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Factors contributing to disease phenotypes in conifers

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Abstract

This thesis investigates the genetic and environmental factors and mechanisms influencing disease development in conifers, focusing on Scots pine and Norway spruce in Sweden. The thesis contributes new knowledge on the threat that diseases pose to forest ecosystems.

I studied two pathogens in pine: the opportunistic necrotroph *Diplodia sapinea* and the biotrophic rust fungus *Melampsora pinitorqua*. Drought stress exacerbates dieback, and the pathogen likely acts as an accelerating factor in decline but surviving trees can recover. An investigation into co-infection by *M. pinitorqua* and *D. sapinea* in Scots pine found no direct link between the infections but evidence for variation in susceptibility to both pathogens. In a subsequent study, genome-wide association studies (GWAS) were used to identify genetic markers associated with disease symptoms caused by *M. pinitorqua* and *D. sapinea*. Markers for reduced susceptibility to *D. sapinea* were validated in a population of progenies from elite trees in the Swedish Scots pine breeding programme, showing potential for resistance breeding in Scots pine.

In the final study, the potential to improve resistance to the necrotrophic root rot fungus *H. parviporum* in Norway spruce through genomic selection was evaluated. The results indicate sufficient genetic variation within the studied Swedish breeding population. Although genomic selection shows great potential, the findings highlight the need for more data to train the models to improve prediction accuracy.

Overall, this thesis demonstrates the importance of understanding host–pathogen interactions and utilising genetic tools to breed disease-resistant conifers, which is essential for ensuring the long-term health and resilience of Nordic forests in the context of climate change.

Keywords: *Pinus sylvestris*, *Picea abies*, Diplodia tip blight, *Sphaeropsis sapinea*, pine twisting rust, QTL, forest tree breeding, pedigree reconstruction, defence mechanisms

Faktorer som bidrar till sjukdomsfenotyper i barrträd.

Sammanfattning

Denna avhandling undersöker genetiska faktorer, miljöfaktorer och de mekanismer som påverkar sjukdomsutveckling i barrträd, med fokus på tall och gran i Sverige. Avhandlingen bidrar med ny kunskap om hotet som skogspatogener utgör mot skogsekosystemen.

Jag studerade två patogener i tall: den opportunistiska nekrotrofen *Diplodia sapinea* och den biotrofa rostsvampen *Melampsora pinitorqua*. Torkstress förvärrar gren- och toppdöd orsakad av *D. sapinea*, och patogenen fungerar sannolikt som en pådrivande faktor i försvagning av träden men överlevande träd kan återhämta sig. En studie av samtidiga infektioner av *M. pinitorqua* och *D. sapinea* i tall fann inga direkta bevis för att *M. pinitorqua* predisponerar träd för *D. sapinea*, men visade att det sannolikt finns variation i mottaglighet för båda patogenerna. I en uppföljande studie användes helgenom-associationsstudier (GWAS) för att identifiera genetiska markörer associerade till sjukdomssymptom av *M. pinitorqua* och *D. sapinea*. Markörer för minskad känslighet för *D. sapinea* validerades i ett avkommematerial, vilket visar potential för resistensförädling hos tall.

I den sista studien utvärderades potentialen att förbättra resistens mot den nekrotrofa rotrötesvampen *H. parviporum* i gran genom genomisk selektion. Resultaten visar på tillräcklig genetisk variation inom den svenska förädlingspopulationen som studerades. Även om genomisk selektion visar potential, understryker resultaten behovet av mer data för att träna modellerna och därmed förbättra precisionen i prediktionerna.

Sammanfattningsvis lyfter denna avhandling vikten av att förstå växt-patogen-interaktioner och att använda genetiska verktyg för att förädla sjukdomsresistenta barrträd, vilket är avgörande för att säkerställa nordiska skogars långsiktiga hälsa och motståndskraft i ett förändrat klimat.

Nyckelord: *Pinus sylvestris*, *Picea abies*, Diplodia-sjuka, *Sphaeropsis sapinea*, knäckesjuka, QTL, skogsträdsförädling, släktskapsanalys, försvarsmekanismer

Preface

“Jag borde blitt nåt som gjorde morsan stolt
Brevbärare eller astronaut
Jag kunde vart rik
En miljonär
Jag kunde vart kär”

Trubbel

Dedication

To Irma

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. Laura Brodde, Matilda Stein Åslund, Malin Elfstrand, Jonàs Oliva, Karin Wågström, Jan Stenlid (2023). *Diplodia sapinea* as a contributing factor in the crown dieback of Scots pine (*Pinus sylvestris*) after a severe drought. Forest Ecology and Management, vol 549. https://doi.org/10.1016/j.foreco.2023.121436

II. Matilda Stein Åslund, Michael Reichelt, Ke Zhang, Carles Castaño, Jan Stenlid, Jonathan Gershenzon, Malin Elfstrand (2025). Scots Pines With Tolerance to *Melampsora pinitorqua* and *Diplodia sapinea* Show Distinct Metabolic Profiles. Plant, Cell & Environment, vol 48, pp. 1479-1493. https://doi.org/10.1111/pce.15218

III. Matilda Stein Åslund, Mikael Brandström-Durling, Pasi Rastas, Carles Castaño, Jan Stenlid, Tanja Pyhäjärvi, Malin Elfstrand. Genetic basis of susceptibility and tolerance to *Melampsora pinitorqua* and *Diplodia sapinea* in *Pinus sylvestris* (manuscript)

IV. Matilda Stein Åslund, Henrik Hallingbäck, Magnus Hertzberg, Zhiqiang Chen, Johannes Wiesenberg, Amelia Tudoran, Andreas Helmersson, Harry X. Wu, Malin Elfstrand. Towards the integration of disease resistance in Norway spruce breeding, can genomic selection support early selection of *Heterobasidion* resistance? (manuscript)

The contribution of Matilda Stein Åslund to the papers included in this thesis was as follows:

I. Contributed to field and laboratory work, data analysis, and the writing and revision of the manuscript. Responsible for journal correspondence.

II. Participated in the study conception. Led the study design, planned and conducted field and molecular work, analysed data with supervisor support, and drafted and revised the manuscript following co-author feedback. Responsible for journal correspondence.

III. Participated in the study conception. Led the study design, planned and conducted field work and part of the molecular work, contributed to data analysis, and drafted and revised the manuscript after co-author feedback.

IV. Contributed to planning, coordination and execution of the experimental work, conducted data analysis and modelling with co-author support, and assisted in drafting the manuscript.

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Abbreviations

ABLUP	Pedigree-based best linear unbiased prediction
ABLUP-AD	ABLUP with additive and dominance effects
ANOVA	Analysis of variance
asreml	Average Standard REML (residual maximum likelihood)
DTB	Diplodia tip blight
DNA	Deoxyribonucleic acid
EBV	Estimated breeding value
ETI	Effector-triggered immunity
FDR	False discovery rate
GAM	Generalised additive model
GBLUP	Genomic best linear unbiased prediction
GBLUP-AD	GBLUP with additive and dominance effects
GEBV	Genomic estimated breeding value
GS	Genomic selection
GWAS	Genome-wide association study
HR	Hypersensitive response
ISR	Induced systemic resistance
ITS	Internal transcribed spacer
LD	Linkage disequilibrium
LL	Lesion length
PA	Predictive ability
PAC	Predicted accuracy
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait loci
RDA	Redundancy analysis
ROS	Reactive oxygen species
SAR	Systemic acquired resistance
SNP	Single nucleotide polymorphism
SWG	Sapwood growth

1. Introduction

Forests are vital ecosystems that provide essential services, including water regulation, biodiversity preservation, carbon sequestration, recreation, cultural heritage preservation, and protection against soil erosion, as well as commercial products such as wood, paper, and biofuel. Forest diseases pose a serious threat to these systems, disrupting their functions and leading to significant economic, social, and ecological consequences (Runyan & Stehm 2020; Williams *et al.* 2023; Hamelin 2024; Perry *et al.* 2024).

Approximately two thirds of Sweden's terrestrial area is covered by forests (SLU 2021). In addition to playing a vital role in sustaining ecosystems, forests are central to Sweden's economy. The country has 23.5 million hectares of productive forest land (SLU 2021), managed primarily for timber and resource extraction by over 320,000 private forest owners (Miljödepartementet 2019). The forest industry accounted for 2% of total employment in Sweden in 2021 (Industriarbetsgivarna & Skogsindustrierna 2021), and the country is one of the world's leading exporters of sawn wood, paper, and pulp (FAO 2021).

Swedish forests face significant challenges from abiotic and biotic stressors that contribute to tree mortality and have been associated with growth declines over the past decade (Carlén *et al.* 2024). Given their importance in Swedish forests, conifers are a major focus in efforts to understand and mitigate forest damage. In this thesis, I examine different hosts, pathogens, and environments, investigating the trees' responses to infection and the genetics behind those responses.

1.1 Conifers in boreal ecosystems and Swedish forestry

Today's conifer species are the surviving remnants of a once ecologically dominant group, with approximately 630 species still in existence (Farjon 2001). These species are classified within the division Pinophyta, class Pinopsida, and order Pinales (or Coniferales). The pine family (Pinaceae) is the largest and most widespread, with approximately 225 species in eleven genera, including pine (Pinus), spruce (*Picea*), larch (*Larix*), and fir (*Abies*) (Farjon 2001; Ran *et al.* 2018). The wide distribution and diversity of

Pinaceae species have driven the evolution of adaptations to a variety of environments. For instance, northern pine and spruce populations are cold-tolerant, have earlier growth cessation, and are slower growing than southern populations, consistent with the gradient in environmental conditions (Rousi *et al.* 2018; Milesi *et al.* 2019; Pyhäjärvi *et al.* 2020; Hallingbäck *et al.* 2021; Ranade & García-Gil 2021).

Swedish forestry is dominated by two conifer species – Norway spruce (*Picea abies* (L.) Karst.) and Scots pine (*Pinus sylvestris* L.). Together, they comprise 80% of the standing volume of forest trees in the country. The sector sustains through the planting of ~375 million Scots pine and Norway spruce seedlings per year (Skogsstyrelsen 2020).

Scots pine (*Pinus sylvestris*) has a wide distribution across Eurasia (Houston Durrant *et al.* 2016) and is of significant commercial importance, particularly in the Nordic countries (Praciak 2013). It occupies a broad ecological niche compared to many other pine species and shows substantial local adaptation throughout its range. Pines show particularly high plasticity in how they allocate biomass to foliage (Delucia *et al.* 2001; Martínez-Vilalta *et al.* 2004; Poyatos *et al.* 2013). Scots pine, in particular, is a strong competitor in nutrient-poor soils and in dry or cold environments (Farjon 2001), and its use in forest regeneration has increased in Sweden over the past decade (Skogsstyrelsen 2024).

Norway spruce (*Picea abies* [L.] Karst.) is the dominant species in boreal and subalpine coniferous forests. Its natural range extends from the Alps and Carpathians in the south, through Scandinavia, and eastward to northern Russia, where it merges with Siberian spruce (*Picea obovata*) (Farjon & Page 1999; Caudullo *et al.* 2016). It is one of the most economically and ecologically important conifers in Europe (Praciak 2013).

1.2 Concepts of forest pathology

Forest pathology is the study of forest diseases (Tainter & Baker 1996). A **pathogen** is an organism that can cause disease (Merriam-Webster 2025c), and the expression of a disease involves the interaction between a pathogen, a susceptible host, and a conducive environment. This concept is commonly distilled into the framework known as the disease triangle (Ray 2024). A tree's response to pathogenic infection is influenced by both characteristics of the pathogen and the tree's **phenotype** – the observable characteristics

resulting from the interaction between its **genotype** (genetic composition) and the environment (Merriam-Webster 2025a; Merriam-Webster 2025b). The **disease phenotype** refers to the physical and biochemical characteristics of the disease (Collins English Dictionary 2025).

Fungi make up a major group of forest pathogens. Recent research has estimated the number of fungal species to be ~2.5 million (Niskanen *et al.* 2023). The precise number of these species classified as forest pathogens remains uncertain; however, their associated diseases are becoming more prevalent. From the years 2000 to 2022, 962 emerging infectious diseases, i.e., new host-pathogen-location interactions, were documented globally (Gougherty 2023).

Fungi exhibit various mechanisms of action. For instance, organisms that grow on dead and decaying plant material (**saprobes** or saprophytes) are differentiated from **symbionts**, which grow on plants and provide a benefit to the host, and **pathogens**, which cause disease in living plants (Oliver 2024). **Endophytes** reside in plant tissues temporarily or permanently, without inducing visible damage or morphological changes in their hosts. They can exist in a latent pathogenic state; under stress conditions – such as nutrient deficiency, climate extremes, or senescence – they may shift to a virulent phase, causing disease symptoms and altering host morphology and physiology (Alam *et al.* 2021). Fungi can manipulate host signals that regulate host defence responses and metabolic processes (Abdullah *et al.* 2017). Their **pathogenicity** – the ability to cause disease in a host plant – can vary significantly, depending on how well they adapt to evade plant defence mechanisms and the conditions that promote their growth and proliferation (Spanu *et al.* 2024).

The traditional classification of biotrophic, hemibiotrophic, and necrotrophic pathogens is based on the state of the host tissue from which they acquire nutrients (Oliver 2024). **Biotrophs** obtain their nutrients from living plant cells. They grow and reproduce on the host without causing obvious local damage, although the plant's overall growth may be severely impacted. A key feature of biotrophs is haustoria, specialised structures through which they absorb nutrients from intact and metabolically active host cells. Biotrophs are often host-specific. **Necrotrophs** obtain their nutrients from dead or dying host cells. They typically secrete toxins and cell-destroying enzymes that kill host tissues, releasing nutrients. Resistance to necrotrophic diseases is often partial. **Hemibiotrophs** exhibit a two-phase

lifestyle, initially obtaining nutrients from living plant cells like biotrophs and then switching to a necrotrophic phase where they kill host cells (Oliver 2024). This traditional pathogen classification system has limitations, as many pathogens don't fit neatly into these categories. A new system based on gene content, particularly carbohydrate-active enzyme genes, classifies pathogens into four major groups based on their nutrient sources and interaction sites with hosts. These include polymertrophs, mesotrophs, monomertrophs (roughly corresponding to necrotrophs, hemibiotrophs, and biotrophs, respectively), and vasculartrophs (causing wilt diseases) (Hane *et al.* 2020). However, some pathogens still do not fit clearly into these groups, and the system's overall utility is still under evaluation (Oliver 2024).

1.3 Defence mechanisms in conifers

The two main strategies against plant pathogens are resistance and tolerance. **Resistance** is an organism's ability to limit pathogen or pest growth, thereby reducing infection (Whitehill *et al.* 2023). It decreases infection intensity by limiting pathogen proliferation, i.e., the growth and spread of a pathogen within the host (Clarke 1986; Strauss & Agrawal 1999; Råberg 2014). **Tolerance** allows organisms to endure infection while minimising health impacts without directly lowering pathogen levels or replication rates (Strauss & Agrawal 1999; Råberg 2014; McCarville & Ayres 2018; Jeger 2023). If disease occurs, the host-pathogen interaction is considered compatible, and the pathogen is classified as **virulent**. Conversely, if the host is resistant to the pathogen strain, the interaction is incompatible, and the pathogen is **avirulent** (Ray 2024).

Conifers are likely to encounter a wide range of aggressors throughout their long lifetime (Feeny 1976) and some level of defence against pathogens is necessary for a tree to survive, reproduce, and pass its genes to the next generation (Mageroy *et al.* 2023). Interactions between trees and pathogens or pests are shaped by past selection pressures, including whether the tree population and the biotic stressor share a co-evolutionary history. Abiotic factors also play a role, as environmental conditions can influence disease resistance by affecting pest or pathogen populations or modifying tree defences (Mageroy *et al.* 2023).

Conifer defences are typically partial and **quantitative**, meaning they are controlled by multiple genes, each providing some degree of resistance rather

than a single gene offering complete protection (Hückelhoven & Pillen 2024).

The primary role of conifer defence mechanisms is to protect the tree's life processes, particularly by maintaining the integrity of the bark, the vascular cambium, and the transpiration stream in the sapwood (Krokene 2015). The defence strategies reflect evolutionary trade-offs (Redmond *et al.* 2019) and vary in their form and significance depending on species, genotype, environmental conditions, seasonal changes, tree age, and tissue (Krokene 2015).

Different plant structures possess specific defences and vulnerabilities. For example, needles have a protective waxy cuticle, but their stomata can be entry points for pathogens (Zeng *et al.* 2010; Fraser *et al.* 2016). The bark's outer lignin-rich layer defends the stem, but lenticels can allow pest entry (Rosner & Führer 2002; Franceschi *et al.* 2005). The phloem contains physical and chemical defences such as lignified sclerenchyma cells and phenolic compounds (Hudgins *et al.* 2004), and the xylem's lignin enhances its resistance against drought and freezing (Davis *et al.* 1999; Song *et al.* 2021). As conifers grow, they allocate different types of chemical defences to specific structures (Nerg *et al.* 1994; Franceschi *et al.* 2005). The mechanisms are broadly categorised as specific or general, mechanical or chemical, local or systemic, and constitutive (preformed) or inducible.

Constitutive defences are formed during the tree's development and morphogenesis. They are always present and provide immediate protection, whereas **inducible** defences activate following an attack, allowing for a more resource-efficient strategy that balances costs with the need for defence (Steppuhn & Baldwin 2008; Cipollini & Heil 2010). While the separation between the different categories provides a useful conceptual framework, in practice, they often overlap (Krokene 2015).

1.3.1 Constitutive defences

Preformed defences serve to prevent or inhibit attacks. They encompass both structural barriers and chemical deterrents, offering continuous but metabolically costly protection (Franceschi *et al.* 2005; Hückelhoven & Schouten 2024).

Constitutive chemical defences include secondary metabolites that inhibit pathogens and deter herbivores. Among the most critical are terpenoids and phenolics (Krokene 2015). Terpenoids, stored in resin ducts present in

conifer stems and needles, are toxic against insects and fungal pathogens (Kopaczyk *et al.* 2020). Phenolics include diverse metabolites derived from the shikimate pathway, such as flavonoids, stilbenes, lignins, and their precursors. The protective mechanisms involve direct toxic effects, inhibition of extracellular enzymes produced by pathogens, or rapid formation of barriers such as lignin (Bennett & Wallsgrove 1994; Fraser *et al.* 2016; Ullah *et al.* 2017). Lignin and suberin deposition enhance resistance to pathogen penetration and tissue degradation, reinforcing the mechanical strength of the bark and vascular tissues (Franceschi *et al.* 2005; Adomas *et al.* 2007). Phenolic compounds are highly concentrated in the secondary phloem. Upon attack, these compounds can be rapidly mobilised to restrict fungal growth (Franceschi *et al.* 2000).

1.3.2 Inducible and localised responses

Inducible defences in conifers are activated in response to pathogen attack or wounding and function to kill or compartmentalise the intruder, seal and repair damaged tissues, and prevent further spread. These defences can be induced both locally and systemically (Franceschi *et al.* 2005; Krokene 2015). One key component of this system is the activity of **phloem parenchyma (PP) cells**, which contribute to defence by accumulating high concentrations of phenolic compounds and altering the phenolic profile (Li *et al.* 2012; Celedon *et al.* 2017). Resin production is another important inducible defence: it can be triggered by wounding or attack, leading to increased activity in existing resin ducts and the formation of new ones, which boosts the supply of terpenoids (Ruel *et al.* 1998; Nagy *et al.* 2000).

The induction of toxic and antifungal compounds, such as terpenoids and phenolics, is also central to the inducible defence response (Fossdal *et al.* 2006; Fossdal *et al.* 2012; Fraser *et al.* 2016; Liu *et al.* 2022a). The phenylpropanoid pathway plays a key role in this process, as it governs the synthesis of compounds such as flavonoids and stilbenes and contributes to lignin deposition (Arnerup 2011; Liu *et al.* 2022a). Secondary metabolites such as catechins, stilbenes, and monoterpenes have been reported as important inhibitors of fungal growth in studies on *H. parviporum* and *Endoconidiophora polonica* (Hammerbacher 2011; Fossdal *et al.* 2012; Jyske *et al.* 2020; Wang *et al.* 2024b). Conifers with strong disease defences often show increased lignin accumulation, elevated levels of flavonoids, terpenes, and phenolics, and upregulation of genes involved in these

biosynthetic pathways (e.g., Hammerbacher 2011; Swedjemark *et al.* 2012; Hammerbacher *et al.* 2019; Kovalchuk *et al.* 2019; Jyske *et al.* 2020; Liu *et al.* 2022a; Liu *et al.* 2022b).

Defence-related proteins such as peroxidases and **reactive oxygen species (ROS)** scavengers play an important role in fungal resistance by preventing oxidative damage (Adomas *et al.* 2007). In addition, resistance to biotrophic fungi often involves **hypersensitive response (HR)**, an induced defence mechanism involving localised cell death at the infection site to restrict pathogen spread (Agrios 2005). The host cell invaded by the haustorium dies, thus depriving the pathogen of nutrients and preventing its survival.

1.3.3 The plant immune system and systemic signalling

The response time for inducible defences varies. Rapid responses, such as changes in gene expression and upregulation of defence-related proteins, can be initiated within hours of an attack (Salzer *et al.* 1996; Lim *et al.* 2021). More complex structural modifications, such as the formation of traumatic resin ducts in the sapwood or the development of a wound periderm, require a longer time frame, often taking weeks to materialise fully (Franceschi *et al.* 2000; Nagy *et al.* 2000).

The plant immune system functions to detect pathogens (i.e., **pathogen recognition**) and initiate a defence response through **pattern-triggered immunity (PTI)** and **effector-triggered immunity (ETI)**. PTI is triggered when pattern recognition receptors (PRRs) on the plant cell surface recognise elicitors. These elicitors can be pathogen-derived, known as microbe-associated molecular patterns (MAMPs), or plant-derived, known as damage-associated molecular patterns (DAMPs). Downstream signalling components are activated, leading to the production of ROS and ion fluxes. These early signalling events lead to the activation of transcription factors that trigger pattern-activated genes, resulting in the expression of defensive proteins and chemical defence compounds.

ETI is triggered by the recognition of pathogen effectors (virulence factors) by plant resistance (R) proteins and often leads to HR. Effectors are commonly pathogen race-specific. PTI and ETI are part of an integrated immune signalling network rather than separate processes, and genetic studies propose that the loss of components in one pathway can limit the efficacy of the other (Hückelhoven & Schouten 2024; Spanu *et al.* 2024).

Hormones are central to plant defence responses, activating key processes such as the biosynthesis of defence compounds and the formation of anatomical barriers. Crosstalk between phytohormone pathways triggers two major forms of systemic immunity: **systemic acquired resistance (SAR)** and **induced systemic resistance (ISR)** (Bürger & Chory 2019). Local pathogen infection can initiate SAR in distal tissues, leading to the accumulation of salicylic acid and systemic expression of pathogenesis-related (PR) genes. SAR provides protection against a broad range of biotrophic and hemibiotrophic pathogens. ISR is another form of long-distance immunity, primarily effective against hemibiotrophic and necrotrophic pathogens, and is largely mediated by jasmonic acid and ethylene (Hückelhoven & Schouten 2024).

The salicylic acid, jasmonic acid, and ethylene pathways are further influenced by other hormones, including abscisic acid, gibberellins, and auxin (Bürger & Chory 2019). Brassinosteroids also regulate plant immune responses by modulating both abiotic and biotic stress signalling, enhancing pathogen resistance through ethylene synthesis and activation of defence-related pathways (Bartwal *et al.* 2013).

Priming, a form of acquired systemic defence involved in SAR and ISR, has become an increasingly studied topic. Priming is reported to enhance the plant's defence responses at sites distant from the initial attack, with a delayed response and lasting effects allowing for a faster and stronger reaction to subsequent attacks (Franceschi *et al.* 2005; Wilkinson *et al.* 2022; Mageroy *et al.* 2023; Hückelhoven & Schouten 2024).

Interactions with one fungus can modify the responses triggered by another. Focusing defence efforts on certain pathogens increases the protection against them but may compromise defences against others (Abdullah *et al.* 2017). These priority effects emerge when an initial infection changes the environment and impacts the host’s susceptibility to subsequent infections (Halliday *et al.* 2018). Furthermore, immune-mediated facilitation may occur as the activation of one immune-signalling pathway leads to the suppression of another, consequently promoting subsequent infections and increasing the frequency of co-infections (Halliday *et al.* 2018).

Recent research indicates that endophytic fungal communities play a crucial role in influencing the defensive outcomes of pine trees by enhancing plant fitness through mechanisms such as defensive priming or the induction

of metabolites (Bullington *et al.* 2018; Ata *et al.* 2023; Bullington *et al.* 2024).

1.4 Pathogens affecting Nordic conifer forests

The emerging pine pathogen *Diplodia sapinea* is a growing concern issue in the Nordic countries (Terhonen 2022; Brodde 2023). Recent reports have highlighted the co-occurrence of *Melampsora pinitorqua* and *D. sapinea* on Scots pine in Sweden (Skogsstyrelsen 2023), emphasising the need to investigate the underlying mechanisms driving these interactions. Meanwhile, *Heterobasidion parviporum*, a major pathogen affecting Norway spruce, continues to cause significant economic losses (Garbelotto & Gonthier 2013). These pathogens threaten the health and productivity of two of Sweden's most economically and ecologically important tree species. Forest damage caused by fungal pathogens can reduce timber yield and quality, weaken stand structure, increase susceptibility to windthrow, and disrupt regeneration (Stenlid 1985; Hellgren & Stenlid 1995; Bendz-Hellgren 1999; Oliva *et al.* 2011). Understanding these pathosystems is therefore crucial for developing effective management strategies and ensuring the sustainability of Swedish forests in the face of both biological threats and ongoing environmental change (Terhonen *et al.* 2019; Gomez-Gallego *et al.* 2022; Krokene *et al.* 2023).

1.4.1 Biology and impact of *Diplodia sapinea*

Diplodia sapinea (Fr.) Fuckel (syn. *Diplodia pinea* (Desm.) Kickx., *Sphaeropsis sapinea* (Fr.: Fr.) Dyko & Sutton) causes Diplodia tip blight (DTB) on conifers, particularly *Pinus* spp. (CABI 2021). Damage caused by *D. sapinea* leads to significant economic losses in forestry, due to tip blight, shoot dieback, cankers, blue stain, and reduced latewood growth, which disrupts crown shape and decreases stem quality (Wright & and Marks 1970; Zwolinski *et al.* 1990). DTB can also result in tree mortality (Smith *et al.* 2002; Caballol *et al.* 2022b). Beyond economic impacts, the disease affects ecosystems by altering species composition and microbiota, reducing carbon sequestration, and increasing fire risk due to crown dieback (Brodde *et al.* 2019; Blumenstein *et al.* 2021b).

Historically, severe impacts of DTB were documented primarily in the southern hemisphere (Swart *et al.* 1985; Swart & Wingfield 1991; Burgess *et al.* 2004). *D. sapinea* is now a major pine pathogen globally, and increased damages have been observed in northern Europe in the last decade (Hanso & Drenkhan 2009; Oliva *et al.* 2013; Brodde *et al.* 2019; Terhonen *et al.* 2021; Brodde 2023). *D. sapinea* was first reported harming pines in Sweden in 2013 (Oliva *et al.* 2013), with the first DTB outbreak in a commercial Scots pine plantation in 2016 (Brodde *et al.* 2019). Since then, DTB symptoms have been detected across the southern half of Sweden (Skogsstyrelsen 2023) and the disease is a growing concern in the country (Brodde *et al.* 2019).

D. sapinea spreads via conidia (asexual spores) (Bihon *et al.* 2011). These conidia are released from asexual pycnidia during moist weather and are dispersed by rain splash and wind-driven rain (Brookhouser & Peterson 1971; Swart 1987). Pycnidia typically form on cones, necrotic shoots, and needles under humid conditions (Punithalingam & Waterston 1970; Peterson 1977). Understory regeneration can become infected through horizontal transmission (Bihon *et al.* 2011; Caballol *et al.* 2022a). Several studies also show that *D. sapinea* is vectored by insects (Luchi *et al.* 2012; Davydenko *et al.* 2017; Davydenko & Baturkin 2020).

D. sapinea persists latently or saprophytically in host tissues. It has repeatedly been reported as an endophyte (e.g., Stanosz *et al.* 1997; Smith *et al.* 2002; Bihon *et al.* 2011; Terhonen *et al.* 2021) and Blumenstein *et al.* (2021a) found the fungus to be dominant in the Scots pine mycobiome, irrespective of host health. *D. sapinea* is frequently described as an opportunistic pathogen that transitions from endophyte to pathogen, often in hosts stressed by drought or heat (e.g., Stanosz *et al.* 2001; Bihon *et al.* 2011). DTB infection is also aided by other abiotic stressors such as hail, wounding, or other mechanical damage (e.g., Zwolinski *et al.* 1990; Swart & Wingfield 1991; Stanosz *et al.* 2001). The introduction of wounds notably elevated the occurrence of symptomatic *D. sapinea* infections in an inoculation trial (Oostlander *et al.* 2023). In the same study, wounding improved the reisolation success of the pathogen, while non-wounded plants primarily showed asymptomatic infections, suggesting that host stress enables *D. sapinea* to shift into an aggressive necrotrophic state.

In the Bulletin of the Royal Station of Plant Pathology's review of phytopathological cases observed in 1941, they reported that *Pinus halepensis* branches showing wilting caused by *Cronartium ribicola* also

exhibited development of *Sphaeropsis ellisii* (a former synonym of *D. sapinea*) and claimed that it caused drying of the younger twigs (Petri 1942), but the co-infection was not further discussed. Zwolinski *et al.* (1995) proposed the potential of cambiophagous insects infesting healthy *P. radiata* tissue to facilitate further colonisation by *D. sapinea*. Since *D. sapinea* can be vectored by beetles (Luchi *et al.* 2012; Davydenko *et al.* 2017; Davydenko & Baturkin 2020), the fungus may exploit insect damage for colonisation. This implies that biotic stress could trigger its transition from an endophytic to a necrotrophic lifestyle.

The necrotrophic lifestyle of *D. sapinea* is supported by toxin production and suppression of host defences, achieved through modifications of host gene expression and key foliar metabolic traits such as phytohormone profiles, cell wall composition, and ROS production (Hu *et al.* 2018; Blumenstein *et al.* 2021b; Roy *et al.* 2022; Hu *et al.* 2023).

These alterations affect both primary and secondary metabolism, leading to localised carbon and nitrogen stress in host tissues during both latent infection and active DTB development (Sherwood *et al.* 2015; Ghosh *et al.* 2022; Hu *et al.* 2023).

Infection triggers bark and wood dehydration, accompanied by the accumulation of hydrogen peroxide (H_2O_2), a type of ROS involved in lignin polymerisation. This accumulation indicates disruption of phytohormone signalling and antioxidative defence mechanisms (Hu *et al.* 2018). *D. sapinea* counters the host's ROS defences by employing fungal catalase activity to degrade H_2O_2, which likely also aids infection under drought conditions (Sherwood *et al.* 2015). Infection induces the build-up of lignin, phenolics, and free amino acids (Wallis *et al.* 2008; Sherwood *et al.* 2015; Hu *et al.* 2023).

In stressed trees, increased levels of free amino acids may promote disease development by supplying *D. sapinea* with nitrogen (Sherwood *et al.* 2015). In a study by Sherwood *et al.* (2015), proline appeared to be a preferred nutrient source for *D. sapinea*. Caballol *et al.* (2022b) suggested that DTB disease severity is influenced by competition for proline between *D. sapinea* and other pine endophytes.

Abiotic stressors also compromise host defences and can further increase the disease severity by suppressing host metabolism and impairing physical defences like resin exudation (Sherwood *et al.* 2015; Blumenstein *et al.*

2022; Ghosh *et al.* 2022). Concurrent stressors amplify *D. sapinea*'s defence-suppressing effects (Sherwood *et al.* 2015; Ghosh *et al.* 2022).

Genetic studies show low global allelic diversity in *D. sapinea*, with clonal genotypes dominating and some found across continents, which points to long-term asexual reproduction and human-assisted spread (Burgess *et al.* 2004; Adamson *et al.* 2021). A more recent study in Spain identified an admixture of *D. sapinea* genotypes across isolates from various stands and tissue types (Vilanova *et al.* 2024).

