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Technologies for early detection and monitoring of new and emerging pathogens

Safeguarding urban and forest trees

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Abstract

Forests and urban trees face growing threats from pathogens, both alien and native, and historical examples have proven invasive forest pathogens capable of reshaping entire ecosystems. Climate change exacerbates the problem by favoring pathogen growth and predisposing trees to infection. The galloping rates at which forest pathogen outbreaks are occurring needs to be addressed, but there is a lack of appropriate detection tools that can be implemented into biosurveillance infrastructures to mitigate these issues. This thesis aimed to acquire new knowledge and improve the applicability of a new generation of detection tools, focusing on analysis of volatile organic compounds for pathogens relevant for Sweden (*Fusarium circinatum* and *Phytophthora*), and high-throughput DNA sequencing of pine needle fungi. In this thesis, volatile analysis could distinguish between species of *Fusarium* and *Phytophthora in vitro*. *In vivo* studies showed that quantitative volatile comparisons can distinguish between *Fusarium circinatum*-inoculated pine trees and mock-inoculated controls, and that beech and oak trees infected with *Phytophthora cinnamomi* or *Phytophthora plurivora* produced volatiles that were qualitatively distinct from controls, though volatiles emitted by the pathogens grown *in vitro* were not detected *in vivo*. Pine needle associated mycobiomes were compared using Illumina and nanopore sequencing technologies which revealed that nanopore identified a larger proportion of the taxa to the species level, while Illumina captured higher species diversity. Both platforms successfully identified pathogenic species in asymptomatic needles, illustrating their capacity for early detection. This work underscores the potential of implementing already available technologies for improving the biosecurity infrastructures for safeguarding future forest health.

Keywords: tree diseases, early detection tools, diagnostics, VOCs, nanopore technology, MinION, high-throughput sequencing, pine needle pathogens, *Fusarium circinatum*, *Phytophthora* spp.

Tekniker för tidig detektion och övervakning av nya och kommande patogener - Skydd för träd i skog- och stadsmiljö

Abstrakt

Att skogar och stadsträd hotas av patogener, både utländska och inhemska, är ett växande problem och historiska exempel har visat att invasiva skogsskadegörare är kapabla till att omvandla hela ekosystem. Klimatförändringar förvärrar problemet ytterligare genom att gynna patogeners tillväxt och predisponera träd för infektion. Skogspatogener orsakar i dagsläget utbrott i en rasande takt som kräver åtgärder, men det saknas lämpliga detektionsverktyg som kan implementeras för att motverka introduktion av nya skadegörare till förmån för biosäkerheten. Syftet med denna avhandling var att samla ny kunskap och förbättra applicerbarheten för en ny generation av detektionsverktyg, med fokus på analys av flyktiga organiska ämnen från patogener med relevans för Sverige (*Fusarium circinatum* och *Phytophthora*), samt high-throughput DNA-sekvensering av tallbarrssvampar. Resultaten visade att analyser av volatiler kunde särskilja *Fusarium*- och *Phytophthora*-arter *in vitro*. Analyser *in vivo* visade att *Fusarium circinatum*-infekterade tallar kan särskiljas från friska genom kvantitativa skillnader i volatiler, samt att bok- och ekträd som var inokulerade med *Phytophthora cinnamomi* eller *Phytophthora plurivora* avger kvalitativt olika volatiler jämfört med friska. Ingen av volatilerna som detekterades *in vitro* kunde ses *in vivo*. Tallbarrs-mykobiom jämfördes med hjälp av Illumina- och nanopore-sekvensering och visade att nanopore identifierar en större andel taxa till art-nivå, medan Illumina kunde fånga en större diversitet. Båda metoderna kunde identifiera patogena arter i asymptomatiska barr, vilket visar deras kapacitet för tidig detektion. Resultaten betonar potentialen för implementering av redan tillgängliga tekniker för att förbättra infrastruktur för biosäkerhet och skydd av framtida skogar.

