al., 2005b; Dong et al., 2020; Blumenstein et al., 2021b). While these approaches have contributed to important findings, they have the disadvantage of not adequately representing the infection process as it occurs *in natura* since it is assumed that new infections of Diplodia tip blight emerge from spores as a source of inoculum.

4.2.1 A standardised sporulation and infection method

In **Paper IV**, I aimed to produce a standardised sporulation method to produce inoculum for controlled infection studies of the pathosystem *D. sapinea* - Scots pine.

To develop a simple and reliable method for the *in vitro* sporulation of *D. sapinea*, the following four different cultivation parameters were tested, which are known to be crucial for *in vitro* sporulation of fungi: media composition, incubation time, light intensity, and the daily duration of light exposure. The parameters were optimised in a stepwise fashion in the above-mentioned order, such that the condition determined as optimal was used in all following experiments

A selection of media commonly used for fungal cultures (CD, LNA, OA, PDA, VMM), as well as *D. sapinea*-specific media, such as water agar and VMM containing ground or cut pine needles were tested for sporulation of *D. sapinea* cultures. Spore yield was highest with lowest variation on VMM without any amendments of Scots pine needles (**Paper IV**, Figure 1). Consistently highest spore yield could be harvested after 21 days, compared to 14, 18, and 25 days. Constant (compared to darkness, long, and short-day treatment) and high light intensity (6000 vs 2000 lx) resulted in the highest spore harvests. Final spore yield of the optimised cultivation resulted in about 1.6 to 1.7 x 10^6 spores per plate (Ø 5.5 cm).

Two observations within these tests were crucial to reach improved spore formation and harvest; **1)** pycnidia in culture released spores in droplets at their top when spores were mature, and **2)** spores were easiest to harvest when pycnidia were not over-grown by mycelium (**Figure 4**).

These two observations were favoured by the low nutrient richness of the medium, constant and high light intensity, and an optimised incubation time that allowed harvesting the mature spores after release, but before droplets began to dry out. The optimal spore harvest was obtained as follows:

- *D. sapinea* was grown in Petri dishes (Ø 5.5 cm) containing Vogel's Minimal Medium (VMM) at ca. 27°C for 21 d.
- Plates were kept in transparent plastic boxes with lids to contain humidity, under constant illumination by constant daylight at an intensity of 5000 – 6000 lx.
- Spores were harvested by pipetting 2 ml of 0.01% (v/v) Tween 20 onto the surface of the culture and rinsing the plate by pipetting the liquid several times.

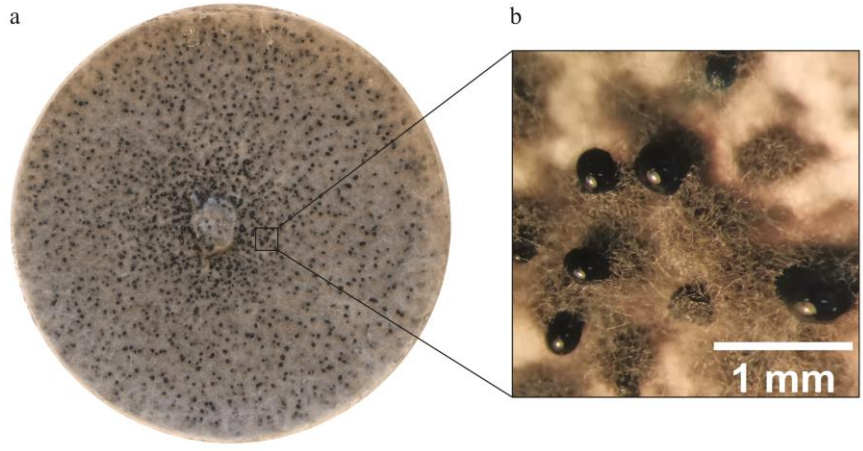

Figure 4 a) *Diplodia sapinea* culture growing on Vogel's minimal medium, incubated under constant light (6000lux) for 21d. **b)** Magnification of pycnidia in culture which formed droplets containing mature spores on top. (**Paper IV**)

Pathogenicity of the spores produced *in vitro* was verified by a greenhouse experiment using Scot's pine seedlings (**Figure 5)**.

The experiment compared five inoculation methods on two sets of pine seedlings in different physiological states: dormant and actively growing. The application method of spores (pipetting vs. spraying), effect of wounding, as well as a comparison of inoculation by spores with commonly used agar plugs, were carried out. Symptoms were scored 4 and 6 weeks post inoculation, and samples for reisolation of *D. sapinea* were taken at the latter and final scoring time point.

Overall, disease incidence was highest in wounded seedlings (vs non-wounded; $p < 0.0001$ in all treatments) inoculated with spores (vs mycelium; $p < 0.05$ in dormant plants), independent of their application method (pipetting vs spraying of spores; $p > 0.5$ in all treatments). The spores produced *in vitro* were not only proven to be pathogenic, but also significantly more efficient than inoculation with agar plugs in actively growing plants. *Diplodia sapinea* was reisolated from almost all symptomatic plants (57 were positive of 60 symptomatic plants). Furthermore, we could observe asymptomatic infections by reisolating *D. sapinea* from healthy-looking plants. Here, inoculation of actively growing seedlings with spores applied by spraying showed a tendency to be most successful in establishing colonisation of *D. sapinea* in its endophytic stage.

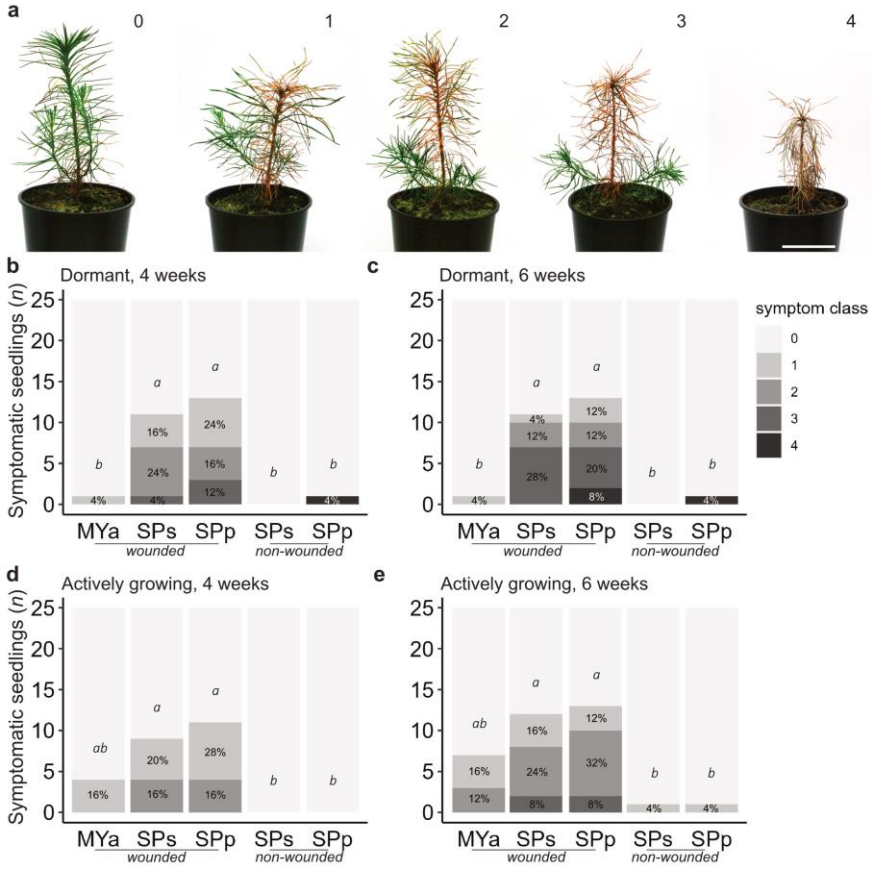

Figure 5 a) Representative examples of disease classes of Scot's pine seedlings infected with *D. sapinea*. Shown are dormant seedlings 4 weeks after inoculation, actively growing seedlings comparable. **0**: asymptomatic, **1**: necrosis of needles and stem asymptomatic, **2**: upper third of stem necrotic, **3**: upper two thirds of stem necrotic, **4**: seedling dead. Frequencies of symptom classes of active (**b**, **c**) and dormant (**d**, **e**) Scots pine seedlings inoculated with *D. sapinea* 4- and 6 -weeks post inoculation. Dormant and actively growing seedlings were assigned to five treatment groups: Spores pipetted (SPp) or sprayed (SPs) on wounded seedlings, mycelium (MYa) on wounded seedlings, and spores pipetted or sprayed on non-wounded seedlings. None of the controls developed visible symptoms (not shown). Same letter above bar indicates no difference between treatments (pairwise fisher's exact test; $\alpha = 0.01$). (**Paper IV**)

5. *Diplodia sapinea* affecting Scots pine in Sweden

5.1 The first detected outbreak in Sweden (**Paper I**)

Large areas of Scots pine infected with Diplodia tip blight had not been recorded in Sweden before 2016. The aim of this project was to describe the northernmost outbreak of Diplodia tip blight on Scots pine to date, and to investigate potential drivers that lead to disease establishment on plantation-level.

5.1.1 Damages on Scots pine caused by *D. sapinea*

In the first detected outbreak of Diplodia tip blight on Scots pine in Sweden (**Figure 6**), almost 90% of pines were affected by tip blight in a 15-ha plantation, mixed with asymptomatic Norway spruce. On average, an affected pine lost a third of its crown, and more than half of all measured pines showed a damaged leader shoot. Based on the number of previous loses in the leader shoot, the epidemic was reconstructed back to 2007. From 2007 onwards, a significant increase of the number of attacks was observed over the years in approximately half of the plantation. These findings indicated an accumulation of symptoms over the previous years, rather than a sudden establishment of infections.

At a tree level, the percentage of dead shoots correlated positively with the number of previous attacks and negatively with tree height. There was a clear spatial pattern of damages within the stand, where trees appeared more severely damaged in the southwest area.

Tree ring growth analysis showed an impact of *D. sapinea* infections in latewood production. Impact on non-structural carbohydrate (NSC) reserves was localised in proximal tissues to the attack area (needles and shoots), but no overall effect was detected in the root system where a large amount of NSCs are stored during winter. *D. sapinea* proved to be a damaging pathogen in economic terms. Not so much because of its impact on growth, as losses were mainly seen on the latewood production (representing a small fraction of the ring), but because of its capacity to kill the leader shoot(s), disrupt the shape of the growing crown, and decrease the quality of the stem.

These severe damages demonstrated that *D. sapinea* has no limitations in becoming a serious pathogen in Northern Europe.

Figure 6: Scots pine (*Pinus sylvestris*) showing typical symptoms of Diplodia tip blight (*Diplodia sapinea*): top shoot dieback with a "hockey-stick" like bending at the tip. Shown is a representative section of first detected outbreak of Diplodia tip blight nearby Arlanda, Sweden, discovered in late summer 2016. (Picture taken by Álvaro Camisón Caballero, February 2017)

5.1.2 A new, more aggressive genotype of *D. sapinea*?

The first hypotheses of what led to the outbreak of Diplodia tip blight in Sweden, was the introduction of a cryptic species, or a new and more aggressive genotype of *D. sapinea*. These hypotheses can be rejected; isolates obtained from the outbreak area corresponded to *D. sapinea* with a 100 % match to the sequence of *Sphaeropsis sapinea* 18S ribosomal RNA gene. Furthermore, the isolates of the outbreak were not genetically different, though slightly less diverse, from isolates previously obtained from asymptomatic forests collected in Sweden 2013. Comparing the Swedish populations with European ones, we found signs of geographic differentiation between European populations. In this study, it is not possible to link the genetic structure with some sort of adaptation to northern latitudes, though certain level of adaptation cannot be fully discarded since there seems to be a geographical pattern in the populations across Europe.

5.1.3 Possible drivers of the outbreak

Severity of damages by *D. sapinea* has been associated with higher temperatures in studies from southern Europe (Fabre et al., 2011; Bosso et al., 2017); however, the role of weather in northern latitudes was unknown. We hypothesised that weather conditions also played a role on the development of the *D. sapinea* outbreak and could show that warm temperatures in May were associated with a lower tree growth and higher disease levels. A nearly significant association was also found between low June temperature and growth ($p = 0.054$). The underlying mechanism behind the association between *D. sapinea* attacks and warm conditions during May and June remains unclear. In May and June, shoots start to develop, but completion ends more towards the end of June, when the correlation was no longer significant. Warm temperatures may enable the pathogen to develop endophytically in the bud immediately before sprouting (Brookhouser et al., 1971). Alternatively, warm temperatures could increase drought stress in the new shoots, a condition that has been found associated with *D. sapinea* damages in our and previous reports (Bachi et al., 1985; Stanosz et al., 2001a).

Spore captures were higher underneath trees with higher percentage of dead shoots. Captured spores tended to be more in the southern part of the stand, both under wet and dry weather conditions. During a week of wet weather, more exposed trees showed higher spore captures, indicating that

for a given level of damage, larger captures were obtained under crowns more exposed to wind and rain. Spore deposition within the same tree may be an important component of the epidemic, as shown by the fact that highly damaged trees were also those with more infections in the past. However, under rainy conditions, crown exposure also favoured dispersal. One possibility is that under an open crown the spore trap captured not only spores from the immediate tree but also from the surroundings. Also, a more open crown may facilitate a better wetting of the crown and a higher number of pycnidia being hydrated and releasing spores.

Comparing pairs of trees defoliated by *D. sapinea* and non-defoliated trees revealed that *D. sapinea* attacks affected mainly latewood production. Heavily defoliated trees were characterised by displaying consistently lower $\delta^{13}C$ values irrespective of whether the year had a warm or cold spring. A more liberal use of water seemed to be a key phenotype associated with susceptibility. Previous studies have brought up the role of the tree phenotype increasing susceptibility, which seems to be relating fast growth with disease in northern regions (Stenlid et al., 2016). This issue is particularly important in northern areas where forest regeneration is mostly done by planting and where growth expectations in high site index areas may carry a higher susceptibility.

A survey of Scots pine stands in the outbreak area showed single symptomatic trees in half of the observed stands in proximity (<5km). Surveying stands of further distance showed 10% of the stands with single symptomatic trees infected by *D. sapinea* in a 30km radius around the outbreak area. The outbreak might have been cause by a localised introduction from infected material, since no stand damages similar to this extent were found in close or a bit further proximity.

To control the outbreak, the pines in the plantation were cut down before reaching a harvestable age. The infected timber was used for wood chipping to burn for bioenergy, leaving the owner with large economical losses.

5.2 Drought induced crown dieback of Scots pine, with contribution of *D. sapinea* on Gotland (**Paper II**)

Given the new occurrence of Diplodia tip blight on Scots pine in Scandinavia, the development of disease of affected trees has been one of the central questions since the discovery.