1.4.2 Biology and impact of *Melampsora pinitorqua*

Pine twisting rust is one of the most common diseases affecting Scots pine in Sweden (Skogsstyrelsen 2023) and is caused by the biotrophic rust fungus *Melampsora pinitorqua* (Braun) Rostrup (syn. *Melampsora populnea* (Pers.) P. Karst.).

Rust fungi (Phylum Basidiomycota, order Pucciniales) make up the largest group of plant fungal pathogens, comprising over 7000 described species (Cummins & Hiratsuka 2003). As obligate biotrophs, rust fungi rely entirely on nutrients from living plant host cells to complete their life cycle (Lorrain *et al.* 2019). During infection, rust fungi develop haustoria that facilitate nutrient uptake and deliver effector proteins into host cells (Rafiqi *et al.* 2012). These effectors enhance pathogen fitness by suppressing host immunity, creating a favourable environment, and inducing a carbon sink in the infected area (Oliva *et al.* 2014; Petre *et al.* 2014; Duplessis *et al.* 2021). Studies of the rust fungi genomes show that host-derived oligopeptides likely serve as critical sources of nitrogen and sulfur, essential for the fungi's growth and development (Lorrain *et al.* 2019; Guerillot *et al.* 2023).

Many rust fungi are heteroecious, requiring two distinct host species to complete their life cycle. This dependency creates vulnerabilities but also enables the exploitation of diverse ecological niches (Savile 1971; McTaggart *et al.* 2016; Duplessis *et al.* 2021). Heteroecism and multiple spore stages contribute to the adaptability of these pathogens (Kolmer *et al.* 2018).

M. pinitorqua distribution is concentrated in temperate Europe (Siwecki 1974; Longo *et al.* 1975; Mattila 2005). Pine twisting rust was mentioned in Swedish literature as early as 1874 as "a malignant fungal attack in 1872-1873 on a 2-3-year-old pine culture on 400-500 acres of fire field in northern Västergötland" (translated) (Wilke 1874).

M. pinitorqua is heteroecious and alternates between Scots pine and European aspen (*Populus tremula*) (Sylvén 1917) and therefore thrives in new pine plantations where the pioneer tree aspen tends to emerge. It has a macrocyclic life cycle with five spore stages and requires Scots pine for its aecial stage and European aspen for its uredinial and telial stages (Siwecki 1974; Mattila 2005). The cycle is initiated by basidiospores infecting young pine shoots in spring (Desprez-Loustau 1986a), penetrating through stomata or directly invading the tissue (Nicola *et al.* 2006). The fungus forms intercellular hyphae and haustoria in the host tissue (Jonsson *et al.* 1978). The infection leads to the formation of aecia (Longo & Longo 1975; Desprez-Loustau 1986b) and about a week after the aecia appear, aeciospores are released and spread to infect aspen leaves. Urediniospores develop and form uredinia, spreading clonally and reinfecting aspen. Finally, teliospores develop in telia and overwinter on fallen aspen leaves, where they germinate in spring to produce new basidiospores (Klingström 1963; Longo *et al.* 1979; Nicola *et al.* 2006).

While the infection duration on pine is short, it results in a canker that can cause shoot bending or breaking. This may lead to deformed or multiple stems and, consequently, reduced growth and timber quality (Sylvén 1917; Siwecki 1974; Longo *et al.* 1975; Mattila 2005). Elongating shoots are most susceptible to basidiospore infections between bud-scale separation and needle emergence (Desprez-Loustau 1990).

M. pinitorqua disease dynamics are influenced by factors such as host proximity, spring drought (which reduces infection), and summer humidity (Siwecki 1974; Longo *et al.* 1975; Mattila 2005). Infection risk can be reduced by increasing the distance between aspen and pines and by managing host density (Mattila 2005).

1.4.3 Biology and impact of *Heterobasidion parviporum*

Heterobasidion annosum s.l., causing root rot on conifers, is widely distributed in coniferous forests of the Northern Hemisphere (Garbelotto & Gonthier 2013). *H. annosum* s.l. was long considered a single species with different intersterility groups (Korhonen 1978). These groups are now formally described as separate species. In Eurasia, the species include *H. annosum* sensu stricto (s.s.), *H. abietinum* Niemelä & Korhonen, and *H. parviporum* Niemelä & Korhonen, while *H. irregulare* (Underw.) Garbel.

& Otrosina and *H. occidentale* Otrosina & Garbel. occur in North America (Garbelotto & Gonthier 2013).

H. parviporum predominantly affects Norway spruce (Oliva *et al.* 2011; Garbelotto & Gonthier 2013). The fungus mainly occurs in boreal and temperate Europe and coincides largely with the natural distribution of Norway spruce (Woodward *et al.* 1998). *H. annosum* s.s., primarily targeting Scots pine, colonises Norway spruce within its distribution range in southern Sweden. However, *H. parviporum* is the most prevalent species on Norway spruce in the country and is responsible for the majority of the forest industry's losses (Thor *et al.* 2007; Garbelotto & Gonthier 2013).

H. parviporum has a saprotrophic-necrotrophic lifestyle. It infects trees via basidiospores on fresh stumps or open wounds (caused by e.g., thinning operations) and spreads locally through root contacts (Oliva *et al.* 2011; Garbelotto & Gonthier 2013). Mycelia of *H. annosum* s.l. can grow at a rate of about 10-30 cm per year within living roots (Bendz-Hellgren 1999) and persist in decaying root systems for decades. Consequently, new generations of trees can become infected when their roots come in contact with decaying roots already colonised by *H. annosum* s.l. (Stenlid & Redfern 1998). The secondary spread driven by root connections creates localised disease centres and long-term persistence of fungal genets (Stenlid 1987; Piri & Korhonen 2007; Oliva *et al.* 2011; Klavina *et al.* 2021).

A *H. parviporum*-infected Norway spruce can survive for many years, but severe decay leads to growth losses as sapwood function is impaired, and the decay reduces timber value and predisposes the trees to windthrow, causing substantial economic losses for the forestry sector (Hellgren & Stenlid 1995; Bendz-Hellgren 1999; Oliva *et al.* 2011; Garbelotto & Gonthier 2013; Klavina *et al.* 2021). A stem decay column in Norway spruce can reach up to 12 m (Stenlid 1985).

Silvicultural practices to mitigate root rot include altering species composition, planting mixed stands with deciduous trees, and delaying thinning (Oliva *et al.* 2011; Garbelotto & Gonthier 2013; Zaļuma *et al.* 2019; Zaļuma *et al.* 2021). Biological control with *Phlebiopsis gigantea* (e.g., Rotstop®) can prevent *H. parviporum* spread, but its effectiveness depends on stump cover and temperature (Pettersson *et al.* 2010; Zaļuma *et al.* 2021). Basinox is a new treatment derived from a strain of an unidentified *Pseudomonas* bacterium (Hantula *et al.* 2024). Additionally, urea-based chemical treatments reduce *H. parviporum* infections by altering pH

(Johansson *et al.* 2002; Zaļuma *et al.* 2021). All these agents offer effective, though incomplete, protection against spore-mediated infection when applied correctly. However, they do not eliminate established root rot fungi, as the existing mycelia in root systems continue to spread despite stump treatment (Hantula *et al.* 2024).

1.4.4 Tree resistance against *D. sapinea*, *M. pinitorqua*, and *H. parviporum*

Diplodia sapinea resistance

Genetic factors, especially in stress response pathways, strongly influence tree tolerance to *D. sapinea*. Several loci associated with pathogen response were identified, indicating a genetic basis for disease tolerance (Hurel *et al.* 2021). Wallis *et al.* (2008) linked elevated levels of phenolic glycosides and stilbenes to reduced DTB susceptibility. Hu *et al.* (2021) showed that *D. sapinea* alters phytohormone profiles in infected conifer needles, further supporting the pathogen's exploitation of host stress response pathways for infection. They observed significant heritability in resistance to *D. sapinea* in *Pinus pinaster* and linked slower growth and drought adaptation to reduced susceptibility.

Melampsora pinitorqua resistance

Research on resistance to *M. pinitorqua* in Scots pine remains limited, and few studies have been conducted. However, it is known that resistance is partly linked to anatomical defences, such as haustorial encasement, which limits the parasite's nutrient access and protects the tree from further invasion (Jonsson *et al.* 1978). Susceptibility to *M. pinitorqua* is positively correlated with tree growth and vigour (Klingström 1963; Martinsson 1985; Desprez-Loustau & Dupuis 1994; Andersson & Danell 1997; Desprez-Loustau & Wagner 1997).

There is genetic variation in resistance to *M. pinitorqua* among Scots pine populations and families, and within families (Andersson & Danell 1997; Quencez & Bastien 2001; Persson *et al.* 2010). The genetic factors controlling the resistance are still poorly understood, but there is evidence that resistance traits show moderate heritability, showing that there is

potential for breeding of more tolerant pine trees (Andersson & Danell 1997; Quencez & Bastien 2001).

The genetic basis of resistance to other tree rust pathogens has been more extensively studied. For example, pathogenesis-related (PR)-10 genes in *Pinus monticola* are upregulated in response to *Cronartium ribicola* infection and stress, and they are associated with antifungal activity and potential quantitative resistance (Ekramoddoullah 2004; Liu & Ekramoddoullah 2004; Liu *et al.* 2021). Similarly, NLR (nucleotide-binding Leucine-rich repeat) genes are fundamental for fusiform rust resistance in *Pinus taeda* (Quesada *et al.* 2014; Ence *et al.* 2021). Resistance to *Chrysomyxa rhododendri* in Norway spruce involves key genetic pathways with antifungal proteins, plant-pathogen interaction genes, MAPK (mitogen-activated protein kinase) signalling, and secondary metabolite biosynthesis genes, enhancing both constitutive and infection-induced defence (Trujillo-Moya *et al.* 2022).

Heterobasidion parviporum resistance

Resistance to *H. parviporum* in Norway spruce is a quantitative trait (Karlsson & Swedjemark 2006; Arnerup *et al.* 2010; Steffenrem *et al.* 2016; Chen *et al.* 2018b). There is significant heritable variation in resistance traits, such as fungal growth and lesion length (Swedjemark *et al.* 2012; Lind *et al.* 2014; Skrøppa *et al.* 2015). Research on *H. parviporum* genetic resistance mapping has revealed significant variability in resistance based on **SNPs (single nucleotide polymorphisms)** and **QTLs (quantitative trait loci)**. A number of loci associated with resistance traits have been reported(Lind *et al.* 2014; Elfstrand *et al.* 2020a; Capador-Barreto *et al.* 2021). LAR (leucoanthocyanidin reductase) catalyses the production of important intermediates in the flavonoid pathway and have been associated disease resistance in spruce (Danielsson *et al.* 2011; Hammerbacher *et al.* 2014; Lind *et al.* 2014). For example, *PaLAR3* is linked to resistance via catechin biosynthesis. It encodes an enzyme for (+)-catechin synthesis and contributes to resistance by inhibiting fungal growth (Danielsson *et al.* 2011; Hammerbacher *et al.* 2014; Nemesio-Gorriz 2015; Nemesio-Gorriz *et al.* 2016; Durodola *et al.* 2024). In addition, *PaLAC5*, a laccase gene involved in induced sapwood defences, is linked to shorter lesion lengths during infection (Elfstrand *et al.* 2020a).

Some studies suggest that Norway spruce clones differing in susceptibility to *H. parviporum* may vary in the timing or magnitude of

defence responses, such as phenolic accumulation (Fossdal *et al.* 2012) and terpene levels (Wang *et al.* 2024b). Similarly, Liu *et al.* (2022a) reported a correlation between reduced susceptibility and increased transcript abundance of genes involved in the terpenoid pathway.

The genetic contribution to phenotypic variation in resistance traits has been reported to range from 11% in full-sib progeny trials (Arnerup *et al.* 2010) to approximately 40% when evaluating large sets of unrelated genotypes (Swedjemark *et al.* 1997; Chen *et al.* 2018b). Genetic components of this size are sufficient to improve resistance in Norway spruce through selective breeding. Additive inheritance and moderate to high heritability support the feasibility of breeding for increased resistance (Swedjemark *et al.* 2012; Skrøppa *et al.* 2015). Selection can be based either on superior parent trees (Chen *et al.* 2018b) or on their progeny (Arnerup *et al.* 2010).

While some studies have reported a trade-off between *H. parviporum* resistance and growth traits (Keriö *et al.* 2015), others have found no negative correlation, implying that selection for resistance is unlikely to adversely affect growth or wood quality (Swedjemark *et al.* 2012; Steffenrem *et al.* 2016; Chen *et al.* 2018b).

1.5 Climate change impacts on host-pathogen dynamics

Due to their long lifespans, trees are inherently exposed to environmental variability over time (Feeny 1976). However, climate change is accelerating the pace of these changes, introducing novel and intensified stressors. This increased environmental instability heightens forest vulnerability to pathogen invasion and other abiotic and biotic threats.

The frequency and effect of drought on global ecosystems have risen over the past century (Chiang *et al.* 2021). Drought can have a substantial negative impact on tree growth and vitality (Anderegg *et al.* 2015; Camarero *et al.* 2018). It weakens tree defences by, for example, reducing the carbon reserves and delaying chemical defences (Terhonen *et al.* 2019; Gomez-Gallego *et al.* 2022; Krokene *et al.* 2023).

Scots pines exposed to drought respond by closing their stomata and losing needles (Dobbertin *et al.* 2010), which can lead to reduced development of the canopy and decreased growth rates (Galiano *et al.* 2011; Poyatos *et al.* 2013). Premature needle shedding reduces whole tree

transpiration (Martínez-Vilalta *et al.* 2004) and is likely a final effort to prevent hydraulic failure (Wolfe *et al.* 2016; Nadal-Sala *et al.* 2021).

Drought stress alters gene expression and carbon reserves in spruce, reducing resistance to *H. annosum* s.l. (Terhonen *et al.* 2019; Krokene *et al.* 2023). Greenhouse experiments confirm that drought magnifies the impact of *Heterobasidion* species on Norway spruce, with increased necrosis in seedlings (Terhonen *et al.* 2019; Yeoh *et al.* 2021). Combined drought and *H. annosum* s.l. infections also result in increased mortality in spruce saplings (Gomez-Gallego *et al.* 2022).

For many forest pathogens, the altered conditions may prove beneficial, facilitating their broader distribution. *D. sapinea*'s impact is expected to intensify with climate change, as rising temperatures and shifting precipitation patterns drive its northward expansion (Fabre *et al.* 2011; Sturrock *et al.* 2011; Woods 2011; Brodde *et al.* 2019; Terhonen *et al.* 2021). Both heat and water stress have been shown to amplify DTB severity, increase necrosis, and enhance *D. sapinea*'s aggressiveness in experimental simulations (Swart *et al.* 1987; Johnson *et al.* 1997; Sherwood *et al.* 2015; Blumenstein *et al.* 2021a; Blumenstein *et al.* 2022; Ghosh *et al.* 2022). Fungi such as *D. sapinea* and *H. parviporum* display faster reproduction and higher overwintering survival under changing climate conditions, which contributes to increased infection levels (Terhonen *et al.* 2021; Yeoh *et al.* 2021; Hu *et al.* 2023; Krokene *et al.* 2023).

An increased prevalence of *H. parviporum* in warmer climates is predicted (Garbelotto & Gonthier 2013), and a warmer and drier climate may increase both the infection and spread of the fungus (Wen *et al.* 2019; Yeoh *et al.* 2021). Furthermore, wetter, milder winters enhance the fungus' survival (Oliva *et al.* 2011; Woods *et al.* 2016).

Mitigating the challenges of climate change requires accurate predictions, which depend on a deeper understanding of the interactions between trees and their pathogens. However, long-term monitoring and modelling efforts remain constrained by limited data on pathogen distributions and their responses to abiotic and biotic factors (Klopfenstein *et al.* 2009).

1.6 Overview of forest tree breeding

The general goal of breeding is to maximise **genetic gain** – the improvement in desirable traits achieved per unit of time through selection (Araus *et al.*

2018). Ideally, this includes developing trees with improved resilience to abiotic and biotic stressors, capable of persisting in the future climate.

1.6.1 The Swedish breeding programmes for Norway spruce and Scots pine

Breeding of Norway spruce and Scots pine in Sweden began in the 1940s, when the first phenotypically superior "plus trees" were selected from natural forests and used for the establishment of seed orchards (Jansson *et al.* 2017). The goal was to identify and utilise trees with desirable traits, such as height, straightness, and health, for reforestation and timber production. These trees formed the foundation for obtaining improved seed sources for genetically improved breeding populations (Gullberg & Kang 1985; Jansson *et al.* 2017).

The second and subsequent generations have progressively deployed improved genetic material by introducing genetic evaluations, such as clonal and progeny testing (Rosvall *et al.* 1998; Lindgren 2009; Capador-Barreto *et al.* 2021). The transition from first-generation phenotypic selection to second-generation breeding has resulted in improved selection accuracy and higher heritability estimates for key traits (Jansson *et al.* 2003; Andersson *et al.* 2007; Abrahamsson *et al.* 2012). Improvements in seed orchard management have focused on enhancing genetic diversity, reducing pollen contamination, and optimising seed crop quality, with the aim of increasing long-term productivity and genetic gain (Moriguchi *et al.* 2008; Rosvall 2019; Heuchel *et al.* 2022). Approximately 85% of planted seedlings in Sweden originate from seed orchards (Skogsstyrelsen 2020) and improved trees are expected to show 10–25% higher growth compared to unimproved trees (Rosvall *et al.* 2016; Skogforsk 2023). Sweden is currently planning for the fourth generation of seed orchards (Skogforsk 2023).

All breeding populations within the Swedish breeding programme have the general purpose of improving vitality (biotic and abiotic resistance), growth (biomass production), and wood quality (stem straightness, branch patterns, fibre characteristics, etc.) (Berlin *et al.* 2012; Rosvall *et al.* 2016). Deployment zones are designed to match plant material with clinal variations in environmental conditions such as latitude, altitude, photoperiod, and temperature sum (Nilsson *et al.* 1991; Andersson *et al.* 2003; Berlin *et al.* 2016). Regional testing and adaptation are crucial for ensuring optimal

deployment in local climates, as well as for improving resilience to climate change (Androsiuk *et al.* 2013; Chen 2016; Chen *et al.* 2021). These strategies are particularly important since latitude and temperature significantly influence growth and survival (Nilsson *et al.* 1991; Berlin *et al.* 2016). Phenotypic performance is tested across different deployment zones in Sweden to capture the environmental variation.

The Swedish breeding programme for Scots pine consists of 24 separate breeding populations (Berlin *et al.* 2012). While growth is a general breeding priority, the emphasis on specific traits varies regionally; in northern zones, for instance, traits such as frost hardiness receive greater attention (Andersson *et al.* 2003; Jansson 2007; Berlin *et al.* 2010). The breeding programme for Norway spruce includes 22 breeding populations (Berlin *et al.* 2012), and as with Scots pine, the focus on particular traits differs across Sweden in response to regional variation in climate and photoperiod adaptation (Androsiuk *et al.* 2013; Liziniewicz *et al.* 2019; Milesi *et al.* 2019; Chen *et al.* 2021). In both programmes, each breeding population comprises approximately 50 clones (Mats Berlin, Skogforsk, personal communication).

Cone production in pine plantations can begin after 5-10 years (Rosvall *et al.* 2016). Completing the full breeding cycle of Scots pine – including parental crosses, progeny testing, and selection – takes approximately 20-25 years (Krakau *et al.* 2013). The breeding cycle for Norway spruce is of similar duration. However, in spruce plantations, cone production typically begins no earlier than ten years and more commonly after 15-20 years, often with long intervals between years of abundant flowering (Krakau *et al.* 2013; Rosvall *et al.* 2016).

To prepare Swedish forestry for a changing climate, there is an increasing emphasis on assisted gene flow, climate-adapted provenancing, and modelling of future conditions (Milesi *et al.* 2019; Chen *et al.* 2021).

1.6.2 Genomic tools for resistance breeding

Breeding for resistance is a promising strategy to reduce the impact of forest pathogens and enhance the resilience of future tree generations. While non-native pests often receive attention due to their severity, native pathogens also contribute significantly to tree mortality and long-term economic losses. Natural genetic resistance exists for many of these threats, but effectively utilising it requires a strong understanding of the ecological, physiological,

and genetic interactions between conifers and their pathogens (Sniezko & Koch 2017).

In Sweden, resistance to *Heterobasidion annosum* s.l., the causal agent of root rot, has not yet been incorporated as a selection trait in the Norway spruce breeding programme, despite the substantial losses it causes. Although knowledge of resistance traits and their distribution in breeding populations is improving, practical tools to translate this knowledge into efficient selection and breeding strategies have, until recently, been limited.

Genotyping in conifer species

Studying conifer genomes is particularly challenging due to their large size (20–30 gigabases) and extensive regions of highly repetitive DNA (De La Torre *et al.* 2014). The large genome size appears to result from the gradual accumulation of diverse long terminal repeat transposable elements (Nystedt *et al.* 2013). This complicates assembly and limits the resolution for identifying genomic regions of interest (Grattapaglia *et al.* 2018; Thistlethwaite *et al.* 2020; Klápště *et al.* 2022). The first conifer genome, a draft of the Norway spruce, was published in 2013 (Nystedt *et al.* 2013). Several reference genomes are now available, including multiple representatives from the genera *Picea* and *Pinus*.

Linkage disequilibrium (LD), the non-random association of alleles at different loci, plays a critical role in genomic studies and breeding strategies. In conifers and other wind-pollinated plants with large population sizes, LD generally decays rapidly over short distances in the genome. However, conifer genomes are known to display considerable heterogeneity in LD patterns (Pyhäjärvi *et al.* 2020). In conifer breeding programmes, LD patterns are crucial for determining marker-trait associations and predicting the genetic value. While population-level LD is typically low, structured breeding populations with reduced effective population sizes may display more extensive LD, improving the feasibility of genomic prediction within such populations (Pyhäjärvi *et al.* 2020).

While whole-genome sequencing (WGS) theoretically provides a higher resolution, the size and complexity of conifer genomes make WGS impractical (Hung *et al.* 2024). Advances in sequencing and bioinformatics tools are now rapidly improving our ability to decode these massive genomes and have led to the development of 30–50kb single nucleotide polymorphism (SNP) arrays for conifer genotyping (e.g., Howe *et al.* 2020; Bernhardsson

et al. 2021; Kastally *et al.* 2021). SNP array genotyping is a method to genotype thousands of SNPs across the genome simultaneously. In short, a SNP array slide is coated with allele-specific probes that target SNP regions, where template DNA binds. Fluorescence signals at each site are then measured to determine the alleles present at the targeted sites (LaFramboise 2009). These arrays facilitate genomic predictions and help select superior parents in conifer breeding programmes (Kastally *et al.* 2021).

Genome-wide association studies (GWAS)

Genome-wide association studies (GWAS) is a method used to identify genetic variants, such as SNPs, associated with specific traits by analysing genetic and phenotypic data from a population. It detects statistical associations between SNPs and traits using association models like mixed linear models to account for population structure and relatedness (Korte & Farlow 2013). GWAS has been successfully used for forest trees with complex genomes, identifying numerous candidate SNPs associated with disease resistance (e.g., Quesada *et al.* 2010; Elfstrand *et al.* 2020a; Elfstrand *et al.* 2020b; Weiss *et al.* 2020; Liu *et al.* 2024; Singh *et al.* 2024). For instance, GWAS analyses have identified SNPs and QTLs associated with key traits in Norway spruce, such as frost tolerance and resistance to pathogens, allowing for greater precision and earlier selection in the breeding programme (Capador-Barreto *et al.* 2021; Chen *et al.* 2021).

The broad implementation of GWAS-derived SNPs in breeding programmes is, however, constrained by population specificity and limited validation of SNPs across populations and environments (Younessi-Hamzekhanlu & Gailing 2022).

Pedigree reconstruction and its use in breeding

The advancements in genotyping technologies can be used for reconstructing pedigrees (Hall *et al.* 2020). Pedigree reconstruction is a pivotal technique in conifer breeding, enabling breeders to determine the genetic relationships among individual trees without the need for controlled crosses. This approach uses molecular markers to infer parentage and relatedness, thereby enhancing the accuracy of genetic evaluations and streamlining breeding programmes (Doerksen & Herbinger 2010; El-Kassaby *et al.* 2011; Bouffier *et al.* 2019).

Pedigree reconstruction facilitates breeding-without-breeding (BwB), an approach that capitalises on natural mating events within breeding populations. By employing genotyping and pedigree reconstruction, breeders can assemble full- and half-sib families from open-pollinated progeny. The BwB strategy has been proposed as an efficient alternative in forest tree breeding, particularly when resources for controlled pollination are limited (El-Kassaby & Lstiburek 2009).

Principles and application of genomic selection (GS)

When correctly applied, the prediction accuracy based on genomic information can match or exceed the prediction accuracies in phenotypic selection while increasing genetic gain (Grattapaglia *et al.* 2018). In genomic selection (GS), a large training population, where individuals are both genotyped and phenotyped, is used to build a regression model that links genetic markers to observed phenotypes. The equation is then applied to predict genomic breeding values for related populations, using only their genotype data (Meuwissen *et al.* 2001; Grattapaglia *et al.* 2018).

GS in conifer breeding is expected to outperform traditional methods by accelerating breeding cycles, increasing genetic gain, and enabling early selection. GS allows for selection at early developmental stages since it can capture within-family variance and eliminates the need for progeny testing (Resende Jr *et al.* 2012; Beaulieu *et al.* 2014b; Lenz *et al.* 2020a; Gamal El-Dien *et al.* 2022). High genotyping costs are often offset by long-term gains through reduced field trials and phenotyping (Grattapaglia, 2022; Klápště et al., 2022; Galeano et al., 2023).

The potential to use GS for growth, wood, and pest resistance traits has been shown for several forest tree species: loblolly pine (Resende Jr *et al.* 2012; Walker *et al.* 2021), maritime pine (Bartholomé *et al.* 2016), white spruce (Beaulieu *et al.* 2014a; Beaulieu *et al.* 2014b), Norway spruce (Chen *et al.* 2018a; Lenz *et al.* 2020b), Scots pine (Calleja-Rodriguez *et al.* 2020; Calleja-Rodriguez *et al.* 2021), and western redcedar (Gamal El-Dien *et al.* 2022; Gamal El-Dien *et al.* 2024).

Evaluating conifer performance is challenging due to long breeding cycles, making within-family genomic selection essential for improving genetic gain and breeding efficiency. **GBLUP** (Genomic Best Linear Unbiased Prediction) and **ABLUP** (pedigree-based BLUP) are statistical models used in plant and animal breeding to estimate genetic parameters and

predict breeding values. GBLUP, based on genomic data, better captures **Mendelian sampling** (the random segregation of alleles during reproduction), recombination events, and rare alleles, leading to more accurate within-family predictions than ABLUP, which relies on pedigree-based matrices (Zapata-Valenzuela *et al.* 2013; Beaulieu *et al.* 2014b; Walker *et al.* 2021). ABLUP can overestimate predictive ability by ignoring segregation, hidden relatedness, and non-additive effects, whereas GBLUP more accurately reflects genetic variance and relatedness structures (Gamal El-Dien *et al.* 2015; Gamal El-Dien *et al.* 2016; Thavamanikumar *et al.* 2020; Thumma *et al.* 2021).

The optimal SNP density for accurate predictions depends on population size, trait heritability, and genetic architecture. Larger training populations can compensate for lower marker densities, while smaller or less diverse populations require denser markers to maintain accuracy (Lauer *et al.* 2021; Papin *et al.* 2024). In general, lower marker densities (~2,000-4,000 SNPs) are sufficient for high-heritability traits, while for low-heritability or polygenic traits, higher densities (~6,000-8,000 SNPs) are necessary (Bartholomé *et al.* 2016; Chen *et al.* 2018a; Lenz *et al.* 2020b).

1.7 Research context and rationale

The preceding sections have described the ecological significance of Nordic conifers, their defence strategies, and their interactions with key pathogens. In the context of climate change and ongoing challenges in breeding for resistance, there is a growing need to develop effective strategies for protecting forest health. Recent advances in genomic tools have improved the potential to identify genetic markers associated with disease resistance, providing new opportunities to support resistance breeding. Building on this background, the following section outlines the specific aims and objectives of this thesis.

2. Objectives

The aim of this thesis is to increase the understanding of genetic and environmental factors and mechanisms – including molecular and physiological processes – contributing to disease phenotypes, development, and severity in conifers.

The specific objectives are to analyse abiotic and biotic influences on the onset of disease and investigate the potential to use the genetic architecture of disease resistance in the Swedish breeding population of the major Swedish conifer species. Four papers address these objectives.

- **Paper I: *D. sapinea* as a contributing factor in the crown dieback of Scots pine (*P. sylvestris*) after a severe drought.** In this study, we examined the effects of *D. sapinea* in drought-affected Scots pine areas, hypothesising that drought and infection hinder tree recovery, with severely damaged trees more likely to die and mildly damaged trees better able to recover. We also expected *D. sapinea* infections in both healthy and symptomatic trees and fungal spore dispersal to correlate with Scots pine dieback.
- **Paper II: Scots pines with tolerance to *Melampsora pinitorqua* and *Diplodia sapinea* show distinct metabolic profiles.** This paper, and **paper III**, explored the responses of Scots pine trees infected by both *M. pinitorqua* and *D. sapinea*, as well as the genetic factors that may confer increased susceptibility to these pathogens in certain trees. In this study, we hypothesised that *M. pinitorqua* infections predispose the trees to DTB, and that disease symptoms from the previous growing season affect tree amino acid and phenolic compound levels, pathogen invasion, and overall health in the next season. We proposed that *M. pinitorqua*-symptomatic tissues differ in compound composition from asymptomatic trees and that *M. pinitorqua*-infected shoots have a distinct phenolic profile compared to those infected by both pathogens.
- **Paper III: Genetic basis of susceptibility and tolerance to *Melampsora pinitorqua* and *Diplodia sapinea* in *Pinus sylvestris*** evaluated whether Scots pine trees heavily affected by *M. pinitorqua* were more prone to *D. sapinea* due to genetic factors influencing disease tolerance. We predicted varying susceptibility among offspring

from different parental trees and distinct allele frequency patterns in more tolerant parents. We also hypothesised that distinct loci are associated with symptoms of each disease independently and that allele status vary between parent trees producing less disease-susceptible offspring versus those with more susceptible progeny.

- **Paper IV**: **Towards the integration of disease resistance in Norway spruce breeding, can genomic selection support early selection of *Heterobasidion* resistance?** This study aimed to evaluate the potential application of genomic selection in enhancing resistance to *H. parviporum* within the Norway spruce breeding programme. We hypothesised that a single breeding population in Sweden possesses sufficient variation in *H. parviporum* resistance to enable enhancement through selective breeding. We further hypothesised that genomic prediction could facilitate the selection of more resistant genotypes or the selection against genotypes with inferior resistance traits.

3. Materials and methods

3.1 Study sites

The experimental sites in **paper I** are located east and northeast of Visby in the area most affected by the 2018 drought on Gotland. Four sites with Scots pine showing crown dieback symptoms (Affected 1–4) and four with healthy-looking trees (Healthy 1–4) were selected (Figures 1 and 2). Most sites had thin soil layers over rock. Affected sites and Healthy 3 are situated on bedrock, while other healthy sites are on sand, gravel, or clay till (SGU 2014). Groundwater levels near affected sites were generally low over the past 60 years, reaching record lows in 2018 and briefly dropping further in 2019 before recovering in 2020 (SGU 2020).