Nyckelord: trädsjukdomar, verktyg för tidig detektion, diagnostik, flyktiga organiska ämnen, MinION, high-throughput sekvensering, tallbarrspatogener, Tallens hartskräfta, *Phytophthora*-algsvampar

Dedication

Till min dotter Ester Solbritt Sofia Sadé

Ingenting får hända dig
Nej vad säger jag
Allt måste hända dig
och det måste vara underbart

- Bodil Malmsten

Till minne av faffan, Karl-Axel Nordström, 1934-2023.

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. Nordström, I.*, Sherwood, P., Bohman, B., Woodward, S., Peterson, D.L., Niño-Sánchez, J., Sánchez-Gómez, T., Díez, J.J., and Cleary, M. (2022). Utilizing volatile organic compounds for early detection of *Fusarium circinatum*. *Scientific reports*, 12, 21661. https://doi.org/10.1038/s41598-022-26078-1

II. Sherwood, P.*, Nordström, I., Woodward, S., Bohman, B., and Cleary, M. (2024). Detecting pathogenic *Phytophthora* species using volatile organic compounds. *Molecules*, 29, 1749. https://doi.org/10.3390/molecules29081749

III. Nordström, I.*, Sherwood, P., and Cleary, M. Illumina versus nanopore sequencing: a comparison of two technologies for detecting pine needle fungi. (manuscript)

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*Corresponding author

The contribution of Ida Nordström to the papers included in this thesis was as follows:

I. **I.N.**, P.S., M.C., J.J.D., B.B. and S.W. developed the research design and formulated the original idea; **I.N.** and P.S. developed the methodology; **I.N.** conducted the greenhouse and lab work; **I.N.**, B.B. and D.L.P. analyzed the GC-MS data and performed statistical analyses; **I.N.** wrote the manuscript together with co-authors.

II. P.S., M.C. and S.W. developed the research design and formulated the original idea; P.S. and **I.N.** developed the methodology; P.S. and **I.N.** conducted the greenhouse and lab work; P.S. analyzed GC-MS data and performed statistical analyses; P.S., **I.N.**, B.B., S.W. and M.C. wrote the manuscript.

III. **I.N.**, P.S. and M.C. developed the research design and formulated the original idea; **I.N.**, P.S. and M.C. developed the methodology; **I.N.** and P.S. conducted the lab work; **I.N.** performed bioinformatic and statistical analyses; **I.N.** wrote the manuscript together with co-authors.

Abbreviations

ASV	Amplicon sequence variant
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
EAB	Emerald ash borer
EMA	Elliott's medium agar
GC-MS	Gas-chromatography mass-spectrometry
IAS	Invasive alien species
ISA	Indicator species analysis
ITS	Internal transcribed spacer
LAMP	Loop-mediated isothermal amplification
MIC	Mock-inoculated control
m/z	Mass to charge ratio
ONT	Oxford Nanopore Technologies
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCR	Polymerase chain reaction
PCoA	Principal coordinate analysis
SPME	Solid-phase micro-extraction
VOCs	Volatile organic compounds

1. Introduction

Trees are of vital ecological, cultural and economic importance and cover about 31% of all global land, but are easily overlooked and often undervalued (Ninan & Inoue 2013; Wingfield *et al.* 2015; FAO & UNEP 2020; BGCI 2021). Due to anthropogenic activities, forests worldwide are facing increasing disturbances that jeopardize system processes and ecosystem services of the earth, such as climate regulation (including carbon sequestration), water cycle regulation, vital resources and habitats for wildlife (Trumbore *et al.* 2015; Cavender-Bares *et al.* 2022; Guégan *et al.* 2023). Many tree species populations are declining; in fact, 42% of European native tree species are threatened with extinction (Rivers 2019). The decline is mainly attributable to invasive plants, deforestation, changes in land use, climate change and either climate-driven increases in, or new introductions of, pests and pathogens (Trumbore *et al.* 2015; Prospero & Cleary 2017; Rivers 2019; BGCI 2021; Guégan *et al.* 2023). Developing systems to assess and monitor forest health, particularly to identify thresholds for rapid decline, is urgent as damage from pests and pathogens can occur swiftly, while restoration of the services forests provide can take decades (Trumbore *et al.* 2015).