In this study, we aimed at describing mortality and recovery of a drought-induced crown dieback in Scots pine under the presence of *D. sapinea*.

5.2.1 Crown dieback in Scots pine initiated by the drought 2018

In summer of 2018, a large-scale drought was recorded all over Europe (Peters et al., 2020), and Scandinavia was among the regions that showed highest temperature anomalies (Moravec et al., 2021). In Sweden, 2018 showed the warmest recorded mean temperatures in May, and a third of the days from of May to August were significantly warmer than average since 1756 (Wilcke et al., 2020). The anomalies in temperature in combination with precipitation deficits impacted soil moisture, while increased total evapotranspiration might have been the driving force in the drought observed during 2018 (Moravec et al., 2021). Although summer droughts are frequent for the calcareous island of Gotland (Lindroos, 2001), the drought of 2018 had a larger impact on the flora and fauna of the island than usual (Johansson et al., 2022). Scots pine dominates both production-, and natural forests on the island, and in late summer of 2018 large areas of Scots pine showing crown dieback were found all over Gotland (**Figure 7**). Trees were showing overall discoloration on the needles, mixed with symptoms of shoot blight in the upper parts of the crowns. The symptoms were consistent with a potential outbreak of Diplodia tip blight, which could be confirmed by examining pycnidia on cones, and symptomatic twigs on affected sites.

Interestingly, severely affected pine stands were close to healthy-looking stands in the same region. Crown dieback development, as well as presence, and abundance of *D. sapinea* were investigated at both site types in the most severely affected region close by Visby.

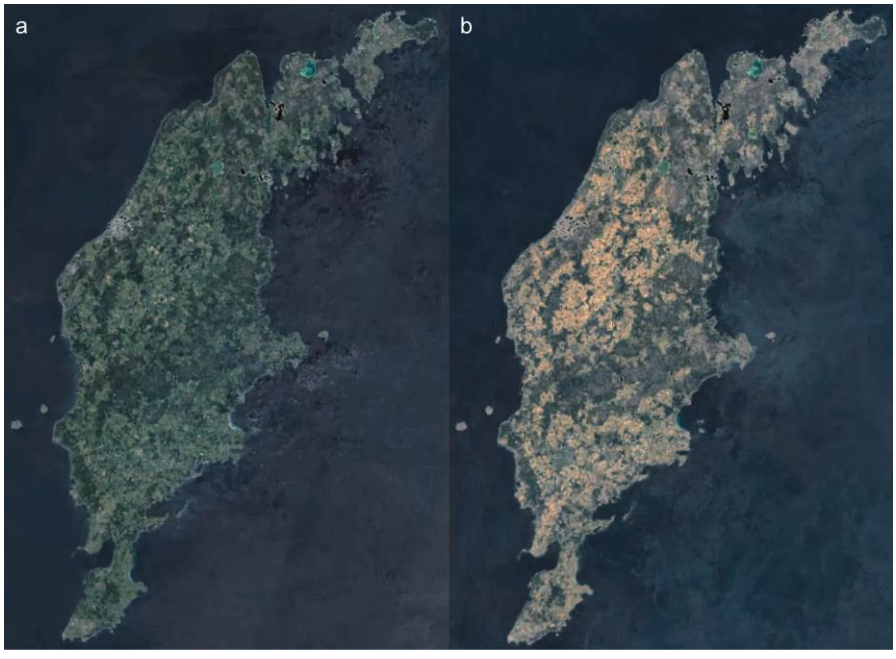

Figure 7 Drought impact on vegetation on Gotland seen from space **a**) in 2017, and **b**) 2018. Pictures taken in July 2017 & 2018 by EUs satellite program Copernicus (Rymstyrelsen, Google/Esa; https://www.rymdstyrelsen.se/rymddata/nyheter/sa-ser-sverige-ut-efter-torkan/)

Scots pine trees on affected sites showed damages with an average of 25% crown dieback in the year of severe drought (Figure 8**a**). The observed damages doubled to above 50% by the end of 2019. It was at this phase that the majority of mortality was observed. Trees with high dieback level in 2018 had an increased likelihood to die before the assessment in 2019. This is consistent with previous studies that reported a delayed response affecting the trees after a drought event occurred (Rebetez et al., 2004; Martínez-Vilalta et al., 2012). Alternatively, observed mortality and increase in crown dieback may have been a consequence of prolonged drought at the sites were the groundwater table was lower than normal also in 2019, even though the intensity of the drought in Scandinavia was lower compared to the drought of 2018 (Moravec et al., 2021; Rakovec et al., 2022).

No significant shift in overall crown dieback occurred during the second year, from 2019 to 2020. However, in 2020 shoots grown after 2018 became clearly visible when necrotic and senesced needles from the previous years

were shed (**Figure 8d**). Recovery of the new crown was apparent in surviving trees, but also in the group of trees classified as severely damaged. In fact, single pines with very severe crown dieback of up to 90% were displaying signs of recovery. This observation is consistent with studies showing that trees even with very high drought-induced crown dieback (>50%) are able to recover once water availability improves (Dobbertin et al 2010; Eilmann et al 2013).

Nevertheless, the increased mortality of highly damaged (>70%) trees was comparable to the mortality of *Pinus* spp. in a hail-storm triggered outbreak of Diplodia tip blight in Spain (Caballol et al., 2022). The presence of *D. sapinea* following the drought-triggered crown dieback might have made a difference for recovery or mortality in medium-damaged trees, where additional loss of needles caused by *D. sapinea* infection could have pushed a tree over the threshold to mortality.

5.2.2 *Diplodia sapinea*'s distribution in the affected region, sites, and trees

Spore trapping, and species specific quantification of *D. sapinea* DNA in Scots pine twigs verified that the opportunistic pathogen was present in the studied region, even on the healthy-looking sites. Investigating asymptomatic and symptomatic trees and twigs revealed *D. sapinea* to be most abundant in symptomatic twigs from symptomatic trees. We could detect *D. sapinea* endophytically in asymptomatic twigs, but in very low amounts. The health status of the tree or the site had no impact on how abundant *D. sapinea* was in asymptomatic twigs.

Monitoring spore dispersal for two years post-drought confirmed spore release was related to precipitation (Brookhouser et al., 1971; Swart et al., 1987). Spores were detected throughout all seasons, without a seasonal pattern in any of the two sampled years, nor an obvious increase of spore dispersal. In agreement with the detection of *D. sapinea* also on healthy sites, pines did develop mild symptoms of Diplodia tip blight during the two years of the study. Regardless, disease incidence was substantially lower compared to the affected sites.

Diplodia sapinea seems to be widespread, but not necessarily abundant, in Scots pine stands in the studied region on Gotland.

Figure 8 Representative pictures of different Scots pine trees on Gotland. Crown dieback estimation was started after severe drought in 2018, trees were revisited in 2019 and 2020. **a) 2018**: A Scots pine tree with 70% crown dieback of upper third of living crown (2018). Browning of needles was found in apical shoots (shoot blight), consistent with symptoms of Diplodia tip blight. **b) 2019**: Scots pine tree with 40% crown dieback, measured as shoot blight and loss of needles (defoliation), as they were difficult to distinguish. **c) 2020**: Scots pine tree with 30% dieback including defoliation. **d) 2020 new growth**: Scots pine tree with 70% crown dieback in the upper third of the crown, *vs* 20% dieback when measuring only shoots grown post-drought, not considering the defoliated, transparent parts of the crown. (**Paper III**)

5.3 Fungal community of Scots pine twigs under drought and the presence of *D. sapinea* (**Paper III**)

As a latent pathogen, *D. sapinea* has an endophytic life stage, inhabiting its host without causing symptoms. Disease occurs in interaction with its host, where a stress-weakened tree is prone to develop symptoms of Diplodia tip blight. Another interactive part in disease establishment is the endophytic fungal community co-inhabiting the host. In this study, we investigated if community composition, or a single species, is associated with asymptomatic or symptomatic tissues, twigs, trees, and sites of the affected Scots pine area found in 2018 (**Paper II**), to explore potential antagonists of *D. sapinea*.

5.3.1 Fungal community differs mainly among sites, but also in relation to tree health (**Paper III**)

The endophytic fungal community we found in this study was in general similar to the communities found in twigs of Scots pine in previous studies (e.g., Sanz-Ros et al., 2015; Bußkamp et al., 2020). Ascomycota dominated the endophyte community comprising 60% of the OTUs. The second largest group of OTUs was classified as Basidiomycota (18%), while 22 % of the OTUs could not be assigned taxonomically. The largest class among all samples was Dothideomycetes with 18% of the OTUs, followed by Leotiomycetes (6.3%) and Sordariomycetes (2.3%). The largest orders were represented by Capnodiales (8.1%), Pleosporales (7.9%), and Helotiales (7.1%). The Capnodiales, including sooty moulds such as *Alternaria sp*. and *Cladosporium sp.*, belong to the Dothideomycetes and represent their second largest order after the Pleosporales (Abdollahzadeh et al., 2020). With epi-, ecto- and endophytes of various hosts, this order includes a wide spectrum of lifestyles. Helotiales contains many species of weak pathogens, saprophytes and mycorrhizal species. Recently, their function as endophytes, and as invasive, virulent pathogens have been notable, with *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya as a prominent representative (Hosoya, 2021). The majority of species in Pleosporales are saprobes, but the order also contains several species of necrotrophic pathogens (Watkinson et al., 2015).

Sampled sites had the strongest effect (R^2 = 0.42, p = 0.035) on the composition of the endophytic fungal community in Scots pine (**Figure 9** & **Paper III**, Table 2). The health status of the sites did not significantly influence the communities, though healthy sites clustered with more narrow variation compared to the affected sites in a principal component analysis (R^2 = 0.09, p = 0.055; **Paper III**, Table 2). Affected sites 1 and 4 seemed to drive the bigger variation between the sites encompassing symptomatic Scots pine.

Within affected sites, health status of the trees (R^2 = 0.03, p = 0.019), as well as health status of the twigs within symptomatic trees (**Figure 10** & **Paper III**, Table 4) did significantly affect fungal communities of the Scots pines. Furthermore, communities differed between the twigs showing Diplodia tip blight symptoms, depending on the health status of the tissue (necrotic vs healthy; R^2 = 0.06, p = 0.001; **Figure 10** & **Paper III**, Table 5).

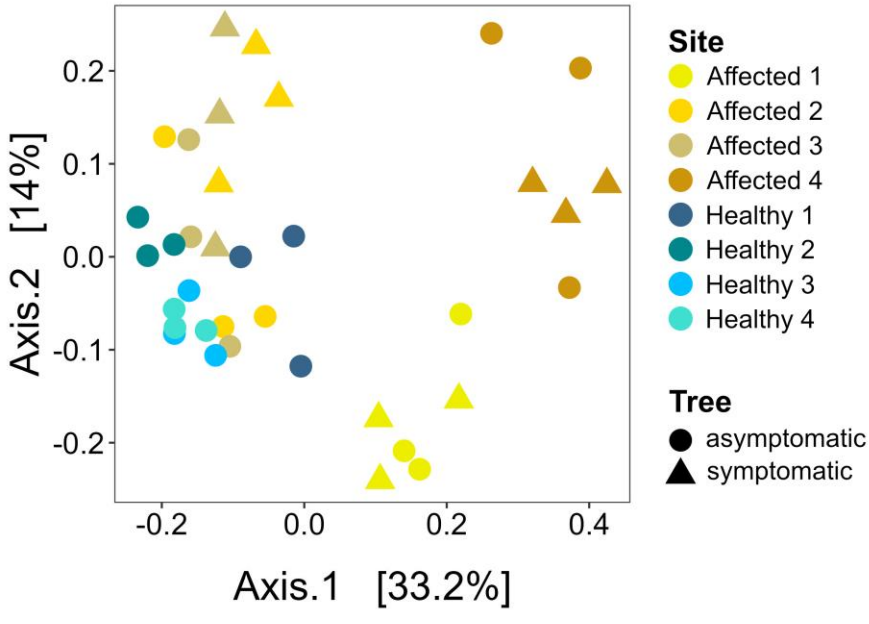

Figure 9 Ordination plot of principal component analysis (PCoA) of the endophytic fungal community sampled from asymptomatic (●) and symptomatic Scots pines showing Diplodia tip blight (▲) on affected and healthy sites. Affected sites 1 to 4 in yellow/orange shades, healthy sites 1 to 4 in blue shades. (**Paper III**)

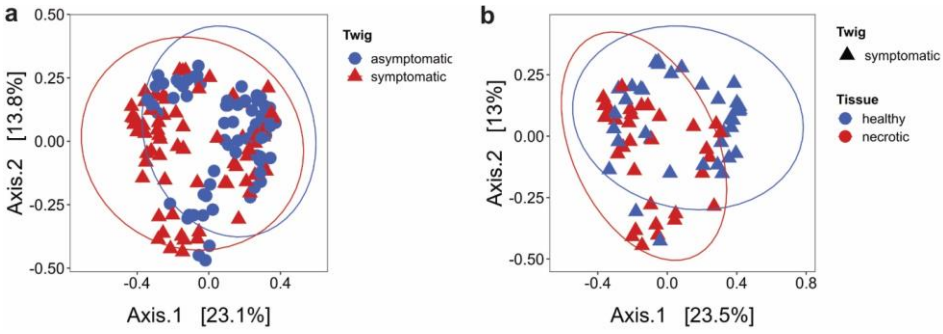

Figure 10 Ordination plot of principal component analysis (PCoA) of the endophytic fungal community sampled from Scots pine showing Diplodia tip blight symptoms on affected sites. a Comparison of symptomatic (▲) and asymptomatic (●) twigs within the affected trees. b) Comparison of healthy (▲) and necrotic tissues (▲) within symptomatic twigs. (**Paper III**)

Diplodia sapinea was found to be strongly associated with the fungal communities of symptomatic trees (IndVal = 0.992, p = 0.0001) and twigs (IndVal = 0.784, p = 0.0001). We did not find a significant tissue preference of *D. sapinea* inside the symptomatic twigs, although it was almost twice as abundant in relation to the total fungal community in necrotic tissue (10 %) at the border of infection compared to the healthy tissue (6.5 %) (**Paper III**, Table 1). *D. sapinea* was not the only fungus that was associated with symptomatic trees, twigs or tissues in the indicator species analysis. For instance, both *Lophium arboricola* (IndVal = 0.64 in twigs | 0.83 in tissue, both $p < 0.001$) and *Therrya pini* (IndVal = 0.83 in twigs | 0.90 in tissue, both $p < 0.01$) were significantly associated with symptomatic twigs and tissues (**Paper III**, Table 3). *Therrya pini* is considered a saprophyte known to associate dead pine twigs, while *L. arboricola* originally was isolated from stem cankers on conifers (Hietala et al 2012, Bugzacki 1972). Taken together, it is evident that *D. sapinea* is associated with symptomatic trees, and specifically with the symptomatic twigs in this study. Even if it was possible to detect sequence reads from *D. sapinea* in 70% of the apparently healthy (asymptomatic) trees, the abundance of the pathogen was 60-fold larger in the communities of trees that display shoot dieback symptoms.