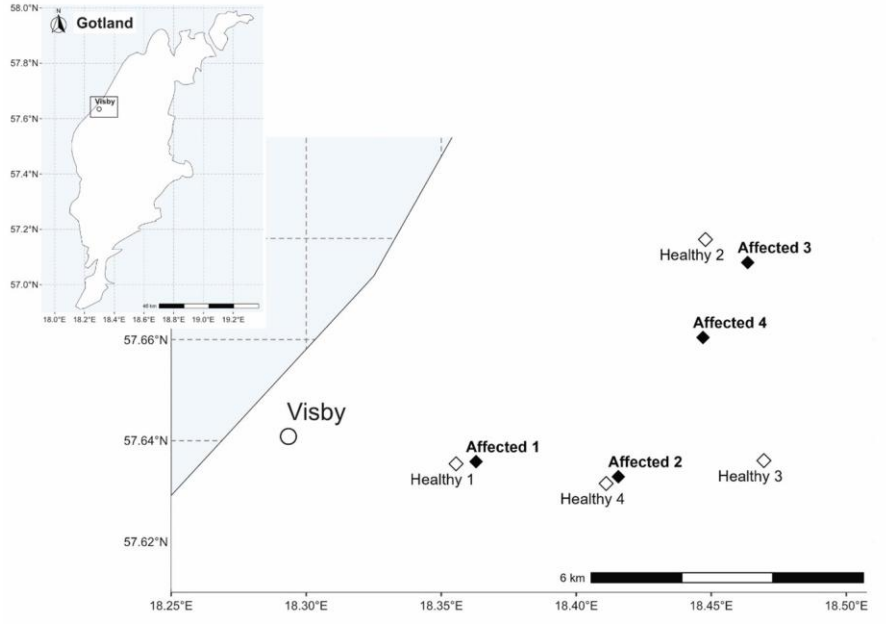

Figure 1. Location of experimental sites in **paper I** on Gotland, Sweden. Severely affected stands (Affected 1–4) and healthy-looking stands (Healthy 1–4). (Map source: rnaturalearth (Massicotte *et al.* 2023)).

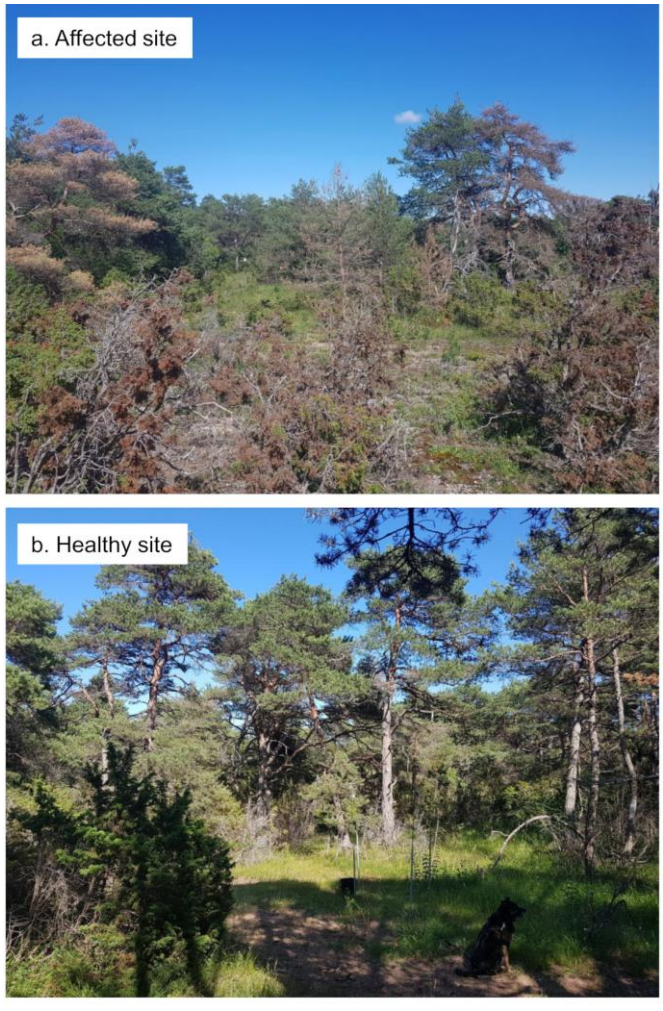

Figure 2. Representative photos of affected and healthy sites in **paper I**. a) Site Affected 1; b) Site Healthy 1. (Photos taken in June 2019).

The experimental site for **papers II** and **III** is an area of ~5,500 m² located outside Ängelsberg, Västmanland, Sweden (N 59.957, E 16.060). In 2014, a severe forest fire consumed all vegetation and most organic material. The site was reforested in 2015 with 1-year-old Scots pine seedlings planted in 20 cm high mineral soil heaps.

The study in **paper IV** was conducted in a greenhouse. Norway spruce seeds were sown in March 2021 at Lugnet nursery (Häggeby, Sweden) and transferred to SLU Ultuna in April 2022. Plants were randomised into three

blocks, repotted in a peat-vermiculite mix (3:1), and grown under a ≥16-hour light photoperiod at 22–25°C. They were watered daily with fertiliser to maintain constant moisture.

3.2 Plant material

In **paper I**, we selected 20–27 Scots pines at each affected site based on their level of defoliation, including trees with 0–100% defoliation of the upper third of the crown. Five trees were randomly selected at each healthy site. The age and origin of the trees are unknown.

The Scots pines planted at the site in Ängelsberg in 2015 and included in **papers II** and **III** were reported to originate from the Hade seed orchard (FP-610). In spring 2021, 567 seven-year-old trees (100–275 cm tall) were surveyed for *M. pinitorqua* and *D. sapinea* symptoms. Based on the survey, 15 trees were selected for detailed investigations in **paper II** and divided into three disease categories based on their symptoms in 2020: healthy-looking (H), *M. pinitorqua*-symptomatic (M), and *M. pinitorqua*- and *D. sapinea*-symptomatic (MD) (Figure 3).

Paper IV was part of a larger project that included a total of 3,000 plants belonging to 105 F1 full-sib families, from four Swedish deployment zones (G4, G5, G6, and G7) (Table 1). F1 refers to the first generation of crosses between plus trees. **Paper IV** centres on the plants from deployment zone G5, specifically plants belonging to the breeding population Gpop11. We selected this population because it represents a single breeding population, in contrast to the plants in G7, which were sampled across several breeding populations within the deployment zone. Gpop11 have been progeny-tested and consists mainly of plus trees from a recent plus tree selection. There are 65 founders in the population (Skogforsk 2021) and the families included in **paper IV** originated from 44 unique parental clones. The paper also includes plants from deployment zone G6, as they were used for model validations.

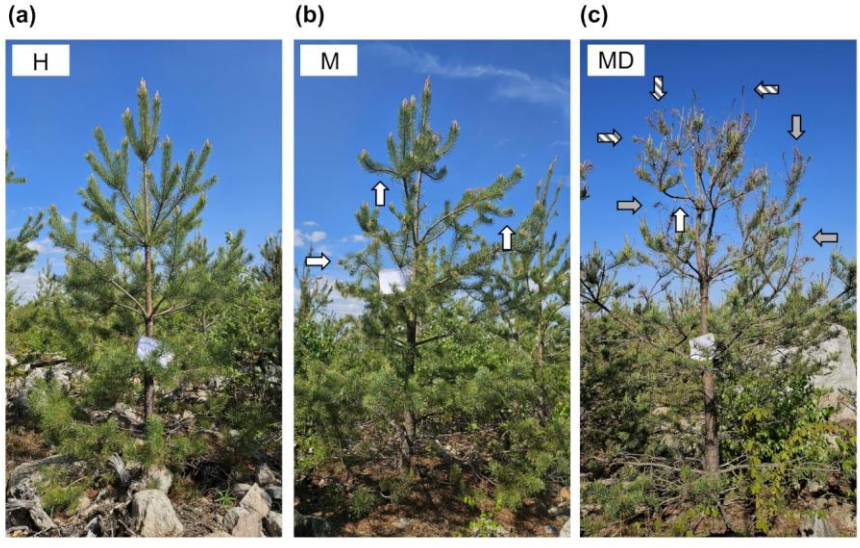

Figure 3. Photos depicting tree disease categories in **paper II** post-2020 growing season: White arrows indicate *M. pinitorqua* symptoms, grey for *D. sapinea*, and striped for both. a) H – healthy-looking; b) M – *M. pinitorqua*-symptomatic with bent shoots; c) MD – symptoms of both *M. pinitorqua* (bent/broken shoots) and *D. sapinea* (stunted/dead shoots).

Table 1. Overview of the plant material used in the project, including the subset covered in **Paper IV**. (SWE – Sweden, BY – Belarus, NFE – Fennoscandia).

Deployment zone	**G7**	**G5 (Gpop11)**	**G6**	**G4**
Origin	all European domains	SWE/BY	SWE/BY	NFE
No. of families	50	50	5	5
Small[a]	40	39	5	5
Large[b]	10	11	0	0
Plants phenotyped	1296	1464	120	119
Phenotype data[c]	1230	1345	112	97
Plants genotyped	900	900	75	75
Genotype data[d]	781	878	66	70

[a] Small families: 4/15 trees phenotyped and genotyped
[b] Large families: 8/30 trees phenotyped and genotyped
[c] High quality phenotype data
[d] High quality genotype data

3.3 Field surveys and disease scoring

3.3.1 Confirmation of *D. sapinea* presence

Visual detection of DTB symptoms on affected sites in **paper I** and the experimental site included in **papers II** and **III** was confirmed by conidia morphology through microscopic examination of conidia from pycnidia found on necrotic shoots and cones. For **papers II** and **III**, *D. sapinea* was also confirmed through isolates from shoots of DTB-symptomatic trees collected in July (see Section 3.4.1, sampling time point 3). Shoots were surface sterilised as follows: 1 min in 70% EtOH, 1 min in 3% NAOCl, 1 min in 70% EtOH, 2 × 1 min in high-purity water. Shoots were cut and placed on Hagem agar plates. Fungal mycelia were reisolated, and DNA was extracted from lyophilised, homogenised pure cultures using the DNeasy Plant Mini Kit (Qiagen), following the manufacturer's protocol. **PCR (polymerase chain reaction)**, a technique used to amplify specific DNA sequences to make their detection possible, was performed. Each reaction contained 12.5 µL DNA. The master mix contained DreamTaq Green Buffer (10×, Thermo Fisher Scientific), 200 µM of each dNTP, 0.2 µM of each primer (ITS1F and ITS4), 1.5 mM $MgCl_2$, and 0.25 µL DreamTaq DNA polymerase (Thermo Fisher Scientific). PCR was run for 35 cycles with an annealing temperature of 58°C and products were purified using the E.Z.N.A. Cycle Pure Kit. The DNA was then sequenced using Sanger.

3.3.2 Survey of defoliation and disease symptoms

In **paper I**, crown dieback was estimated on 20–27 Scots pines at each affected site, beginning in December 2018. The level of crown dieback was estimated based on the proportion of twigs with shoot blight and dead twigs in the upper third of the living crown. Trees were revisited in October 2019 and November 2020. In 2020, separate estimates were made for overall crown dieback and for dieback in shoots that had developed after the drought, excluding defoliated or transparent parts. Five asymptomatic trees on each healthy site were also measured for crown dieback across the three years. Tree height, diameter at breast height, and stem bifurcation were recorded in 2018 for all trees at affected sites.

For **papers II** and **III**, the surveys of symptoms differed between *M. pinitorqua* and *D. sapinea*. In spring 2021, we surveyed trees for *M. pinitorqua* infection in the top whorl of 2020 and the second whorl (15 randomly selected shoots) and assessed infection in the top whorl of 2019. In autumn 2021, we repeated the survey, but for the 2021 shoots. We recorded the number of shoots with DTB symptoms in the entire crown in spring (for the 2020 growing season) and autumn 2021 (2021 growing season). Height growth between nodes was measured annually from 2018 to 2021, along with total tree height at the end of the study. In autumn 2021, tree condition was scored on a scale from 0 to 5 based on visible damage caused by the two pathogens.

In **paper II**, trees were grouped into vitality classes – fully vital (scores 0–1), mildly affected (2–3), and severely affected (4–5) – while in **paper III**, the damage score was used as a continuous variable. The score reflected deviations from a typical seven-year-old pine crown – no self-pruning, straight stem, clear leader shoot, and balanced side branches. It aligns with Desprez-Loustau and Wagner (1997), who assessed damage from a forester's perspective. Coordinates were recorded for every 15th surveyed pine, along with the number of aspen trees within a 2.84 m radius. Additional coordinates were taken for corner trees and some along the plot's edges (n = 47). The symptom variables used for analyses in **paper III** were the mean percentages of *M. pinitorqua*-infected shoots (referred to as Mp^{W1} for the top whorl and Mp^{W2} for the second whorl) and the mean number of DTB-symptomatic shoots (Ds) across the surveyed years, as well as the mean height growth for 2018-2021.

3.3.3 Inoculation trials and phenotyping

Plants included in **paper IV** were inoculated in August and September 2022 following the method described in Arnerup *et al.* (2010) with *H. parviporum* Rb175. The fungus was grown on media for three weeks before the experiment, along with 5 mm Norway spruce wood dowels. At inoculation, bark was removed with a cork borer and a colonised wooden dowel was placed at the wound and covered with Parafilm (Figure 4a). The plants were harvested and phenotyped after three weeks: lesion length (LL) on the inside of the bark was recorded (Figure 4b), and fungal growth in the sapwood (SWG) was measured following Arnerup *et al.* (2010) by cutting the inoculated stem into 5 mm discs, incubating them in humid conditions for

one week, and observing the presence of *H. parviporum* conidiophores under a stereomicroscope (Figure 4c).

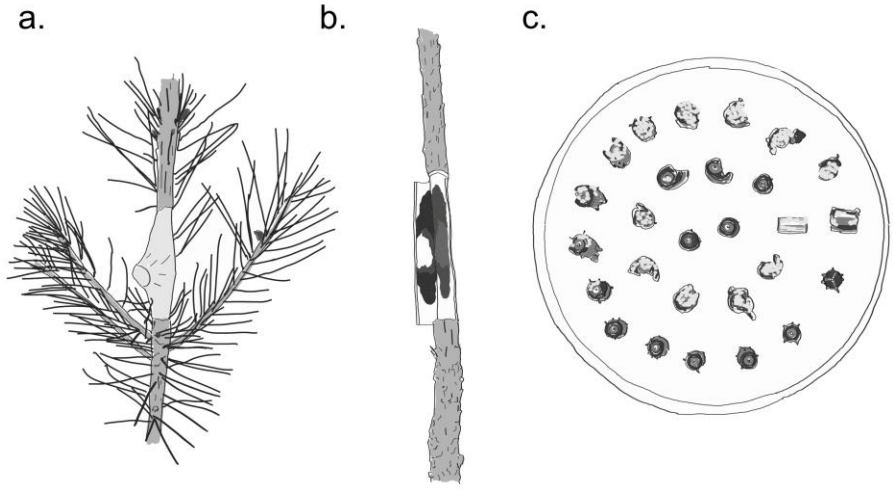

Figure 4. Phenotyping of resistance traits in *H. parviporum*-inoculated Norway spruce seedlings in **paper IV**. a) Inoculated seedling. b) Lesion length (LL) assessed by measuring the lesion under the bark. c) Fungal growth in the sapwood (SWG) evaluated by observing *H. parviporum* conidiophores under a stereomicroscope.

3.4 Sample collection

3.4.1 Sampling of plant tissue for quantification of fungal biomass and metabolites and tree genotyping

In **paper I**, Scots pine twigs were collected in December 2018. Three healthy twigs were collected from all trees, and if a tree showed symptoms, three additional twigs with tip blight were sampled. On healthy sites, three trees were randomly chosen, while on affected sites, three symptomatic and three asymptomatic trees were selected based on crown dieback estimations (Table 2). Healthy twigs were sampled from the 2018 growth, while symptomatic ones were sampled from the infection border (2008–2017).

For quantification of fungal biomass, phenolic compounds, and amino acids in **paper II**, shoot tissue samples were collected at three time points in 2021, reflecting early (June 2), mid (June 10), and late (July 8) infection

stages by *M. pinitorqua* (Figure 5). No *M. pinitorqua*-symptomatic shoots were observed in category H during the first two time points, so only asymptomatic samples were collected. No DTB symptoms were detected in shoots during any of the time points. Eighty samples were prepared for metabolite and fungal biomass analysis. A 10–20 mg subsample was used for metabolite analysis, and the rest for DNA isolation.

Table 2. Scots pine twig sampling design for **paper I**, including site status (affected or healthy), tree status (symptomatic or asymptomatic), and twig status (symptomatic or asymptomatic). Twigs were collected in December 2018.

Site	**Affected**			**Healthy**
No. sites	4			4
Tree	Symptomatic		Asymptomatic	
No. trees per site	3		3	3
Twig	Symptomatic	Asymptomatic		
No. twigs per tree × symptom	3	3	3	3
No. samples per category	36	36	36	36
Total number of samples	144			

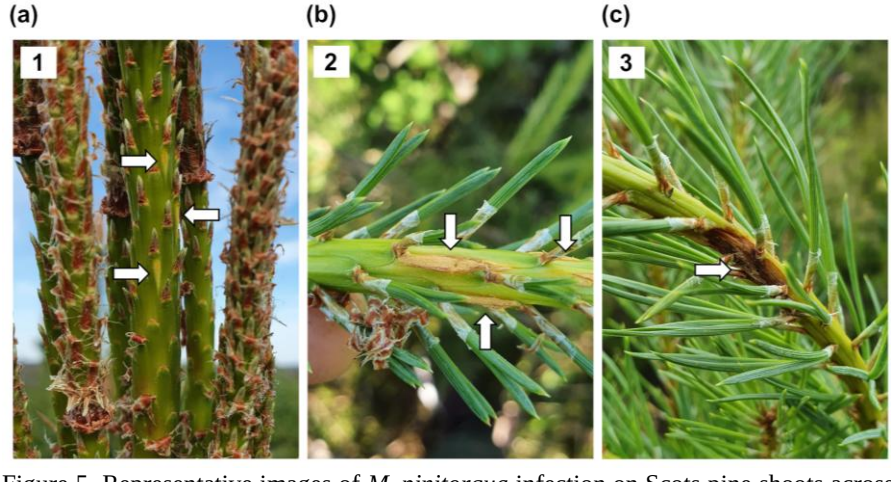

Figure 5. Representative images of *M. pinitorqua* infection on Scots pine shoots across the three sampling time points in **paper II**. White arrows indicate symptoms. (a) June 2, 2021 – early aecia development; (b) June 10, 2021 – aeciospores visible on the infection site; (c) July 8, 2021 – infection inactive, with canker healing underway.

Bud samples for genotyping were collected from each surveyed individual included in **paper III** and the parental trees in the seed orchard, and newly emerged fresh needles from all seedlings were sampled for genotyping in **paper IV**.

3.4.2 Spore trapping for quantification of *D. sapinea* spore DNA

To monitor *D. sapinea* spore release in **paper I**, four spore traps were placed at the centre of each site. The traps consisted of horizontally fixed filter papers treated with TE buffer. Filters were exposed for two consecutive seven-day sampling periods per season from January 2019 to October 2020 to capture seasonal variations in spore release.

3.5 Biochemical and molecular analyses

3.5.1 DNA extraction

For quantification of fungal DNA in **paper I**, DNA was extracted using the E.Z.N.A® SP Plant DNA Kit (Omega Biotek). Spore DNA from filter paper traps was extracted with the Macherey-Nagel NucleoSpin Plant II kit following the manufacturer's protocol. Fungal and tree DNA for the studies addressed in **papers II** and **III** were isolated using the Qiagen DNeasy Plant mini kit with protocol. In **paper IV**, total genomic DNA was extracted from the homogenised tissue using the E.Z.N.A. ® SP Plant DNA Kit (Omega Bio-tek) DNA extraction protocol.

In all studies, buffer volumes were adjusted as needed to facilitate pellet resuspension or account for the amount of plant material.

3.5.2 Quantification of fungal DNA (qPCR)

D. sapinea DNA from twigs and spore traps in **paper I,** as well as DNA from *D. sapinea, M. pinitorqua,* and Scots pine from shoots in **paper II**, were quantified using qPCR.

Quantitative PCR (qPCR) builds on PCR by amplifying and simultaneously measuring DNA in real-time, allowing for both detection and quantification. It uses fluorescent dyes or probes that emit signals proportional to the amount of PCR product generated, allowing for the

quantification of the target DNA. The increase in fluorescence during the amplification process helps determine the initial concentration of the template (Dymond 2013).

Primers and probes for quantification of *D. sapinea* DNA (**papers I** and **II**) were designed by Luchi *et al.* (2005). Primers for *M. pinitorqua* (**paper II**) were designed in-house. A GH3 homologue designed by Heller *et al.* (2012) was used for the determination of Scots pine biomass (**paper II**). Standard curves for qPCRs were generated from PCR products of *D. sapinea* (**papers I** and **II**), *M. pinitorqua*, and Scots pine (**paper II**), and the PCR products' desired lengths were confirmed by gel electrophoresis.

qPCR reactions for *M. pinitorqua* and Scots pine used SsoFast EvaGreen Supermix, while SsoAdvanced™ Universal Probes Supermix was used for *D. sapinea*. All reactions were carried out on the CFX Maestro qPCR detection system. Each assay included a standard curve of serial dilutions (1×10^7 to 1×10^2 copies), DNA templates in triplicates, and three non-template controls. The iQ™5 Optical System software was used for analysis. Samples with a Ct standard deviation > 0.5 were excluded, while those below the detection limit were assigned an SQmean = 0.01 (**paper I**) or 0 (**paper II**) depending on the requirements of the respective statistical models. For analysis in **paper II**, *D. sapinea* and *M. pinitorqua* DNA was normalised based on the amount of Scots pine DNA in the same sample.

3.5.3 Metabolite analysis (LC-MS/MS)

Extraction and quantification of phenolic compunds and amino acids in **paper II** were conducted using LC/MS-MS.

LC-MS/MS (Liquid Chromatography-Tandem Mass Spectrometry) is a technique used for separating, identifying, and quantifying complex analytes. First, liquid chromatography (LC) separates the sample components as it passes through a column, where molecules are separated based on their properties. After separation, analytes are ionised (via electrospray ionisation) to convert them into gas-phase ions. The ions are analysed based on their mass-to-charge ratio (m/z) in a mass spectrometer. In tandem mass spectrometry (MS/MS), the ions undergo multiple stages of analysis. The first stage selects a precursor ion, which then collides with an inert gas to fragment into product ions. These product ions are selected for detection in the final stage. The detector measures the abundance of the product ions, which correlates to the analyte's concentration (Pitt 2009).

Phenolic compounds were identified based on retention time and mass spectra, with quantification performed using standards where available. Compounds for which standards were not available were identified and relatively quantified. Most phenolics had been previously reported in pine species (Slimestad 2003). Amino acids were quantified using labelled internal standards.

3.5.4 SNP genotyping and data filtering

Scots pine (**paper III**) and Norway spruce (**paper IV**) samples were genotyped using the PiSy50K (Kastally *et al.* 2021) and Piab50K (Bernhardsson *et al.* 2021) SNP arrays, respectively. SNPs were filtered based on call rate, minor allele frequency, and missing data. In **paper III**, LD pruning was performed using the SNPRelate package in R (Zheng *et al.* 2012; R Core Team 2021), and SNPs were sorted based on the PiSy50K marker order. In **paper IV**, missing data were imputed using Beagle v4.0 (Browning & Browning 2007). Filtered datasets were used for downstream GWAS and genomic prediction.

3.6 Statistical analysis

3.6.1 Kinship estimation

In **paper III**, PCA was performed on the genotype data using the snpgdsPCA function from SNPRelate (Zheng *et al.* 2012) to explore SNP structure. Kinship was estimated with PLINK's MoM in SNPRelate, creating a kinship matrix to identify first-degree relationships (coefficient > 0.177) among parental trees. Mendelian errors were counted to infer parentage. The population structure of unknown seed source trees was analysed with the sNMF function in package *LEA* (Frichot *et al.* 2014) in R, fitting models for K = 1:20 and selecting the best K based on the lowest entropy, with admixture coefficients extracted and plotted.

3.6.2 GWAS

Genome-wide association studies (GWAS) were conducted in **paper III** on the unknown seed source population and the LD pruned SNP data using

GEMMA (Zhou & Stephens 2012). *D. sapinea*-symptomatic shoots (Ds) were log(x+0.5) transformed, and damage was $\sqrt{x}$-transformed for variance stabilisation and improved normality. Univariate GWAS models were fitted for Ds, Mp^{W1} (percentage of *M. pinitorqua*-infected shoots in the top whorl), Mp^{W2} (second whorl), damage, and height growth using the linear model by Zhou and Stephens (2012) without covariates. Multivariate models followed the specifications in Zhou and Stephens (2014). A relatedness matrix from SNP data was calculated in GEMMA, and models included Ds × Mp^{W2}, Ds × damage, Ds × Mp^{W2} × damage, Mp^{W2} × damage, and Mp^{W1} × damage. Wald test p-values were adjusted using the Benjamini-Hochberg method, retaining significant SNPs with FDR thresholds of 0.05 and 0.10.

3.6.3 Genomic selection modelling

Data processing

Statistical analyses of phenotypic and genotypic data for genomic selection in **paper IV** were performed using the *asreml* package in R (Butler *et al.* 2023). Further filtering removed SNPs with MAF < 0.03, those called in < 15% of individuals, and individuals with < 20% genotyping success.

Genomic (G) and pedigree-based (A) relationship matrices were constructed using the VanRaden (2008) approach and according to the ASReml manual (Gilmour 2015), respectively. Pedigree correction was applied for the G5 population by examining the genomic relationship matrix to identify and correct inconsistencies between the recorded pedigree and the genomic data. This resulted in the removal of some individuals and the reassignment of others to different families. No pedigree correction was performed for the individuals from deployment zone G6.

Dominance relationship matrices, both pedigree-based ($Adom$) and genomic ($Gdom$), were generated and inverted (according to Amadeu *et al.* (2023) and Vitezica *et al.* (2013), respectively) to capture the non-additive genetic effects arising from interactions between alleles at the same locus (i.e., dominance effects). The lesion length (LL) data was natural logarithm transformed (log(1 + x)) to address deviations from a normal distribution.

Full asreml models were fitted to the data, including block effects as fixed factors and additive and dominance genetic effects as random factors, using either the pedigree-based (ABLUP-AD/ABLUP) or genome-based (GBLUP-AD/GBLUP) relationship matrices. The full asreml model was:

$$y_{ij} = \mu + b_j + u_{ij} + d_{ij} + e_{ij} \quad (1)$$

where y_{ij} is the observed phenotype for the i-th genotype nested within the j-th block, μ is the fixed effect of the overall mean, b_j is the fixed effect of block j, u_{ij} is the random genetic effect based on relationship matrix A or G (for ABLUP or GBLUP), d_{ij} is the dominance effect based on $Adom$ or $Gdom$ (for ABLUP-AD or GBLUP-AD), and e_{ij} is the residual error.

Dominance effects were found to be statistically significant for LL but not for SWG, so only additive effects were included in the models for SWG. LL and SWG were adjusted for block effects. The final asreml ABLUP-AD and GBLUP-AD model was:

$$y'_i = \mu + u_i + d_i + e_i \quad (2)$$

while the final ABLUP and GBLUP asreml model was:

$$y'_i = \mu + u_i + e_i \quad (3)$$

where y'_i is the adjusted phenotype value for the i-th genotype.

Data analysis, modelling, and validation

Phenotypic correlations were calculated using Pearson's correlation coefficient, while genetic correlations were estimated using a multivariate asreml model.

Cross-validation was performed with 100 replicates for all asreml models using different ratios of training and validation sets (3:2, 7:3, 4:1, and 9:1) to evaluate model performance. In addition to using the complete set of filtered SNPs, GS models were also built using random subsets of 3,000, 6,000, and 9,000 SNPs for each trait.

Variance components (additive genetic variance (σ_A^2), dominance genetic variance (σ_D^2, for lesion length only), phenotypic variance (σ_P^2), and residual error variance (σ_e^2)) were estimated from the ABLUP models. Narrow-sense heritability (h^2) was calculated as the ratio of additive genetic variance to total phenotypic variance:

$$h^2 = \frac{\sigma_A^2}{\sigma_P^2} \quad (4)$$

Coefficients of variation for additive genetic variance (CV_A) and phenotypic variance (CV_P) were calculated as:

$$CV_X = \left(\frac{\sqrt{\sigma_X^2}}{mean\ phenotype} \right) \times 100 \quad (5)$$

where the mean phenotype is the average of the observed trait in the dataset.

The predicted breeding value (EBV for ABLUP, GEBV for GBLUP) for each individual was calculated as the sum of the model mean (μ) and the predicted additive genetic effect (u). Predictive ability (PA) was determined as the correlation between adjusted phenotypic values (y') and predicted breeding values, and predictive accuracy (PAC) was calculated as PA divided by the square root of the heritability.

Finally, the predictive ability and accuracy of the models were tested on the genetically unrelated individuals from deployment zone G6 (n = 66), using the entire G5 dataset as the training set.

3.6.4 Regression, multivariate, and correlation analyses

R Statistical Software (R Core Team 2021) was used for all statistical analyses and figure creation, except for the GWAS analysis in **paper III**.

In **paper I**, ANOVA was used to compare crown dieback and *D. sapinea* DNA from twigs (package *AICcmodavg* (Mazerolle 2020)), followed by Tukey's post hoc tests. Linear mixed-effects models (package *nlme* (Pinheiro *et al.* 2021)) tested effects of initial dieback, tree height, and bifurcation. Recovery was analysed using pairwise t-tests (package *rcompanion* (Mangiafico 2022)). Repeated measures regression (*nlme*) assessed *D. sapinea* DNA quantities from spore traps, with post hoc comparisons using package *emmeans* (Lenth 2023).

In **paper II**, redundancy analysis (RDA) (package *vegan* (Oksanen *et al.* 2022)) examined the effects of time point, shoot symptom, and disease category on metabolite profiles, with significance assessed by permutational ANOVA. Kruskal-Wallis and Wilcoxon tests analysed metabolite data, followed by Dunn's post hoc tests. Indicator species analysis was performed using package *indicspecies* (Cáceres & Legendre 2009) to identify metabolites linked to time point, symptom, and disease category. Generalised additive models (GAMs) (package *mgcv* (Wood 2017)) were used for metabolite levels and pathogen biomass, and Chi-square tests evaluated relationships between tree vitality and prior disease symptoms.

In **paper III**, GAMs (*mgcv* (Wood 2003; Wood 2017)) analysed the relationships between tree geographic position, growth, damage, and shoot infection. Pearson correlations assessed phenotypic trait relationships. Linear models examined trait interactions, and Type III ANOVAs (package *car* (Fox & Weisberg 2019)) were used to assess family effects, accounting for group size imbalance. Candidate SNPs linked to Ds from univariate GWAS were validated via linear regression on progeny and parental data, coding genotypes as integers.

In **paper IV**, ANOVA and Tukey's HSD post hoc tests evaluated family and parental tree differences in adjusted LL and SWG values.

3.6.5 SNP annotation

Affymetrix probe sequences for SNPs significantly associated with *D. sapinea* susceptibility (FDR $p < 0.05$) were used as queries in BLASTN searches. Searches were conducted against the NCBI nucleotide database (restricted to Pinaceae) and the *P. abies* v1 gene catalog (PlantGenIE.org), retrieving significant hits (e-value $< 1\times10^{-8}$). Descriptions, PFAM annotations, and closest *Arabidopsis* orthologs were collected. To characterise loci within significant QTLs, the same probe sequences were also BLASTed against the *Pinus tabuliformis* genome (BioProject: PRJNA784915), retrieving 2,000–4,000 bp sequences. When multiple hits were found, the one with the lowest score was selected for further analysis.

3.6.6 Data visualisation

Data visualisation was performed primarily using the R package *ggplot2* (Wickham 2016), along with *UpSetR* for set-based visualisations (Gehlenborg 2019). Additional illustrations were created as drawings using a Samsung S7 tablet.

4. Results and discussion

The results are presented in the order of the four papers, each focusing on a specific aspect of conifer–pathogen interactions and their relevance for resistance breeding in Swedish forestry.

4.1 *Diplodia sapinea* and drought stress in Scots pine (paper I)

The emergence of Diplodia tip blight (DTB) as a forest disease in Fennoscandia has drawn significant attention to the progression of disease in affected pines. **Paper I** provides insights into the recovery of drought-affected pines and the contribution of *D. sapinea* to crown dieback in this process.