1.1 Invasive alien species - an urgent threat

We are living in an era of globalization, a phenomenon that brings both positive and negative implications depending on one's perspective. On the positive side, globalization has contributed to poverty reduction in some developing countries (Dollar & Kraay 2004; Bergh & Nilsson 2014; Gopinath 2019). However, arguably, the negative consequences are more readily apparent, including significant emissions from the transportation

sector, excessive consumption, and ecological and economic disasters resulting from the unintentional introduction of invasive alien species (IAS) to new regions (Carneiro *et al.* 2024). The latter, along with emerging pathogens resulting from changed conditions of host, pathogen, management or environment, are the issues that this thesis aims to address.

International trade is the most significant pathway for accidental movement of alien species, and there is a clear correlation between increasingly globalized economies and the rates of biological invasions (Santini *et al.* 2013; Pysek *et al.* 2020; Haubrock *et al.* 2021). In 2019, the estimated global annual economic cost of biological invasions was US$423 billion (Roy 2023), but estimating the economic consequences of IAS is challenging, and only the known and reported costs are accounted for (Bradshaw *et al.* 2016). Despite being grossly underestimated due to reporting gaps (Diagne *et al.* 2021; Fantle-Lepczyk *et al.* 2021), the yearly estimated costs of IAS globally far exceeds those of the losses from all natural disasters in a given year (Simpson 2004). The total costs of IAS have increased exponentially (Haubrock *et al.* 2021) with a consistent threefold increase over the past decades (Diagne *et al.* 2021), because new species are continually introduced, established, and spread (Seebens *et al.* 2017), which further complicates precise estimations.

In forest-related industries, the costs of IAS are associated to the loss of end-product value and increased management costs for the removal of damaged trees (Haight *et al.* 2011). As an example, the most costly biological invasion in US history is believed to be caused by the emerald ash borer (EAB), a wood-boring insect that was accidentally introduced to North America from Asia (Kovacs *et al.* 2010). Throughout its secondary range where it is still actively spreading, EAB has killed more than 99% of the ash (*Fraxinus*) trees, which are some of the most commonly planted tree species in urban and suburban landscapes in the US (Herms & McCullough 2014).

For some countries, the economy and society largely depend on forestry. For example, 70% of the landscape in Sweden is covered by forests, and forest related industries provide 200,000 jobs and 10% of the sawn timber, pulp, and paper traded on the global market (Gray 2018). Consequently, there are huge socio-economic values to protect by maintaining healthy forests. Beyond economic consequences, IAS can significantly impact ecosystems and ecosystem services, causing devastating and sometimes irreversible

damage, including species extinction, not only for the trees but also their associated biodiversity (Reaser *et al.* 2007; Hultberg *et al.* 2020; Kumar Rai & Singh 2020; Carneiro *et al.* 2024), which represents a value loss beyond any monetary measure. Well-known examples of disastrous consequences following the accidental introduction of forest pathogens include the functional extinction of American chestnut due to chestnut blight, caused by the fungal pathogen *Cryphonectria parasitica* from East Asia (Rigling & Prospero 2018), and the decline and mortality of numerous endemic tree species (and associated biodiversity) in southern Europe and in western Australia due to the oomycete pathogen *Phytophthora cinnamomi* (Hardham & Blackman 2018).

In Europe, invasive forest pathogens have increased exponentially over the last century, mainly as a result of trade in roundwood and wood products, and the trade in live plants for planting which are the main introduction pathways (Santini *et al.* 2013; Prospero & Cleary 2017). Alarmingly, only 1% of recorded alien forest pathogens in Europe have been successfully eradicated, suggesting that eradication is nearly impossible and highlighting the need for preventive regulations and better tools for the rapid and early detection of new threats (Santini *et al.* 2013).