An indicator species analysis identified five OTUs that were associated to asymptomatic twigs, and four OTUs associated to tissues in symptomatic trees. Among these were two relatively abundant OTUs in the order Phaeothecales. These fungi were associated to both asymptomatic twigs and asymptomatic tissues in symptomatic twigs. Isolates of *Phaeoteca dimorphospora* have been explored because of their capacity to inhibit the growth of a wide range of tree pathogens such as *Ophiostoma ulmi*, *Gremmeniella* spp., *Sphaerulina musiva*, and *Heterobasidion annosum* s.l. *in vitro* and *in planta* (Roy et al 2001; Yang et al 1994; Yang et al 1995). Isolates of *P. dimorphospora* have been shown to produce diffusible metabolites with capacities to inhibit the growth of pathogenic fungi (Roy et al 2001, Yang et al 1993) indicating that these fungi may use antibiosis to keep competing fungi at bay *in planta*.

Another fungus that was associated to asymptomatic trees, twigs and tissues was identified as *Neophaeothecoidea proteae* (IndVal = 0.67, p = 0.0024). Interestingly this species was identified as one of five indicator species for disease class 0 i.e., healthy Scots pine trees in an outbreak of Diplodia tip blight in Germany in 2018 (Blumenstein et al., 2021a). However, *N. proteae* was relatively rare in the endophyte community, in both the present study (<1% of the reads) and in the work of Blumenstein et al. (2021a). It can be speculated that this taxon is rather excluded from necrotic tissues than a potential antagonist to *D. sapinea*.

6. Conclusions and perspectives

In this thesis, I showed that *Diplodia sapinea* is not only present in Sweden but has no restriction to cause damages on Scots pine in the Nordic climate. The main conclusions of the thesis are:

- *Diplodia sapinea* can cause disease in boreal conditions. (**Paper I**)
- The first detected outbreak in Sweden built up over the course of years, rather than being a sudden outbreak of infection. (**Paper I**)
- *Diplodia sapinea* is neither a recently introduced pathogen, nor does the Swedish population substantially differ from European populations. (**Paper I**)
- Warmer spring temperatures, as well as phenotypic variation in drought adaptation of pines are potential drivers of disease establishment. (**Paper I**)
- Pines showing severe symptoms of Diplodia tip blight can recover when factors stressing the host, such as drought, are released. (**Paper II**)
- *Diplodia sapinea* was detected in asymptomatic trees, but it was only found in high abundance in symptomatic twigs in the studied region on Gotland. (**Paper II**)
- The endophytic community of symptomatic and asymptomatic trees, twigs, and tissues differs, with four fungal species being associated with healthy twigs and tissues in symptomatic trees. (**Paper III**)

- *In vitro* sporulation of *D. sapinea* is favoured by low nutrient media, and constant high light intensity. (**Paper IV**)
- The choice of inoculation technique of Scots pine seedlings with *in vitro* produced pycnidiospores of *D. sapinea* influences the frequency of symptomatic, compared to asymptomatic infections. (**Paper IV**)

Overall, this thesis provides new knowledge of the range expansion of Diplodia tip blight further north. How widespread *Diplodia sapinea* is in Swedish forest lands remains to be explored.

Ever since the first observation of *D. sapinea* infections of cones on asymptomatic *Pinus* spp. (Oliva et al., 2013), several incidences of Diplodia tip blight have been recorded in the southern half of Sweden (**Figure 11**, **Table S1**). A lack of records earlier than 2013 might have been caused by misidentification. More common pathogens, as *Melampsora sp.* Castagne or *Gremmeniella abietina* (Lagerb.) M. Morelet, cause shoot dieback similar to Diplodia tip blight. Alternatively, or in combination with misidentification, there could have been a very low frequency of symptomatic infections of *D. sapinea* in Scots pine stands in Sweden during the last century. At the time of the description of the first outbreak in 2016, awareness, and disease symptoms might have been increased.

Paper I indicated that the first detected outbreak of Diplodia tip blight in Sweden was unlikely a sudden outbreak of infection, but rather an accumulation of symptoms throughout a decade before the discovery. Former symptomatic infections with lower severity across the whole plantation might have remained undiscovered given the location of the forest. Next to a highway (E4), and with no street or path connecting populated areas crossing though the forest, frequency of passing people who could have discovered the symptoms was probably low. In contrast, the crown dieback of Scots pine on Gotland (**Paper II**) developed rapidly. Dieback symptoms developed within less than a couple of months and received direct attention by the public in relation to the severe drought of 2018 (*pers. commun*. Karin Wågström, Skogsstyrelsen). Why are there no previous records of large-scale crown dieback in Scot pine related to *D. sapinea* on Gotland? It is possible that the outbreak on Gotland was caught right at the beginning of the

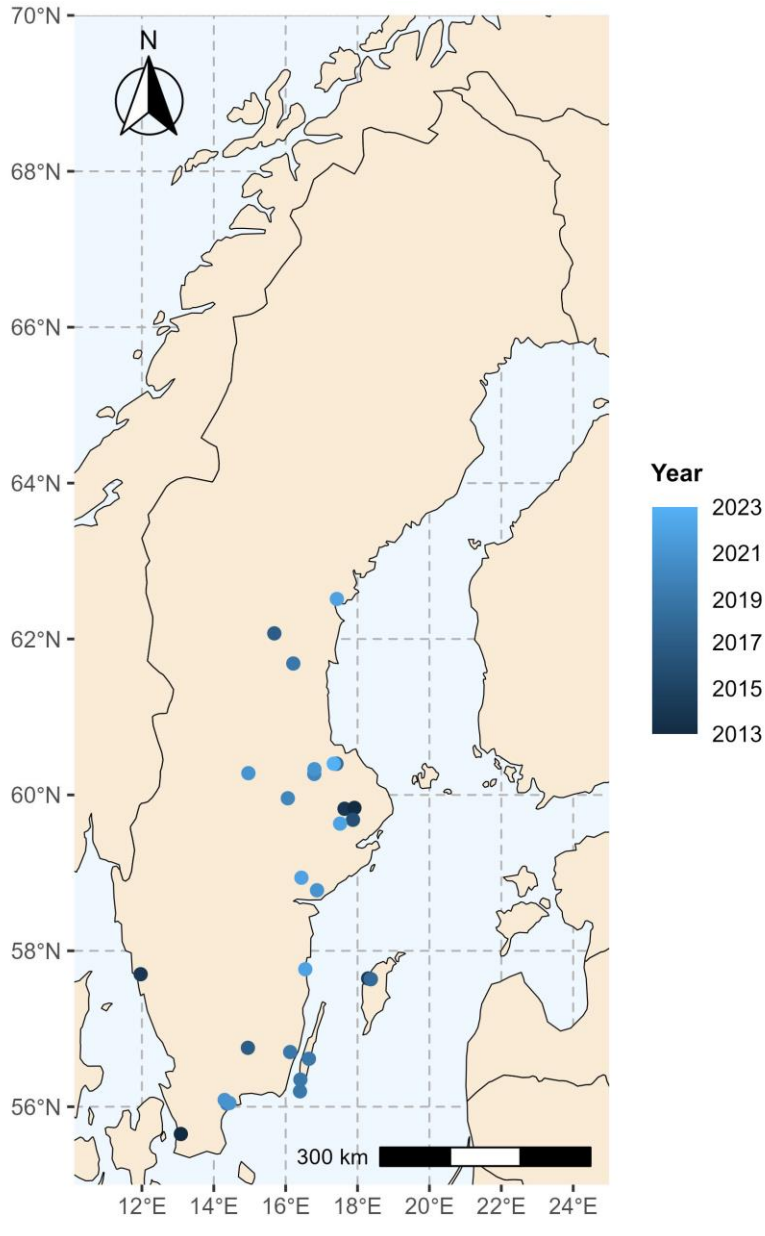

Figure 11 Incidence records of *D. sapinea* in Sweden 2013 - 2023. Information collected from colleagues at the Dept. of Forest Mycology and Plant Pathology (SLU), and Skogsstyrelsen. The Year of incidence is indicated with a gradient from **dark blue** (2013) to **light blue** (2023).

accumulative phase of *D. sapinea*, while it was found to be already locally endemic throughout pine stand of **Paper I**. On the other hand, pines on Gotland are well adapted to stress conditions to survive in the harsh conditions on this island. Only now, that trees were exposed to a drought with a severity they had not experienced in the last 200 years, could symptoms of Diplodia tip blight develop on a large scale.

In any case, drought was very likely a key driver for the sudden development of Diplodia tip blight on Gotland, while the underlying cause of disease development at the first detected outbreak remains unclear. It is not unlikely that the fungus was introduced with the planted seedlings of the pine stand described in **Paper I**. Nevertheless, conditions at this site must have affected tree health to a level that symptomatic infections by *D. sapinea* could reoccur over the years.

To my knowledge, *D. sapinea* has not been found in any of the previous fungal community studies in Scots pine carried out in Sweden. Studies often focused on needles (e.g., Millberg, 2015) or roots (e.g.,Varenius et al., 2016). Nevertheless, I suspect *D. sapinea* to be rather widespread at least in the southern part of Sweden, but endophytically and in very low abundances. Systematic sampling campaigns of Scots pine twigs, where endophytic infections are more likely to occur, such as those carried out by Bußkamp (2018), are needed to clarify how widespread *D. sapinea* actually is in Swedish forests.

In **Paper I**, and **II** climate has been a re-occurring factor in disease establishment. In Fennoscandia, climate is expected to change to milder winters with increasing heavy rainfalls, and dryer and warmer summers (SMHI, 2015). Consequences of more extreme and longer lasting droughts to tree health is already observed with increasing tree mortality world-wide, and appears likely to further rise with progressing change of climate (Hartmann et al., 2022).

Fabre et al. (2011) found a direct positive effect of a warming climate to the occurrence of *D. sapinea* on *Pinus* spp. in France. If *D. sapinea* is already widespread in Sweden, even if in low abundancy, accumulation of symptoms might lead to a build-up of inoculum followed by increased pressure to tree health by this opportunistic pathogen. These findings, together with previous

knowledge, underline the importance of reducing stress factors in plantations, by e.g., careful site selection, to prevent large-scale outbreaks.

A limited number of studies have investigated the endophytic community of Scots pine under the presence of *D. sapinea* (Bußkamp et al., 2020; Blumenstein et al., 2021b; Oliva et al., 2021). **Paper III** contributes insights with a more detailed sampling approach that allows for a refined analysis of potential antagonists and synergistic members of the endophyte community under the presence of *D. sapinea*. Using this approach, potential antagonistic endophytes have been found in asymptomatic tissues. The antagonistic potential of these candidates should be further investigated. *In vitro* antagonism tests, but also *in planta* co-infection studies of e.g. *Phaeoteca* spp. or other potential antagonists and *D. sapinea* could provide insights on a potential biocontrol agent. Biocontrol agents could be applied on commercially produced seeds or seedlings to reduce further human-driven spread of this latent pathogen in forests. The standardised sporulation method developed in **Paper IV** provides insights for possibly carrying out large-scale infection studies which would be needed to verify potential effects of biocontrol agents.

Even though triggers of Diplodia tip blight are well studied, the detailed infection mechanism, especially the switch from endophyte to pathogen, remains unclear. The developed method for *in vitro* sporulation (**Paper IV**) is not only applicable in large-scale infection studies, but also for further molecular studies of the infection morphology. With this provided method, the *Diplodia sapinea* – Scots pine pathosystem could be further utilised to understand dynamics and underlying mechanisms of disease outbreaks by stress related forest pathogens.

Taken together, I conclude that increasing damages caused by *Diplodia sapinea* in our forests are symptomatic for the global climate change, and most likely not caused by the simple presence, or range expansion of this opportunistic pathogen. Prevention and management actions against Diplodia tip blight will at best only reduce direct damages. They do not tackle the underlying cause of the human-driven change of the global climate which puts many organisms in an environment they have not had the chance to adapted to in the past millennia.

Further research, and especially political actions are needed to mitigate damages to our forests, not only to secure production capacities, but to protect ourselves together with all the living creatures that are affected by us human beings.

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Popular science summary

Under the current unprecedented climate crisis, plants are exposed to significant changes in their environment, such as a higher probability of hot, dry summers, and mild, wet winters. Plants are stationary organisms which cannot change their location. Climate is changing at a much faster pace than any adaptation through evolutionary adjustment could occur. This exposes the trees of our forests to a variety of new stress factors that makes them susceptible to stress-related diseases.

Three aspects are crucial in the development of disease: 1. the pathogen, 2. the host, and 3. environmental factors, ranging from climate to species competing with the pathogen or host. Diplodia shoot dieback in conifers is characterized by the death of newly grown shoots in the upper crown, the formation of stem cankers and blue discolouration inside the stems. Its causal agent is the mould *Diplodia sapinea*, but *D. sapinea* can also infect its host tree without causing symptoms of the disease. The decisive factor for the outbreak of the disease is a stressed host by, for example, drought, heat or mechanical damage.

This thesis focuses on *D. sapinea* in the highly susceptible host species, Scots pine. Scots pine (*Pinus sylvestris*) is the 2nd most common tree species in Sweden and accounts for 40% of the total forest area. Within the framework of my doctoral thesis, the first, and to date the northernmost large-scale outbreak of Diplodia shoot dieback was described. The aim of my work was to gain new knowledge about the establishment and spread of Diplodia shoot dieback in Sweden.

One factor in the outbreak of Diplodia shoot dieback in Sweden was the climate. Warm June and May temperatures favoured an accumulation of Diplodia shoot dieback over the last 10 years in the pine stand of the first

large-scale outbreak (**Paper I**). However, genetics of *D. sapinea* individuals from the outbreak in Sweden did not differ from those derived from surrounding less-affected areas or the rest of Europe, indicating that the outbreak was caused by fungi already present in Sweden.

A second region of pine stands on Gotland showed crown dieback accompanied by symptoms of Diplodia shoot dieback after severe drought and heat in summer 2018 (**Paper II**). Within two years without extreme drought, even trees heavily affected by *D. sapinea* showed new shoot growth and thus regeneration. Drought in combination with the location and resilience of the individual pines were crucial for an outbreak of Diplodia shoot dieback.