The study, conducted on the island Gotland in Sweden, followed a significant drought in 2018. The drought caused substantial crown dieback of Scots pine on the island, with symptoms consistent with a DTB outbreak. We confirmed the presence of *D. sapinea* on affected sites by spore morphology. Increased DTB severity is expected when *D. sapinea* infections occur in combination with other stressors (Swart *et al.* 1987; Johnson *et al.* 1997; Sherwood *et al.* 2015; Blumenstein *et al.* 2021a; Blumenstein *et al.* 2022; Ghosh *et al.* 2022). The effects of drought and *D. sapinea* in combination had not been studied in the Nordic countries, a risk that will likely be significantly increased by the changing climate (Sturrock *et al.* 2011).

Tree recovery and mortality

Affected Scots pines in **paper I** experienced a significant increase in crown dieback between 2018 and 2019, with the average nearly doubling (Figure 6a). However, between 2019 and 2020, the overall dieback levels generally stabilised or showed only minor changes compared to the previous year. Interestingly, new shoots that grew in 2020 showed noticeably lower dieback symptoms, often aligning with the initial damage levels observed in 2018. Healthy sites initially showed no crown dieback in 2018 but experienced a minor increase in the following years (Figure 6b).

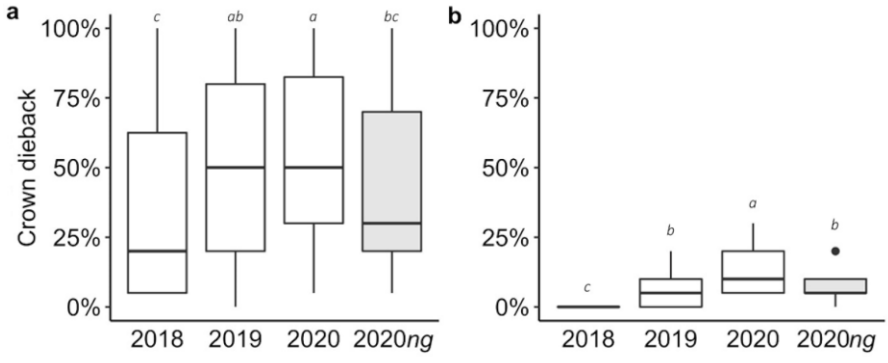

Figure 6. Crown dieback of Scots pine in **paper I** at a) four affected sites (97 trees) and b) four healthy sites (20 trees) on Gotland during 2018-2020. *D. sapinea* was confirmed to be present on the affected sites. 2020ng refers to the estimation of dieback observed in shoots newly grown (ng) post-drought. Significances indicated by different letters (ANOVA and Tukey's HSD post-hoc test).

The initial level of crown dieback in 2018 had a notable influence on both the overall crown dieback and the dieback in the new shoots by 2020. Notably, even trees with very high dieback levels in 2019 demonstrated recovery in their new shoots by 2020. The initial dieback severity did not affect whether a tree recovered, stagnated, or declined in overall crown health by 2020. Trees that were initially moderately damaged showed the highest rate of recovery in their new shoots, although this wasn't significantly different from severely damaged trees. Mortality was low in trees with low and medium initial dieback but was considerably higher (over 50%) in severely damaged trees, with most deaths occurring in the first year of observation (Figure 7).

Scots pine recovery after drought is known to occur once water stress is relieved (Dobbertin *et al.* 2010; Eilmann *et al.* 2013). However, severe dieback can have long-term consequences on tree vitality and growth (Galiano *et al.* 2011). We found that despite initial significant dieback and subsequent increase in the first year, surviving trees showed a clear recovery in the second year, even those with severe initial damage. This recovery was independent of the initial dieback levels, possibly reflecting the opportunistic nature of *D. sapinea*, whose impact may weaken once the primary stressor is alleviated.

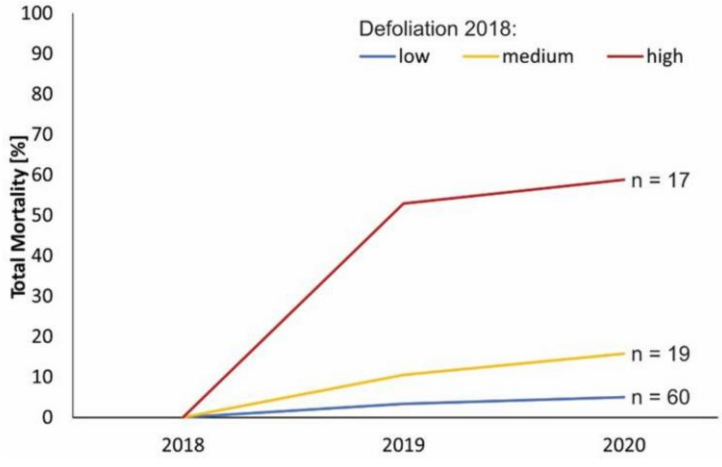

Figure 7. Total mortality of Scots pine in **paper I** two years following an initial drought in 2018. Trees were categorised in low (< 30%; blue), medium (40–70%; yellow), and high (> 70%; red) crown dieback based on the initial estimation. Number of selected trees indicated per category, total n = 96.

The mortality levels in highly damaged trees were comparable to those seen in a *D. sapinea* outbreak triggered by a hailstorm in Spain (Oliva *et al.* 2020), suggesting that the fungus can significantly contribute to mortality under stress. *D. sapinea* might have played a crucial role by increasing the dieback and pushing trees past the mortality threshold.

Pathogen biomass and symptom patterns

Significantly higher quantities of *D. sapinea* DNA were detected in symptomatic twigs from symptomatic trees on affected sites compared to asymptomatic twigs from the same trees (Figure 8). Notably, healthy-looking twigs, regardless of whether they originated from symptomatic or asymptomatic trees on affected or healthy sites, contained similarly low amounts of *D. sapinea* DNA. Thus, *D. sapinea* was most abundant in symptomatic tissues, while low levels of endophytic colonisation were also detected in healthy twigs, regardless of tree or site condition. This is consistent with a study by Oliva *et al.* (2020). However, in contrast to our findings, they observed significantly higher *D. sapinea* quantities in asymptomatic trees from affected sites compared to those from healthy sites.

This indicates differences in colonisation patterns depending on the history of DTB in the region.

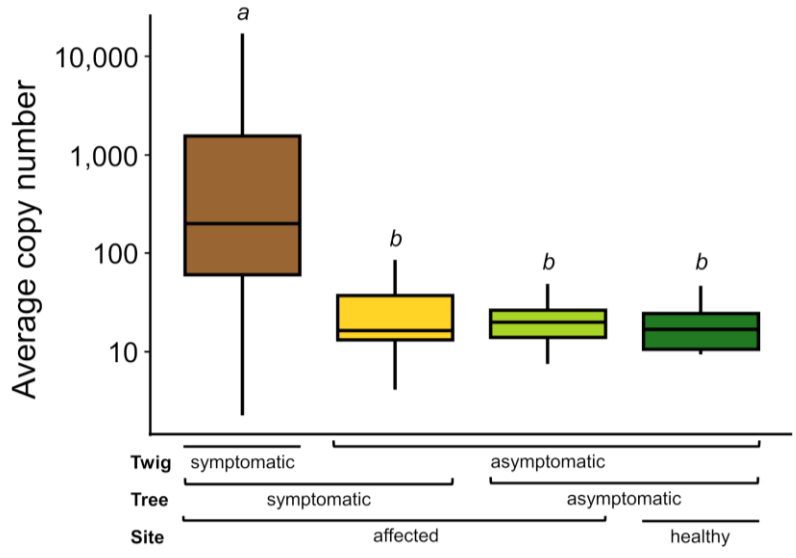

Figure 8. Quantification of *D. sapinea* DNA (qPCR) in Scots pine twigs in **paper I**. The scale is logarithmic. Symptomatic (brown) and asymptomatic (yellow) twigs from symptomatic trees on affected sites; asymptomatic twigs from asymptomatic trees on affected sites (light green); asymptomatic twigs from asymptomatic trees on healthy sites (dark green). 12 trees per category, three twigs analysed per tree. Significances indicated by different letters (ANOVA).

Spore dispersal and site effects

D. sapinea DNA was consistently detectable in spore traps on both affected and healthy sites throughout the two years of the study. The amount of *D. sapinea* DNA did not exhibit a clear seasonal pattern or an increase in the second year of sampling. A significant positive correlation was found between the quantity of *D. sapinea* DNA and high mean precipitation during the week of spore trap exposure. No significant correlations were found with mean temperature or mean wind speed. While there was a trend of higher DNA quantities on affected sites compared to healthy sites, this difference was statistically significant only for two sample weeks. The quantity of DNA varied considerably among traps within the same site.

The presence of *D. sapinea* spores on both affected and healthy sites and the development of mild symptoms on healthy pines indicate the widespread

presence of the pathogen. The correlation between spore dispersal and precipitation supports earlier research (Brookhouser & Peterson 1971; Swart *et al.* 1987). However, the lack of a clear seasonal pattern in spore dispersal in this study contrasts with some previous findings (Kuntzmann *et al.* 2009), potentially due to the limited sampling period, which may not adequately reflect dispersal dynamics in northern latitudes. It is also possible that this is an indication of that *D. sapinea* is sporulating throughout the year, when conditions allow, reflecting its opportunistic lifestyle.

Site properties likely played a significant role in the contrasting dieback patterns between affected and healthy sites, as previously shown by Munck *et al.* (2009). The bedrock soil with thin organic layers on affected sites likely experienced more severe drought impacts compared to the deeper soils with potentially higher water-holding capacity on healthy sites.

Conclusion paper I

Separating the individual effects of drought and DTB on the observed crown dieback is challenging. Nevertheless, our results suggest that *D. sapinea* likely contributed to the dieback and potentially influenced post-drought recovery. With drought frequency expected to increase due to climate change, drought may become a recurring stressor – potentially leading to further Scots pine decline, with *D. sapinea* acting as an accelerating factor.

A key finding was that surviving trees showed clear recovery in their new needles by 2020, and this recovery was independent of initial dieback levels. This shows that once the primary stressor (drought) is relieved, the impact of the opportunistic pathogen *D. sapinea* may decrease. It also implies that immediate sanitary cuttings in infested stands on poor sites might not be the best strategy, as tree removal could increase soil erosion. In addition, reducing the canopy cover by removing trees could lead to an even drier microclimate, creating harsher conditions for understory and ground-layer vegetation (Kovács *et al.* 2020). However, it is also important to consider the economic losses associated with reduced wood quality and growth in DTB-affected trees.

Further research on the prevalence of endophytic *D. sapinea* and the mechanisms of symptomatic infections is needed to be able to make accurate predictions of the impact of DTB in Swedish forests in a changing climate.

4.2 Co-infection by *M. pinitorqua* and *D. sapinea* in Scots pine

In **papers II** and **III**, we investigated the tree responses to simultaneous infections by *M. pinitorqua* and *D. sapinea*, and the genetics behind susceptibility to both pathogens. We used a site in Västmanland, central Sweden, that was established in 2015. Damage caused by *M. pinitorqua* was reported by forest managers in 2017, and DTB was confirmed in affected trees in 2020. This motivated an in-depth study of the possible triggering effect of *M. pinitorqua* infection on DTB development. DTB is mainly associated with trees experiencing abiotic stress (e.g., Zwolinski *et al.* 1990; Swart & Wingfield 1991; Stanosz *et al.* 2001; Bihon *et al.* 2011), but based on visual assessment, the trees in the studied stand did not exhibit any indicators of abiotic stress. On the contrary, these trees demonstrated robust growth rates and showed no evidence of drought stress or mechanical damage. However, since various other stressors have been suggested to facilitate DTB (Zwolinski *et al.* 1995; Luchi *et al.* 2012; Davydenko *et al.* 2017; Davydenko & Baturkin 2020), we hypothesised that the presence of *M. pinitorqua* may also contribute to conditions that favour DTB development, either by releasing endophytic infection into active pathogenicity or by providing entry points for spore infection.

4.2.1 Scots pine responses to infection by *M. pinitorqua* and *D. sapinea* (paper II)

Symptom development and pathogen load

The disease categories established based on 2020 symptoms remained consistent throughout the study period in **paper II**. Healthy-looking (H) trees consistently had the fewest *M. pinitorqua*-infected shoots and remained principally free of DTB. *M. pinitorqua*-symptomatic (M) trees showed high levels of pine twisting rust but low DTB incidence, while *M. pinitorqua* and *D. sapinea*-symptomatic (MD) trees had high levels of both. Furthermore, trees that were healthy in 2020 got infected by *M. pinitorqua* later in 2021 compared to trees in the other categories.

M. pinitorqua biomass was higher in symptomatic shoots and decreased from the first to the third time point. The biomass of *D. sapinea* increased significantly over the three time points, and there was no significant difference in its abundance between *M. pinitorqua*-symptomatic and

asymptomatic shoots (Figure 9a). Trees in the H category had lower *M. pinitorqua* DNA copy numbers compared to M and MD trees. However, there was no significant difference in *D. sapinea* DNA abundance between the disease categories (Figure 9b).

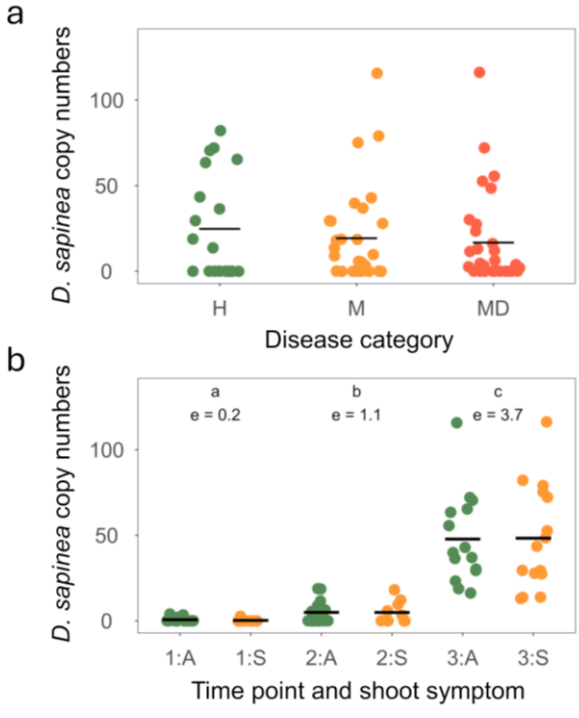

Figure 9. Abundance of *D. sapinea* DNA in **paper II** (qPCR copy numbers normalised to Scots pine DNA). a) By disease category based on 2020 symptoms: H – healthy-looking (green), M – *M. pinitorqua*-symptomatic (yellow), MD – symptomatic for both pathogens (red). b) By time point (1 – June 2; 2 – June 10; 3 – July 8, 2021) and shoot symptom status: A – asymptomatic (green), S – *M. pinitorqua*-symptomatic (yellow). Black lines show means; different letters indicate significant group differences (TukeyHSD, $p < 0.05$). e shows estimated marginal means of log(1+x).

The lower *M. pinitorqua* infection rates and DNA levels in healthy-looking trees suggest a higher level of tolerance within this population. This variation in susceptibility might be linked to "defeated resistance (R) genes", i.e., resistance genes that have been overcome by the pathogen but may still confer residual protection (Dowkiw & Bastien 2006).

The similar levels of *D. sapinea* DNA in symptomatic and asymptomatic shoots indicate that the lesions resulting from *M. pinitorqua* infections were

not specific entry points for *D. sapinea*, and local stress or healing did not obviously facilitate *D. sapinea* colonisation. *D. sapinea* was long believed to be a wound-infecting pathogen, often connected to especially hail-damage. However, since it is now acknowledged as a common endophyte in healthy pines, becoming virulent under stress, the wound-infecting theory is considered outdated. Tree mortality after wounding is likely caused by the activation of endophytic infections (Wingfield *et al.* 2024).

Metabolic responses

Both amino acid and phenolic compound profiles showed significant differences across the three sampling time points in **paper II**. Several metabolites were identified as indicators for each specific time point.

At the first sampling time point, the composition of phenolic compounds in asymptomatic shoots differed significantly between the disease categories (Figure 10). Specifically, the total concentration of phenolics was lower in asymptomatic shoots of H category trees. No such association was found for the amino acid profile at this time.

The amino acid profile differed between *M. pinitorqua*-symptomatic and asymptomatic tissue, with aspartate, leucine, and phenylalanine being indicators of symptomatic shoots. The differences were more pronounced for phenolic compounds, with proanthocyanidin B1, catechin, piceid, astringin, matairesinol, and taxifolin associated with symptomatic shoots, while kaempferol-3-O-glucoside and quercetin-glucoside were linked to asymptomatic shoots. Total amino acids and phenolics were higher in symptomatic shoots at the third time point.

The biomass of individual pathogens was not directly linked to the composition of amino acids or the concentration of individual metabolites. However, the interaction between the pathogens was connected to the composition of phenolics, and this effect was more pronounced when considering the interaction between *M. pinitorqua* symptoms and *D. sapinea* DNA abundance.

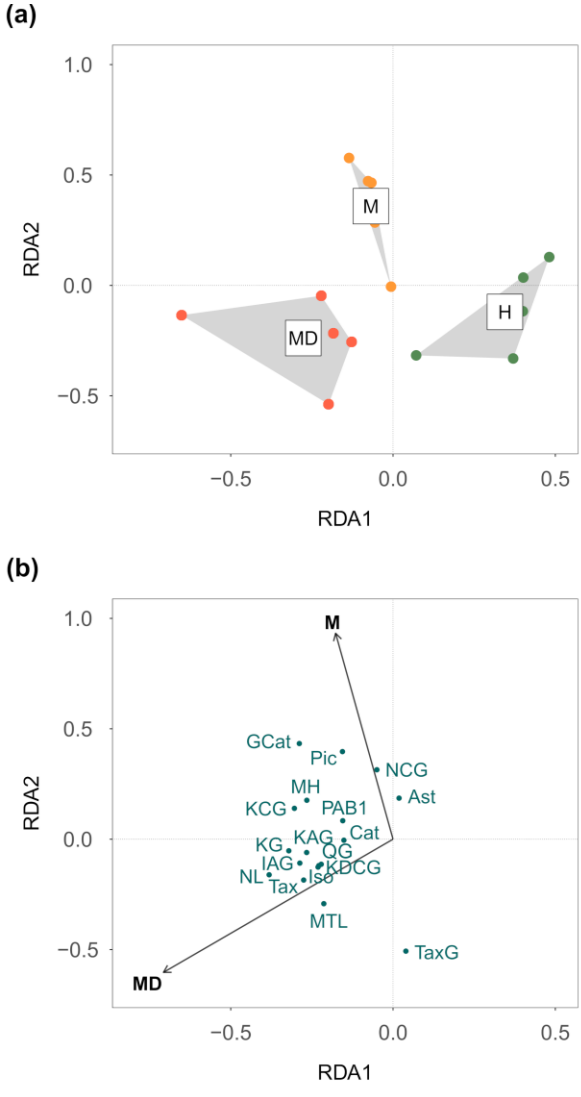

Figure 10. Composition of phenolic compounds in asymptomatic shoots at time point 1 (June 2, 2021) in **paper II**, grouped by disease category based on 2020 symptoms: H – healthy-looking, M – *M. pinitorqua*-symptomatic, MD – symptomatic for both pathogens. a) RDA with hulls connecting samples within each category. b) RDA with compound loadings. Disease category was significantly associated with phenolic composition (RDA; permutational ANOVA). For full compound names, see Stein Åslund *et al.* (2025).

The fact that *M. pinitorqua* symptoms had a stronger effect on the shoot metabolites than the actual biomass of the fungus suggests that the tree's response may be triggered by the recognition of the infection rather than the quantity of the pathogen present.

The *D. sapinea* infections were likely latent or presymptomatic during the sampling period, and the characteristic DTB symptoms were mainly observed later in the autumn. The more pronounced metabolic changes associated with *D. sapinea* reported in other studies (Sherwood *et al.* 2015; Caballol *et al.* 2022b; Ghosh *et al.* 2022) might be linked to its transition to a necrotrophic lifestyle rather than the initial host response.

Tree vitality

In **paper II**, a clear association was found between tree vitality in 2021 and the disease symptoms observed in 2020. All H trees were fully vital in 2021. M trees were either fully vital or mildly affected, while the majority of MD trees were severely affected. These patterns support the hypothesis that prior disease impacts the tree's subsequent health. Combined with the sporulation patterns reported in **paper I**, it is plausible that previously infected trees are more likely to develop fruiting structures, potentially resulting in a higher spore load than uninfected individuals. This may contribute to a positive feedback loop that reinforces further infection.

The poorer vitality of MD trees could be due to the combined effects of both pathogens, potentially through altered microbiome composition, toxin production, or suppressed immunity (Liu *et al.* 2023). The differences in total phenolics in asymptomatic tissues at the first time point across disease categories could be a consequence of systemic phenolic accumulation induced by previous pathogen challenges or a genetic effect (Halliday *et al.* 2018).

Conclusion paper II

In conclusion, we did not find direct support for the hypothesis that *M. pinitorqua* infection predisposes Scots pines to DTB by stressing the host or altering metabolite profiles in a way that directly facilitates *D. sapinea* colonisation. However, we observed that some trees remained largely unaffected by either pathogen, implying that certain individuals may possess mechanisms that limit pathogen development or reduce symptom expression. This indicates variation in susceptibility within the Scots pine

population, with some trees exhibiting lower sensitivity to *M. pinitorqua* and *D. sapinea*. This variation deserves further attention.

4.2.2 Genetics behind susceptibility to *M. pinitorqua* and *D. sapinea* in Scots pine (paper III)

The study presented in **paper III** aimed to investigate the genetic basis of variation in susceptibility to *M. pinitorqua* and *D. sapinea* in Scots pine. Based on the assumption that these traits have a genetic component, we aimed to identify genetic markers and genomic regions associated with reduced disease severity, evaluate their potential functional roles, and validate their effects.

Disease symptoms and spatial factors

High levels of *M. pinitorqua* infections were observed across all surveyed trees. No significant spatial patterns were found for disease symptoms, damage, or growth (Figure 11). Aspen tree density did not correlate with *M. pinitorqua* infection. Growth showed a significant positive relationship with top whorl *M. pinitorqua* infection (Mp^{W1}) and a significant negative relationship with second whorl infection (Mp^{W2}), both with small effect sizes. Significant positive effects of damage on *D. sapinea* symptoms (Ds) and of the interaction between Mp^{W2} and damage on Ds were observed.

The consistently high *M. pinitorqua* infection rate across the site, likely due to uniformly high aspen density, resulted in no observed spatial effects on rust symptoms. The similar planting conditions likely contributed to the lack of spatial effects on other traits as well, making the site well-suited for genetic studies. The lower variation in Mp^{W1} compared to Mp^{W2} might be due to significant shoot loss in the top whorl. Faster-growing pines have been linked to increased susceptibility to *M. pinitorqua* (Klingström 1963; Martinsson 1985; Desprez-Loustau & Dupuis 1994; Desprez-Loustau & Wagner 1997), though the pathogen can also negatively impact tree growth (Martinsson 1985). The contradictory relationship between growth and *M. pinitorqua* susceptibility observed in previous studies might explain the minor and opposite effects found.

The stronger effect of the Mp^{W2} × damage interaction on Ds demonstrates *M. pinitorqua*'s involvement in the *Ds*-damage relationship, although it does not play a major role. The observed positive correlations between damage and each of the traits, Ds and Mp^{W2}, support the conclusion that both

pathogens independently affect tree health. While their interaction does not seem to be a major driver, these findings suggest that the presence of either pathogen is associated with increased levels of damage, emphasising their individual roles in influencing overall tree vitality.

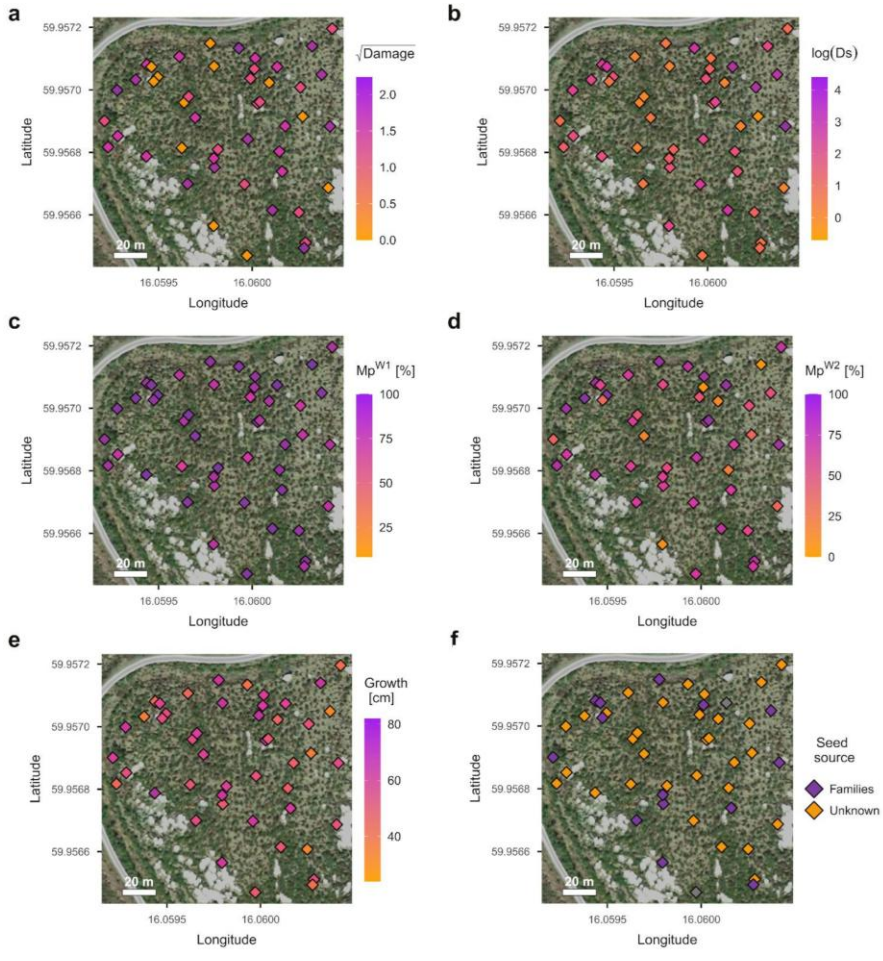

Figure 11. Spatial distribution of a) square root of the damage score (damage), b) number of *D. sapinea*-symptomatic shoots (Ds) on a logarithmic scale, c) percentage of *M. pinitorqua*-infected shoots in the top whorl (Mp^{W1}), d) percentage of *M. pinitorqua*-infected shoots among 15 random shoots in the second whorl (Mp^{W2}), e) height growth, and f) seed source for the 47 trees for which coordinates were recorded in **papers II** and **III**. Missing values are shown as grey symbols.

While complete resistance to *M. pinitorqua* was not observed, variations in symptoms and damage indicated differences in tolerance. The case of *D. sapinea* presents a more nuanced pattern of disease development. In **paper II**, *D. sapinea* was detected at low levels across both symptomatic and asymptomatic tissues early in the season, and **paper I** showed similarly low biomass in asymptomatic shoots and at healthy sites. However, only some trees and shoots later developed symptoms, pointing to a host-mediated difference in disease progression (Badet *et al.* 2019). If *D. sapinea* biomass increases across all trees, but symptoms only develop in some, this would imply that certain trees allow uncontrolled fungal growth – while others do not. Rather than reflecting tolerance, this pattern aligns more closely with partial resistance, where the host limits fungal development before symptom expression (Rowe & Kliebenstein 2008; Badet *et al.* 2019).

This observation aligns with the biology of necrotrophs and hemibiotrophs. Many such pathogens exhibit latent, endophytic, or biotrophic-like stages before initiating host cell death, making the boundary between hemibiotrophy and necrotrophy difficult to define (Corwin & Kliebenstein 2017; Badet *et al.* 2019; Petrasch *et al.* 2021). Resistance against these pathogens is often quantitative, shaped by a combination of structural barriers and inducible defences (Rowe & Kliebenstein 2008; Corwin & Kliebenstein 2017; Badet *et al.* 2019). Given this, describing asymptomatic trees as less susceptible to *D. sapinea*, rather than tolerant or resistant, may better reflect the biology of the interaction and support a more accurate interpretation of disease outcomes in Scots pine.

Pedigree reconstruction

Genotyping was successful for 542 individuals from the experimental site and 100 parental trees, and 20,654 SNPs were retained for analysis. We observed no genetic clustering explaining differences in disease symptoms, which supports the interpretation that resistance is governed by quantitative traits.

The pedigree reconstruction revealed two distinct populations: 179 individuals with at least one inferred parent from the seed orchard and 363 unrelated individuals. Nineteen families with more than three individuals were identified. The unexpected discovery of two distinct populations within the same environment provided a unique opportunity for comparison and validation.

Phenotypic variation among reconstructed families

Reconstructed families showed significantly different levels of disease and damage (Figure 12). Five families had consistently high values for damage, Mp^{W2}, and Ds. Three families consistently exhibited low values for these traits. Significant differences in the relationships between Ds and Mp^{W2}, Ds and damage, and Mp^{W2} and damage were found among specific families compared to the unrelated population.

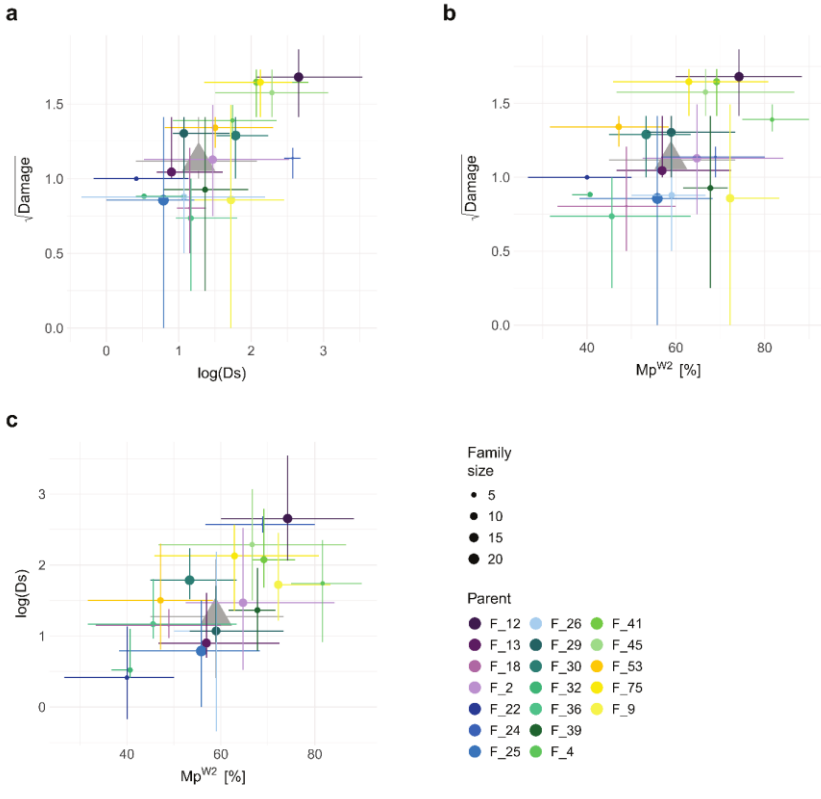

Figure 12. Scatterplots showing phenotypic variation among families in **paper III**, with coloured points scaled by family size. Grey triangles represent data from a population of unknown origin. Plots display: a) mean number of *D. sapinea*-symptomatic shoots (Ds, log scale) vs mean damage score (square root), b) *M. pinitorqua*-infected shoots in the second whorl (Mp^{W2}) vs damage, and c) Mp^{W2} vs Ds. Error bars show 25th and 75th percentiles. Significant differences between families were found for all traits, and relationships between traits varied by family (ANOVA).

Significant differences in disease symptoms and damage levels among the reconstructed families highlight the genetic component of susceptibility to both diseases, supporting the idea of quantitative resistance. Families exhibiting consistently high or low susceptibility to both pathogens point to underlying genetic factors influencing overall vulnerability. The identification of such families and their parental trees has implications for breeding programmes, since integrating disease resistance alongside traits like growth and climate adaptability can contribute to the development of more robust populations.