The possibility of non-native pathogens establishing in new areas depends on their ability to perform a so-called “host jump”, to infect a naïve host (Woolhouse *et al.* 2005; Prospero & Cleary 2017). Then, there is typically a prolonged lag phase during which the pathogens exist in small, localized populations, as visualized in the generalized invasion curve (Figure 1). During this phase, they may cause limited damage and can remain undetected for extended periods, sometimes years or even decades in the case of microbial pathogens. The opportunity for eradication is typically lost when IAS overcome barriers such as host or habitat competency which potentially limits their reproduction, allowing them to spread (Paap *et al.* 2022). Based on historical examples of biological invasions gone wrong, by the time the public takes notice, the invasive species’ population is far beyond the point where eradication measures are likely to succeed. Both the area occupied by, and costs for controlling, IAS increase substantially over time (Figure 1). Therefore, investments are needed for preventing the entry of pests and pathogens through pre-border regulatory policies and at/post-border surveillance systems (Hulme 2014). Such surveillance systems should include cost effective detection and diagnostic tools that would allow

for rapid identification and responses to forest biosecurity threats. This would reduce long-term economic losses and help safeguard future forest resources (Epanchin-Niell *et al.* 2012; Harris *et al.* 2018).

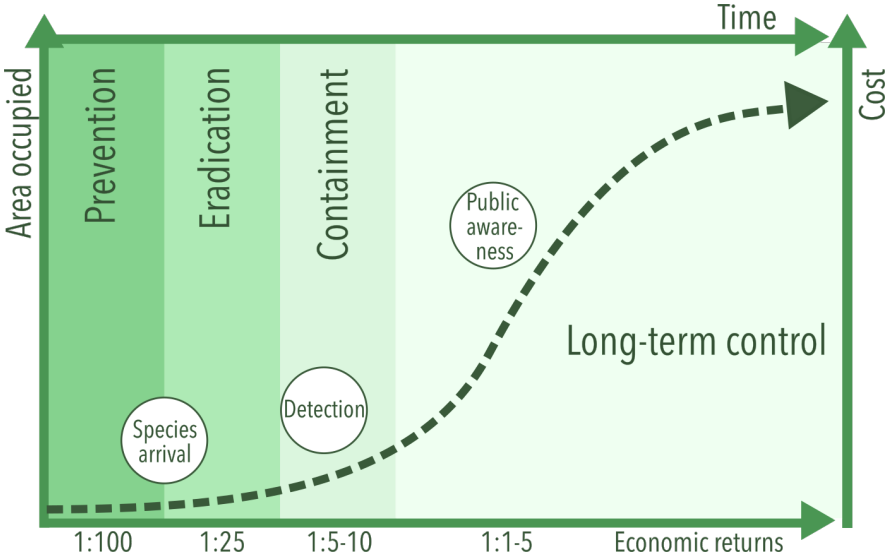

Figure 1. Generalized invasion curve: A schematic overview of the stages of invasive species introduction and establishment, from pre-arrival to long-term management. After the introduction and establishment of a species, management costs increase substantially over time, while economic returns on expenditures decrease. Eradication is only feasible when populations are localized, so as the area occupied by the invader increases, the likelihood of eradication success decreases. Usually, by the time the general public become aware about the problem, the invader is already widely established and efforts are aimed at preventing further spread to limit losses. Investments in better prevention will give greater economic returns than long-term management of the species. Adapted from Harris *et al*. (2018).