A molecular investigation of the pines within the affected area indicated a locally increased occurrence of *D. sapinea* in diseased tissue of affected pines. The potentially harmful fungus was also found in healthy pine stands, but only in very small amounts. *D. sapinea* is spread via spores that are formed in fruiting bodies on infested cones, and dead twigs and needles. Tracking of *D. sapinea* spores in the air on attacked stands confirmed it disperses only short distances (**Paper I**) and its spread is accelerated by precipitation (**Paper II**).

Fungi can colonise their hosts internally (endophytic), or superficially (epiphytic). In this work, the internal, endophytic fungal community in the microbiome of healthy and diseased trees, twigs and wood tissues was investigated (**Paper III**). The endophytic fungal communities differed from tissue to site level depending on the health status. Four fungal species were found only in healthy wood tissue. This probably indicates that fungi present in the endophytic community of pine can act as antagonists of *D. sapinea* and could possibly be considered as control agents.

Future research on Diplodia shoot dieback will benefit from the development of a *D. sapinea*-pine laboratory system. This thesis partly achieved this via the development of a method for the spores formation in the laboratory (**Paper IV**). This work contributes to the knowledge of the spread of Diplodia tip blight to the north and underline the importance of studying stress-related forest diseases for maintaining forest health in the face of climate change.

Populärvetenskaplig sammanfattning

I samband med klimatförändringarna utsätts växterna, som är stationära organismer och inte kan förflytta sig från ogynnsamma förhållanden, för påtagliga förändringar i sin miljö, t.ex. större sannolikhet för torra och varma somrar, eller milda och blöta vintrar. Klimatet förändras i en mycket snabbare takt än vad växter skulle kunna göra genom evolutionär anpassning. Det betyder att träden i våra skogar utsätts för nya stressfaktorer som de är inte anpassade till och som gör dem mottagliga för stressrelaterade sjukdomar.

För att sjukdom ska uppstå måste tre faktorer vara på plats: 1. Skadesvampen, 2. en mottaglig värdväxt, och 3. miljöfaktorer som gynnar sjukdom. Miljöfaktorer kan vara allt från klimat till arter som konkurrerar med skadesvampen eller värden.

Diplodia sapinea är orsaken till Diplodiasjukan hos barrträd. Karaktäristiska symptom är att nytillkomna skott dör i den övre delen av kronan, det bildas stamsår och veden blir blåfärgad. *Diplodia sapinea* kan dock infektera värdträdet utan att orsaka sjukdomssymptom. Man säger då att sjukdomen är latent, eller att svampen växer endofytiskt. Den avgörande faktorn för att sjukdomen ska bryta ut är att värdträdet är stressat av t.ex. torka, värme eller mekaniska skador.

Sveriges vanliga tall *Pinus sylvestris* står för 40 % av totala skogsarealen. Tall är dessutom ett av de trädslag som är mest mottagliga för att drabbas av Diploidasjukan. I min avhandling beskriver jag det första och hittills nordligaste storskaliga utbrottet av Diplodiasjukan. Syftet med mitt arbete har varit att samla ny kunskap om hur Diplodia har etablerat och spridit sig i Sverige.

En bidragande faktor till utbrott av Diplodiasjukan i Sverige har varit klimatet. Under de tio åren som ledde fram till det första kända storskaliga utbrottet i ett svenskt tallbestånd, har varma temperaturer i juni och maj visats gynna en ackumulering av Diplodia angrepp (**Artikel I**). Det arbetet visade också att det var flera olika genetiska individer av *D. sapinea* som orsakade utbrottet i Sverige och att de liknade andra grupper av skadesvampen i Europa rent genetiskt. Dessutom tyder resultaten att samma *D. sapinea* individer funnits på plats i Sverige redan innan utbrottet uppmärksammades.

Efter den svåra torkan och värmen sommaren 2018 uppvisade tallbestånd på Gotland kronurglesning tillsammans med symptom av Diplodiasjukan (**Artikel II**). Vi följde de här bestånden två år, som kännetecknades av extrem torka. Under den här perioden återhämtade de flesta träden sig och uppvisade ny skottillväxt, även träd som drabbats hårt av *D. sapinea*. Torka i kombination med växtplatsen och de enskilda tallarnas motståndskraft är avgörande för ett utbrott av Diplodiasjukan.

I arbetet med de Diplodiaangripna bestånden på Gotland använde vi molekylärbiologiska metoder för att mäta tillväxten av Diplodia inuti tallarna. Resultaten från det arbetet visade att det fanns mycket *D. sapinea* i synligt sjuk vävnad från drabbade tallar. Svampen hittades också i vitala tallbestånd, men endast i mycket små mängder. *Diplodia sapinea* sprids via sporer från fruktkroppar som kan finnas på angripna kottar, döda kvistar och barr. Detektion av luftburna sporer från *D. sapinea* inom angripna bestånd bekräftade antagandet att sporerna kan spridas relativt korta avstånd (**Artikel I**) och när det regnar (**Artikel II**).

Svampar kan som sagt kolonisera insidan på sina värdar (endofytiskt) eller på värdens yta (epifytiskt). I den här avhandlingen undersöktes svampsamhället, mikrobiomet, inuti friska och sjuka träd, kvistar och trävävnad (**Artikel III**). De endofytiska svampsamhällena skiljde sig åt mellan bestånd och mellan olika tallvävnader beroende på hälsostatus. Fyra svamparter hittades endast i frisk tallvävnad. Detta skulle kunna betyda att svampar som finns i det endofytiska samhället hos tall kan vara antagonister till *D. sapinea* och detta skulle kunna undersökas vidare för att potentiellt utveckla biologiska bekämpningmetoder.

Dessutom har en metod för att bilda sporer i laboratoriet vidareutvecklats för att underlätta framtida forskning om samspelet mellan *D. sapinea* och tall, och därmed av Diplodiasjukan.

Resultaten av detta arbete bidrar till kunskapen om spridningen av Diplodiasjukan norrut och understryker vikten av att forska på stressrelaterade skogssjukdomar för att upprätthålla skogens hälsa i samband med klimatförändringarna.

Populärwissenschaftliche Zusammenfassung

Im Zuge des Klimawandels sind Pflanzen, die als stationäre Lebewesen nicht ihren Standort wechseln können, einer erheblichen Veränderung ihrer Umwelt ausgesetzt, wie z.B. eine höhere Wahrscheinlichkeit fuer trockene und heisse Sommer, und milde und feuchte Winter. Das Klima verändert sich in einem vielfach schnelleren Tempo, als jegliche Anspassung durch evolutionäre Anpassung geschehen könnte. Hierdurch werden die Bäume unserer Wälder einer Vielzahl von neuen Stressfaktoren aussgesetzt, die sie anfällig fuer stressbedingte Krankheiten macht.

In der Entwicklung einer Krankheit sind drei Askepte ausschlaggebend; 1. der Krankheitserreger, 2. der Wirt und 3. Umwelteinfluesse, welche vom Klima bis zu ggf. mit dem Kankheitserreger oder Wirt konkurierenden Arten reichen.

Diplodia sapinea, ein Schlauchpilz (umgspr. ”Schimmelpilz”), ist der Erreger vom Diplodia Triebsterben in Nadelbäumen. Charakteristische Symptome sind das Absterben der neugewachsenen Triebe in der oberen Braumkrone, die Formation von Stammkrebs und blauverfärbung im inneren des Stammes. *D. sapinea* kann jedoch den Wirtsbaum infizieren, ohne Symptome der Krankheit zu verursachen. Ausschlaggebend fuer den Ausbruch der Krankheit ist, dass der Wirt durch beispielsweise Trockenheit, Hitze oder mechanischen Schaden gestresst wird.

Die gemeine Waldkiefer (*Pinus sylvestris*) ist die 2. häufigste Baumart in Schweden und macht 40% der Wälder aus. Darueber hinaus is die gemeine Waldkiefer eine der anfälligsten Baumarten, die vom Diploida Triebsterben betoffen sein kann.

Im Rahmen meiner Doktorarbeit wurde der erste, und bis *dato* nördlichste grossflächige Ausbruch vom Diplodia Triebsterben beschrieben. Das Ziel

meiner Arbeit war es, neues Wissen ueber die Etablierung und Ausbreitung vom Diplodia Triebsterben in Schweden zu Erlangen.

Ein Faktor fuer den Ausbruch vom Diplodia Triebsterben in Schweden war das Klima. Warme Juni und Mai Tempereaturen beguenstigten eine Akkumulation vom Diplodia Triebsterben ueber die letzten 10 Jahre im Kiefernbestand des ersten grossflächigen Ausbruchs (**Paper I**). Studien der Verbreitung von genetischen Individuen von *D. sapinea* in Schweden und Europa zeigten, dass der Ausbruch von mehreren, unteranderem in Schweden schon vorhandenen Individien des Pilzes verursacht wurde.

Eine zweite Region von Kiefernbeständen auf Gotland zeigte ein Absterben der Kronen, einhergehend mit Sympotmen von Diplodia Triebsterben nach starker Trockenheit und Hitze im Sommer 2018 (**Paper II**). Innerhalb von zwei Jahren ohne extreme Trockenheit, zeigten auch von *D. sapinea* befallene Bäume neues Wachstum von Trieben und somit Regeneration. Trockenheit in Kombination mit dem Standort und die jeweilige Resillienz der einzelnen Kiefer sind auschlaggebend fuer einen Ausbruch vom Diplodia Triebsterben.

Eine molekulare Untersuchung von Kiefern im Gebiet der betroffenen Bestände wies auf ein lokal vermehrtes Vorkommen von *D. sapinea* in kranken Gewebe von betroffenen Kiefern hin – der potenzielle Schadpilz wurde auch in vitalen Kiefernbeständen gefunden, aber nur in sehr geringen Mengen. *D. sapinea* wird ueber Sporen verbreitet, die sich in Fruchtköpern auf befallenen Zapfen und abgestorbenen Zweigen und Nadeln bilden. Die Verfolgung von *D. sapinea* Sporen in der Luft von angegriffenen Beständen bestätigten die Annahme von eine Verbreitung auf kurzer Distanz (**Paper I**) in Verbindung mit Niederschlag (**Paper II**).

Pilze können ihre Wirte im Inneren (endophytisch), oder oberflächlich (epiphytisch) besiedeln. Im Rahmen dieser Arbeit wurde die Pilzgesellschaft im Mikrobom von gesunden und gerkrankten Bäumen, Zweigen und Holzgewebe untersucht(**Paper III**). Die endophytischen Pilzgemeinschaften unterschieden sich je nach Gesundheitszustand vom Standort bis zur Gewebe-Ebene. Vier Pilzarten kamen nur in gesundem Holzgewebe vor. Dies weist vermutlich daruaf hin, dass Pilze in der endophytischen Gemeinschaft der Kiefer vorkommen, die Gegenspieler von *D. sapinea* sind und eventuell als Bekämpfungsmittel in Frage kommen könnten.

Desweiteren wurde eine Methode fuer die Bildung von Sporen im Labor weiterentwickelt, um künftige Forschungen des Zusammenspiels von *D. sapinea* – Kiefer, und der Entwicklung vom Diplodia Triebsterben zu erleichtern.

Die Ergebnisse dieser Arbeit tragen zum Wissen über die Ausbreitung von Diplodia tip blight in den Norden bei und unterstreichen die Bedeutung vom Erforschen von stressbedingten Waldkrankheiten für die Erhaltung der Waldgesundheit im Zuge des Klimawandels.

Acknowledgements

Everything in live is shaped and influenced by interactions. One could even say that no organism can be considered independently from its environment, and other interacting organisms. Also, intangible things in life such as ideas, science and success would not be accomplishable without interactions. This thesis is the result of a countless number of interactions that all influenced and shaped this work. Each and every one of them led to the final result which would have looked very different, if just a single one had happened at a different time or in a different way. I am incredibly thankful for all the small and big, brief and long-lasting, and for the high diversity of interactions that led to the accomplishment of finishing this thesis.

I want to thank all my supervisors who let me enter this medley of interactions, a PhD project. **Jonás**, thank you so much for taking me on this journey many years ago. You introduced me to the fantastic world of forest mycology and Mykopat. You always inspired me with your curiosity and your unlimited drive. **Jan**, thank you for leading me through this journey and for encouraging my scientific development with your creative and open mind. **Malin**, thank you for always supporting me, for the countless pep talks and for pushing me over the finish line. I would not have made it without you. **Alyona**, thank you for your unconditional help, curiosity, and always checking up on me.

I also want to thank all the collaborators on the different projects of the last years. **Anne** and **André**, thank you so much for reviving my scientific spirit when I walked through the valley of the motivation curve which is fluctuating throughout a PhD's life. **Karin**, **Rasmus**, and **Miguel**, thank you for your indispensable help, encouragement, and patience. **Matilda**, thank you for being such a motivated, sharp minded scientist and for bringing **Irma**

and **Marie** to field work - you were the *icing of the cake* on Gotland! **Silvia**, thank you for your hard work at the lab and your dedication for details that made my life so much easier. Thank you to all the amazing students that have helped me at the field, greenhouse, and lab!

Thank you for all the proofreading; **Maia**, **Kat**, **Grace** and **Robbie** (English), **Rebecca** & **Malin** (Swedish) and **Mara** & **Johannes** (German).

I felt very lucky finding myself as a PhD student at **Mykopat**. I experienced the environment of this department as incredibly welcoming, caring, and inspiring for a young scientist. A big thank you to **EVERYBODY** for exciting seminars, stimulating journal clubs, fun department days, uplifting fika rounds and heart-warming small talks. You all really make a difference! An especially big thank you goes to **Katta**, **Maria J, Rena** and **Yasi** for irreplaceable help at the lab and field. Thank you for your patience while answering my 1001 questions.

Thank you to the amazing people that were part of the Mykopat's **PhD group** during the last years. Thank you for all the fun, support and ventilating about PhD-life! **Hernàn**, you were the reason I made it to the office when things felt impossible. Thank you so much for your love and friendship. Thank you for sharing the burden of the tough days and for sharing love and joy of the happy days. **Rebecca**, thank you for your open heart and endless support. You made lab-work during the pandemic and learning Swedish to my favourite activities.

Outside the work-environment, I am incredibly thankful for the awesome people I can call my friends and family. Thank you for holding me up, for your support, encouragement, joy, and love. **Wouter**, **Rike**, **Caitlin**, **Robbie**, **Hannah, Hernàn, Camilla** and **Nemi**: thank you for all the bike rides, hikes, camping trips, climbs, laughs, cries, talks, and beers. I would have gone crazy without you. **Felix**, we developed our academic dreams together and were anchors for each other during all the turbulences life comes with. Thank you for being a soulmate. **Anne**, **Flo**, **Fibi**, **Martin**, **Jonny**, **Nina**, and especially **Jessi**: thank you for always being there for me, for endless discussions about life and everything and for always making me laugh, no matter what live throws at us.