Genetic associations with disease traits and QTL validation

GWAS on the unrelated population identified 26 SNPs associated with Ds, 21 with Mp^{W1}, and one with Mp^{W2} at an FDR p-value < 0.05. One SNP was associated with both Ds and Mp^{W1}. Five SNPs were associated with the interaction between Mp^{W1} and damage, and one SNP with Mp^{W2} × damage. Two SNPs were associated with Mp^{W2} × Ds × damage. No SNPs passed the significance threshold for damage alone. Among the SNPs identified in the univariate GWAS on Ds, one aligned with a WUSCHEL homeobox protein (WOX3), and others with leucine-rich repeat-containing proteins.

Analyses on the parental genotype showed significant associations between mean family Ds and three SNPs. Analyses on progeny genotypes revealed significant associations between individual Ds levels and four SNPs. One SNP was significant in both parental and progeny analyses. Among the Ds-associated SNPs validated in the reconstructed families, two were in or near genes with predicted proteins similar to a member of the ubiquitin-specific protease gene family, and a near-significant SNP for the mean family Ds in the parental analysis aligned with a Scots pine aspartate transaminase gene.

The identification of QTLs primarily associated with individual disease symptoms and damage traits in the GWAS implies that these phenotypes are governed by distinct genetic controls. The validation of several QTLs for *D. sapinea* symptoms (Ds) in the reconstructed families strengthens the relevance of these loci.

Detection of significant SNPs in or near genes involved in protein deubiquitination (UBP family), amino acid metabolism (aspartate transaminase), pathogen recognition (leucine-rich repeat proteins), and developmental processes (WOX3) provides interesting insights into potential mechanisms of disease resistance. The involvement of a UBP

protein suggests a role for post-translational regulation in stress responses (Zhou *et al.* 2017). The association with aspartate transaminase aligns with previous findings linking free amino acids to DTB development (Sherwood *et al.* 2015; Oliva *et al.* 2020; Hu *et al.* 2023). The identification of putative receptor proteins indicates a potential importance of pathogen recognition in quantitative disease resistance (Weiss *et al.* 2020; Liu *et al.* 2024). The association with WOX3, involved in stress responses in *P. massoniana* (Wang *et al.* 2024a), warrants further investigation in the context of *D. sapinea* interaction in Scots pine.

Conclusion paper III

Paper **III** provides valuable insights into the genetic factors influencing susceptibility to *M. pinitorqua* and *D. sapinea* in Scots pine, and highlights families that may be useful in future breeding efforts. Functional validation of the identified SNPs, along with gene expression analyses, is needed to further elucidate the mechanisms underlying variation in susceptibility and resistance. Additional studies across diverse Scots pine populations are also necessary to clarify the genetic architecture of resistance and the role of *D. sapinea* in interaction with other stressors.

4.3 Genomic selection to identify Norway spruce progenies resistant to *H. parviporum* (paper IV)

To assess the potential of genomic selection for resistance breeding, we applied genomic selection (GS) methods to predict resistance to *H. parviporum* in Norway spruce. We phenotyped 1,464 plants from deployment zone G5 and 120 from G6, with 876 and 66 genotyped individuals retained after quality filtering, respectively. Pedigree correction in the G5 population, based on the genomic relationship matrix, resulted in the exclusion or reassignment of several individuals and families, yielding 763 individuals across 46 families for the final analyses (Figure 13). After initial filtering, 45,253 SNPs were retained for genomic analyses in G5.

Variation in resistance traits

We observed considerable variation in both the extent of lesions (LL) and fungal growth in the sapwood (SWG) within the studied population. Significant differences were found between families in their average levels of both lesion length and sapwood growth.

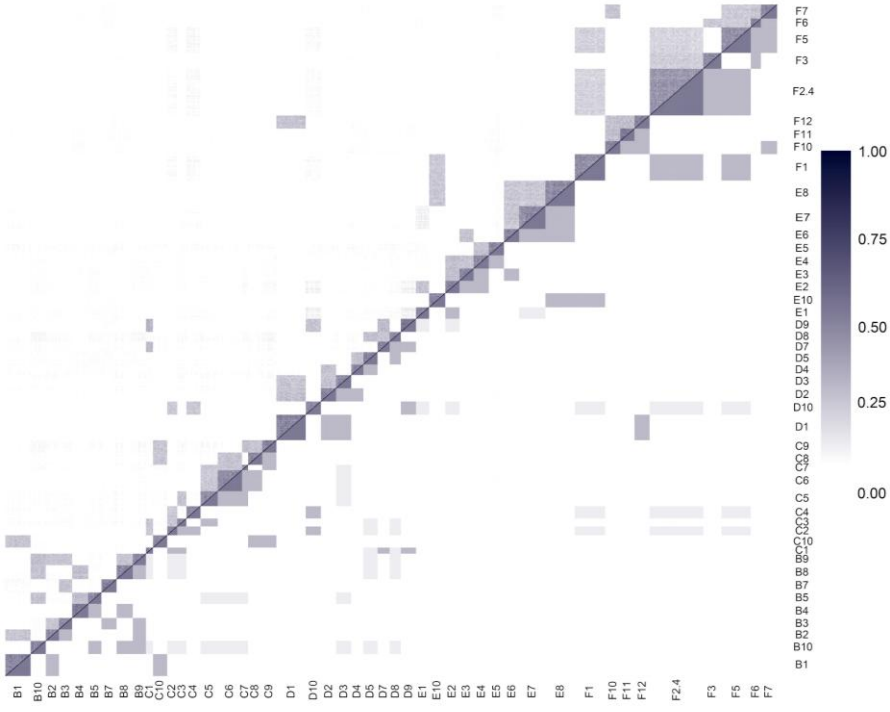

Figure 13. Heat map of relationship coefficients among individuals in **paper IV** (n = 763) grouped by families (n = 46) using the pedigree-derived relationship (A, below diagonal) and the genomic relationship (G, above the diagonal) after pedigree correction.

Similarly, the estimated breeding values for families and parent trees also showed significant variation. We identified families exhibiting limited pathogen spread and favourable breeding values for both LL and SWG, while others showed conflicting patterns, such as short sapwood colonisation but extensive lesion development. Progenies from certain parent trees had consistently higher average values and less desirable breeding values, whereas others displayed the opposite trend. This implies that the population possesses the genetic diversity needed to potentially improve resistance through selective breeding.

Heritability estimates for both lesion length and sapwood growth were found to be in the lower range of previously reported values (Karlsson & Swedjemark 2006; Arnerup *et al.* 2010; Steffenrem *et al.* 2016; Chen *et al.* 2018b). Genome-based (GBLUP) heritability estimates were slightly lower than pedigree-based (ABLUP) heritability estimates, which aligns with other

studies (Nadeau *et al.* 2023). The levels of additive genetic and phenotypic variation for lesion length were comparable to or stronger than previous studies (Steffenrem *et al.* 2016; Chen *et al.* 2018b), indicating strong potential for genetic gain for this trait through selection. While the variation for sapwood growth was somewhat weaker, the overall findings still suggest that meaningful improvements in resistance can be achieved. The ability to identify both families and individual parent trees with significantly different levels of resistance further supports this conclusion.

Prediction models and cross-validation

Prediction models showed that a 9:1 training-to-validation split yielded the highest predictive accuracy for both lesion length and sapwood growth, although these results were somewhat unstable. A training-to-validation set ratio of 7:3 provided similar and more stable predictive ability for both pedigree-based and SNP-based methods when predicting within the original population for both traits. The predictive accuracy also showed similar values between the two methods (Table 3). The comparable predictive abilities and accuracy estimates of the pedigree-based and genome-based models have been observed also in other GS studies on conifers (Tan *et al.* 2017; Chen *et al.* 2018a; Calleja-Rodriguez *et al.* 2020; Chen *et al.* 2019; Lenz *et al.* 2020b; Nadeau *et al.* 2023) and demonstrate that genome-based models effectively capture the pedigree structure and the genetic variation relevant to these traits.

Table 3. Predictive ability (PA), predictive accuracy (PAC), and rank correlation (Spearman), with standard errors (SE), estimated using ABLUP and GBLUP based on 100 cross-validations (training:validation ratio 7:3) in **paper IV**.

Trait	**Model**	**h^2**	**PA**	**SE_{PA}**	**PAC**	**SE_{PAC}**	**Rank Cor**	**$SE_{RankCor}$**
LL	ABLUP	0.437	0.399	0.004	0.603	0.007	0.395	0.004
	GBLUP		0.379	0.004	0.574	0.007	0.380	0.004
	ABLUP-AD	0.302	0.399	0.004	0.726	0.008	0.394	0.004
	GBLUP-AD		0.377	0.004	0.687	0.008	0.378	0.004
SWG	ABLUP	0.209	0.256	0.005	0.559	0.010	0.261	0.005
	GBLUP		0.250	0.005	0.546	0.011	0.256	0.005

This supports the potential for genomic selection as a valuable tool for integrating resistance breeding into Norway spruce breeding programmes, especially considering the challenges of traditional resistance testing. However, when the models trained on the original population were used to predict resistance in an unrelated population, the predictive ability significantly decreased for both lesion length and sapwood growth. Similar patterns have been seen in other GS studies (Shalizi *et al.* 2022; Chen *et al.* 2023; Nadeau *et al.* 2023). Notably, the reduction was less pronounced for sapwood growth compared to lesion length. This significant reduction highlights the importance of genetic relatedness between the training and prediction populations for genomic selection to be effective. The relatively smaller decline in predictive ability for sapwood growth suggests that models for this trait might be somewhat more robust across different genetic backgrounds. One possible explanation is that lesion length, but not sapwood growth, has been shown to vary significantly across different origins in Norway spruce (Capador-Barreto *et al.* 2021).

Alternative models using smaller, random sets of SNPs showed predictive abilities and accuracy estimates for lesion length and sapwood growth that were comparable to those obtained using the entire SNP dataset. This indicates that a reduced number of markers can capture most of the relevant genetic variation for resistance in this population, consistent with findings from several other studies (Bartholomé *et al.* 2016; Chen *et al.* 2018a; Lenz *et al.* 2020b). Marker reduction can also lower the risk of overfitting in genomic prediction models (Tan *et al.* 2017; Chen *et al.* 2018a; Stocks *et al.* 2019; Cappa *et al.* 2022; Chen *et al.* 2023). Maintaining an appropriate balance between marker density and sample size is essential to preserve statistical power, which is crucial for effective GS modelling (Crossa *et al.* 2017). The use of relatively small families – some with as few as six individuals after pedigree correction (Figure 13) – likely reduced model accuracy. According to Papin *et al.* (2024), training sets should include 1,600-2,000 individuals, with at least 40–65 per full-sib family for within-family predictions. Within-family selection is the ultimate goal since it improves genetic gain through precise selection among relatives while enabling the preservation of genetic diversity. Larger family sizes or more precise phenotyping, such as repeated inoculations on clones of each progeny, could potentially improve the prediction ability and accuracy of the models.

The study design for **paper IV**, outlined in 2020, predated the publications of more recent research highlighting the necessity of larger training populations for achieving accurate predictions in GS. This acknowledges the rapid advancements in the field of genomics and the ongoing learning process in optimising GS strategies for forest trees. In addition, we experienced a significant loss of individuals after pedigree correction, which was attributed to unexpectedly complex population structures. This points out a common challenge in genetic studies of forest populations, where intricate structures can complicate the accuracy of pedigrees, which is crucial for many genetic analyses, including genomic selection.

Conclusion paper IV

In conclusion, our study demonstrates that sufficient genetic variation exists within the studied Norway spruce breeding population to improve resistance to *H. parviporum*. It also shows that genomic selection is a promising tool for supporting the integration of resistance breeding into the programme, but improvements in training population size and experimental design – particularly the inclusion of more biological replicates – are needed to enhance model prediction accuracy. Furthermore, our results underline the need to evaluate the genetic robustness of LL and SWG across different populations and develop selection indices that incorporate disease resistance to enable its inclusion in forest tree breeding strategies.

5. Conclusion

5.1 Summary of key findings

This thesis contributes to a deeper understanding of the genetic and environmental factors and mechanisms influencing disease development in conifers. It highlights the role of stress in exacerbating pathogen effects, explores the interactions between different pathogens and potential mechanisms underlying variation in disease susceptibility, and identifies genetic factors associated with disease susceptibility. Furthermore, it investigates the application of genomic selection as a tool for breeding disease-resistant conifers, while also noting the need for continued research and refinement of these approaches.

A central theme in the studies on *D. sapinea* (**papers I-III**) is the presence of the fungus in asymptomatic tissue. *D. sapinea* is primarily investigated when it causes noticeable symptoms. The prevalence of latent *D. sapinea* infections in Sweden is not well known, but it is not unlikely that the fungus could be present in forest stands throughout Sweden without causing disease. This highlights the importance of understanding the factors that can weaken conifers and increase their susceptibility to opportunistic pathogens. It also raises broader questions about fungal strategies in forest pathosystems and our understanding of epidemiology, susceptibility, and resistance as applied in risk assessments, management, and breeding strategies. The existence of hemibiotrophic and latent necrotrophic phases suggests that building up biomass before initiating host cell death is an evolutionarily successful infection strategy (Corwin & Kliebenstein 2017; Badet *et al.* 2019). However, latent or endophytic stages make accurate assessments of outbreak risks, as well as selection in resistance breeding programmes, more complex and uncertain.

Paper I demonstrates the association between environmental stress and an opportunistic pathogen in Scots pine and underscores that stressed trees are more susceptible to the damaging effects of *D. sapinea*. **Papers II** and **III** investigate the interactions between *M. pinitorqua* and *D. sapinea*, as well as the genetic variation underlying Scots pine responses to co-infection. In **paper II**, we found that disease symptoms caused by both pathogens remained consistent over time, and previous symptoms influenced the tree's

metabolic state. We did not find direct evidence that *M. pinitorqua* infection predisposes Scots pines to *D. sapinea* infection by increasing stress or altering metabolites. However, we identified healthy-looking trees that exhibited *M. pinitorqua* tolerance and reduced susceptibility to *D. sapinea*. Building on this, **paper III** showed that genetic factors are significantly associated with symptom severity of both pathogens, as reflected in the varying disease levels observed among reconstructed families. We identified specific genetic markers and genomic regions associated with symptoms of both diseases, including genes potentially involved in stress responses, pathogen recognition, and metabolic processes. These findings indicate that breeding programmes could potentially select for Scots pine with enhanced tolerance to these diseases. Overall, the studies on *D. sapinea* highlight the necessity of understanding not only the host's vitality and stress physiology but also the pathogen's biology, behaviour, biological interactions, and distribution in the landscape.

Paper IV focused on the potential of genomic selection (GS) for improving resistance to *H. parviporum* in Norway spruce. We demonstrated that sufficient genetic variation exists within the studied Swedish breeding population to allow for improvement in resistance through selective breeding. Our results display that genomic selection is a promising tool for integrating resistance breeding into Norway spruce programmes but demonstrate that study design is essential: phenotyping precision and larger training populations are needed to enhance the accuracy of genomic selection models for *Heterobasidion* resistance.

5.2 Methodological challenges, reflections, and implications for future research

Building on the findings of this thesis, several promising directions emerge for future research and breeding applications.

Temporal and spatial heterogeneity in disease symptom expression

The majority of the studies in this thesis were conducted in a natural environment, which led to various unforeseen challenges for which adaptation in study design and data collection was required. This emphasises the dynamic nature of ecological research and the importance of flexibility in experimental design to address unexpected observations. For example, the

surveys of defoliation in current-year shoots in **paper I** and damage assessments in **papers II** and **III** were not initially planned but were implemented due to unforeseen manifestations of disease symptoms.

Moreover, the findings presented in **papers II** and **III** are based on observations from the Ängelsberg site, which experienced unusually high levels of co-infection by *M. pinitorqua* and *D. sapinea*. While this created a valuable context for studying natural variation in susceptibility, the site itself is atypical in several ways. It had been previously affected by fire, featured sparse ground vegetation and was characterised by high local aspen density – all factors that may have influenced pathogen pressure and disease expression. As such, the site may be considered an ecological outlier within the Swedish forest landscape. Despite this, the results clearly demonstrate genetic variation in susceptibility under severe natural infection and provide a strong foundation for identifying resistant genotypes and understanding host-pathogen interactions in Scots pine.

Understanding the latent-to-pathogenic transition

To better understand what triggers the switch from latent colonisation to symptomatic disease in Scots pine, future studies should combine *D. sapinea* inoculation with different combinations of abiotic and biotic stress treatments. Trials should include time-course sampling to monitor fungal biomass, symptom progression, and host responses across different stress conditions. Gene expression profiling, histological examination of tissue damage, and ROS staining could be used to trace changes in host defence activity and pathogen behaviour over time. Metabolite profiling and hormone measurements may also provide insights into whether systemic responses (e.g., SAR or ISR) are involved in limiting disease development.

Paper II raises an ecological question about the dynamics of *D. sapinea* colonisation during the summer months preceding visible DTB symptoms. Since DTB symptoms typically appear in late summer or autumn, the increase in *D. sapinea* DNA observed in June and July indicates that the fungus is already active during a presymptomatic phase. What remains unresolved is whether this biomass accumulation reflects benign endophytic growth, a gradual transition toward pathogenicity, or the early, asymptomatic phase of disease development. Recent work by Oostlander *et al.* (2024), which visualised fluorescently tagged *D. sapinea* in symptomless pine tissues, showed that this early phase involves spatially organised hyphal growth rather than static presence. Their transformation protocol may be

useful for future studies aiming to clarify whether symptom development is preceded by gradual fungal activity or triggered by a more abrupt switch.

Since we could not find direct evidence that *M. pinitorqua* predisposes trees to DTB but observed an association with later disease development, further investigation is needed to determine how the biotroph affects the trees in a way that benefits *D. sapinea*. This could be investigated through gene expression analysis, hormone profiling, and histological examination of rust-infected shoots. Ideally, such studies would include controlled inoculations of *M. pinitorqua*, although this would require collecting basidiospores from aspen leaves and carefully timing inoculation during pine shoot flushing.

By identifying the conditions under which *D. sapinea* becomes pathogenic and clarifying the type of infection and resistance observed, future research can provide a stronger foundation for effective resistance screening and selection of Scots pine genotypes with lower susceptibility.

Marker validation for breeding

The markers identified as associated with disease severity in Scots pine in **paper III** should be further validated in larger, more genetically diverse populations and across different environments to assess genotype × environment interactions. Combining genomic data with repeated field observations could help refine estimates of trait heritability and stability over time. For *D. sapinea*, studies that link genotypic variation in the host to pathogen biomass at different infection stages would help clarify whether resistance acts through limiting colonisation, delaying symptom onset, or reducing fungal growth. Additionally, identifying transcriptomic or metabolic markers associated with reduced symptom severity could support the development of early selection tools. Together, these approaches could contribute to a more robust genetic framework for integrating resistance traits into Scots pine breeding.

While **paper III** does not represent a full breeding-without-breeding (BwB) approach, it demonstrates that informative family-level variation in resistance can be recovered through genotyping even when pedigree records are incomplete. This approach could be applied in future studies involving open-pollinated or partially documented populations to support selection efforts. The findings on *M. pinitorqua* show that the phenotyping method used can reveal genetic differences in tolerance under natural infection and may help identify tolerant genotypes in broader, less well-documented plant material.

Integration of genomic selection in conifer breeding

El-Kassaby *et al.* (2024) notes that climate change is constantly altering breeding zones, which may reduce the relevance of existing test sites for identifying genotypes tolerant to emerging stresses. Therefore, it is crucial to continue testing for resistance on a large scale and across diverse environments to ensure the identification and deployment of conifer varieties that can withstand future climate conditions. This reinforces the importance of utilising genomic selection for early selection in the context of the ongoing climate change.

Despite several methodological and unforeseen study design issues, **Paper IV** demonstrated the potential of genomic selection (GS) to improve resistance to *H. parviporum* in Norway spruce. To support its integration into operational breeding, GS models should be trained on large, genetically diverse datasets with sufficient family sizes. Increasing the number of genotyped and phenotyped individuals per family will improve model accuracy and allow for robust within-family predictions – an essential step for selecting top-performing individuals while maintaining genetic diversity. Testing GS models across deployment zones and environmental conditions will also be important to ensure their broad applicability and reliability.

Both resistance traits assessed in **paper IV** – lesion length (LL) and sapwood growth (SWG) – represent distinct but complementary aspects of the host response to *H. parviporum*. LL showed greater variation among families and higher predictive ability within the population, making it a promising candidate for early selection. SWG, on the other hand, was more consistent across unrelated individuals, indicating it may offer advantages in broader or cross-population applications. Including both traits in selection may allow for a more complete assessment of resistance and improve the identification of robust, disease-tolerant genotypes.

Finally, *Heterobasidion* resistance traits should be incorporated into selection indices alongside growth and wood quality, ensuring that disease resistance can be integrated into practical breeding goals without compromising other priorities.

Closing reflections

The synopsis of these observations reflects the complexity of studying conifer diseases – from the latent behaviour of some pathogens and the influence of environmental stress to the need for flexible research

approaches, the limitations of current biological tools, and the continued development of methods like genomic selection. The findings collectively suggest that breeding for disease resistance in conifers is a viable strategy but requires a deeper understanding of the underlying genetic mechanisms and consideration of environmental factors and pathogen interactions. Importantly, my thesis reveals the pressing need for continued research and large-scale testing in the face of climate change to ensure the long-term health and resilience of conifer forests.

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Popular science summary

Forests are critical for biodiversity, climate regulation, and economic sustainability. In Sweden, coniferous forests dominated by Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) are central to both the country's ecosystem services and its forestry sector. However, they are increasingly threatened by fungal pathogens (fungi that can cause disease), whose impact is intensifying under a changing climate.

This thesis examines how conifer–pathogen interactions unfold in Swedish forests, with a focus on the genetic, physiological, and environmental factors that influence disease susceptibility. The work centres on three fungal pathogens of relevance in the Nordic region: *Diplodia sapinea*, an opportunistic necrotroph causing Diplodia tip blight linked to drought-stress in Scots pine; *Melampsora pinitorqua*, a rust fungus causing pine twisting rust; and *Heterobasidion parviporum*, the primary agent of root rot in Norway spruce.

The impact of drought stress on Scots pine susceptibility to *D. sapinea* was explored. Field studies confirmed that the fungus often occurs without visible symptoms, consistent with previous findings. However, when conditions are favourable, the fungus can become pathogenic. Observations from drought-affected stands showed that while severely damaged trees had high mortality, surviving individuals were able to recover. These results suggest that drought increases susceptibility to *D. sapinea*, with the fungus likely acting as an accelerating factor in tree decline.

An investigation into co-infection by *M. pinitorqua* and *D. sapinea* in Scots pine was carried out in trees that showed no signs of abiotic stress. It was hypothesised that biotic stress from rust infection might facilitate *D. sapinea* colonisation or disease development. However, no direct evidence was found that *M. pinitorqua* predisposes trees to *D. sapinea* by increasing stress or altering metabolites. The pattern of disease remained stable over multiple seasons, and disease symptoms in the preceding season were linked to changes in the tree's metabolic state. Genetic variation seemed to play a role in disease outcomes, with some trees apparently less sensitive to both pine twisting rust and Diplodia tip blight. Genetic analyses of the Scots pine population identified significant variation in susceptibility to both pathogens. Genome-wide association studies (GWAS) revealed loci (specific genetic regions) linked to disease symptoms, including genes

involved in pathogen recognition, stress response, and metabolism. Some of these loci could be validated in progeny from the seed orchard. The results suggest that variation in disease susceptibility is controlled by many genes and could be reduced through breeding.

In the last study of the thesis, genomic selection (GS) was evaluated as a practical breeding tool for improving disease resistance. GS is a method that uses DNA data to predict the levels of resistance in an individual. In Norway spruce, models built from both genetic and disease data showed good predictive ability for resistance to *H. parviporum* within a breeding population. This suggests that incorporating disease resistance into breeding programmes is feasible. However, the models were less accurate when applied to unrelated spruce populations, highlighting the importance of using representative data when developing predictive tools.

Together, these studies demonstrate that fungal pathogens can exploit both environmental stress and genetic susceptibility in trees, but also that there is meaningful variation in host responses. By combining ecology, forest pathology, and population genetics, this thesis contributes to a better understanding of forest disease dynamics and outlines practical strategies for breeding more resilient conifer populations. In the context of climate change, such approaches are essential for safeguarding the ecological and economic value of boreal forests.

Populärvetenskaplig sammanfattning

Skogar är avgörande för biologisk mångfald, klimatreglering och ekonomisk hållbarhet. I Sverige är de boreala barrskogarna, som domineras av tall (*Pinus sylvestris*) och gran (*Picea abies*), viktiga för både ekosystemtjänster och skogsbruket. Våra skogar hotas dock i allt högre grad av svampsjukdomar, vars skadeeffekter förvärras i takt med att effekterna från klimatförändringarna blir mer kännbara.

Den här avhandlingen undersöker hur interaktioner mellan barrträd och patogener (mikroorganismer som kan orsaka sjukdom) utvecklas i svenska skogar, med fokus på genetiska, fysiologiska och miljöfaktorer som påverkar sjukdomsmottagligheten hos träden. I avhandlingen studeras interaktionerna med tre svamppatogener som är relevanta i Norden: *Diplodia sapinea*, en opportunistisk nekrotrof som orsakar Diplodia-sjuka och är kopplad till torkstress i tall; *Melampsora pinitorqua*, en rostsvamp som orsakar knäckesjuka; och *Heterobasidion parviporum*, den vanligaste orsaken till rotröta i gran.

I den första delstudien studerades effekten av torkstress på tallens mottaglighet för *D. sapinea*. Studien bekräftade att svampen ofta förekommer i tallar även utan synliga symptom, vilket stämmer överens med tidigare studier. Under förhållanden som är gynnsamma för svampen kan den dock bli bli patogen. Observationer från torkdrabbade bestånd i vår studie visade att svårt skadade träd hade hög dödlighet, men att överlevande individer kunde återhämta sig. Resultaten tyder på att torka ökar mottagligheten för *D. sapinea*, där svampen sannolikt fungerar som en pådrivande faktor i trädens försämrade vitalitet.

Den andra delstudien handlade om samtidiga infektioner med *M. pinitorqua* och *D. sapinea* i tallar, planterade utanför Ängelsberg. Tallarna visade inga tecken på abiotisk stress. Hypotesen var att så kallad biotisk stress från rostinfektionen kunde underlätta kolonisering eller sjukdomsutveckling av *D. sapinea*. Inga direkta bevis hittades dock för att så var fallet. Sjukdomsbilden för varje enskilt träd förblev stabil över flera säsonger, och symptom från föregående säsong kunde kopplas till förändringar i trädens metabolitprofiler. Sannolikt påverkade genetisk variation sjukdomsutfallet, där vissa träd uppvisade lägre känslighet för både knäckesjuka och Diplodia-sjuka. Den möjligheten följdes upp i en separat studie. Genetiska analyser av tallpopulationen i Ängelsberg identifierade

betydande variation i mottaglighet för båda patogenerna. Helgenom-associationsstudier (GWAS) visade på flera loci (specifika genetiska regioner) som associerar med symptom på Diplodia-sjuka, inklusive gener involverade i signalering, stressresponser och metabolism. Ett antal av dessa loci kunde valideras i avkommor från fröplantaget Hade. Sammantaget tyder den här studien på att variationen i mottaglighet styrs av många gener och att den kan minskas genom förädling.

I avhandlingens sista delstudie utvärderades genomisk selektion (GS) som ett praktiskt verktyg i förädlingsarbete för att förbättra sjukdomsresistens. GS är en metod som använder DNA-profiler för att förutsäga individers resistensnivåer. I gran visade modeller baserade på både genetiska och symptomrelaterade data god förmåga att förutsäga resistens mot *H. parviporum* inom en förädlingspopulation. Detta tyder på att det är möjligt att inkludera sjukdomsresistens i framtida förädlingsprogram. Dock var modellerna mindre träffsäkra när de tillämpades på obesläktade granpopulationer, vilket understryker vikten av representativa data vid utveckling av prediktiva verktyg.

Tillsammans visar dessa studier att svamppatogener kan utnyttja både yttre stressfaktorer och genetisk mottaglighet hos träd, men också att det finns betydande variation i värdträdets respons. Genom att kombinera ekologi, skogpatologi och populationsgenetik bidrar denna avhandling till en ökad förståelse för skogssjukdomars dynamik och föreslår praktiska strategier för att förädla mer motståndskraftiga barrträdsbestånd. I ett föränderligt klimat är sådana angreppssätt avgörande för att skydda boreala skogars ekologiska och ekonomiska värde.

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I

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Diplodia sapinea as a contributing factor in the crown dieback of Scots pine (*Pinus* sylvestris) after a severe drought

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ABSTRACT

The frequency and impact of drought on global ecosystems have increased within the last century, while drought has affected tree health in many regions. *Diplodia sapinea* is a widespread, opportunistic pathogen infecting most conifers, causing Diplodia tip blight, thriving on hosts impacted by stress such as drought, heat, or mechanical wounding. In summer of 2018, a large-scale drought was recorded all over Europe. In late summer, pine trees all over Gotland showed crown dieback, where necrotic twigs and needles were found, especially in the upper part of the crowns. Symptoms were consistent with a potential outbreak of *D. sapinea*. Effects of the combination of drought and Diplodia tip blight on mortality or recovery of Scots pine in Nordic conditions are unknown. This study confirmed the presence and potential contribution of *D. sapinea* in the observed damages of Scots pine. Shoot blight and drought led to crown defoliation which was observed one year post-drought, while trees showed a clear recovery of newly grown shoots within the second year. Severely affected pines (>70% of the upper third of the crown with shoot blight) showed increased mortality. Recovery of the surviving trees was independent of previous dieback levels. *Diplodia sapinea* was most abundant in twigs with shoot blight of the symptomatic trees compared to healthy-looking twigs from the same trees and asymptomatic trees in affected and healthy pine stands. Sampling on affected and healthy sites showed possible endophytic infections with low abundance within healthy-looking twigs. Spore deposition of *D. sapinea* was monitored on healthy and affected sites for two consecutive years after crown damages occurred to confirm the presence of the opportunistic pathogen in the affected region. Spore deposition was observed during all seasons and correlated with high precipitation during sampling. Our observations provide insights into the emergence of Diplodia tip blight in the Northern countries and underline the potential impact of *D. sapinea* on tree health in the course of a changing climate.

1. Introduction

The frequency and thereby impact of drought on global ecosystems have increased within the last century (Chiang et al., 2021). Drought can significantly affect tree growth and vitality (Anderegg et al., 2015; Camarero et al., 2018). Tree growth could be partitioned in needle and leaf elongation, or shoot and stem growth, which are all influenced by climate, especially by temperature and water availability (Dobbertin et al., 2010). Drought-exposed Scots pines (*Pinus sylvestris* L.) react with closing stomata and loss of needle biomass (Dobbertin et al., 2010; Galiano et al., 2010), which can be related to reduced canopy development (Poyatos et al., 2013) and low growth rates (Galiano et al., 2011). Estimates of crown vitality can serve as a proxy for tree vigour (e.g. Rebetez and Dobbertin, 2004). Different measurements of crown vitality can provide distinct angles on the health status of a tree. Evaluating crown dieback involves estimating the ratio of deceased branches in relation to the entire healthy crown. This measurement serves as an early indication of reduced vigour and growth potential due to recent stresses or damage, including severe defoliation caused by factors like drought or defoliating agents. Crown transparency, on the other hand, quantifies the extent of a tree's healthy crown that becomes absent due to early needle loss. This transparency can be influenced by a range of causes, including diseases, environmental stresses, declining tree vigour, and decreased needle retention resulting from events like insect

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outbreaks or drought. Foliage transparency is a dynamic variable, rapidly changing within tree crowns and acting as a measure of defoliation or stress (Schomaker, 2007). By combining these approaches, a more comprehensive understanding of the tree's health, performance, and recovery trajectory can be achieved.

Pines are particularly plastic in their patterns of biomass allocation to leaves (Delucia et al., 2000; Martínez-Vilalta et al., 2004; Poyatos et al., 2013). Premature shedding of needles reduces whole tree transpiration (Whitehead et al., 1984; Martínez-Vilalta et al., 2014) and is presumably a last-chance mechanism to avoid hydraulic failure (Wolfe et al., 2016; Nadal-Sala et al., 2021). Crown dieback and associated symptoms can therefore be considered as indicators of reduced or poor vigour in Scots pine trees exposed to drought stress. Once drought stress is relieved the canopy can recover even in trees with relatively high rates of defoliation (Dobbertin et al., 2010; Eilmann et al., 2013).