1.2 Climate change aggravates the threat of forest pathogens

Forests are increasingly coping with anthropogenic stressors (Trumbore *et al.* 2015; Cavender-Bares *et al.* 2022; Guégan *et al.* 2023). During 2023-2024, global temperatures remained at record highs (SMHI 2024a) and all scenarios of global climate predictions indicate that this will continue to worsen and cause, not only temperatures increases, but also severe storms, fires, flooding, and other extreme climate-related events (IPCC 2024). Apart from the direct effects posed to forests by these environmental challenges, the indirect effects of climate-induced changes in host resistance and other community interactions will also occur (Ayres & Lombardero 2000;

Sturrock *et al.* 2011; Raza *et al.* 2015). A fundamental principle in plant pathology is often illustrated by the "disease triangle", a conceptual paradigm underlining the three needed components for disease to occur: a virulent pathogen, a susceptible host and conducive environment (Stevens 1960; Francl 2001; Scholthof 2007; Bonello *et al.* 2020; Leveau 2024) (Figure 2). Conversely, plant disease can be prevented by eliminating any of these three causal components. The disease triangle serves as a guide to manage, predict and prevent plant disease (Leveau 2024).

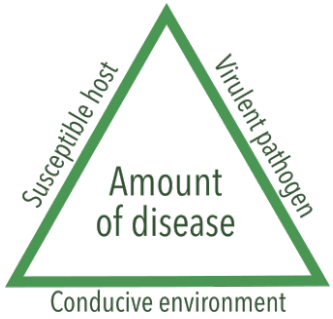

Figure 2. The phytopathological disease triangle. Each side of the triangle represents a necessary component for disease to occur. The extent of disease is determined by the interaction of each side, *e.g.* the environment may be permissive, but not favorable, plants of the same species often possess varying levels of quantitative resistance, and isolates of the same pathogen can differ in virulence.

Environmental barriers, historically keeping some IAS at bay, are likely now being removed by climate change. This enables species to thrive in new regions, and extreme weather events (*e.g.* drought) offer opportunities for their establishment as stressed trees are generally more susceptible to attack (Bergot *et al.* 2004; Garrett *et al.* 2006; Buotte *et al.* 2017; Pysek *et al.* 2020). Even mild variations in temperature and precipitation can trigger forest pathogen outbreaks (Teshome *et al.* 2020), which also applies to native pathogens. Studies indicate that species with long lifecycles, such as trees, have a limited adaptability and are expected to face greater difficulty than tree pathogens in light of climate change (Raza *et al.* 2015; Teshome *et al.* 2020). Meanwhile, many fungal species have plastic lifecycles, and changing environmental conditions can allow endophytes or weak/latent pathogens to become opportunistic and cause disease, especially when the vitality of the host is reduced because of abiotic stress (Slippers & Wingfield 2007; Langer *et al.* 2021). Two examples where weather and possible climatic triggers have caused recent disease emergence in forests are the fungal pathogens

attacking *Pinus* spp., namely *Dothistroma* spp. (Woods *et al.* 2016) and *Diplodia sapinea* (Brodde 2023), further mentioned in a later section.

1.3 Current biosecurity infrastructure

Globalization and trade entails the movement of goods and people, offering key pathways for the introduction of IAS, including invasive forest pathogens (Santini *et al.* 2013). In particular, the trade in plants for planting is a major pathway by which invasive forest pathogens have entered Europe, but many other plant-derived commodities, such as wood and seeds harbor a potential to bring along unwelcome hitchhikers (Rabitsch 2010; Santini *et al.* 2013; Roques *et al.* 2016; Cleary *et al.* 2019b; Franić *et al.* 2019; Pysek *et al.* 2020). Though challenging given the current global mobility and huge volumes of transported goods, prevention of new IAS introductions by rapid, early and applicable diagnostic methods for detection of potentially harmful organisms seems to be the only logical way forward (Santini *et al.* 2013).

The required infrastructure to support biosecurity in plant trade is, however, largely missing in most countries, as there is often insufficient resources, expertise, and equipment for effective and high-throughput diagnostics (Roques 2010; Early *et al.* 2016; Avtzis 2019; Zamora-Ballesteros *et al.* 2019b). Traditional approaches of disease detection are insufficient to meet the demands of screening larger volumes of plants; in an ideal world, cost-effective surveillance systems, that weigh the infrastructure costs against the economic consequences of failed prevention of IAS introductions, should be constructed (Epanchin-Niell *et al.* 2012).