Mama, your support is truly infinite ever since the beginning of my life, and especially during the last months when you moved in with us to support me and my little, new family at the finish line of my PhD. Thank you for always believing in me, for encouraging me, and for your unconditional love. Thank you to my siblings, **Camilla**, **Lydia**, **Pia**, and **Robert** for believing in, challenging, and encouraging me at just the right moments. **Håkan** & **Lena**, thank you for welcoming me into your family, for always backing me up, and for being awesome grandparents to Henning!

Kajsa, words just cannot describe how grateful I am to have you at my side. Throughout the past years, you went with me through all the PhD ups and downs. I can always rely on you to be there for me, and I am so excited to go with you through the next adventures in our lives! **Henning**, thank you for your patience with your stressed-out Mama, and for always reminding me about the important things in life.

Love to everybody!

Appendix

Table S1 Information about incidence records of *D. sapinea* in Sweden 2013 - 2023

Location	Coordinates	Host	Trees symptomatic or asymtomatic	Examined samples	Researchers	Year	Publication
Visby, Gotland	57.645519, 18.294956	*Pinus nigra*	symptomatic	cones	Jonás Oliver, Johanna Boberg	2013/ 2014	Brodde et al., 2019
Alnarp, Lomma	55.65, 13.083333	*Pinus nigra*	symptomatic	cones	Jonás Oliver, Johanna Boberg	2013	Oliva et al. 2013; Brodde et al., 2019
Uppsala (Gula stigen)	59.822157, 17.637605	*Pinus sylvestris*	symptomatic	cones	Jonás Oliver, Johanna Boberg	2013/ 2014	Brodde et al., 2019
Gothenburg	57.699814, 11.965243	*Pinus mugo*	symptomatic	cones	Jonás Oliver, Johanna Boberg	2013/ 2014	Brodde et al., 2019
Uppsala, Fjällnora	59.833347, 17.912831	*Pinus sylvestris*	symptomatic	cones	Jonás Oliver, Johanna Boberg	2013	Oliva et al. 2013; Brodde et al., 2019
Håsta Hage (Arlanda/ Märsta)	59.680889, 17.872444	*Pinus sylvestris*	symptomatic	twigs, spore traps	Jonás Oliver, Jan Stenlid, Laura Brodde	2016	Brodde et al., 2019
Tierp, several stands	60.400389, 17.410611	*Pinus sylvestris*	symptomatic	na	Jonás Oliver, Jan Stenlid	2017	/
Tierp	60.400389, 17.410611	*Pinus sylvestris*	symptomatic	twigs, cones	Jan Stenlid, Laura Brodde	2018	/
near by Visby, Gotland	57.636583, 18.368361	*Pinus sylvestris*	symptomatic/ asymptomatic	twigs, cones, spore traps	Laura Brodde, Matilda Stein Åslund, Jan Stenlid	2018	Paper II/III
near by Visby, Gotland	57.636583, 18.368362	*Juniperus communis*	symptomatic	twigs	Matilda Stein Åslund, Laura Brodde, Jan Stenlid	2018	/
Öland	56.614949, 16.646482	*Pinus sylvestris*	symptomatic	twigs	Matilda Stein Åslund, Anna Bowen, Thora Bergsveinsdóttir, Rán Finnsdóttir	2019	/

Öland	56.348033, 16.409613	*Pinus sylvestris*	symptomatic	twigs	Matilda Stein Åslund, Anna Bowen, Thora Bergsveinsdóttir, Rán Finnsdóttir	2019	/
Öland; Ottenby fågelstation	56.197338, 16.398744	*Pinus sylvestris*	symptomatic	twigs	Matilda Stein Åslund	2019	/
Ängelsberg	59.957023, 16.060091	*Pinus sylvestris*	symptomatic	twigs	Jan Stenlid, Matilda Stein Åslund	2020/2021	/
Västervik	57.763565, 16.546145	*Pinus sylvestris*	symptomatic	no	Matilda Stein Åslund	2022	/
Nässja plantskola	60.269071, 16.795708	*Pinus sylvestris*	symptomatic	seedling shoots	Audrius Menkis	2020	/
Stakhedels plantskola	60.27885, 14.962703	*Pinus contorta*	symptomatic	seedling shoots	Audrius Menkis	2021	/
Lugnet plantskola	59.63211, 17.514015	*Pinus sylvestris*	asymptomatic	seedling shoots	Audrius Menkis, Rebecca Larsson	2019	Larsson et al., 2021
NorrPlant	62.513663, 17.424575	*Pinus contorta*	symptomatic	seedling needles	Audrius Menkis	2022	/
Trekanten plantskola	56.70141, 16.12482	*Pinus sylvestris*	symptomatic	seedling shoots	Audrius Menkis, Rebecca Larsson, Åke Olson	2019	Larsson et al., 2021
Gotthardsberg seed orchard	58.93773, 16.43825	*Pinus sylvestris*	symptomatic	seeds, cones, shoots	Rebecca Larsson, Audrius Menkis, Åke Olson	2022	/
Larslund seed orchard	58.77667, 16.87195	*Pinus contorta*	symptomatic	seeds, cones, shoots	Rebecca Larsson, Audrius Menkis, Åke Olson	2021	/
Lundsved-Harsbo	60.400495, 17.337809	*Pinus sp.*	symptomatic	na	private, Lars-Erik Vindeland	2023	/
Österfärnebo	60.329817, 16.800173	*Pinus sp.*	symptomatic	na	private, Katarina Calamaras	2021	/

Ingelstad	56.75576, 14.949301	*Pinus sylvestris*	symptomatic	na	Skogsstyrelsen, Anders Henriksson	2017	/
Gualöv, Skåne	56.041437, 14.367261	*Pinus sylvestris*	symptomatic	twigs	Gunnar Isacsson, Matilda Stein Åslund, Jan Stenlid, Malin Elfstrand	2019-2022	/
Bromölla, at E22, Skåne	56.047475, 14.433899	*Pinus mugo*	symptomatic	twigs	Gunnar Isacsson, Matilda Stein Åslund, Jan Stenlid, Malin Elfstrand	2021	/
Karsholm, Skåne	56.086775, 14.302028	*Pinus sylvestris*	symptomatic	twigs	Gunnar Isacsson, Matilda Stein Åslund, Jan Stenlid, Malin Elfstrand	2021	/
Ljusdal/ Lobsteråsen	62.072418, 15.683274	*Pinus sylvestris*	symptomatic	twig/ needles	Jan Stenlid, Laura Brodde, Nils Frank	2017	/
Järvsjö	61.685809, 16.212044	na	symptomatic	na	Jan Stenlid	na	/

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 09 January 2019
doi: 10.3389/fpls.2018.01818

Diplodia Tip Blight on Its Way to the North: Drivers of Disease Emergence in Northern Europe

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OPEN ACCESS

Edited by:
Kristian Forbes,
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Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: *15 July 2018*
Accepted: *22 November 2018*
Published: *09 January 2019*

Citation:
Brodde L, Adamson K, Camarero JJ, Castaño C, Drenkhan R, Lehtijärvi A, Luchi N, Migliorini D, Sánchez-Miranda Á, Stenlid J, Özdağ Ş and Oliva J (2019) Diplodia Tip Blight on Its Way to the North: Drivers of Disease Emergence in Northern Europe. Front. Plant Sci. 9:1818. doi: 10.3389/fpls.2018.01818

Disease emergence in northern and boreal forests has been mostly due to tree-pathogen encounters lacking a co-evolutionary past. However, outbreaks involving novel interactions of the host or the pathogen with the environment have been less well documented. Following an increase of records in Northern Europe, the first large outbreak of *Diplodia sapinea* on *Pinus sylvestris* was discovered in Sweden in 2016. By reconstructing the development of the epidemic, we found that the attacks started approx. 10 years back from several isolated trees in the stand and ended up affecting almost 90% of the trees in 2016. Limited damage was observed in other plantations in the surroundings of the affected stand, pointing to a new introduced pathogen as the cause of the outbreak. Nevertheless, no genetic differences based on SSR markers were found between isolates of the outbreak area and other Swedish isolates predating the outbreak or from other populations in Europe and Asia Minor. On a temporal scale, we saw that warm May and June temperatures were associated with higher damage and low tree growth, while cold and rainy conditions seemed to favor growth and deter disease. At a spatial scale, we saw that spread occurred predominantly in the SW aspect-area of the stand. Within that area and based on tree-ring and isotope ($\delta^{13}C$) analyses, we saw that disease occurred on trees that over the years had shown a lower water-use efficiency (WUE). Spore traps showed that highly infected trees were those producing the largest amount of inoculum. *D. sapinea* impaired latewood growth and reduced C reserves in needles and branches. *D. sapinea* attacks can cause serious economic damage by killing new shoots, disrupting the crown, and affecting the quality of stems. Our results show that *D. sapinea* has no limitations in becoming a serious pathogen in Northern Europe. Management should focus on reducing inoculum, especially since climate change may bring more favorable conditions for this pathogen. Seedlings for planting should be carefully inspected as *D. sapinea* may be present in a latent stage in asymptomatic tissues.

Keywords: carbon isotopes, dendroecology, water-use efficiency, latewood, earlywood, vascular wilt pathogen

INTRODUCTION

Globalization and climate change are driving forest pathogen invasions and disease emergence worldwide (Stenlid and Oliva, 2016). While reports on human-mediated movements of pathogens continue to cumulate (Liebhold et al., 2012; Jung et al., 2016; Redondo et al., 2018b), few cases where new outbreaks are appearing in connection with climate change have been documented (La Porta et al., 2008). Climate may limit disease by means of different, and often interacting, mechanisms, making climate-change driven outbreaks difficult to understand and predict (Sturrock et al., 2011). One possibility is that climate is limiting the distribution of the pathogen. A good example of such mechanism is the cold winters presumably limiting the northwards expansion of *Phytophthora x alni* (Brasier and S.A. Kirk) Husson, Ioos and Marçais on alder (*Alnus glutinosa* (L.) Gaertn.) (Redondo et al., 2015). The second possibility is that the pathogen is present, but climate is limiting the capacity of the pathogen to cause disease for instance by reducing its capacity to build inoculum. A good example of such mechanism was seen for *Dothistroma septosporum* (Dorogin) M. Morelet in British Columbia where large damages occurred on lodgepole pine forests (*Pinus contorta* Douglas ex Loudon) along with an increase of summer rainfall during the previous decades (Woods et al., 2005). The third and less well-documented possibility is when climate is limiting host susceptibility. In that case, outbreak etiology is complex and may involve maladaptive phenotypes (Stenlid and Oliva, 2016), changes in stress regimes (Hanso and Drenkhan, 2013; Oliva et al., 2014), changes in phenology, or the combination of both (Françoise et al., 2015).

Predicting new forest disease outbreaks also needs to consider their geographic location. Climate change for instance has been suggested to increase forest damage in northern latitudes (Sturrock et al., 2011). However, still little is known about the particular processes behind disease emergence under these conditions. For instance, short growing seasons may make trees growing in northern locations more sensitive to needle loss than their southern counterparts (Oliva et al., 2016; Stenlid and Oliva, 2016). Also, a longer daytime during summer may also extend the periods when trees photosynthesize and expose themselves to water stress. Nevertheless, more cases are needed to depict general patterns. New and, so far, innocuous pathogen encounters are increasingly reported in Northern areas (Millberg et al., 2016; Redondo et al., 2018a,b), and we lack tools to predict their potential impact.

The case of *Diplodia sapinea* (Fr.) Fuckel (syn. *Diplodia pinea* (Desm.) Kickx., *Sphaeropsis sapinea* (Fr.: Fr.) Dyko and Sutton) in Sweden represents a good example of emergence in Northern forests. The pathogen was reported for the first time in 2013 and was regarded innocuous as no associated damages were observed (Oliva et al., 2013). However, 3 years after the first observation, *D. sapinea* was found associated with an unprecedented outbreak on Scots pine (*Pinus sylvestris* L.). In August 2016, stand-level damages affected a circa (ca.) 15 ha plantation north from Stockholm, where hundreds of 20-year-old Scots pine trees appeared severely damaged. Well-developed trees had lost completely all current year's shoots, and some of them were dead. The incidence was high, with the majority of trees affected, in most cases having lost their main leader shoot. A closer look in the stems revealed that most trees were either bifurcated or displayed bushy crowns, suggesting that they had probably suffered *D. sapinea* attacks in the past.

The outbreak in Sweden not only represented a qualitative change to the previous behavior of this pathogen in Northern Europe but also at a global scale. *Diplodia sapinea* had been historically reported in Southern and Central Europe causing shoot dieback, canker, blue stain, and root disease on pines (Fabre et al., 2011; Luchi et al., 2014). Mirroring the situation in Europe, in the Southern Hemisphere, *D. sapinea* had caused outbreaks in areas with mild climates, such as South Africa and New Zealand, where mostly non-native pine plantations have been established (Burgess et al., 2004). The pathogen had also been detected in relatively cold areas, but recent detections in Estonia (Hanso and Drenkhan, 2009), Finland (Müller et al., 2018) and north-western Russia (Adamson et al., 2015) seemed to suggest an ongoing range expansion to the north. Following that pattern, pycnidia of *D. sapinea* were observed for the first time in 2013 (59°N) on a Scots pine cone in Sweden (Oliva et al., 2013). Further samplings done in that same year found pycnidia in cones all over Southern Sweden, in areas such as Uppsala, Gothenburg, Malmö, and Visby, on both non-native *Pinus nigra* Arnold and Scots pine. In spring 2014, a small group of trees displaying shoot blight damages from previous year (2013) were found north from Gothenburg, though as in previous cases, symptoms corresponded to isolated branches or trees.

In this research, we aimed at understanding the causes of the *D. sapinea* outbreak in Sweden, as a way to improve our capacity to predict disease emergence in northern conifer forests. We did so by testing five explicit hypotheses on disease emergence. The first hypothesis concerned the origin of the pathogen. Until 2016, the steady increase of reports in Northern Europe fitted well in the picture of a pathogen slowly expanding its range. However, the isolated nature of the outbreak and the high severity of damage over several hectares raised the question of whether the observed damages could be the result of introduction of a new aggressive strain or a cryptic species of the pathogen (Hypothesis 1). We tested hypothesis 1, by studying the population structure of the pathogen in the outbreak area in comparison with that of isolates previously discovered in Sweden in areas with no apparent damage and with isolates of other parts in Europe, such as Estonia, Spain, and Italy, as well as from Turkey.