In summer 2018, a large-scale drought was recorded all over Europe (Peters et al., 2020), and Scandinavia was among the regions that showed the highest temperature anomalies (Moravec et al., 2021). In Sweden, 2018 showed the warmest recorded mean temperatures in May. A third of the days from May to August were significantly warmer than the average since 1756 (Wilcke et al., 2020). The anomalies in temperature in combination with precipitation deficits impacted soil moisture, while increased 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. Trees were showing an overall discolouration of 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 caused by *Diplodia sapinea* (Fr.) Fuckel (syn. *Diplodia pinea* (Desm.) Kickx., *Sphaeropsis sapinea* (Fr.: Fr.) Dyko & Sutton).

Diplodia sapinea is an opportunistic forest pathogen, causing Diplodia tip blight on conifers. As a latent pathogen, it can be present in asymptomatic *Pinus spp*. (Luchi et al., 2011). Symptoms develop when its host is challenged by stress such as drought, heat, or mechanical wounding (e.g. Stanosz et al., 2001; Smith et al., 2002). It is well known from field observations (e.g. Swart et al., 1987a), as well as greenhouse studies (e.g. Johnson et al., 1997), that symptomatic infections by *D. sapinea* increase in severity under drought stress of the host. The fungus spreads via asexual spores (conidia) (Bihon et al., 2011), which are released from asexual pycnidia in moist weather, and are supposed to be dispersed by wind-driven rain and rain splash (Brookhouser and Peterson, 1971; Swart et al., 1987b). Pycnidia develop on cones, necrotic shoots, and needles in relation to moist conditions (Peterson, 1977). In central Europe, tracking of conidia from closely related *Diplodia* species illustrated a release mainly during the vegetative period with peaks in late summer and early autumn (Kuntzmann et al., 2009). Historically, severe impacts on forestry have been reported from the southern hemisphere (Swart et al., 1985; Swart and Wingfield, 1991; Burgess et al., 2004), whereas an increase of damages by Diplodia tip blight further North were recognised during the last decades (Hanso and Drenkhan, 2009; Oliva et al., 2013; Adamson et al., 2015; Brodde et al., 2019; Blumenstein et al., 2021; Terhonen et al., 2021). Diplodia tip blight is considered as a relatively new disease in the Nordic countries; ten years ago, *D. sapinea* was found for the first time on healthy and single, symptomatic pines in Sweden (Oliva et al., 2013). In 2016, the first outbreak in a commercial Scots pine plantation in Sweden was discovered (Brodde et al., 2019). Diplodia tip blight has been shown to be associated with drought, though effects of the combination of drought and Diplodia tip blight on mortality or recovery of Scots pine in Nordic conditions are unknown.

In this study, we aimed at describing the mortality and recovery of drought-induced crown dieback and the relation with *D. sapinea* in Scots pine. We observed the development of Scots pine displaying crown dieback and apparently healthy trees within affected and healthy areas for two consecutive years after the initial drought in 2018. We also investigated if *D. sapinea* was present locally only in symptomatic trees of the affected sites or also in healthy-looking trees, as well as on both affected and healthy sites. Furthermore, we tested if the presence could be classified as a local or systemic infection within the trees. By local infection, we mean present only in tissues exhibiting Diplodia tip blight symptoms, in contrast to systemic, where the level of *D. sapinea* colonisation is similar in symptomatic and apparently healthy tissues of affected trees. Additionally, the spore dispersal of *D. sapinea* was analysed each season for two years to monitor inoculum on all studied sites.

We investigated the presence of *D. sapinea* in the affected area of Scots pine and hypothesised that drought effects in combination with *D. sapinea* infection limit recovery of the trees, so that i) severely damaged trees (>70%) show higher mortality rates and ii) mildly damaged trees (<30%) show a higher probability of recovery. We also predicted that iii) *D. sapinea* is abundant in symptomatic and asymptomatic trees on affected and healthy sites and that iv) the spore dispersal of *D. sapinea* is associated with the occurrence of dieback of Scots pine at the sample site (affected vs. healthy sites). We could show that a reduction of crown dieback occurred within two years after the drought, even under the presence of *D. sapinea,* where i) severely damaged Scots pine (>70%) showed higher mortality rates two years post-drought, but ii) recovery could be observed independently from initial crown dieback levels. iii) *D. sapinea* was endophytically present with low abundance in healthy twigs of symptomatic and asymptomatic trees on affected and healthy sites and abundant only in necrotic twigs of symptomatic trees. iv) Spore deposition of *D. sapinea* was correlated with precipitation during sampling but not with the health status of the site.

2. Methods

2.1. Experimental sites

All experimental sites are located east and northeast of Visby (Fig. 1) within an area most affected after the drought of 2018 on Gotland. Four sites with Scots pine showing symptoms of crown dieback (Affected 1 to 4) and four sites with healthy-looking Scots pine (Healthy 1 to 4) were selected (representative picture: Fig. S1). The soil type on Gotland is Inceptisol, where the beginning of profile development is visible (Brady and Weil, 2008). All affected sites and site Healthy 3 are located on bedrock, where available growth substrate consists mainly of organic matter in cracks in the rock. The asymptomatic sites Healthy 1 and 4 are located on post-glacial sand/gravel, and Healthy 2 is located on clay till (SGU, 2014). In general, all sites but Healthy 4 had very thin soil layers on top of the rock layers (Soil depths map, SGU 2014, 2017). Groundwater levels measured close to the affected sites during the last 60 years were generally low, while 2018 showed the lowest values during a longer-than-average time in the last 30 years. In 2019, even lower groundwater levels were reached for a short period, while levels recovered to magnitudes recorded pre-drought in 2020 (data obtained from SGI, see Fig. S2).

2.2. Detection of D. sapinea

Visual detection of Diplodia tip blight symptoms on affected sites was confirmed by spore morphology. For this purpose, necrotic shoots in the upper part of the crown and cones infested with pycnidia morphologically matching *D. sapinea*, were collected. Three to nine shoots and six to nine cones were collected on each site. Spores from pycnidia were microscopically examined from three needles of each shoot and two scales of each cone. No presence of other potent pathogens capable of inducing the observed symptoms was identified in symptomatic pine

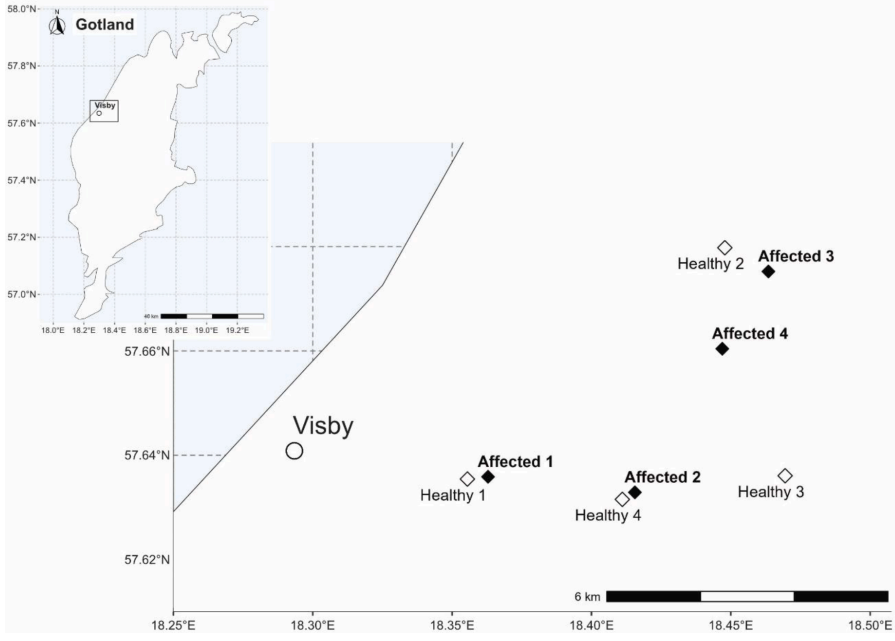

Fig. 1. Location of experimental sites within an affected region of Scots pine showing crown dieback after a severe drought in summer 2018 on Gotland, Sweden. Severely affected stands (Affected 1–4) were located near healthy-looking stands (Healthy 1–4). Map source: rnaturalearth (Massicotte and South, 2023).

twigs at the sites (Brodde, 2023).

2.3. Crown dieback estimation

Crown dieback of 20–27 Scots pines was estimated on each affected site for the first time in December 2018 (Table 1). The level of crown dieback was estimated in 10% steps as the proportion of twigs with shoot blight and dead twigs in the upper third of the living crown in relation to a completely healthy tree in the same population. Selection of trees was based on the level of crown dieback, with the aim of including trees covering a span from very low (<30%) to very high crown damages (<70%). Selected trees showed a minimum of 5% and a maximum of 100% crown dieback. Measured trees were revisited in October 2019 and November 2020. In 2019, shoot blight and loss of needles (crown transparency) were difficult to distinguish and estimated as one overall measurement of crown dieback as carried out in 2018. By 2020, crown transparency had increased to such a high level that two separate estimations were carried out; one overall estimate of crown dieback as carried out in 2018 and 2019, and one estimate of dieback focused on the shoots grown post-drought, not considering the defoliated, transparent parts of the crown. Five asymptomatic trees on each healthy site were randomly selected and measured for crown dieback in 2018, 2019, and 2020. For all trees selected at affected sites, height, diameter at breast height (DBH, 1.3 m), and bifurcation of the stem (absence or presence) were recorded in 2018.

Table 1
Overview of experimental site properties. Soil type SUG, 2014; soil depth SUG, 2017.

Site	Coordinates (SWEREF99 TM)	Soil type	Measured trees [n]	Mean tree height [m] ± 1SD
Affected 1	57.63587, 18.36288	bedrock	20	5.1 ± 1.6
Affected 2	57.63289, 18.41557	bedrock	24	9.7 ± 2.2
Affected 3	57.67524, 18.46347	bedrock	25	9 ± 1.7
Affected 4	57.66042, 18.44692	bedrock	27	8.3 ± 1.2
Healthy 1 *	57.63544, 18.35548	post-glacial sand/ gravel	5	8.2 ± 0.6
Healthy 2	57.67979, 18.44784	clay till	5	11.4 ± 2.4
Healthy 3	57.63607, 18.46947	bedrock	5	9.3 ± 1.4
Healthy 4	57.63154, 18.41107	post-glacial sand/ gravel	5	12.1 ± 1.5

* Site destroyed in 2020.

2.4. Sampling of Scots pine twigs and spore traps

Scots pine twigs were collected in December 2018. Twigs were sampled with a telescopic pruner (5 m long) as high as possible, aiming for light-exposed twigs at the upper half of the crown. Three healthy twigs were collected from all sampled trees. If a tree was symptomatic, three additional twigs showing tip blight were sampled. On each healthy site, three trees were randomly chosen. On affected sites, three symptomatic and three asymptomatic trees were selected based on the crown dieback estimations. Healthy trees showed ≤ 10%, and symptomatic trees showed ≥ 20% crown dieback.

Twigs were transported at < 4 °C and stored at −20C within five days. Surface sterilisation was carried out according to Bußkamp (2018) with minor modifications. In brief, needles were removed, stems were brushed under running tap water and then surface-sterilised under a sterile hood. Twigs were incubated for 1 min in 70% ethanol, 5 min in

3% NaOCl, and 1 min in 70% ethanol. A final washing step in ddH2O for ca. 15 sec was added to the protocol. A surface print of a sterilised twig on 2% malt extract agar was carried out each day of sample preparation to check the efficiency of surface sterilisation; fungal growth was observed in 5% of the cultures (n = 20). Sampling of stem tissue was carried out with a sterile scalpel. Each sample contained 1 cm twig tissue which was either healthy (asymptomatic twigs) or from the border of an infection, including necrotic and healthy tissue (symptomatic twigs). Healthy twigs could be sampled for the 2018 year's growth, while symptomatic twigs were sampled at the growth year where the infection border was localised, reaching from year 2008 to 2017. Twig samples were cut into thin sections, transferred into a 2 mL screw cap tube containing one 5 mm and two 2 mm glass beads, and stored at −20 °C until lyophilisation. Freeze-dried samples were homogenised using the Precellys® 24 Tissue homogenizer (Bertin Instruments), 2x 25 s at 5000 rpm. DNA extraction was carried out according to instructions with the E.Z.N.A® SP Plant DNA Kit (Omega Biotek).

To monitor *D. sapinea* spore release, four spore traps were placed in the centre of each site (Table 2). One healthy site was lost at the beginning of the second sampling year due to destruction of the whole site. The traps consisted of one horizontally fixed filter paper (Munktell, Ahlström; 90 mm diameter) treated with 4x TE buffer (for detailed description, see Zhang et al., 2022) at the height of 1.2–1.5 m. Filter papers were exposed during two consecutive seven-day sampling periods per season from January 2019 to October 2020. The sampling periods were chosen to capture potential seasonal variations in spore release. Filters were collected in 50 mL Falcon tubes, kept on ice during transportation, and stored at −20 °C within 4 hrs and until DNA extraction.

For spore DNA extraction, 20 mL SDS buffer (0.05 M Tris pH 8, 0.05 M EDTA pH 8, 0.104 M SDS, 1 M NaCl, dissolved by incubation at 60 °C for two days) was added to the Falcon tubes containing the filter papers followed by incubation at 65 °C for 90 mins. After removal of the filter papers, 20 mL 2-propanol was added to the SDS extract. The samples were mixed thoroughly and incubated at RT overnight. The following day, the samples were centrifuged at 7000 rpm for 10 mins at RT before the supernatant was removed. The pellet was resuspended in 700 μL lysis buffer PL2 (Macherey-Nagel, Düren, Germany) and transferred to 2 mL screw-cap tubes containing ~ 130 mg 0.2 mm glass beads, ~200 mg 0.4 mm glass beads, ~200 mg 3 mm glass beads, and ~ 4 mg diatomaceous earth (powdered siliceous sedimentary rock). The samples were lysed for 30 s at 5000 rpm using Precellys® 24 Tissue homogenizer (Bertin Instruments). Extractions were performed using the Macherey-Nagel NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol, with increased volumes of buffer PL3 and buffer PC proportionally to the volume of lysis buffer PL2 used for resuspension of the pellet.

Table 2
Spore trap sample weeks (time points) and number of successfully analysed filter papers per sample week. Samples with failed qPCR (no signal) were excluded (in total n = 14). Filter papers were left on site for seven consecutive days. Year 2019: four filter papers per week on four affected (n = 16) and four healthy sites (n = 16). Year 2020: four filter papers per week on four affected (n = 16) and three healthy sites (n = 12); site "Healthy 1" lost by destruction at beginning of 2020.

Week	Start	Season	Year	Analysed samples (n)
1	21.01.	winter	2019	31
2	29.01.	winter	2019	31
3	10.04.	spring	2019	32
4	17.04.	spring	2019	27
5	15.07.	summer	2019	32
6	22.07.	summer	2019	31
7	14.10.	autumn	2019	31
8	21.10.	autumn	2019	30
9	13.01.	winter	2020	28
10	20.01.	winter	2020	28
11	09.04.	spring	2020	28
12	16.04.	spring	2020	28
13	06.07.	summer	2020	27
14	13.07.	summer	2020	26
15	15.10.	autumn	2020	28
16	22.10.	autumn	2020	28

2.5. Quantification of *D. sapinea* DNA from twigs and spore traps

The amount of *D. sapinea* DNA from twigs and spore traps was quantified using qPCR. A standard curve was produced by PCR amplification on DNA template from a *D. sapinea* isolate and the PCR product's desired length (79 bp) was confirmed through gel electrophoresis. DNA from the PCR product was precipitated, quantified using a Qubit Fluorometer, and the highest concentration replicate was chosen for serial dilutions to generate a standard curve. The primers used for PCR and qPCR, and the probe used for qPCR, were designed by Luchi et al. (2005). The qPCR master mix was prepared following their methodology with the following modifications: 1x SsoAdvancedTM Universal Probes Supermix (BioRad), 250 nM each of forward and reverse primer, and 200 nM probe. Reactions were set up in triplicates, including the standard curve from 1×10^6 to 1×10^2 copies per μL and non-template controls (nuclease-free water). Each reaction consisted of 15 μL master mix and 5 μL 1:1 dilutions of the spore trap DNA extractions or 5 μL twig DNA (diluted so that the total amount of DNA was 5 ng per reaction). The qPCR program was 2 min at 95 °C followed by 40 cycles of 10 s at 95 °C and 15 s at 60 °C. iQ™5 Optical System software (BioRad) was used to analyse the qPCR data. Samples were excluded if the triplicate cycle threshold (Ct) value's standard deviation (stDev) was > 0.5. Samples below the linear detection limit (37 Ct) of the assay were included in the analysis with SQmean = 0.01 even if the stDev was > 0.5.

Sixty-four of 466 DNA samples extracted from spore trap filter paper were below the detection limit for *D. sapinea*, and 17 of 466 were excluded due to non-reliable reads. Nine of 141 DNA extractions from twigs were below the detection limit.

2.6. Statistical analysis

All analyses were performed using R Statistical Software (v4.1.1; R Core Team 2021). Crown dieback estimations per year and qPCR data from twig samples were compared by ANOVA using AICcmodavg R package (v.2.3–1) followed by post hoc Tukey's honest significance test. Effects of initial dieback, tree height, and bifurcation on crown dieback in new growth, as well as the overall dieback in the upper third of the crown of pines in 2020, were tested in a linear mixed-effects model using the lme function of the nlme R package (v.3.1.157). Site and tree identity were used as random factors to account for repeated measurement of the trees and within-site variability. The effect of low (<30%), medium (40–70%) or high (>70%) initial crown dieback on recovery (<-10% difference in dieback), stagnation (+/-10%), or decline (>10%) of the pines was tested by a pairwise *t*-test using the rcompanion R package (v.2.4.15).

Differences in *D. sapinea* DNA quantifications from spore traps between affected and healthy sites during the two sampled years were analysed with a repeated measures linear regression model using the lme function of the nlme R package (v. 3.1.157). Fixed effects were site type (healthy/affected) and sampling week (time point, 1 to 16). Mean values were computed for each site type and sample week in combination to analyse the development of *D. sapinea* quantity over time. Repeated measurement of the same traps was accounted for by the correlation of the error term by sample week for each trap. Site number and spore trap ID were treated as random factors. Post hoc pairwise comparisons of *D. sapinea* DNA quantities per site type were carried out by sample week using the emmeans R package (v. 1.7.3).

3. Results

The exceptional drought of 2018 was followed by severe crown dieback of Scots pine on Gotland. The observed symptoms were consistent with a potential outbreak of *D. sapinea* (Fig. 2a). The presence of *D. sapinea* on the affected sites could be confirmed by spore morphology and on average ca. 60% of the examined shoots and cones from the affected sites carried fruiting bodies of *D. sapinea* (Table S1).

3.1. Crown dieback development

In 2018, Scots pine trees in the affected sites showed an average of 25% crown dieback (Fig. 3a). Mean crown dieback of the trees doubled from 2018 to 2019, from ca. 25% to > 50% (Fig. 3a). No significant shift in overall crown dieback occurred during the second year, from 2019 to 2020 (Fig. 3a). However, in 2020 shoots grown after 2018 became clearly visible when necrotic and senescent needles from the previous years were shed (Fig. 2d, Fig. 3a). The assessment of dieback in shoots that developed post-drought revealed a reduction in the proportion of impaired shoots. This decline was in line with the levels of crown dieback observed in 2018 when the initial damages occurred, highlighting the cumulative defoliation and subsequent foliage recovery dynamics (Fig. 3a).

Scots pine on healthy sites showed no crown dieback in 2018 (Fig. 3b), with a slight increase of mean dieback to ca. 10% in 2019 and ca. 17% in 2020. Shoots grown after 2018 showed lower crown dieback than the overall estimate, with a mean value of ca. 12%.

Fig. 4 visualises the crown dieback development of single trees in 2019 and 2020 in relation to their previous year's crown dieback. Trees falling on the plotted line of equality (x_1, y_1 = (0, 0), x_2, y_2 = (100, 100)), +/-10% in × and y stagnated in their level of crown dieback between the two compared years. Most pines showed increased crown dieback of 20–50% within the first year after the drought of summer 2018 (Fig. 4a). An evident decrease in crown dieback compared to the conditions observed in 2018 was recorded in only two cases. The remaining pines displayed a stagnation in crown dieback (within the +/-10% blue area in Fig. 4a). By 2020, two years after the drought, dieback levels of the overall crown stagnated or changed within +/-20% compared to 2019 for the majority of the pines (Fig. 4b). Crown dieback estimation of shoots grown post-drought (Fig. 4c) showed a strong reduction of symptomatic shoots. Most pines exhibited<40% dieback in their new shoots. The extent of crown dieback in 2018 had a notable impact on both the overall crown's dieback and the dieback observed in the newly grown shoots by 2020 (Table S2). Bifurcation and tree height in 2018 had no significant effect on crown dieback development until 2020. Notable were five trees with a crown dieback level of 90% in 2019, which showed recovery in the newly grown shoots at the last estimation in 2020 (Fig. 4c).

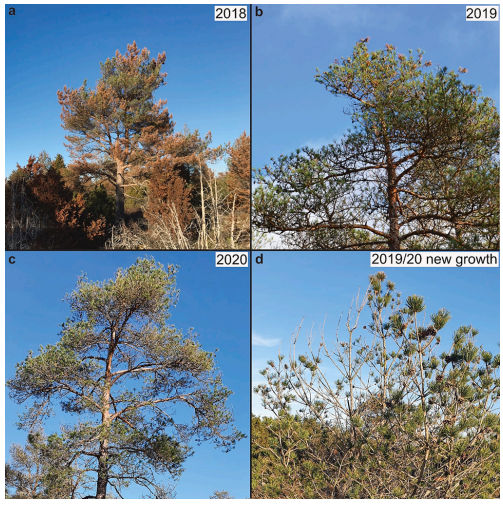

Fig. 2. Representative pictures of different Scots pine trees on Gotland. Crown dieback estimation started after a severe drought in 2018; trees were revisited in 2019 and 2020. a) 2018: A Scots pine tree with 70% crown dieback of upper third of living crown (2018). Browning of needles was found in apical 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 total crown dieback of 70% vs. 20% dieback when taking shoots grown post-drought into account, not considering the defoliated, transparent parts of the crown.

Classes of trees with initially low (<30%), medium (40–70%), or high (>70%) crown dieback showed no significant difference in frequencies of recovery, stagnation, or decline by 2020 considering the overall crown estimation (Table S3a). The highest frequency of recovery was recorded in newly grown shoots of initially medium-damaged trees but with no significant difference to highly damaged trees (Table S3b).

Taken together, the observed crown dieback of Scots pine in the affected sites started with severe needle losses, which doubled during the first year. Surviving trees showed clear recovery in 2020 compared to 2019, independently of their initial crown dieback.

Among the trees classified as having medium- and low levels of crown dieback, total mortality observed was low (5–10%, Fig. 5). However, in severely damaged trees (>70% crown dieback) total mortality exceeded 50%. Almost all mortality among the trees in the study was recorded during the first year of the study whereafter only limited additional mortality was recorded (Fig. 5). Trees were more likely to die when crown dieback exceeded 70% and within the first year after the drought event.

3.2. Quantification of D. sapinea DNA in Scots pine twigs.

To localise symptomatic as well as asymptomatic infections of *D. sapinea* in declining Scots pine, we quantified *D. sapinea* DNA in symptomatic and asymptomatic twigs of symptomatic and asymptomatic trees on affected sites and asymptomatic twigs of asymptomatic trees on healthy sites (Fig. 6). Significantly higher amounts of *D. sapinea* DNA were found in symptomatic twigs from symptomatic trees on affected sites compared to asymptomatic twigs. Healthy-looking twigs contained similar low amounts of *D. sapinea* DNA, whether they originated from symptomatic or asymptomatic trees on affected or healthy sites.

Overall, *D. sapinea* was most abundant in symptomatic twigs, while detection in asymptomatic twigs was independent of the tree's or site's health status.

3.3. Quantification of D. sapinea DNA in spore traps.

Diplodia sapinea spore deposition was sampled on affected and healthy sites to monitor inoculum levels for two years after the initial crown dieback occurred. *D. sapinea* DNA was detectable in filter paper spore traps throughout the two years on affected and healthy sites. The quantity of *D. sapinea* DNA showed neither a seasonal pattern nor an increase by the second year (Fig. 7a, b). A significant correlation was found between *D. sapinea* DNA quantity and high mean precipitation during spore trap exposure ($R^2 = 0.66, p = 0.005$), but not between DNA quantity and mean temperature or mean wind speed during sampling (Fig. 7c, Table S5).

We observed a trend of higher DNA quantities at affected compared

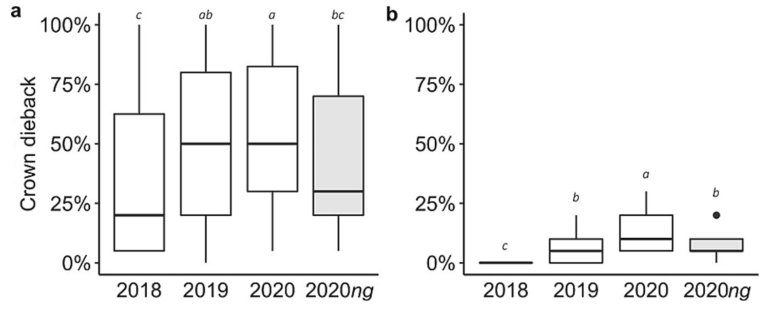

Fig. 3. Crown dieback of Scots pine on a) four affected sites (97 trees) and b) four healthy sites (20 trees) during the exceptional drought of 2018 and the two following years, 2019 and 2020, on Gotland. D. sapinea was confirmed to be present on the affected sites. Crown dieback was assessed by estimating the percentage of symptomatic shoots in the upper third of the living crown at the end of each vegetation period. 2020 ng refers to the estimation of dieback observed in shoots newly grown (ng) post-drought. Significances indicated by different letters: (ANOVA, post hoc Tukey's honest significance test).

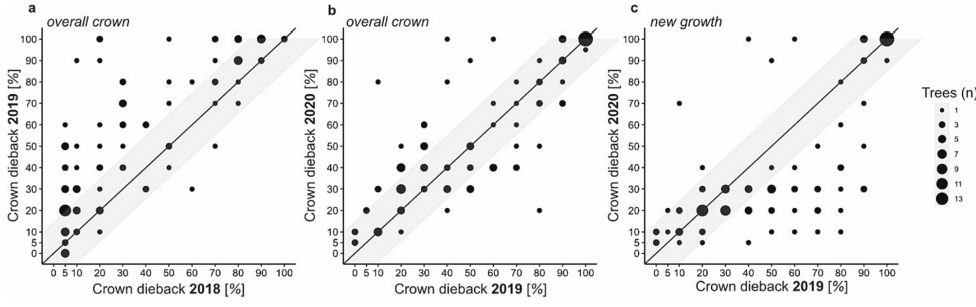

Fig. 4. Crown dieback development of Scots pine on Gotland. Shown are comparisons of crown dieback level of the crowns of single trees between years a) 2018–2019, b) 2019–2020 overall, c) 2019–2020 new growth. Trees on the line of equality (solid black line), within the +/-10% blue area, did not change in crown dieback. Trees above the blue area showed increased crown dieback and trees below showed less crown dieback in the latter year. Initial crown dieback of 2018 was observed after extreme drought and heat during summer 2018. Overall crown dieback was measured in % of symptomatic shoots in the upper third of the crown. Crown dieback of newly grown shoots of 2019 and 2020 (new growth) was measured in % of symptomatic shoots in the upper third of the crown. Scots pines (n = 97) were distributed among four affected sites.

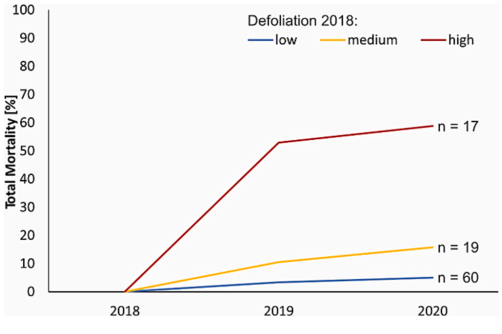

Fig. 5. Total mortality of Scots pine in two years (2019–2020), after initial crown dieback in Scots pine, 2018. Trees were categorised in low (<30%; blue), medium (40–70%; yellow), and high (>70%; red) crown dieback based on the initial estimation. Number of selected trees indicated per category, total n = 96. Pines located on four affected sites near Visby, Gotland.

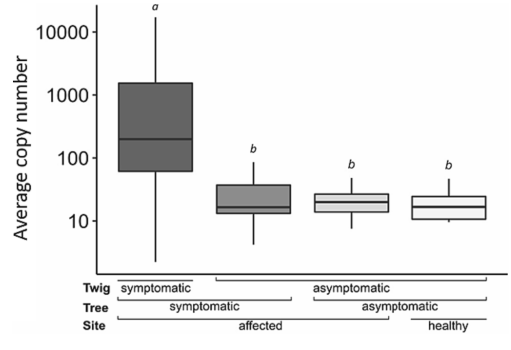

Fig. 6. Quantification of D. sapinea DNA (qPCR) in Scots pine twigs. The scale is logarithmic. 1) symptomatic and 2) asymptomatic twigs from symptomatic trees on affected sites; 3) asymptomatic twigs from asymptomatic trees on affected sites; 4) asymptomatic twigs from asymptomatic trees on healthy sites. n = 12 trees for each category; analysed were three twigs per tree. Same letter above box indicates no difference between twig category (ANOVA; α = 0.01).

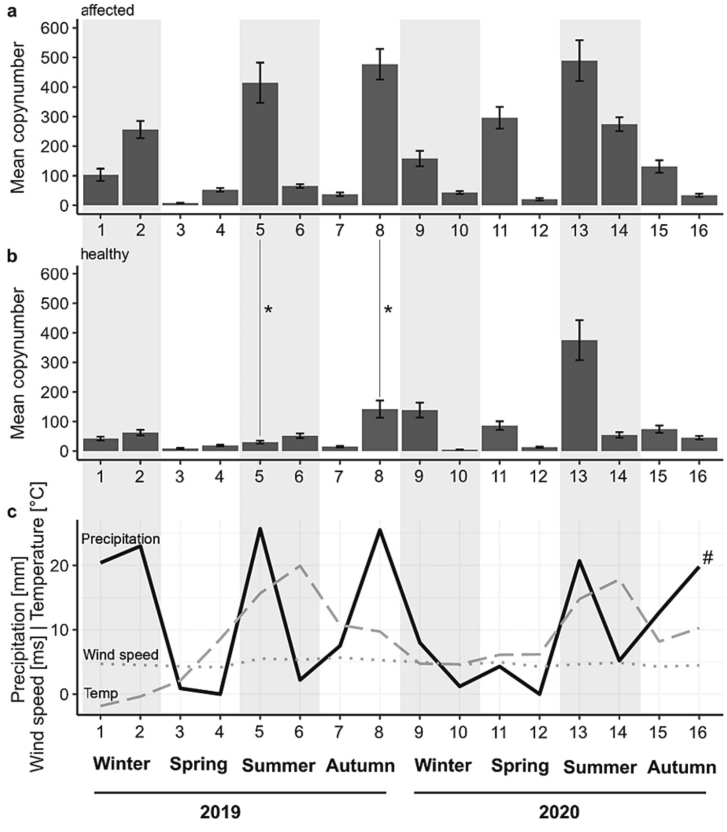

Fig. 7. Mean aerial spore load on the affected sites in relation to climate conditions during a total of 16 sample weeks, 2019 and 2020, on Gotland, Sweden. Mean copy number of D. sapinea DNA (qPCR) extracted from filter paper spore traps. Pairwise comparison of D. sapinea DNA quantity between a) affected and b) healthy sites. Significant difference ($p < 0.05$) labelled with *. Each filter was exposed for one week; four traps were installed on four affected sites per week. c) Total precipitation, mean wind speed, and mean temperature during each sampling week. Significant correlation of precipitation with D. sapinea DNA quantification labelled with # ($R2 = 0.66$, $p = 0.005$).

to healthy sites. However, detected DNA quantities varied greatly between the traps within a site. A statistically significant higher quantity of DNA at affected sites was only found in sample weeks 5 and 8 (Fig. 7, Table S4).