A regional survey of other attacks was also attempted in order to gain insights on the origin of the outbreak. Confirming an isolated nature of the outbreak (Hypothesis 2) could support the idea that infected planting stock was perhaps used in that particular stand. In forest nurseries in Wisconsin (USA), up to 27% of red pine seedlings were found to carry latent infections (Stanosz et al., 1997), and we know that in the 1950s, *D. sapinea* was present in Swedish forest nurseries (Molin et al., 1961). Thus, in order to test hypothesis 2, we surveyed the surroundings of the outbreak area for stands also affected by *D. sapinea*.

The role of weather on disease development was also explored. *D. sapinea* enters the host through stomata of elongating needles at expanding shoots and injured tissue (Brookhouser and Peterson, 1971). *D. sapinea* remains in a latent stage in asymptomatic trees (Smith et al., 1996), while disease is often induced by stress factors such as drought (Bachi and Peterson, 1985; Stanosz et al.,

2001), hail (Zwolinski et al., 1990), or mechanical wounding (Chou, 1987). At regional scale, severity of damages has been associated with higher temperatures in Italy (Bosso et al., 2017) and France (Fabre et al., 2011); however, the role of weather in northern latitudes is unknown. We hypothesized that weather conditions played a role on the development of the *D. sapinea* outbreak (Hypothesis 3). In order to test hypothesis 3, we reconstructed the *D. sapinea* epidemic in the stand by inferring the time when trees had lost the leader shoot. We modeled the development of the epidemic and correlated departures from the expected disease progression curve with tree growth and monthly weather conditions.

Damages were not homogeneous across the stand, pointing to disease development being modulated by spatial features. Across the outbreak, there was also variation in terms of damage among trees. Within the same plot, highly damaged trees were found nearby asymptomatic trees, also pointing to the role of some tree features in the development of the outbreak. Disease in northern latitudes has been sometimes associated with maladaptive phenotypes (Stenlid and Oliva, 2016), such as pine trees planted in fertile sites that would normally correspond to Norway spruce (*Picea abies* (L.) Karst.) (Witzell and Karlman, 2000). Given the link between drought and Diplodia shoot blight, we hypothesized that Scots pine phenotypes with lower water use efficiency (WUE hereafter) would be more susceptible to *D. sapinea* (Hypothesis 4). In order to test hypothesis 4, we reconstructed radial growth (earlywood and latewood widths), quantified non-structural carbohydrate (NSC hereafter) concentrations in several tissues (branch, needles), and measured wood C isotope discrimination ($\delta^{13}C$) of trees severely attacked by *D. sapinea* in comparison with asymptomatic trees growing nearby.

Understanding the spatiotemporal development of the outbreak was also attempted by focusing on the spread of the pathogen. *Diplodia sapinea* has large spores that are released from pycnidia by raindrop splashes (Brookhouser and Peterson, 1971; Swart et al., 1987), thus we expected that *D. sapinea* would disperse in short distances in the studied stand (Hypothesis 5). We tested hypothesis 5 by comparing spore captures underneath diseased trees along a range of severity of symptoms, and between wet and dry periods. We also explored the use of qPCR instead of time-consuming spore counting measures for future monitoring purposes.

MATERIALS AND METHODS

Population Structure Studies

The affected stand was located next to a highway (E4), northwest from the Stockholm Arlanda Airport (59°40′51.2″N, 17°52′20.8″E), and it corresponded to a ca.15 ha Scots pine plantation established in 2000 around some older Norway spruce forest patches.

In order to confirm the identity of the pathogen causing the outbreak, three attacked shoots per tree from 40 trees were collected (**Figure 1**). Five wood pieces were taken from the margins of necrotic tissues of each shoot samples and were placed in a plate containing 2% malt extract agar. After a two-day incubation at 21°C, fungal colonies were randomly picked up from three of the wood pieces, and they were transferred to a new plate. After two weeks, one of the three isolates was chosen by its morphological similarity with the rest and recultured a second time for DNA extraction. After 5 days at 21°C, two pieces (~0.5 cm^2) of mycelium were harvested and stored at −20°C. DNA from all 40 isolates was extracted with a NaOH/Tris-HCl-based method described in Wang et al. (1993).

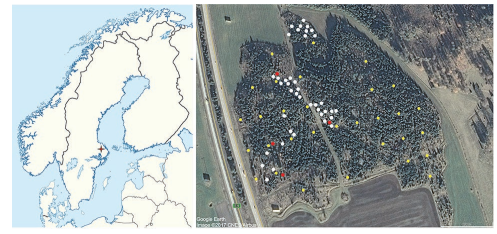

FIGURE 1 | Location of 40 trees used for isolation and spore trapping (white), 31 plots used for damage assessment and dendrochronology (yellow), and location of the four plots where the 24 trees used for physiological measurements were located within the affected stand (red). Image on the left modified from NordNordWest - own work, using World Data Base II data, CC BY-SA 3.0. Image on the right modified from Google Earth Image © 2017 CNES/Airbus.

We searched for signs of genetic differentiation between *D. sapinea* isolates in the outbreak area and isolates from Sweden, Europe, and Asia Minor (Turkey). *D. sapinea* population from Sweden consisted of 92 isolates. The collection was obtained in an isolation campaign carried out in 2013, when single-spore isolates were obtained from infected pine cones from five different locations: Visby (four *P. nigra* cones), Lomma (four *P. nigra* cones), Gothenburg (four *P. mugo* Turra cones), Uppsala (one *P. sylvestris* cone), and Fjällnora (one *P. sylvestris* cone). Single-spore isolates were taken by collecting pycnidia from cones and dissolving them in water and plating them in water agar. Individual germlings were retransferred to malt extract agar. For comparison with the rest of Europe and Asia Minor, a total of 65 isolates from Estonia (two locations, 11 isolates), Spain (five locations, 23 isolates), Italy (two locations, 15 isolates), and Turkey (two locations, 16 isolates) were included. The isolates in Spain, Italy, and Turkey were obtained in a similar scheme as Swedish ones from 2013, that is, single-spore isolates from cones. Estonian isolates were collected by direct isolation from symptomatic shoots and cones.

Isolates used for population structure were confirmed to be *D. sapinea* by being positive based on the specific PCR essay from Smith and Stanosz (2006). No major morphological differences were observed among the isolates of the outbreak area; therefore, a subsample of seven out of 40 was sequenced. Sequencing targeted the internal transcribed spacer (ITS) 1 and 2 and the 5.8S ribosomal RNA gene with the ITS1f and ITS4 primers (Gardes and Bruns, 1993). The purified PCR products were sequenced by Macrogen (Macrogen Inc., Seoul, Korea), edited in SeqMan pro (DNAStar, Inc., Madison, WI, USA; version 12.0.0), and blasted in GenBank (Genbank accession numbers: MK120100 - MK120106).

We searched for genetic differentiation among populations by looking for allelic variation in 10 simple sequence repeat (SSR) markers (Burgess et al., 2001; Bihon et al., 2011). The original panel of 16 markers

was shortlisted to the ones that showed any variability. SSRs were amplified following Burgess et al. (2001). Amplification products were diluted 10- to 200-fold and send to Uppsala Genome Centre for fragment size analysis. SSR alleles were discriminated with the GeneMarker software (Softgenetics, State College, PA). Population structure analyses were carried out with the package POPPR (Kamvar et al., 2014) for R version 3.0.3 (The R Foundation for Statistical Computing). Combining the data of the 10 SSR markers, a multilocus genotype (MLG) was determined for each isolate. Swedish populations collected in 2013 and 2016 and the rest of European populations were compared in terms of rarefied numbers of MLGs and Simpson's index of diversity. Geographical differentiation among European countries was tested by means of a MANOVA analysis, calculated based on D-Jost distance, and visualized with a minimum spanning network. D-Jost values and statistical significance were calculated by bootstrapping with and without clone correction in the R package DEMEtics v.0.7-8.

In order to further support species identification, a sporulation test was conducted for two isolates from the outbreak area belonging to two different haplotypes. Isolates were cultivated on autoclaved pine needle extract (*P. sylvestris* needles ground on liquid nitrogen, 2% Agar) (Luchi et al., 2007). Cultures were kept under constant fluorescent light at 28°C. Pycnidia formed after one week on the surface of the agar and pine needle debris at the ground of the culture. After two weeks, five mature pycnidia per isolate were harvested. Conidial length and width, color, septation, and wall texture of 62 spores were measured from digital images recorded with a Leica DFC285 camera (Leica Microsystems, Switzerland) attached to a microscope (Axioplan, Carl Zeiss AG, Germany) and processed with the NIH imageJ software (version 1.52b, http://rsb.info.nih.gov/ij/). Morphological and morphotype identification followed the observations and identification key of Palmer et al. (1987) and Phillips et al. (2013).

In October 2016, thirteen Scots pine stands situated in a radius of 5 km from the outbreak area were surveyed. A second survey was undertaken in May 2017 including 28 Scots stands with a similar age as the plantation of the initial outbreak in a radius of 30 km. Shoots displaying symptoms were examined for characteristic pycnidia and microscopically screened for spores of *D. sapinea*, but no isolations were undertaken. Isolates from surveys around the infected area in 2016 were not included in the population structure analysis.

Tree Measures and Assessment of Damage

In October 2016, a systematic tree assessment was carried out across the outbreak area. A total of 264 pines within 31 plots were measured (**Figure 1**). Plots were circular and had a 2 m radius (12.6 m^2). In each plot, stem girth, height, and infection level of all trees with a girth larger than 3 cm at breast height were measured. Signs of bifurcation in the stem as well as the infection of the leader shoot were recorded. Stem girth was measured at breast height. The infection level per tree was estimated as the percentage of infected shoots proportional to all shoots from the upper third of the living crown. Signs of previous bifurcations were present all over the stand, thus timing of former attacks was inferred by recording the number of internodes (years) after the bifurcation, that is, after the leader shoot was lost. Additionally, a measure of exposure of the crown to wind and rain was taken in a scale from 0 to 4; according to which, a value of 4 indicated that the tree had a fully exposed crown, while a value of 0 indicated that the tree was completely surrounded by other trees. We extracted two increment wood cores from each tree at breast height using a Pressler increment borer. Wood cores were air-dried and their surface was carefully sanded until tree-ring boundaries were clearly visible. Then, tree rings were visually cross-dated and earlywood and latewood widths were separately measured with precision of 0.001 mm using a binocular microscope and the LINTAB package (Rinntech, Heidelberg, Germany). Earlywood and latewood widths were visually distinguished by experienced dendrochronologists based on the change in wood color and tracheid transversal dimensions. Tree-ring widths were transformed into basal area increments (BAI), assuming a circular shape of the stem. The COFECHA program (Holmes, 1983) was used to evaluate the visual cross-dating of tree-ring series.

Physiological Comparison Between *D. sapinea* Defoliated and Non-defoliated Trees

In February 2017, six pairs of defoliated and non-defoliated trees were selected in four different plots within the outbreak (n = 24 trees). In each plot, defoliated/non-defoliated tree pairs were stratified by size, so two big-diameter trees, two medium-sized, and two small trees were measured. Trees were chosen to be as close as possible (max. distance of 5 m). Trees were felled and discs were cut from the base and below the living crown. Tree growth was obtained from wood discs as done from increment wood cores. To couple growth measures with water-use efficiency, we compared carbon isotope ratios ($^{13}C/^{12}C$, $\delta^{13}C$) in wood formed in the years 2012, 2013, 2014, and 2016 between healthy and infected trees yielding a total of 96 wood samples. The years 2012 and 2014 corresponded to years with colder-than-normal spring temperatures, while spring temperatures in 2013 and 2016 were warmer-than-normal. The wood samples for $\delta^{13}C$ analyses were dried in the oven at 70°C for 48 h, then whole annual tree rings were separated using scalpels, and the resulting wood samples were ground to a fine powder. Wood aliquots (0.001 g) were weighed on a balance (AX205 Mettler Toledo, OH, USA) into tin foil capsules and combusted using a Flash EA-1112 elemental analyzer interfaced with a C isotope ratio mass spectrometer (Thermo Fisher Scientific Inc., MA, USA). Isotope analyses were conducted at the UC Davis Stable Isotope Facility (Davis, USA). Stable isotope ratios were expressed relative to Vienna Pee Dee Belemnite (VPDB). The standard deviation was better than 0.1‰.

Recently formed (one-year old) needles, branch sapwood, and root samples were also taken in order to assess whether NSC concentrations differed between damaged and undamaged trees. From damaged trees, samples were taken from needles and branches proximal to the damaged area, so comparison with the effects of *D. sapinea* defoliation with distal tissues, such as roots, could be possible. Samples were transported to the laboratory in a portable cooler, where they were frozen and stored at −20°C until freeze-dried. Samples were weighed and milled to a fine and homogeneous powder using a ball mill (Retsch Mixer MM301, Leeds, UK). Soluble sugars were extracted with 80% (v/v) ethanol, and their

concentration was colorimetrically determined using the phenol-sulfuric method (Buysse and Merckx, 1993). Starch and complex sugars remaining after ethanol extraction were enzymatically digested with an enzyme mixture containing amyloglucosidase to reduce glucose as described in Palacio et al. (2007). NSC measured after ethanol extractions were regarded as soluble sugars, whereas carbohydrates measured after enzymatic digestion were considered to be mostly starch. The NSC concentration was calculated as the sum of soluble sugars and starch concentration.

Quantification of Spore Production Under Infected Trees

Forty spore traps were placed directly underneath forty trees with varying levels of damage (**Figure 1**). Spore traps had a height of about 50 cm and consisted of one horizontally fixed filter paper (Munktell, Ahlstrom; Ø90 mm) and one microscopy slide covered with two stripes of tape coated with permanent adhesive on both sides (Scotch® Double Sided Office Tape). The first set of traps was placed in 2016, from 30th of September to the 10th of October during a period with little rain (total precipitation 3.6 mm, 3 days of rain, average temperature 8.0°C); the second set was placed from the 21st of October to the 31st, during a rainy period (total precipitation 24.4 mm, 7 days of rain, average temperature 5.4°C). For spore counting, microscopy slides were divided into 22 rectangles of 0.5 cm × 1.3 cm each. Half of those 22 rectangles were screened under the microscope, where *D. sapinea* spores were distinguished by morphological characteristics (Cheng-guo et al., 1985). In order to evaluate the possibilities of monitoring *D. sapinea* by qPCR, we compared the number of spores counted in the microscope slide with the quantity obtained in terms of copy numbers from qPCR. For that, DNA was extracted from filter papers, by placing the entire filter in a 50-ml falcon tube under sterile conditions. After addition of 20 ml SDS buffer, filters were incubated for 90 min at 65°C. Next, the filter paper was removed and 20 ml of isopropanol was added and incubated overnight. On the following day, the sample was centrifuged at 7000 rpm for 10 min and the supernatant removed. From there and on, DNA extraction was continued with the NucleoSpin® Macherey-Nagel Soil Kit, following manufacturer's instructions. qPCR was done following the TaqMan™ setup designed by Luchi et al. (2005). Each 20 µl reaction consisted of a final concentration of 1× SsoAdvanced™ Universal Probes Supermix (BioRad), 250 nM of each primer, 200 nM probe, and 1 µl DNA extract/1 µl sterile water as non-template control. The qPCR program consisted of 2 min at 95°C, followed by 40 cycles of 10 s at 95°C and 15 s at 60°C. Copy numbers were obtained by averaging three technical replicates of each sample.

Data Analysis

The spatial association between disease and growth was examined by correlating the number of attacked trees and the relative X and Y coordinates of the plots ($n = 31$). A composite variable representing a gradient from SW to NE was calculated as the product of the coordinates. The development of the disease in each plot in time was modeled as linear function ($n = 9$ years), and the significance of that correlation was calculated. The correlation was used as a measure of disease increment and was also regressed against the SW-NE location of the plot. When modeling tree features associated with damage, we ran a stepwise selection to reduce the number of variables, always including the plot as a random factor.

We performed an epidemiological analysis in relation to tree growth and weather. We reconstructed the epidemic by pooling the number of records of attacks among all trees in the stand ($n = 264$). Development with time was modeled with a linear function ($n = 9$ years). BAI measures were integrated for all trees in the stand ($n = 264$) and were also regressed against time with a linear function ($n = 9$ years). Departures from a linear increase in both the epidemiological and the growth model were transformed into studentized residuals, which were then correlated with month's daily average temperature and precipitation sum for the period 2007 to 2016. Weather data for the outbreak stand were obtained by the interpolation tool provided by the Swedish Meteorological Institute and Hydrological Institute and available on the web.[1]

Earlywood and latewood widths were compared between pairs of trees defoliated by *D. sapinea* ($n = 12$) and non-defoliated trees ($n = 12$) separately for every year from 2007 to 2016. $\delta^{13}C$ comparisons were done similarly, but in that case all years were considered a factor in the analysis and the particular tree was included as a random factor in a mixed model. The degrees of freedom were adjusted by a Kenward-Roger approximation. Comparisons of NSCs and their different fractions between defoliated ($n = 12$) and non-defoliated trees ($n = 12$) were done by an ANOVA analysis including the plot as a blocking factor in the model.

The association between spore captures and tree features was done by regression ($n = 40$ and 39 traps in wet period and dry period respectively). Inclusion of variables in the model was done by stepwise selection. The association between spore captures and location was calculated by correlating the number of spores with the coordinates of each particular spore trap obtained by GPS *in situ*. Correlation between qPCR values and spore counts was done on data of 77 traps as some paper filters were lost during the rainy week. We tested whether the correlation between gene copies number (qPCR of filter paper trap) and spores (sticky traps) was different during the rainy and the dry week by including "week" as a factor in the model, both in the intercept and the slope "week × spore number." All analyses were carried out in Minitab® 18.1 for Windows.

RESULTS

Genetic Background of the Isolates in the Outbreak Area

Diplodia-like colonies represented a 76% of the isolates obtained from symptomatic tissues in the outbreak area. Isolates showed first a fast-growing white mycelia which turned to gray/black as cultures became old. Isolates obtained from the outbreak area corresponded to *D. sapinea* with a 100 % match to the sequence of *Sphaeropsis sapinea* 18S ribosomal RNA gene (GenBank: JF440618.1). Those, and the rest of isolates from Europe, included in the population structure analysis were positive for *D. sapinea* based on specific primers.

[1] luftweb.smhi.se

Based on 10 SSR markers, the European population of *D. sapinea* was highly clonal, as only 28 MLG were found among 197 isolates. Rarefied numbers of MLG showed between 3 and 5 MLG (per 10 isolates) among all studied countries with the exception of Turkey, whose isolates displayed a distinct and much higher diversity than the other areas (9 MLG per 10 isolates) (**Table 1**). We found signs of geographic differentiation between European populations, accounting for 24% of the genetic variance ($p < 0.001$) (**Figure 1**). The Estonian population was the least differentiated from the rest (average D-distance of 0.05), while Swedish populations was the most dissimilar from the others (average distance 0.15), in particular from southern Spanish and Turkish populations (**Table 1**). Minimum spanning network showed a cluster of Turkish isolates highly differentiated from the rest (**Figure 1**). When using clone correction, significant differentiation was only found between Sweden and Turkey (D-Jost = 0.14, $p = 0.003$). No differentiation was found among Swedish populations (**Table 1**). The isolates of the outbreak were not genetically different from isolates previously obtained in Sweden (**Figure 2**), although they appeared to be slightly less diverse. The most abundant MLG in the outbreak, arbitrarily named G28, was also relatively abundant in 2013. That same haplotype was not found anywhere else in Europe. Spore size of G28 was similar to that of the haplotype G19, the most abundant in Sweden before the outbreak (length: 40.0 vs. 40.9 µm, $p = 0.08$; 15.4 vs. 15.5 µm, $p = 0.85$, respectively). In both cases, conidia produced *in vitro* were non-septated and showed a rough wall texture. Spore size, shape, and number of septa matched those of morphotype A.

Spatiotemporal Spread of the Epidemic

Symptomatic trees with similar symptoms were found in 7 of 13 stands in the vicinity of the outbreak area (<5 km). Further away (<30 km), incidence was much lower, with 3 of 28 stands with signs of *D. sapinea*. In any of the surveyed stands, damage severity resembled the one observed in the outbreak area. Across the outbreak, 85% of the trees were infected, while 53 % showed damage on the leader shoot. Among attacked trees, almost a third of the shoots in the upper third of the crown were affected (28%). There was a clear spatial pattern across the stand, where severity appeared to be higher in the southwest area of the stand (**Figure 3**). Based on the number of previous loses in the leader shoot, we reconstructed the epidemic back to 2007. Three plots situated in the north/north-western area of the stand had the oldest attacks (**Figure 3**). From 2007 onwards, we observed a significant ($p < 0.05$) increase of the number of attacks over the years in ca. half of the plots (48%). The largest increments were observed in the southwest area of the stand ($R^2 = 0.13$, $p = 0.043$). At plot level, no association between disease progression or damage and diameter, height, exposure, or tree growth was found. At tree level, the percentage of dead shoots correlated with both the number of previous attacks ($p < 0.001$) and tree height ($p = 0.006$).

Weather Conditions Associated With Tree Growth and Disease Increase

The number of putative *D. sapinea* attacks increased linearly over time (**Figure 4A**). In 2013 or 2016, the number of attacks was higher-than-expected, while in 2012, 2014, and 2015 the epidemic declined. In the years with largest attacks, trees grew less than expected (**Figure 4B**), and there was a strong negative correlation ($p < 0.001$) between disease increment and radial growth over time (**Figure 4C**). The same negative association between growth and *D. sapinea* was found in relation to weather conditions. Warm temperatures in May were associated with a lower tree growth ($p = 0.004$) and higher disease levels ($p = 0.042$) (**Figure 4D**). A nearly significant association was also found between low June temperature and growth ($p = 0.054$). No significant association between tree growth, disease, and precipitation was found (**Figure 4E**).

TABLE 1 | Population structure among *Diplodia sapinea* isolates from five different locations in Sweden collected in 2013 and from the location of the outbreak, and genetic distance among Sweden's *D. sapinea* population and other four populations in Europe.

Population	*n*	MLG	eMLG	Simpson's index corrected	Jost's D genetic distance				
Sweden					**Arlanda**	**Fjällnora**	**Gothenburg**	**Gula Stigen**	**Lomma**
Arlanda, 2016	40	4	2.62	0.387					
Fjällnora, 2013	7	2	2	0.286	–0.05				
Gothenburg, 2013	9	2	2	0.223	**0.03**	0.00			
Gula Stigen, 2013	31	2	1.93	0.322	0.02	–0.01	–0.04		
Lomma, 2013	20	3	2.98	0.679	0.00	–0.02	0.00	–0.02	
Visby, 2013	25	4	2.99	0.510	**0.04**	0.02	–0.01	-0.03	–0.02
Europe					**Sweden**	**Estonia**	**Spain**	**Turkey**	
Sweden	132	6	3.19	0.620					
Estonia	11	5	5.00	0.782	**0.12**				
Spain	23	6	5.01	0.810	**0.14**	0.01			
Turkey	16	12	9.17	0.966	**0.19**	**0.07**	**0.07**		
Italy	15	4	3.47	0.657	**0.16**	0.02	**0.06**	**0.07**	

Data based on 10 SSR loci. The number of isolates (n), multilocus genotypes (MLG), multilocus genotypes rarefied to 10 isolates (eMLG), and Simpson index corrected for different sample size are shown. Jost's D genetic distance calculated without clone correction. Jost's D-values significantly different from 0 are shown in boldface.

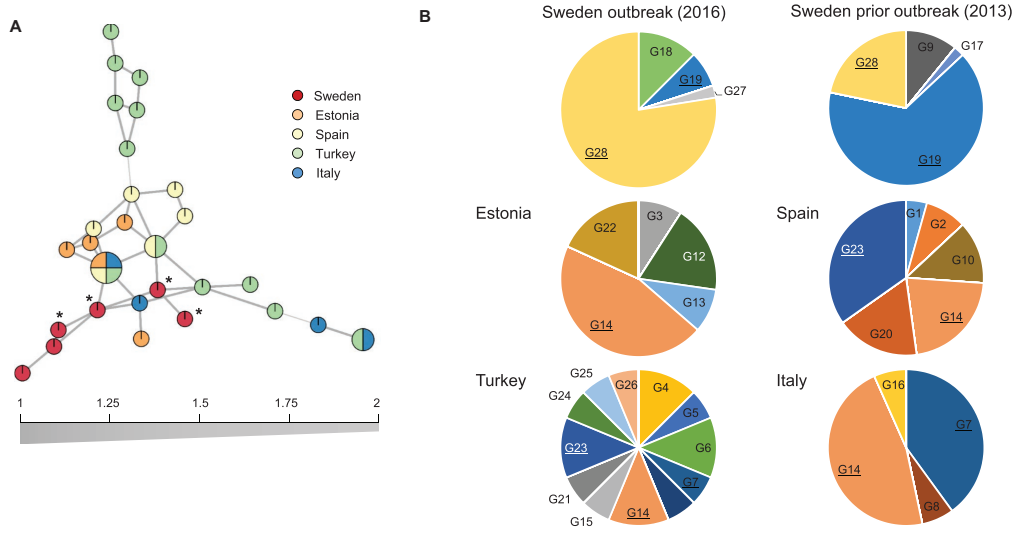

FIGURE 2 | Minimum spanning network among *D. sapinea* haplotypes in Europe **(A)**. Haplotypes from the outbreak area are marked with an (*). Pie charts with relative proportion of haplotypes among populations **(B)**. Underlined haplotypes are found in more than one location.

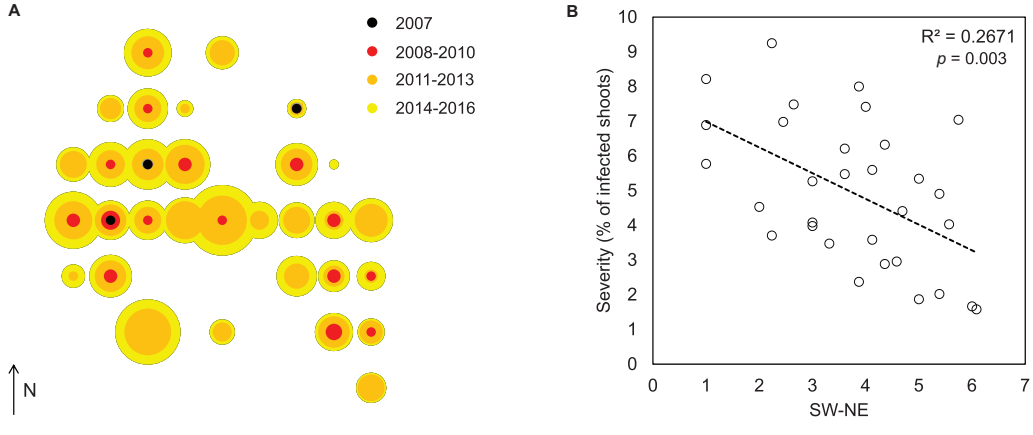

FIGURE 3 | Spatial and temporal spread of *D. sapinea* within the affected Scots pine stand **(A)** and association between severity in 2016 and location (n = 31 stands) **(B)**. SW-NE was calculated as the product of the relative X and Y coordinates of the sampling plots; higher values indicate NE locations, while low values indicate SW locations. Both severity and SW-NE location are plotted following square-root transformation.

Physiology of Attacked Trees

Comparing pairs of trees defoliated by *D. sapinea* and non-defoliated trees revealed that *D. sapinea* attacks affected mainly latewood production (**Figure 5A**). The year of the outbreak (2016) latewood was halved (0.74 vs. 0.35, p = 0.011), while no differences in terms of earlywood production were observed (**Figure 5B**). Heavily defoliated trees were characterized by displaying consistently lower $\delta^{13}C$ values irrespective of whether the year had a warm or cold spring (**Figure 5C**). Defoliated trees had lower NSC concentrations in needles and branch sapwood during the winter after the outbreak (**Figure 5D**), but no differences were found in root NSCs.

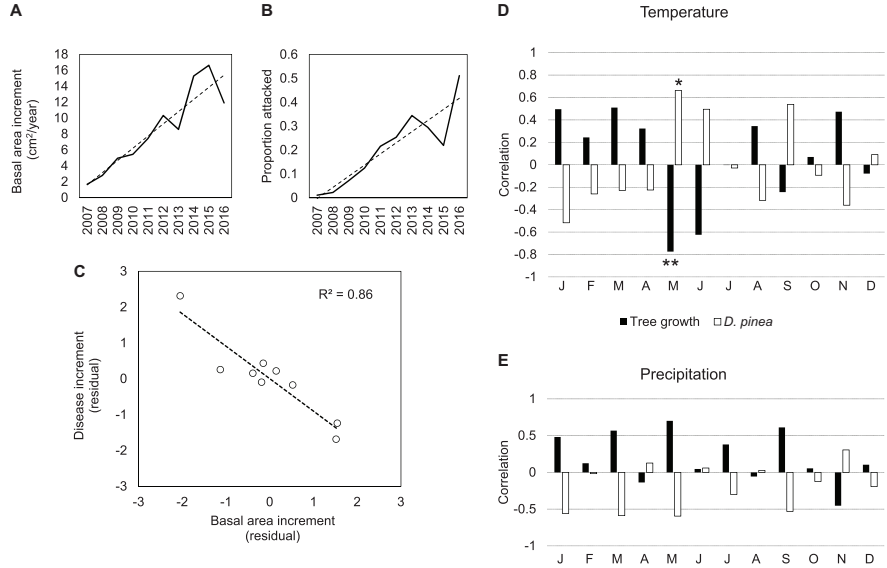

FIGURE 4 | Temporal association between tree growth, *D. sapinea* epidemic, and weather conditions. **(A)** Tree radial growth based on basal area increments (BAI) and development of *D. sapinea* in the stand from 2007 to 2016 (n = 264 trees) **(B)**, correlation between standardized residuals from *D. sapinea* increments and BAI of 264 trees over 9 years **(C)**, correlation between average monthly temperatures **(D)** and precipitation **(E)** from 2007 to 2016 (n = 9 years) and standardized residuals from *D. sapinea* increments and BAI from 264 trees. Dashed lines in a, b, and c show adjusted linear regression. Significance levels in bar plots: $^{**}p < 0.01$; $^{*}p < 0.05$.