4. Discussion

Diplodia tip blight has raised attention as an emerging forest disease in Fennoscandia with observations of single symptomatic infections in the northern Baltics since 2007 (Adamson et al., 2015; Müller et al., 2019), followed by the first detected outbreak in a Scots pine stand in Sweden in 2016 (Brodde et al., 2019). Given the new occurrence of *D. sapinea* infections in Northwest Europe, the development of affected pines has been one of the central questions since then. In this study, we provide data on how pines affected by a drought event recover and which potential role *D. sapinea* played in crown dieback in that process.

We set up a study of Scots pine in eight sites on Gotland in 2018, during one of the most severe droughts recorded in the last 200 years (Moravec et al., 2021). We studied the effects of drought and Diplodia tip blight on crown dieback and mortality or recovery of Scots pine.

Scots pine trees undergoing drought-induced crown dieback have been shown to be able to recover as soon as water-stress is released (Dobbertin et al., 2010; Eilmann et al., 2013). However, high crown dieback under drought poses a long-term risk for the future performance of the tree, where loss of needles is associated with reduced carbon uptake up to four years post-drought, as well as increased mortality in occurrence of a new drought event (Galiano et al., 2011). Similarly, repeated attacks by the dieback fungus *Gremmeniella abietina* can result in severely reduced crown recovery and increased mortality ten years post the initial outbreak (Oliva et al., 2016).

Crowns of trees at the affected sites had lost about 25% of their foliage during the period of our study. After the first year, the average crown dieback of the trees doubled with limited signs of recovery. It was at this phase that the majority of mortality was observed. Trees with high dieback levels in 2018 showed an increased likelihood of dying in 2019. This is consistent with previous studies that reported a delayed response affecting the trees after a drought event (Rebetez and Dobbertin, 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 where the groundwater table was lower

than normal also in 2019, though the intensity of the drought in Scandinavia was lower compared to the drought of 2018 (Moravec et al., 2021; Rakovec et al., 2022).

Two years after the initial drought, a recovery of the new crown was apparent in surviving trees, also in the group of trees classified as severely damaged. In fact, single pines with very severe crown dieback of up to 90% were showing signs of recovery, which is consistent with studies that show 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). In general, recovery was not correlated with initial dieback levels. Biases in the method of visual crown dieback estimation cannot be excluded. A likely error rate in the estimates of approximately +/-10% makes differentiating low crown dieback levels (<30%) difficult. Anyhow, recovery independent of initial dieback might be in line with the opportunistic nature of *D. sapinea*. As soon as the major factor stressing the host is released, the impact of *D. sapinea* decreases.

The spore trapping verified that *D. sapinea* was present in the studied region on Gotland, also on healthy sites. In agreement with this observation, pines on healthy sites did develop mild symptoms of Diplodia tip blight during the two years of the study, though disease incidence was substantially lower compared to the affected sites (see Fig. 3a, b). In earlier studies, spore dispersal of *D. sapinea* was shown to be related to precipitation (Brookhouser and Peterson, 1971; Swart et al., 1987b). The observation in the current study, that there is an association with precipitation during sampling, agrees with these studies. However, no significant seasonal pattern in spore dispersal could be observed, in contrast to previous studies (e.g. Kuntzmann et al. (2009)). The absence of such a pattern could be an effect of the limited number of sampled weeks. Alternatively, the sample period might not fully reflect patterns of spore dispersal of *D. sapinea* in Northern latitudes. At least the first monitored year showed atypical weather for Scandinavia.

It is likely that site properties contributed to the contrasting patterns of crown dieback establishment between affected and healthy sites. The soil type has previously been shown to influence the incidence and abundance of *D. sapinea* (Munck et al., 2009). All healthy sites were located on, or on the edge of, soil types with higher water-holding capacities, while all affected sites were located on bedrock. Consequently, it is not impossible that the drought's impact was more severe at the affected sites.

D. sapinea was found to be associated with symptomatic infections in Scots pines showing crown dieback. Endophytic infections of *D. sapinea* could be detected in healthy twigs, independently of the health status of the tree or site. However, the analyses indicated very low levels of endophytic *D. sapinea* colonisation. A comparable study by Oliva et al. (2021) investigated the abundance of *D. sapinea* DNA in *Pinus* spp., which developed Diplodia tip blight symptoms after a hailstorm in summer 2018 in Spain. That study also found the highest amounts of *D. sapinea* in the symptomatic trees on an affected site. Furthermore, asymptomatic trees on the affected and healthy sites showed an endophytic presence of *D. sapinea* with generally lower levels. Nevertheless, asymptomatic trees on the affected site showed significantly higher quantities of *D. sapinea* DNA compared to asymptomatic trees on the healthy sites in the study by Oliva et al. (2021). This might reflect different patterns in colonisation of asymptomatic trees within the affected site studied in Spain compared to the results of the present study. Further studies are needed to investigate colonisation patterns of *D. sapinea* in pines within and outside areas exposed to stress factors. Comparing regions where Diplodia tip blight has been recorded for a long time with regions showing a recent emergence of this stress-related disease might improve damage predictions of forest health.

It is not possible to separate the effect of drought from the impacts of Diplodia tip blight in the observed crown dieback of Scots pine. Nevertheless, the combined effect leading to 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). In the drought-triggered crown dieback, the presence of *D. sapinea* might have made a difference in 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.

In the present study, we showed that *D. sapinea* is likely to have contributed to the observed crown dieback of Scots pine after a severe drought. The opportunistic pathogen possibly had an impact on the affected trees in their ability to recover post-drought. Nevertheless, we could show that trees can recover, even if damages by drought and *D. sapinea* infections are high. Predictions of future drought frequencies depend on the respective region, season, and emission scenario (Spinoni et al., 2018), though an increasing impact of drought, not only on tree health, is generally expected as a consequence of climate change (Schär et al., 2004; Bellard et al., 2012). If severe drought frequently reoccurs in already affected regions, e.g. on the island of Gotland, drought could be considered a predisposing, as well as an inciting, factor of a potential further decline of Scots pine. *D. sapinea,* as a contributing factor, could accelerate the potential decline, leading to even higher mortality among pines similar to what was reported for *G. abietina* attacks (Oliva et al., 2016). At the same time, our findings reveal that the trees can recover from severe stress, suggesting that immediate sanitary cuttings of surviving trees in infested stands may not be the most viable management choice for stands on sites with poor site properties, as removal of trees could lead to increased soil erosion. However, when deciding on management options, one also has to take timber quality into account and severely affected trees with dieback of leader shoots may not develop into raw material for high-quality timber. Anyhow, further studies of the spread of endophytic *D. sapinea* infections in areas formerly unaffected by Diplodia tip blight, as well as a better understanding of the mechanisms behind symptomatic infections in the pathosystem *D. sapinea* – (Scots) pine, are needed to contribute to more precise predictions of forest health development in a changing climate.

Author contributions

JO, LB, and JS conceptualised the project. LB, JO, and JS designed the experiments. LB coordinated, and LB and MSÅ carried out field and laboratory work. KW coordinated spore trap sampling. ME supported laboratory work. LB and MSÅ analysed data. LB wrote the first manuscript draft. All authors contributed to writing and reviewing the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foreco.2023.121436.

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ORIGINAL ARTICLE OPEN ACCESS

Scots Pines With Tolerance to *Melampsora pinitorqua* and *Diplodia sapinea* Show Distinct Metabolic Profiles

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ABSTRACT

Diplodia sapinea causes Diplodia tip blight (DTB) and is recognised as an opportunistic necrotrophic pathogen affecting conifers. While DTB is associated with abiotic stress, the impact of biotic stress in the host on *D. sapinea*'s lifestyle shift is unknown. Observed co-occurrences of *D. sapinea* and *Melampsora pinitorqua*, causing pine twisting rust on Scots pine (*Pinus sylvestris*), instigated an investigation into their interaction with and influence on the defence mechanisms of the host. We hypothesised that *M. pinitorqua* infections predispose the trees to *D. sapinea* by stressing the host and altering the shoot metabolites. Pines in a plantation were sampled over time to study pathogen biomass and host metabolites. Symptoms of both pathogens were consistent over years, and the preceding season's symptoms affected the metabolic profiles pre-infection and *M. pinitorqua*'s proliferation. Symptoms of *M. pinitorqua* altered shoot metabolites more than fungal biomass, with co-symptomatic trees exhibiting elevated *M. pinitorqua* biomass. Specific phenolic compounds had a strong positive association with the shoot symptom × *D. sapinea* interaction. *D. sapinea*'s biomass presymptoms was independent of previous disease symptoms and infection by *M. pinitorqua*. Some trees showed disease tolerance, with delayed rust infections and minimal DTB symptoms. Further investigations on this trait are needed.

1 | Introduction

Plant diseases have a profound impact on plant metabolism and physiology (Berens et al. 2017). In natural habitats, plants often face multiple pathogens with distinct modes of action (Tollenaere, Susi, and Laine 2016). It is not uncommon for them to encounter opportunistic pathogens, which thrive during altered physiological states and stress within the host. Opportunistic fungi can reside as asymptomatic endophytes, remaining latent until specific host and environmental factors convert them into aggressive necrotrophs (Slippers and Wingfield 2007). The molecular and metabolic processes driving the vulnerability of trees to opportunistic fungi, triggered by stress, are still largely unknown. This gap in knowledge makes predicting the dynamics of these pathosystems a challenging task (Ghosh et al. 2022).

Diplodia sapinea (Fr.) Fuckel (syn. *Diplodia pinea* (Desm.) Kickx., *Sphaeropsis sapinea* (Fr.: Fr.) Dyko & Sutton) causes Diplodia tip blight (DTB) and has repeatedly been reported to be an opportunistic necrotrophic pathogen on conifers (Blodgett, Kruger, and Stanosz 1997; Blumenstein et al. 2021; Brodde et al. 2023; Stanosz et al. 2001; Swart, 1991; Zwolinski, Swart, and Wingfield 1995), in particularly on *Pinus* spp. (CABI 2021). *Diplodia sapinea* is a major pine pathogen globally, but the reports of damages have increased in northern

Europe over the past decade (Brodde 2023; Brodde et al. 2019; Terhonen et al. 2021), with its impact expected to escalate due to climate change (Fabre et al. 2011; Sturrock et al. 2011). It is known that DTB develops when the tree is under the influence of abiotic stressors such as drought, hail, or mechanical damage (Blodgett, Kruger, and Stanosz 1997; Brodde et al. 2023; Sherwood et al. 2015; Stanosz et al. 2001; Swart, 1991; Zwolinski, Swart, and Wingfield 1995). In artificial inoculation experiments on Scots pine (*Pinus sylvestris* L.), wounding facilitated a higher incidence of symptomatic *D. sapinea* infections and significantly increased the success of pathogen re-isolation, while non-wounded plants predominantly exhibited asymptomatic infections (Oostlander et al. 2023), indicating that stress in the host may allow *D. sapinea* to transition into an aggressive necrotroph.

Both latent *Diplodia sapinea* infection and acute DTB may induce alterations in both primary and secondary metabolism of the host (Ghosh et al. 2022; Hu et al. 2023), causing local carbon and nitrogen stress in the host tissues (Ghosh et al. 2022; Sherwood et al. 2015). Infection by *D. sapinea* on pines is associated with the accumulation of lignin, phenolics and free amino acids (Hu et al. 2023; Sherwood et al. 2015; Wallis et al. 2008). The accumulation of phenolic glycosides and stilbenes has shown a negative correlation with disease susceptibility (Wallis et al. 2008). In a study on *D. sapinea* in hail-damaged and non-hail-damaged pine stands, Caballol et al. (2022) proposed a potential proline competition between other endophytes and *D. sapinea*, influencing the disease outcome in pines affected by hail. Among the free amino acids, proline has been reported to be a preferred nitrogen source by *D. sapinea* (Sherwood et al. 2015). Therefore, Sherwood et al. (2015) suggest that nitrogen availability may play a pivotal role in shaping the outcome of the interaction between pine and *D. sapinea* and that the availability or increased abundance of free amino acids in stressed trees may contribute to disease development by providing *D. sapinea* with nitrogen. In their paper, Zwolinski, Swart and Wingfield (1995) suggested the potential of cambiophagous insects to infest healthy radiata pine (*P. radiata*) tissue and facilitate further colonisation by *D. sapinea*, indicating that biotic stress may also allow *D. sapinea* to change from an endophytic to a necrotrophic lifestyle.

Pathogens can alter signals that modify host defence responses and host metabolism. Reactions triggered by one pathogen can also be changed in the presence of another (Abdullah et al. 2017). Priority effects stem from the host's immune responses to earlier pathogen infections and may arise when a previous infection changes the susceptibility to subsequent infections (Halliday, Umbanhowar, and Mitchell 2018). Prioritising defence against certain pathogens increases investment in defending against them but may weaken defences against others (Abdullah et al. 2017). The process of immune-mediated facilitation can thereby occur when one immune-signalling pathway's upregulation leads to another's downregulation, facilitating subsequent infections and increasing co-infection frequency (Halliday, Umbanhowar, and Mitchell 2018).

The defence mechanisms of conifers are multifaceted, functioning at different stages of infection and disease progression. The defences are constitutive or induced, chemical or mechanical, and systemic or local (Fraser et al. 2016). Conifers allocate different types of chemical defences in designated structures as they grow (Franceschi et al. 2005; Nerg et al. 1994). They also often activate multiple defences, including various phenolic compounds, as a response to pathogen attacks (Fraser et al. 2016; Villari et al. 2014). Phenolics encompass a wide array of metabolites originating from the shikimate pathway, like flavonoids, stilbenes, and lignins and their precursors. The mode of action is through direct toxic effects, inhibition of extracellular enzymes generated by pathogens, or the prompt deposition of barriers like lignin (Bennett and Wallsgrove 1994; Fraser et al. 2016; Ullah et al. 2017). It has been reported that phenolic compounds may act as a reservoir for the synthesis of other phenolic compounds when the phenylpropanoid metabolism is activated in induced defences (Keinänen et al. 1999; Lamara et al. 2018). While this may allow a faster response to environmental threats, it may also potentially influence the host's defence responses against other attackers.

Scots pine account for approximately 40% of the standing volume in Sweden (SLU 2023). One of the most prevalent diseases affecting Scots pine in the country is pine twisting rust (Skogsstyrelsen 2023), caused by the biotrophic rust fungus *Melampsora pinitorqua* (Braun) Rostrup (syn. *Melampsora populnea* (Pers.) P. Karst.). This fungus alternates between European aspen (*Populus tremula* L.) and Scots pine, thriving particularly in newly established pine plantations where aspen often emerges. *M. pinitorqua* teliospores overwinter on aspen leaves on the ground, forming basidiospores in spring that infect flushing pine shoots (Klingström 1963). The duration of infection on pine is brief, but it leaves behind a canker that often induces the shoot to bend or break, and if the leader shoot is affected, it can lead to deformed or multiple stems. Susceptibility to *M. pinitorqua* positively correlates with tree growth and vigour (Desprez-Loustau and Wagner 1997; Desprez-Loustau and Dupuis 1994; Klingström 1963; Martinsson 1985). Recent reports have indicated the co-occurrence of *D. sapinea* and *M. pinitorqua* on Scots pine in Sweden, stressing the need to investigate underlying mechanisms (Skogsstyrelsen 2023).

Rust fungi depend entirely on energy and nutrients from living plant host cells to complete their lifecycle (Lorrain et al. 2019). These fungi, including *M. pinitorqua*, employ effector proteins to suppress host defence responses and extract carbon directly from living cells, creating a local carbon sink (Oliva, Stenlid, and Martínez-Vilalta 2014). The signatures in their genome indicate that host oligopeptides are a source of essential nitrogen and sulfur for the rust fungi (Guerillot et al. 2023; Lorrain et al. 2019). Recently, it was shown that changes in the content of specific amino acids, flavonoids and terpenoids in crabapple leaves following infections with the rust fungus *Gymnosporangium yamadae* is associated with an increased abundance of specific taxonomic groups, such as *Venturiaceae*, which includes several plant pathogens, in the host mycobiome (Zhang et al. 2023). Considering our understanding of the metabolic cues that trigger the lifestyle shift in *D. sapinea* and rust fungi's impact on host metabolism and defence responses, we anticipated that the defence responses triggered by *M. pinitorqua* could potentially facilitate infection by *D. sapinea*.

In this study, we aimed to understand the interactions between Scots pine and two prevalent fungal pathogens and investigate

how they influence the tree defence mechanisms. We used a site in Västmanland, central Sweden, that was established in 2015. Two years later, forest managers began reporting significant issues with *M. pinitorqua* on the otherwise vital and well-growing pines in the area, and in 2020, at a closer inspection, it was discovered that *D. sapinea* infected many *M. pinitorqua*-symptomatic trees. Those findings led us to hypothesise that *M. pinitorqua* infection predisposed the trees to new infections by *D. sapinea* existing in the environment, and we additionally formulated three more specific hypotheses: 1) the disease symptoms of the tree in the preceding growing season impact the amino acids and phenolics profile, pathogen biomass abundance, and the tree's vitality during the following growing season, 2) the composition of amino acids and phenolics differs between *M. pinitorqua*-symptomatic and asymptomatic tissue, and that 3) tissues from shoots colonised by *M. pinitorqua* but not by *D. sapinea* exhibit a distinct set of phenolic compounds compared to tissues colonised by both pathogens. To test these hypotheses, we selected 15 trees based on their disease symptoms in 2020: five healthy-looking, five with *M. pinitorqua* symptoms, and five with both *M. pinitorqua* and *D. sapinea* symptoms. We sampled symptomatic and asymptomatic shoots at three time points, comparing amino acid and phenolic profiles and the abundances of *M. pinitorqua* and *D. sapinea*.

2 | Materials and Methods

2.1 | Experimental Site and Plant Material

The experimental site is located outside Ängelsberg, Västmanland, Sweden (N 59.956820, E 16.059968). The site experienced an extensive forest fire in 2014 that consumed all the surface vegetation and even fractured the bedrock beneath. In 2015, the area was reforested with 1-year-old Scots pine seedlings, each placed in a heap of mineral soil (approximately 20 cm high). In the spring of 2021, 567 trees with heights ranging from 100 to 275 cm were surveyed for *M. pinitorqua* and *D. sapinea* symptoms. Based on the survey, five of the healthiest trees (with the lowest percentage of *M. pinitorqua*-infected shoots and negligible or no *D. sapinea* infections, disease category H), five of the trees with the greatest number of *M. pinitorqua* infections but few or no visually discernible *D. sapinea* infections (disease category M), and five of the trees with the highest percentage of *M. pinitorqua*-infected shoots and greatest number of infections by *D. sapinea* (disease category MD) were chosen for detailed investigations. Representative photos of trees from the three disease categories are presented in Supporting Information S1: Figure S1, and the phenotyping data is summarised in Table S1.

2.2 | Survey of *M. pinitorqua* and *D. sapinea* Symptoms

2.2.1 | *Melampsora pinitorqua* Symptoms

In spring 2021, we surveyed the trees for the percentage of shoots in the top whorl of the previous year (2020) and the percentage of previous year shoots in the second whorl (15 random shoots surveyed) showing infections by *M. pinitorqua*. The percentage of shoots infected by *M. pinitorqua* in the top whorl of 2019 was also estimated.

In autumn 2021, the survey was repeated, recording the percentage of shoots in the top whorl of 2021 and the percentage of current year shoots in the second whorl (15 random shoots surveyed) infected by *M. pinitorqua*.

2.2.2 | *Diplodia sapinea* Symptoms

Shoot samples were collected from twelve trees in September 2020 to confirm the presence of *D. sapinea* based on the morphology of the conidia using a microscope. For all trees included in the study, the presence/absence of *D. sapinea* infections in the top shoot and the total number of visible *D. sapinea* infections (based on typical symptoms) were recorded in spring 2021 (for shoots from 2020) and in autumn 2021 (for shoots from 2021).

2.2.3 | Growth and Vitality

Measurements were taken for tree height at the end of the growing season in 2021, as well as height growth from mid-node to mid-node for the years 2018 through 2021.

In autumn 2021, the general condition of each tree was scored based on how affected their appearance and growth were due to symptoms by the two pathogens. A scale of 0–5 was used, where 0—fully vital, no signs of disease, 1—fully vital, 2—mildly affected, 3—affected, 4—clearly affected, and 5—severely affected. The trees were later grouped into three vitality classes: fully vital (0–1), mildly affected (2–3), and severely affected (4–5). Representative photos of trees from the three vitality classes are presented in Supporting Information S1: Figure S2.

2.3 | Sampling of Scots Pine Shoot Tissue for Quantification of Metabolites, *M. pinitorqua* DNA, and *D. sapinea* DNA

Sampling was done during three time points in 2021; when *M. pinitorqua* infections were first visible on the shoots (2 June), when *M. pinitorqua* aeciospores were visible (10 June), and when the trees had started healing the wounds caused by the infections (8 July). No signs of *D. sapinea* infections were visible in young shoots at the time of sampling. Representative photos of *M. pinitorqua* infection phases from the time points are presented in Figure 1. The sampling scheme is presented in Supporting Information S1: Figure S3. The phenotyping data is summarised in Table S1, and the number of samples per time point, group, and shoot symptom is presented in Table S2.

From category M and MD, *M. pinitorqua* symptomatic and asymptomatic shoots from the same branch (where possible, otherwise from the same side of the tree) on the second branch-whorl were cut using secateurs disinfected with 70% EtOH. No symptomatic shoots were observed on category H during the first and second time points; hence, only asymptomatic samples

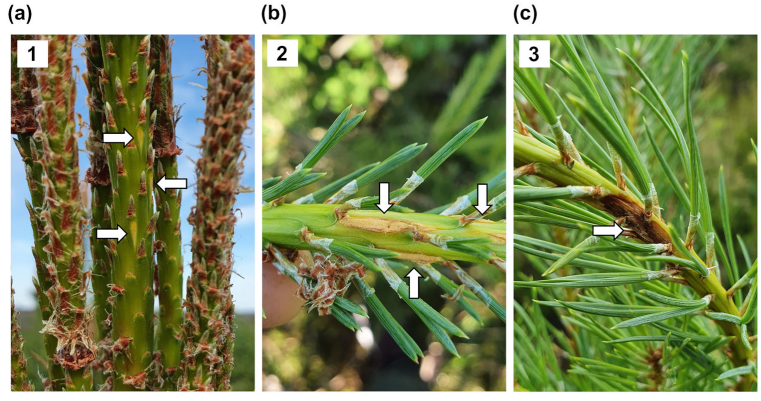

FIGURE 1 | Representative photos of the *M. pinitorqua* infection phase on Scots pine shoots at the different sampling time points. White arrows point to symptoms of *M. pinitorqua*. (a) Time point 1 (2 June 2021)—early signs of aecidia on the shoots, (b) time point 2 (10 June 2021)—aeciospores on the surface of the infection site, (c) time point 3 (8 July 2021)—infection inactive and the canker has started to heal.

were collected at those time points. The samples were put straight in a cooler (filled with clamps frozen at −80°C), kept below 0°C during transport, and stored at −80°C until handling. Eighty samples were included in the metabolite and fungal biomass analyses. To prepare the samples, needles were removed using scissors, and the top of each shoot was removed to fit the sample in a 15 mL Falcon tube. The shoot surface was washed by adding ~12 mL 0.01% Tween, shaking the tubes and then rinsing the samples in ddH_2O three times. Each sample was cut using a disinfected scalpel adjacent to and above the *M. pinitorqua* infection site to a length of ~25 mm. Asymptomatic samples were cut at the corresponding height and to the same length. The samples were lyophilised and homogenised in 2 mL screw-cap tubes containing an M6 screw in the bottom and an M6 nut on top by beating it in a Precellys (Bertin technologies) for 20 s at 4000 rpm as many times as needed (1-8 times) until the sample was homogenised. A subsample of 10–20 mg of each sample was weighed and used for metabolite analysis.

2.4 | Extraction and Quantification of *D. sapinea* and *M. pinitorqua* DNA

DNA was isolated from the remaining homogenised sample using the Qiagen DNeasy Plant mini kit with the following modifications to the protocol: (i) 700 µL AP1 and 225 µL P3 were added for tissue lysis; (ii) 450 µL lysate were transferred to the QIAshredder spin column; and (iii) DNA was eluted twice.

Primers for *M. pinitorqua* diagnostics and quantification were designed in-house (F: 5′ CCC TCG GCT TTA ACA CTT TCT A-3′, R: 5′-CGA TAC GAC CAA AGA CCA TCT C-3′). Briefly, the genus-specific region was identified in an alignment of the ITS1-5.8S-ITS2 region of *Melampsora* spp. and other closely related rust species and used to design the primers. The primers amplify a 168-bp fragment in the ITS2 region of *Melampsora* spp. The specificity was confirmed with (1) NCBI primer blast: among the 251 returned results, 96% were *Melampsora* spp, and the remainder sequences were uncultured fungi except one (GQ479878, labelled as nematode); (2) standard PCR with common rust fungi, Scots pine pathogens and endophytes: *Cronartium pini, Coleosporium* sp., *Gymnosporangium* sp., *M. larci-epitea, M. pinitorqua, Puccinia triticina, P. graminis, Thekopsora areolata, Aequabiliella palatina, Cladosporium* sp., *Sarea coeloplata, and Sydowia polyspora*. Only *Melampsora* spp. produced products with the expected size. The GH3 homologue for the determination of Scots pine biomass in qPCR reactions was designed by Heller et al. (2012). For the determination of *D. sapinea* biomass, the assay used for qPCR was designed by Luchi et al. (2005).

Standard curves for qPCRs for *M. pinitorqua*, *D. sapinea*, and Scots pine were produced by PCR amplification on DNA templates from *M. pinitorqua* (aeciospore sample), *D. sapinea*, and Scots pine. The PCR products' desired lengths (*M. pinitorqua* 168 bp, *D. sapinea* 79 bp, Scots pine ~100 bp) were confirmed through gel electrophoresis. DNA from the PCR products were precipitated, quantified using a Qubit Fluorometer, and used for tenfold serial dilutions to generate standard curves for assays for each organism. The qPCR reactions for quantification of *M. pinitorqua* and Scots pine contained 1× SsoFast EvaGreen Supermix (Bio-Rad) and 500 nM of forward and reverse primer, respectively. The qPCR reactions for quantification of *D. sapinea* followed the protocol by Luchi et al. (2005) with the following modifications: 1× SsoAdvanced™ Universal Probes Supermix (Bio-Rad), 250 nM each of forward and reverse primer, and 200 nM probe. The reaction volumes were 15 µL, using either 12.5 ng of DNA template or ddH_2O as a non-template control. All assays were conducted using the CFX Maestro qPCR detection system (BioRad) with the same cycling conditions: 2 min at 95°C and 40 cycles at 95°C for 10 s and 60°C for 15 s. Each assay included a standard curve of serial dilutions from 1×10^7 to 1×10^2 copies per reaction (in duplicates) and three non-template controls. qPCR efficiencies ranged from 94.5% to 97.3% for Scots pine, 90.2%–96.9% for *M. pinitorqua* and 90.2%–96.7% for *D. sapinea*. All assays had an

$R^2 > 0.99$. CFX Maestro software (version 5.3.022.1030) (BioRad) was used to analyse the qPCR data. Samples were excluded if the cycle threshold (Ct) value's standard deviation (stDev) among replicates was > 0.5 (occurring only for *M. pinitorqua* assays, $n = 2$). Samples below the assay's linear detection limit (37.0 Ct) were included in the analysis with SQmean = 0, even if the stDev was > 0.5.

The qPCR copy numbers for *M. pinitorqua* and *D. sapinea* were normalised based on the amount of Scots pine DNA in the same sample (the desired amount of DNA per reaction was 12.5 ng, corresponding to ~625 haploid pine genomes).

2.5 | Extraction and Quantification of Amino Acids and Phenolic Compounds

2.5.1 | Phenolic Compounds

The methanol extracts (1 mL methanol per sample (10–20 mg) containing 10 μg/mL apigenin-7-glucoside as an internal standard) were first run on an LC-UV-Ion-Trap-MS (1100 series equipment (Agilent Technologies, Germany)) coupled to an Esquire 6000 ESI-Ion Trap mass spectrometer (Bruker Daltonics, Germany) to find peaks that absorb at 280 or 330 nm and to determine their molecular weights. Later the samples were analysed for quantification by LC-MS/MS. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies, Boeblingen, Germany). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 μm, Agilent Technologies). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0.0–1.0 min 0%; 1.0–7.0 min, 0%–65% B; 7.0–7.01 min, 65%–100% B; 7.01–8.0 min 100% B, and 8.01–10.0 min 0% B. The mobile phase flow rate was 1.1 mL/min. The column temperature was maintained at 25°C.

An API 3200 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbospray ion source was operated in negative ionisation mode. The ion spray voltage was maintained at −4200 eV. The turbo gas temperature was set at 600°C. Nebulising gas was set at 60 psi, curtain gas at 30 psi, heating gas at 60 psi, and collision gas at 6 psi. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode; details of the instrument parameters can be found in Table S3. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for data acquisition and processing. Linearity in ionisation efficiencies was verified by analysing the dilution series of standard mixtures.

The following compounds were quantified absolutely from available standards and determined response factors: catechin, taxifolin, astringin, proanthocyanidin B1, isorhamnetin, piceid, quercetin-glucoside, taxifolin-glucoside, and kaempferol-3-glucoside. The other compounds were tentatively identified and were relatively quantified as normalised peak area/g dw: naringenin-6-C-glucoside, kaempferol-3-(6”-acetyl-glucoside), isorhamnetin-acetyl-glucoside, kaempferol-3-(6”-coumaroyl-glucoside), kaempferol-3-(3”,6”-di-coumaroyl-glucoside), 479-316 (compound identity unknown; myricetin-hexoside, molecular formula $C_{21}H_{20}O_{13}$), gallocatechin, neolignan, and matairesinol. A majority of the compounds were previously described from pine species (Slimestad 2003).

2.5.2 | Amino Acids

Amino acids were quantified with an LC-MS/MS using a C18-column (XDB-C18, 50 × 4.6 mm × 1.8 μm; Agilent, Santa Clara, CA, USA) after diluting the methanol raw extracts (used for phenolic compound analysis, see above) at 1:10 (v:v) with water containing 10 μg/mL of a mixture of 15 N, 13C-labelled amino acids (Isotec, Miamisburg, OH, USA) and 5 μM of D5-tryptophane (Cambridge Isotope Laboratories, Inc.; Andover, MA). For details on the chromatography and mass spectrometry (Agilent 1260 LC system (Agilent Technologies, Santa Clara, CA, USA) coupled with a QTRAP 6500 tandem mass spectrometer (AB Sciex, Darmstadt, Germany)), see Crocoll et al. (2016) and Table S4. The mass spectrometer was operated in positive ionisation mode in multiple reaction monitoring mode. All amino acids were quantified relative to the peak area of the corresponding labelled compound, except for asparagine (using aspartate and a response factor of 1.0).

The analysed metabolites are presented in Table S5a,b.