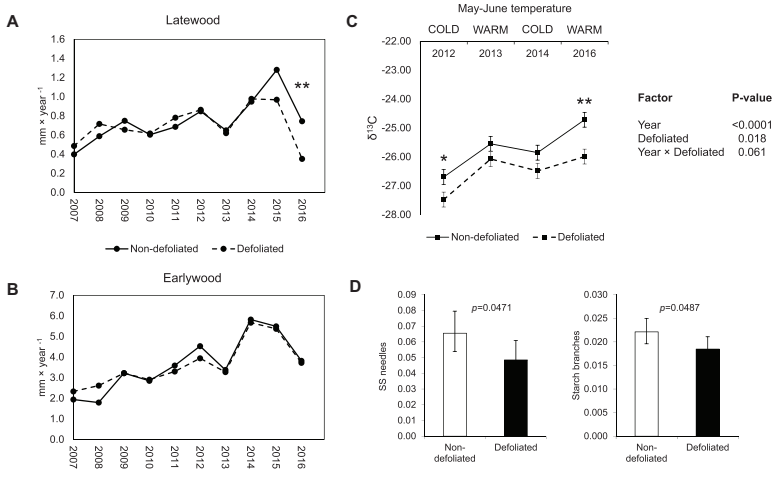

FIGURE 5 | Comparative physiological performance between trees defoliated by *D. sapinea* (n = 12 trees) and non-defoliated trees (n = 12 trees). Latewood **(A)** and earlywood **(B)** widths in the period 2007–2016. **(C)** Carbon isotope ratio ($\delta^{13}C$) comparison between defoliated and non-defoliated trees across years when tree growth was high and spring conditions were cold (2012 to 2014) and years when spring was warm and growth was low (2013 and 2016). **(D)** Soluble sugar (SS) and starch concentration (% of dry weight) differences between defoliated and non-defoliated trees in needles and branch sapwood. Significant levels: $^{**}p < 0.01$, $^{*}p < 0.05$.

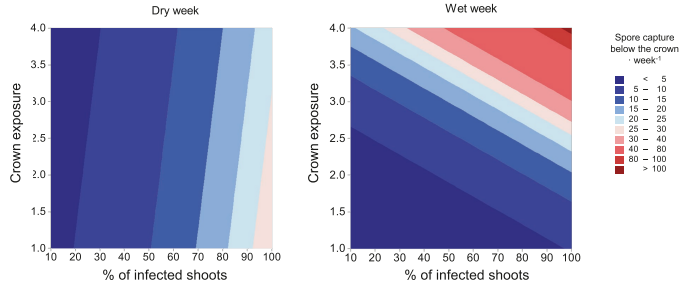

FIGURE 6 | Weekly spore captures depending on the rain conditions, tree exposure, and percentage of infected shoots. Spores were trapped under each tree (n = 40 trees/traps during dry period, and n = 39 during wet period). Tree exposure ranges from 0 to 4 and measures how exposed to rain and wind was the crown of the tree: 0, tree surrounded by other trees; 4, tree growing in open space. No spore trap was placed under a tree with less than 10% defoliation or with a lower exposure than 1.

Spore Dynamics Within the Outbreak

Spores collected during a wet period correlated with the amount of spores collected during dry conditions indicating a spatial consistency (R^2 = 0.59, p < 0.001). Spore captures tended to be larger in the southern part of the stand both under wet (R^2 = 0.25, p = 0.001) and dry weather conditions (R^2 = 0.59, p = 0.013). When considering the association between the amount of captured spores and the characteristics of the tree above the trap, we found a positive correlation with the percentage of dead shoots (p = 0.006) (**Figure 6**). During the wet week, also the exposure was associated with spore captures (p = 0.031 and p = 0.033, respectively for dead shoots and exposure), indicating that for a given level of damage, larger captures were obtained under crowns more exposed to wind and rain. Copy numbers obtained by qPCR on filter traps correlated significantly with spore captures on sticky slides (R^2 = 0.24, p < 0.001). However, the association tended to be lower ("week × spore number," p = 0.060) during wet periods (4115 copies detected by qPCR/spore detected in the sticky trap) than during dry periods (1904 copies/spore).

DISCUSSION

Disease emergence has been traditionally associated with the lack of co-evolution between host and pathogen. However, the Swedish outbreak of *D. sapinea* was illustrative of how a pathogen can establish in a new area and emerge as a new endemic disease. Isolates found in the outbreak area were not genetically different from isolates obtained in previous surveys on asymptomatic forests in Sweden. The most dominant clone found in 2016 was already present in Sweden in 2013, initially discarding our first hypothesis of the outbreak being caused by a novel and more aggressive strain. The Swedish *D. sapinea* outbreak did not seem to fit either into the traditional picture of an introduced pathogen having problems to adapt to novel environmental conditions. Rather than causing intermittent boom-and-bust outbreaks, *D. sapinea* showed a good adaptation to a new environment, and its population displayed a steady increase over several years in the studied stand. Warm May and June temperatures apparently favored disease, which became more severe in the SW area of the stand, more exposed to sun and presumably warmer. Within that area, defoliation appeared on trees with a lower WUE, as indicated by their $\delta^{13}C$ values. Symptomatic trees were the ones contributing more to the spread of the pathogen. Spores were captured during both wet and dry conditions; however, spore discharge seemed to be larger when affected crowns were more exposed to rain and wind. Our main finding is that northern conditions pose no apparent limitation for Diplodia tip blight. Nevertheless, further investigations should focus on understanding the origin of the inoculum of the outbreak.

We can only speculate with the origin of the inoculum of the outbreak area. One possible scenario is that *D. sapinea* was introduced in the stand with planting material bearing latent infections. We know that *D. sapinea* was present in Swedish nurseries during the 1960s (Molin et al., 1961) although the general prevalence in planting material is unknown. Work in Wisconsin (USA) has shown that incidence of *D. sapinea* could be as high as 30% of asymptomatic nursery stock (Stanosz et al., 2007) and that disease would only appear after seedlings would be subjected to water stress once out-planted. The absence of symptoms in the vicinity of the damaged stand fits well in the picture of a possible localized introduction from infected material in the stand of the outbreak; however, further investigations must be carried out to confirm that. Isolated trees showing severe symptoms were found in the immediacy of the outbreak, but those seem to fit better as part of the natural spread from the main stand, than as part of a widespread introduction. Recent surveys on spruce and pine seedlings in Sweden have not detected the pathogen (Menkis et al., 2016), so it seems that the current risk of *D. sapinea* being spread all over Sweden is rather low, although further surveys on the areas where seedlings from the same batch used in the outbreak area were planted should be undertaken.

D. sapinea seems to find highly favorable conditions to cause disease in Sweden, which is at odds with the absence of symptoms in the past. One possibility is that the disease has been misidentified, or perhaps confused with damages caused by *Melampsora* sp. Castagne, *Gremmeniella abietina* (Lagerb.) M. Morelet, or *Lophodermium seditiosum* Minter, Staley and Millar. In fact, and based on the reconstruction of damages, severity should have

been high in previous years, and no one reported them. Overlooking severe damages in the forest may be not so unlikely after all. The outbreak in 2016 affected trees that were next to the highway, and no one noticed the damages even severity was ca. 90%. Another possibility is that *D. sapinea* outbreak in 2016 responded to very special conditions that precluded an explosion of damages. However, 2016 did not seem to be extreme regarding any of the weather variables considered. The possibility of a local haze storm or other phenomena cannot be discarded. However, the reconstruction of the epidemic based on tree bifurcations seems to bring the origin of the outbreak way back in time.

It is unclear which could be the underlying mechanism behind the association between *D. sapinea* attacks and warm conditions during May and June. In May and June, shoots start to develop, but completion ends more towards end of June, when correlation was no longer significant. Warm temperatures may enable the pathogen to develop endophytically in the bud immediately before sprouting (Brookhouser and Peterson, 1970). Alternatively, warm temperatures could increase drought stress in the new shoots, a condition that has been found associated with *D. sapinea* damages in ours and in previous reports (Bachi and Peterson, 1985; Stanosz et al., 2001). We observed that warm conditions in spring seemed to be detrimental for tree growth, pointing to the fact that high temperatures during shoot elongation may pose some stress to the tree. The importance of thermal conditions of May and June could also be associated with day length and photosynthetic activity. During summer, in northern locations, trees may be active for longer hours every day than in southern locations, extending the period of time when shoots may become susceptible due to lower water potentials caused by photosynthesis. As an example, the nearby city of Stockholm has an average of 18 hours of daylight on 1st June. A lack of lignification during early shoot development could also be associated with a higher susceptibility (Jalkanen and Kurkela, 1984; Petäistö and Repo, 1988; Petäistö, 1999).

The clonal structure found across Europe supports the idea of a predominantly asexual reproduction in *D. sapinea* in the continent. The low variability observed among our populations may be due to the low resolution of the markers. However, genotypic diversity in our study was similar to that found in other studies in South Africa (Burgess et al., 2001; Bihon et al., 2011). That and the fact that markers were able to detect a diversity hotspot in Turkey seems to reject resolution limitations. A certain level of adaptation cannot be fully discarded since there seems to be a geographical pattern across Europe, as observed in more localized studies (Luchi et al., 2014). However, without phenotypic data on the isolates, it is not possible to link the genetic structure with some sort of adaptation to northern latitudes, as done for other pathogens such as *Heterobasidion parviporum* Niemelä and Korhonen (Müller et al., 2015). Further investigations on survival and pathogenicity of northern *D. sapinea* isolates should be carried out.

A more liberal use of water seemed to a key phenotype associated with susceptibility. Even though there was some variation in terms of $\delta^{13}C$ across years, no association with high or low temperatures was found, and rather it seemed that the differences in terms of WUE were consistent between symptomatic and asymptomatic trees irrespective of growing under favorable/unfavorable conditions. Previous studies have brought up the role of tree phenotype increasing susceptibility (Witzell and Karlman, 2000; Oliva et al., 2014, 2016), which, in northern conditions, seems to be relating fast growth with disease (Stenlid and Oliva, 2016). This issue is particularly important in northern areas where forest regeneration is mostly done by planting and where growth expectations in high site index areas may carry a higher susceptibility. In Scandinavia, Scots pine and Norway spruce may be alternatively planted in the same stand (as in the case of the outbreak area), and therefore, provenances carrying more resistant phenotypes could be used as a prevention strategy in areas with high inoculum pressure.

Spore captures showed that spread is possible under wet or dry conditions, in contrast with previous studies where a more seasonal dispersal was found (Brookhouser and Peterson, 1971; Swart et al., 1987). We only sampled two weeks in autumn; thus, further experiments should be carried out to find whether these findings hold during summer or spring. Nevertheless, our results pointed to a different spore dynamic during rainy and dry weather. In both, captures were larger under more damaged trees. Spore deposition within the same tree may be an important component of the epidemic, as shown by the fact that highly damaged trees were also those with more attacks in the past. However under rainy conditions, crown exposure also favored dispersal. One possibility is that under an open crown the spore trap captured not only spores from the immediate tree but also from the surroundings. Also, a more open crown may facilitate a better wetting of the crown and a higher number of pycnidia being hydrated and releasing spores. Combining spore trapping on filter papers with qPCR seemed to be a powerful tool to monitor *D. sapinea*, although the lower efficiency under rainy conditions needs to be considered.

D. sapinea proved to be a damaging pathogen in economic terms, not so much because of its impact on growth, as losses were mainly seen on the latewood production (representing a small fraction of the ring), but because of its capacity to kill the leader shoot(s), disrupt the shape of the growing crown, and decrease the quality of the stem. Impact on NSC reserves was localized in proximal tissues to the attack area (needles and shoots), but no overall effect was detected in the root system where a large amount of NSCs are stored during winter. *D. sapinea* seemed to be able to survive well under nearly boreal conditions; therefore, disease management should focus on reducing inoculum. Inoculum build-up seems to be favored by warm conditions in spring, which may have implications under future climate projections. In Sweden, spring temperatures have shown a steady increase over the last decades, while summer, autumn, and winter temperatures seem more stable.[2] Seedlings and also seeds should be carefully inspected as *D. sapinea* may be present in a latent stage in asymptomatic tissues.

AUTHOR CONTRIBUTIONS

JO discovered the outbreak, coordinated the project, performed field work, analyzed the data, and drafted a first version of the manuscript. LB performed field measures, isolations, and molecular work, including population structure analyses. JC and ÁS-M did the dendrochronological, NSC, and isotopic measures. KA, CC,

[2] www.smhi.se

RD, NL, DM, AL, RD, and ŞÖ sampled *D. sapinea* in their respective countries and provided isolates or DNA. JS contributed designing the experiment. JO, JS, and RD funded the project. All authors contributed to the manuscript writing.

FUNDING

This research was partly funded by the grants 2016-02021 and 215-2012-1255 from the Swedish Research Council FORMAS and by the Estonian Science Foundation grants PSG136 and IUT21-04. Financial support for ŞÖ by Süleyman Demirel University (grant BAP 3826-YL1-13) and Turkish Scientific Council (TÜBİTAK –TOVAG Project No: 114O138) is gratefully acknowledged.

ACKNOWLEDGMENTS

The field research assistance by Alvaro Camisón and Hernán Capador is acknowledged.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Doctoral Thesis No. 2023:43

The aim of this thesis was to generate knowledge about the emergence of Diplodia tip blight in Sweden, and showed that *Diplodia sapinea* has no apparent restrictions to cause damages on Scots pine in the Nordic climate. The disease was studied from a landscape – perspective, down to stand –, tree –, twig, and tissue – level. Furthermore, the disease complex was transferred to the laboratory to improve the methodology of infection assays. The findings in this thesis underline the importance of managing stress-related forest pathogens to maintain forest health in the ongoing climate change.

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ISSN 1652-6880
ISBN (print version) 978-91-8046-138-2
ISBN (electronic version) 978-91-8046-139-9