2.6 | Statistical Analysis

Data processing, analyses and visualisation were done using R software (v. 4.1.2; R Core Team 2021). One of the samples showed extreme concentrations of several amino acids and was therefore removed from the amino acid data set. It did not appear to be an outlier in the data for phenolic compounds. When motivated, this outlier was removed from the analyses, e.g., when samples were analysed across metabolite categories. The effect of time point, shoot symptom and disease category on the amino acid and phenolic compound concentrations was tested and visualised using redundancy analysis (RDA) in the *vegan* package (Oksanen et al. 2022), with 79 independent observations and a response matrix of 19 dimensions for amino acids and 80 independent observations and a response matrix of 18 dimensions for the phenolic compounds. The RDAs were run with metabolites scaled proportionally to eigenvalues and conditioned on (i.e., statistically controlling for) time point, tree individual and shoot symptom to remove the effect of those variables when appropriate. Significances for groups were tested using permutational ANOVA. The relationship between total amino acids or phenolics and disease category time point 1 was assessed using Kruskal–Wallis rank sum test with Dunn's post hoc test performed with the kruskal.test and dunn.test functions from the packages *stats* and *dunn.test* (base R implemenation and Dinno [2017], respectively). The association between total amino acids or phenolics and shoot symptoms at each time point was evaluated using the Wilcoxon rank sum test (wilcox.test function in the package *stats*, base R implementation). Indicator Species Analysis (ISA), using the function multipatt from the package *indicspecies* (Cáceres and Legendre 2009), was then performed on rescaled data with 999 permutations to further investigate the metabolites associated

with time point, shoot symptom, and disease category. Heat maps were created with the *pheatmap* package (Kolde 2019) on *Z*-score normalised data using the complete clustering method. The effect of time point, shoot symptom, and disease category on the abundance of *M. pinitorqua* and *D. sapinea* DNA was tested using ANOVA (function aov in package *stats*, base R) on linear models. A transformation with the natural logarithm was performed on the DNA copy number data due to the deviation from a normal distribution with 1 added to each value (log $(1+x)$). Estimated marginal means (package *emmeans* [Lenth 2023]) using multivariate *t* distribution adjustment were computed as post hoc tests for comparison between groups, and significance letters were assigned using the function cld in the package *multcomp* (Hothorn, Bretz, and Westfall 2008). To assess the relationship between individual metabolite concentration levels and pathogen biomass, generalised linear models (GLMs) with a Gamma distribution and log-link function were fitted, including time point and tree individual as covariates, using the glm function (package *stats*, base R implementation). The association between tree vitality in 2021 and disease symptoms in 2020 was tested using a chi-square test (chisq.test function, base R implementation). Figures were created using the *ggplot2* package (Wickham 2016) and the *pheatmap* package (Kolde 2019) in R Studio (R Core Team 2021).

3 | Results

3.1 | Impacts of Disease History and Co-Infection on Phenolic Profile, Tree Vitality and Pathogen Dynamics

The disease categories assigned based on the trees' symptoms after the 2020 growing season remained stable throughout the study. The healthy-looking (H) trees consistently had the lowest number of *M. pinitorqua*-infected shoots from 2019 to 2021 and remained virtually free of DTB. The *M. pinitorqua*-symptomatic (M) trees exhibited a high incidence of *M. pinitorqua* symptoms but had a low number of DTB-symptomatic shoots during the same period. The *M. pinitorqua*- and *D. sapinea*-symptomatic (MD) trees persistently had a high ratio of *M. pinitorqua*-infected shoots and the highest incidence of DTB-symptomatic shoots (Table S1).

Trees that were healthy-looking in 2020 (disease category H) got infected by *M pinitorqua* later than trees that were *M. pinitorqua*-symptomatic in 2020 (M) and trees that were *M. pinitorqua*- and *D. sapinea*-symptomatic in 2020 (MD). The trees in disease category H had no *M. pinitorqua* symptoms on shoots of the top whorl in 2021 in early June (time points 1 and 2). In early July (time point 3), symptomatic shoots were found in all trees, although the frequency of infected shoots was lower in trees from disease category H than in other trees (Supporting Information S1: Figure S3, Tables S1, and S2).

For samples in the MD category, both the total amount and composition of phenolic compounds in asymptomatic shoots differed from the other two categories at the first time point (Figure 2 and Table S6a,b). The total concentration of phenolics was lower in asymptomatic shoots in the H category (Table S6b). Neither the total amount nor the amino acid profile in asymptomatic shoots at time point 1 was associated with the disease categories (Supporting Information S1: Figure S4 and Table S6a,b).

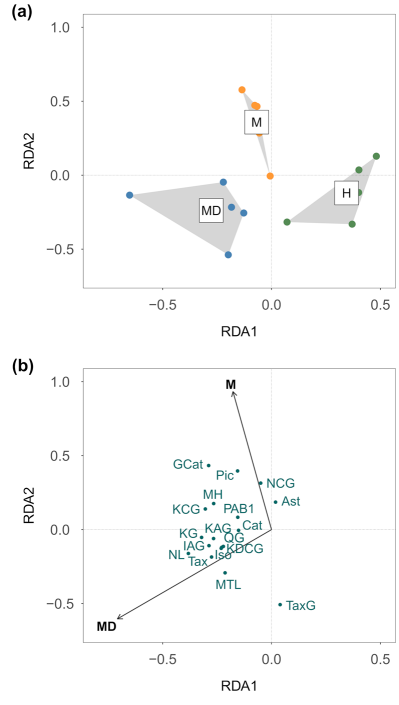

FIGURE 2 | Composition of phenolic compounds in asymptomatic shoots at time point 1 (2 June 2021) explained by disease category based on the tree's symptoms in 2020; H—healthy-looking, M—*M. pinitorqua*-symptomatic, and MD—*M. pinitorqua*- and *D. sapinea*-symptomatic. (a) Sample plot presented with hulls connecting the samples from each disease category. (b) The RDA presented with loadings. The disease category was associated with the composition of phenolic compounds (RDA; permutational ANOVA; $^{***}p = 0.001$, adj. $R^2 = 0.217$). For full compound names, please refer to Table 1.

Trees in disease category H showed lower numbers of *M. pinitorqua* DNA copies than trees in the other two categories (Figure 3a and Table S6c). There was no significant difference in the abundance of *D. sapinea* DNA between trees from different disease categories (Figure 3b, Table S6d).

The tree vitality in 2021 was significantly associated with disease symptoms in 2020 ($\chi^2 = 86$, df = 4, $^{***}p < 0.001$). All trees categorised as healthy-looking (H) in 2020 were in the vitality class "fully vital" in 2021. Of trees categorised as *M. pinitorqua*-symptomatic in 2020 (M), two trees were in the vitality class "fully vital" and three trees in the vitality class "mildly affected" in 2021. One of the trees categorised as *M. pinitorqua*- and *D. sapinea*-symptomatic in 2020 (MD) was in the vitality class "mildly affected" in 2021, while the four remaining trees were in the vitality class "severely affected" (Table S1).

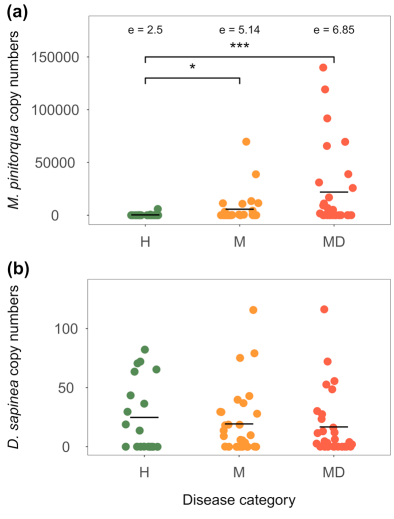

FIGURE 3 | Abundance of (a) *M. pinitorqua* DNA and (b) *D. sapinea* DNA (raw copy numbers quantified using qPCR and normalised on the amount of Scots pine DNA in the sample) per disease category based on the tree's symptoms in 2020; H—healthy-looking trees (green points), M—*M. pinitorqua*-symptomatic trees (yellow points) and MD—*M. pinitorqua*- and *D. sapinea*-symptomatic trees (red points). Black lines represent mean values, asterisks show level of significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), ∈ shows estimated marginal means of $\log(1 + x)$.

3.2 | Metabolic Responses to Infection by *M. pinitorqua* and *D. sapinea*

Both amino acid and phenolic compound profiles showed dissimilarities between time points (Supporting Information S1: Figure S5 and Table S6e). Thirty-three out of the 37 metabolites contributed to the variation (Table 1). Five amino acids were identified as indicators for time point 1, two amino acids and one phenolic compound were identified for time point 2, and six phenolic compounds were identified for time point 3 (see Table 1 for details). The analysed metabolites and their concentrations are presented in Table S5a,b.

The RDA conditioned on time point showed that the amino acid profile differed between *M. pinitorqua*-symptomatic and asymptomatic tissue (Figure 4a, Table S6f), with the strongest indicators for symptomatic shoots being aspartate, leucine and phenylalanine (Table 1). The differences between asymptomatic and symptomatic shoots were more pronounced for the phenolic compounds than for the amino acids (Figure 4b, Table S6g). The phenolic compounds that contributed most to the differentiation were proanthocyanidin B1 (PAB1), catechin, piceid, astringin, matairesinol and taxifolin, all identified as indicator metabolites for symptomatic shoots. Kaempferol-3-O-glucoside and quercetin-glucoside were indicators for asymptomatic shoots (Table 1). Both total amino acids and phenolics were higher in *M. pinitorqua*-symptomatic shoots than in asymptomatic shoots at time point 3 (***$p < 0.001$, Table S6h). There was no association between shoot symptom and total amino acids or phenolics at earlier time points (Table S6h).

The biomass of *M. pinitorqua*, measured as qPCR copy numbers of ITS, was higher in *M. pinitorqua*-symptomatic shoots than in asymptomatic shoots and differed between time points; the number of DNA copies was lower at time point 3 than at both time point 1 and time point 2 (Figure 5a and Table S6c).

There was no significant deviation in *D. sapinea* biomass between *M. pinitorqua*-symptomatic and asymptomatic shoots (Figure 5b and Table S6d). However, although no fruiting structures of *D. sapinea* were observed on the current-year shoots, *D. sapinea* biomass increased significantly with each subsequent time point (Figure 5b and Table S6d).

Neither the abundance of *M. pinitorqua* and *D. sapinea* DNA nor the interaction between their abundances was linked to the composition of amino acids or the concentration of individual amino acids or phenolics. The biomass of the individual pathogens did not relate to the phenolic compound profile, although the interaction between the pathogens was connected to the composition of phenolics. This effect was more pronounced for the interaction between *M. pinitorqua* symptom and the abundance of *D. sapinea* DNA; both shoot symptom and the interaction influenced the phenolic compounds (Table S6i). The phenolic compounds with the strongest positive association with the shoot symptom × *D. sapinea* interaction were PAB1, catechin, piceid, and astringin. Matairesinol was the metabolite with the strongest association with symptomatic shoots (Figure 6).

4 | Discussion

In this study, we showed that the disease symptom severity caused by *M. pinitorqua* and *D. sapinea* in Scots pine trees was consistent between years. In addition, the symptoms of the preceding season affected the metabolite profiles at the beginning of the season and were associated with *M. pinitorqua*'s proliferation in the tree. *D. sapinea*'s biomass during the sampling period was independent of previous disease symptoms and infection by *M. pinitorqua*. The composition of amino acids and phenolics differed between *M. pinitorqua*-symptomatic and asymptomatic tissue, and tissues from shoots colonised by *M. pinitorqua* but not by *D. sapinea* exhibited a distinct set of phenolic compounds compared to tissues colonised by both pathogens. However, the *M. pinitorqua* symptom had a stronger effect on the shoot metabolites than the *M. pinitorqua* biomass.

4.1 | The Disease Categories Predict the Pathogen Dynamics, Phenolic Profiles and Tree Vitality

In 2021, trees that were healthy-looking in 2020 (disease category H) got infected by *M. pinitorqua* later than trees that were classified as *M. pinitorqua*-symptomatic in 2020 (M) and trees that were *M. pinitorqua*- and *D. sapinea*-symptomatic in 2020 (MD). Factors such as the length of the unprotected shoot or trees falling below the height threshold for susceptibility to *M.*

TABLE 1 | Results of the indicator metabolite analyses for factors with a statistically significant multivariate effect on metabolite composition.

		Time point					Shoot symptom (*M. pinitorqua*)				Disease category (asymptomatic shoots time point 1)				
Metabolite	**Abbreviation**	**1**	**2**	**3**	**Stat**	***p***	**A**	**S**	**Stat**	***p***	**H**	**M**	**MD**	**Stat**	***p***
Alanine	Ala	X			0.734	0.014									
Arginine	Arg										*No statistically significant multivariate effect of disease category was found for amino acids.*				
Asparagine	Asn	X	X		0.485	0.014									
Aspartate	Asp							X	0.302	0.016					
γ-aminobutyric acid	GABA	X			0.602	0.014									
Glutamine	Gln	X			0.491	0.014									
Glutamate	Glu	X	X		0.412	0.025									
Histidine	His	X	X		0.584	0.014									
Isoleucine	Ile	X	X		0.601	0.014									
Leucine	Leu		X		0.328	0.014		X	0.216	0.044					
Lysine	Lys		X		0.542	0.014									
Methionine	Met	X			0.518	0.014									
Phenylalanine	Phe	X	X		0.524	0.014		X	0.219	0.033					
Proline	Pro	X			0.459	0.014									
Serine	Ser	X	X		0.703	0.014									
Threonine	Thr	X	X		0.522	0.014									
Tryptophan	Trp	X	X		0.558	0.014									
Tyrosine	Tyr	X	X		0.635	0.014									
Valine	Val	X	X		0.565	0.014									
Astringin	Ast	X		X	0.297	0.045		X	0.325	0.017					
Catechin	Cat			X	0.414	0.026		X	0.426	0.017					
Gallocatechin	GCat	X	X		0.714	0.014						X	X	0.749	0.003
Isorhamnetin	Iso			X	0.560	0.014									
Isorhamnetin-(acetyl-glucoside)	IAG	X	X		0.544	0.014									
Kaempferol-3-(3”,6”-di-coumaroyl-glucoside)	KDCG		X		0.845	0.014									
Kaempferol-3-(6”-acetyl-glucoside)	KAG	X	X		0.544	0.014									

(Continues)

TABLE 1 | (Continued)

Metabolite	Abbreviation	Time point					Shoot symptom (*M. pinitorqua*)				Disease category (asymptomatic shoots time point 1)				
		1	2	3	Stat	*p*	A	S	Stat	*p*	H	M	MD	Stat	*p*
Kaempferol-3-(6”-coumaroyl-glucoside)	KCG	X	X		0.573	0.014						X	X	0.668	0.026
Kaempferol-3-O-glucoside	KG	X	X		0.757	0.014	X		0.225	0.030		X	X	0.629	0.032
Matairesinol	MTL							X	0.241	0.027					
479–316[a]	MH	X	X		0.636	0.014						X	X	0.604	0.04
Neolignan	NL			X	0.656	0.014							X	0.741	0.005
Naringenin-6-C-glucoside	NCG	X	X		0.466	0.039									
Proanthocyanidin B1	PAB1			X	0.495	0.026		X	0.499	0.017					
Piceid	Pic							X	0.421	0.017					
Quercetin-glucoside	QG	X	X		0.696	0.014	X		0.202	0.042					
Taxifolin	Tax			X	0.856	0.014		X	0.178	0.031					
Taxifolin-7-glucoside	TaxG			X	0.857	0.014									

Note: Displayed are the metabolites responsible for the differences between metabolite profiles per time point (1—2 June 2021, 2—10 June 2021, 3 – 8 July 2021), *M. pinitorqua* symptom on the analysed shoot (A—asymptomatic, S—symptomatic), and disease category (based on the tree's symptoms in 2020; H—healthy-looking, M—*M. pinitorqua*-symptomatic, and MD—*M. pinitorqua*- and *D. sapinea*-symptomatic), with their corresponding correlation statistics and permutation-based *p*-values (*n* permutations = 999).

[a]Compound identity unknown; myricetin-hexoside.

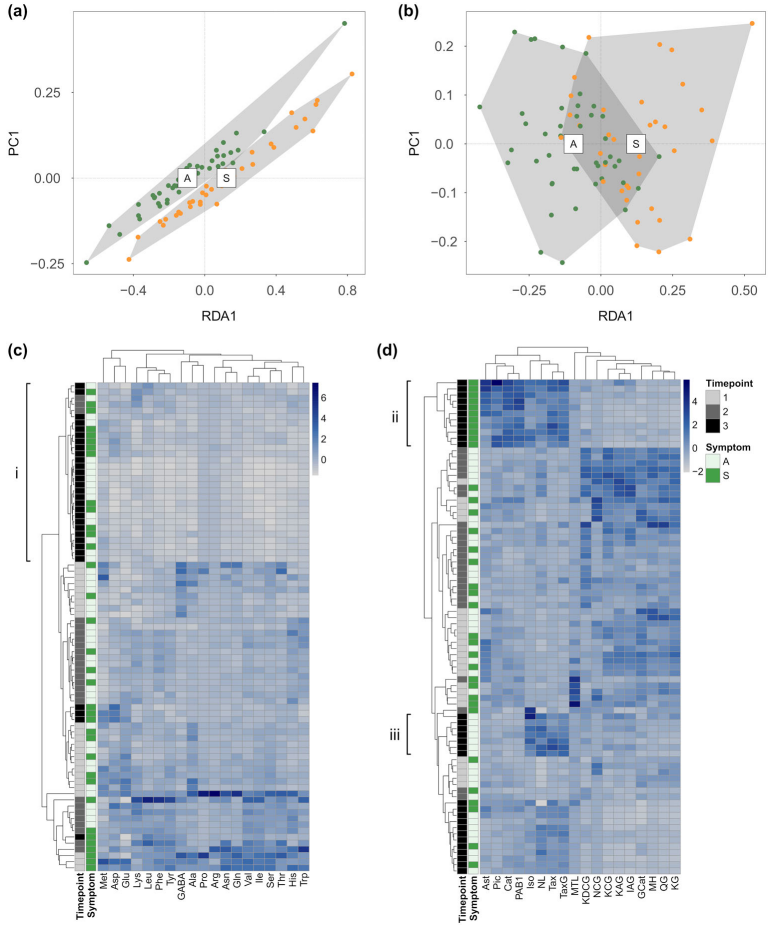

FIGURE 4 | Composition of (a) amino acids and (b) phenolic compounds explained by *M. pinitorqua* symptom on the analysed shoot (A—asymptomatic, S—symptomatic). *M. pinitorqua* symptom causes significant differences in the metabolite profiles (RDA conditioned on time point and individual tree. Permutational ANOVA; $p_{\text{amino acids}} = 0.006^{**}$, adj. $R^2_{\text{amino acids}} = 0.033$, $p_{\text{phenolics}} = 0.001^{***}$, adj. $R^2_{\text{phenolics}} = 0.057$). Clustering of (c) amino acids and (d) phenolic compounds using Euclidean distance as the similarity measure on *Z*-score normalised concentrations, with one pronounced cluster of amino acids in time point 3 (i) and two pronounced clusters of phenolic compounds for *M. pinitorqua*-symptomatic shoots at time point 3 (ii) and asymptomatic shoots at time point 3 (iii). For full compound names, please refer to Table 1.

pinitorqua have been proposed as contributing to variation in *M. pinitorqua* infections (Desprez-Loustau and Wagner 1997). However, there was no difference in tree height in 2021 or height growth in the years 2018 through 2021 between trees from different disease categories (data not shown). Consequently, the lower number of infected shoots on the healthy-looking trees could not be explained by a difference in the amount of *M. pinitorqua*-susceptible tissue between categories. The trees in disease category H also showed lower *M. pinitorqua* DNA copy numbers than trees in categories M and MD, further indicating a higher resistance in those trees and supporting our hypothesis that the abundance of pathogen biomass is impacted by the symptom status of the tree the previous year. Therefore, the population showed a quantitative variation in colonisation and symptoms, with the healthy-looking trees (disease category H) displaying no or very few symptoms. It is possible that this observed variation is due to beneficial effects of defeated resistance (R) genes (Dowkiw and Bastien 2006). Defeated R genes refer to resistance genes previously overcome by pathogens. These genes may still confer a residual level of protection against pathogen attacks, allowing the trees that carry them to better control the disease. Furthermore, although there was no difference in *D. sapinea* biomass between trees from different disease categories, H trees showed consistently low numbers of *D. sapinea* symptoms. The H trees were the most vital trees at the end of the study. Thereby, none of the fungi were

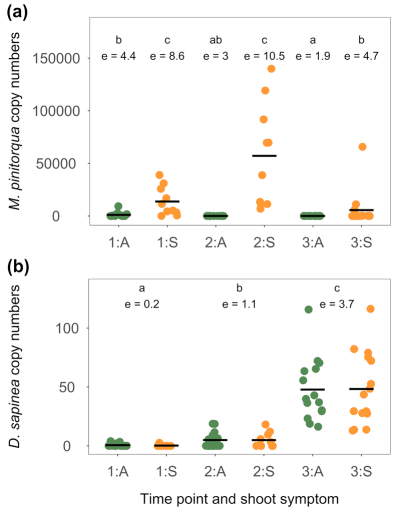

FIGURE 5 | Abundance of (a) *M. pinitorqua* DNA and (b) *D. sapinea* DNA (raw copy numbers quantified using qPCR and normalised on the amount of Scots pine DNA in the sample) per time point (1—2 June 2021, 2—10 June 2021, 3—8 July 2021), and *M. pinitorqua* symptom on the analysed shoot; A—asymptomatic (green points), S—symptomatic (yellow points). Black lines represent mean values, different letters above panels indicate significant differences between groups (Tukey HSD; $p < 0.05$), $\ominus$ shows estimated marginal means of log $(1 + x)$.

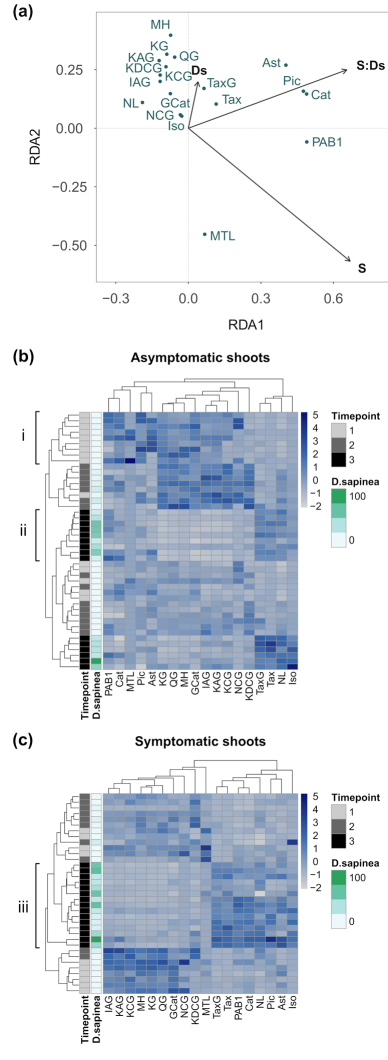

FIGURE 6 | (a) Composition of phenolic compounds explained by the interaction between *M. pinitorqua* symptom (S) and abundance of *D. sapinea* DNA (Ds). Both *M. pinitorqua* symptom and the interaction with *D. sapinea* abundance influenced the phenolic compounds (RDA conditioned on time point and individual tree. Permutational ANOVA; $p_S = 0.001$ ***, $p_{S \times Ds} = 0.001$ ***, adj. $R^2 = 0.106$). (b and c) Clustering of phenolic compounds in (b) asymptomatic shoots and (c) *M. pinitorqua*-symptomatic shoots using Euclidean distance as the similarity measure on *Z*-score normalised concentrations. For full compound names, please refer to Table 1.

particularly successful in the H trees. These observations collectively demonstrate a higher level of disease resistance in the trees classified as healthy-looking.

In contrast, the MD trees, which displayed symptoms of both diseases, were also the most affected at the end of the study, backing our hypothesis that the disease symptoms of the tree in the preceding growing season impact the tree's vitality during the following growing season. There are different potential explanations for this. Higher levels of *M. pinitorqua* biomass in combination with *D. sapinea* colonisation in MD trees may lead to exacerbated disease symptoms as a consequence of alterations in microbiome composition, production of toxins, or suppression of the host's immune response (Liu et al. 2023), ultimately resulting in DTB symptoms. Alternatively, MD trees may be inherently more susceptible to DTB than M trees, even when exposed to similar levels of *D. sapinea*, either due to genetic factors or physiological differences that make MD trees more vulnerable to the disease. This possibility is reflected in the observation that trees in different disease categories had different levels of total phenolics in asymptomatic tissues at time point one. This could be a genetic effect or a consequence of the disease levels in the previous season, as pathogen challenges may induce systemic accumulation of phenolics (Fossdal et al. 2012; Wallis et al. 2008). The presence of both pathogens may alter the microenvironment within the tree, creating conditions that are more favourable for DTB development. For example, the higher pathogen pressure of *M. pinitorqua* in MD trees may weaken or drain their defences (Zaman et al. 2023), making them more sensitive to *D. sapinea*, allowing the fungus to change its physiology.

4.2 | Co-Infections Alter the Metabolite Composition in the Shoots, But *M. pinitorqua* Infection Does Not Directly Impact *D. sapinea* Colonisation

The profiles of free amino acids in Scots pine tissues change over the season and in response to changes in the tree's environment, such as fertilisation, drought, infections, or mechanical wounding (Caballol et al. 2022; Gezelius 1986; Nasholm and Ericsson 1990; Pietiläinen and Lähdesmäki 1986). Similarly, the phenolic compound profiles change during shoot development when trees undergo rapid physiological changes to support new growth, including the synthesis of essential metabolites involved in cell division, elongation and lignification (Ghimire et al. 2019; Nerg et al. 1994). Although the first two sampling times occurred just 1 week apart, in early June, they showed distinct differences in metabolite composition. These differences are likely attributed to changes occurring during shoot development as well as during the progression of *M. pinitorqua* infections. This assumption is supported by the observed variations in both amino acid and phenolic compound compositions between tissues showing symptoms of *M. pinitorqua* and those that are asymptomatic, a finding that aligns with our hypothesis that symptoms induced by *M. pinitorqua* influence the metabolite composition within the tissue. Particularly, our analyses identified the flavonoid glucosides kaempferol-3-glucoside and quercetin-glucoside as indicators for asymptomatic tissue. Flavonoids and flavonoid glucosides are normally abundant in Scots pine tissues (Laracine-Pittet and Lebreton 1988). The flavonoid glucosides can be metabolised to aglycones, for example, quercetin-glucoside may serve as a reservoir for quercetin or other metabolites, which can then be rapidly deployed upon pathogen attack (Keinänen et al. 1999; Lamara et al. 2018). Under such a scenario, the concentration of these compounds may decrease when symptoms appear and the metabolites are used.

The phenolic compounds contributing to the difference in the profiles were mostly indicators for symptomatic tissue, showing that the *M. pinitorqua* infection in the shoot is associated with a local accumulation of phenolic compounds. The indicator phenolics for *M. pinitorqua*-symptomatic tissue belonged to several classes of phenolics: stilbenes (piceid, astringin), lignans (matairesinol), and flavonoids (taxifolin, catechin, PAB1). These metabolites and metabolite classes are well known to associate with defence responses in Pinaceae (Brignolas et al. 1995; Brignolas et al. 1995; Ganthaler et al. 2017; Hammerbacher et al. 2019; Harju, Venäläinen, Laakso, & Saranpää, 2009) and can be directly fungicidal or reinforce the plant cell wall, making it difficult for the pathogen to spread in the tissue (Nagy et al. 2022; Ullah et al. 2017). In short, *M. pinitorqua* symptoms locally altered metabolite composition in shoots, primarily increasing metabolite concentrations in symptomatic ones. However, the *M. pinitorqua* biomass quantity did not influence metabolite profile compositions, indicating that the responses to an active or recent *M. pinitorqua* infection may derive from the simple recognition of the infection rather than the amount of the pathogen present.

The colonisation pattern of the two pathogens differed across the sampling points. More *M. pinitorqua* biomass was consistently found in shoots with symptoms of ongoing infections at the two first sampling times, while the largest amount of *D. sapinea* biomass was detected at the third sampling point. Our sampling relied on visual assessments of disease symptoms in situ to identify and categorise *M. pinitorqua* infections. There was a good agreement between these visual assessments and the determination of *M. pinitorqua* biomass with qPCR-based measurement of the fungal biomass; the *M. pinitorqua* biomass was generally low in asymptomatic shoots. Furthermore, the *M. pinitorqua* biomass was lowest at time point three, independent of whether the shoots were *M. pinitorqua*-symptomatic or not. This suggests that the local infections by *M. pinitorqua* terminated after the production of aeciospores (Mattila 2005), which generally occurred between the second and third time points.

The biomass of *D. sapinea* was the highest at the third time point. Moreover, the finding that *M. pinitorqua*-symptomatic and asymptomatic shoots contained similar levels of *D. sapinea* DNA shows that the lesions caused by the rust infections are unlikely specific entry points for *D. sapinea*, despite the frequent reports on DTB symptoms in mechanically wounded trees (Caballol et al. 2022; Oostlander et al. 2023; Smith, Wingfied, and Coutinho 2002; Zwolinski, Swart, and Wingfield 1995). The local stress responses related to *M. pinitorqua* infection, or the following healing process, also do not appear to influence *D. sapinea*'s ability to colonise the shoots. Consequently, we found no evidence that *M. pinitorqua* infections directly predisposed the trees to *D. sapinea* infection. Instead, these results support that, once *D. sapinea* is established as an endophyte, tree physiology is altered under certain conditions in a way that influences resistance to other pathogens. (Blodgett, Kruger, and Stanosz 1997; Blumenstein et al. 2021; Brodde et al. 2023; Stanosz et al. 2001; Swart, 1991; Zwolinski, Swart, and Wingfield 1995).

We expected tissues from shoots colonised by *M. pinitorqua* but not by *D. sapinea* to show a distinct set of phenolic compounds compared to tissues colonised by both pathogens, a hypothesis that proved to be true. However, the effect on the phenolic compound profile of *M. pinitorqua* symptoms was stronger than the presence of the fungus itself. The presence of *D. sapinea* in shoots with *M. pinitorqua* symptoms impacted the metabolite profiles significantly. Despite proline's and glutamate's reported importance for *D. sapinea* and the tree's defence responses (Caballol et al. 2022; Ghosh et al. 2022; Sherwood et al. 2015), we did not see any distinct patterns in the concentrations of those amino acids. The colonisation by *D. sapinea* in *M. pinitorqua*-symptomatic tissue influenced the phenolic profiles, resulting in different concentrations of phenolic compounds compared to symptomatic tissue without *D. sapinea*. This indicates that although *M. pinitorqua* infections do not directly predispose the trees to *D. sapinea* infection, there is a local interaction between the infections shaping the profiles of phenolic compounds in the tissue when *D. sapinea* is present.

It should be emphasised that the *D. sapinea* infections appeared latent or presymptomatic when the study was carried out, and characteristic DTB symptoms were present in particular on trees in the MD category only during surveys in autumn. It is possible that the distinct changes in amino acids and phenolic compounds

reported in the literature (Caballol et al. 2022; Ghosh et al. 2022; Sherwood et al. 2015) are more intimately tied to the shift in *D. sapinea*'s physiology than the direct response of the host tree to active infections. However, latent or presymptomatic infections have also been shown to induce host defence responses (e.g., Vornam et al. 2019; Hu et al. 2023). These responses can involve the expression of genes related to oxidative stress defence, lignification and flavonoid synthesis (Vornam et al. 2019) and repression of photosynthesis Hu et al. (2023).

5 | Conclusion

Our study found no direct support for our hypothesis that *M. pinitorqua* infections predispose the trees to DTB by stressing the host and altering the composition of metabolites in infected tissues. The presence of *M. pinitorqua* symptoms on the shoot was a stronger predictor for changes in metabolite profiles than the fungal biomass. Due to the biotrophic nature of the pathogen, its recognition may be more important for the activation of the tree's defence mechanisms than the biomass. *D. sapinea* colonisation was independent of the *M. pinitorqua* biomass in the tissues. However, we also found that trees with high vitality retained vital characteristics throughout the surveyed time period and appeared to possess tolerance to *M. pinitorqua*, with delayed rust infections and minimal DTB symptoms, suggesting a more complex relationship between the host and the fungi. This potential tolerance is an interesting observation that should be followed up on in future studies, as it may be a trait that improves the resilience of young pine plantations.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

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Supporting Information

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This thesis investigates genetic, environmental, and mechanistic factors influencing conifer disease development, focusing on Scots pine and Norway spruce in Sweden. It examines *Diplodia sapinea*, *Melampsora pinitorqua*, and *Heterobasidion parviporum* infections, exploring drought effects, co-infection outcomes, and host genetic variation. Genome-wide association studies identified markers linked to disease, and genomic selection showed potential for improving resistance. The findings underscore the importance of understanding host–pathogen interactions and using genetic tools to ensure long-term forest health and resilience under climate change.

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