It has been established that stricter border control helps prevent establishment of pathogens (Sikes *et al.* 2018) and pests (Bacon *et al.* 2012). In New Zealand, a world-leading example of biosecurity infrastructure (Stone 2021), the introduction rate of IAS has plateaued since the 1980s, despite exponentially increasing rates of import volume and human travel (Sikes *et al.* 2018). About 0.3% of New Zealand gross domestic product is invested in biosecurity measures (New Zealand Treasury 2014) such as border surveillance, phytosanitary inspection and quarantine; a cost justified by the fact that biosecurity measures cost less than the economic and ecological costs of IAS incursions (Sikes *et al.* 2018). This underscores the urgent need for enhanced biosecurity infrastructure globally and the need for

developing accurate, high-throughput, cost-effective, and user-friendly tools to rapidly detect, intercept, and prevent the introduction and spread of invasive forest pathogens (Martinelli *et al.* 2015; Seebens *et al.* 2017; Venbrux *et al.* 2023).

1.4 New and emerging forest pathogens of concern

There is a growing concern for, and an increased research interest in, forest pathogens threatening economically and ecologically important host trees globally (Aglietti *et al.* 2021; Paap *et al.* 2022). The situation is especially worrisome for countries that are heavily dependent on forest related industries, like Sweden. Climate change facilitates the range expansion of both native and alien pathogens, and affects the resistance of trees to pathogens (Desprez-Loustau *et al.* 2006; Jactel *et al.* 2012; Ramsfield *et al.* 2016), enabling outbreaks of both new and emerging forest diseases.

Diplodia tip blight disease, caused by the ascomycete pathogen *Diplodia sapinea*, is emerging in many parts of northern Europe (Brodde *et al.* 2019). *Pinus* is the main host genus of the pathogen that is globally distributed and responsible for substantial losses in global forest industry (Fabre *et al.* 2011; Brodde 2023). In Sweden, a large outbreak of *D. sapinea* was discovered for the first time in 2016 in a stand of Scots pine (*Pinus sylvestris*) north of Stockholm, and the pathogen appears to be expanding its range northward with more recent discoveries of diseased lodgepole pine (*P. contorta*) in northern Sweden (Matsiakh 2024). The typical symptoms of Diplodia tip blight disease are apical shoot dieback and cankers (Brodde 2023), and its symptoms are often confused with other pine pathogens including *Gremmeniella abietina* and *Melampsora pinitorqua*. Drought is a trigger for disease outbreaks, so *D. sapinea* damage is expected to worsen with climate change.

Another emerging disease on pine is brown spot needle blight, caused by the ascomycete fungus *Lecanosticta acicola*. In Sweden, the pathogen was first discovered in the Alnarp park arboretum on mugo pine (*Pinus mugo*), an exotic species commonly planted in urban environments and gardens (Cleary *et al.* 2019a). The pathogen was likely unintentionally introduced through infected nursery material, and in the European Union (EU) is listed as a regulated non-quarantine pest (European and Mediterranean Plant Protection

Organization 2024) because of its potential to cause severe disease to *Pinus* spp. The symptoms of the disease include brown spots and necrotic bands on needles that will prematurely shed (van der Nest *et al.* 2019). Its symptoms are often confused with other pine needle pathogens including *Dothistroma pini* and *D. septosporum*, *Cyclaneusma minus*, *Lophodermium* spp., and *Sydowia polyspora* (Behnke-Borowczyk *et al.* 2019; van der Nest *et al.* 2019; Beram & Demiröz 2024). Several of the pine needle pathogens cause symptoms that closely resemble one another, making diagnoses rather difficult.

Fusarium circinatum, the causal agent of pine pitch canker disease, is a deadly, invasive pathogen, causing severe damage on many pine (*Pinus*) species and Douglas fir (*Pseudotsuga menziesii*) (Zamora-Ballesteros *et al.* 2019b; Drenkhan *et al.* 2020). Scots pine (*P. sylvestris*) is the most widely distributed pine species in the world (Durrant et al. 2016), and is known to be susceptible to *F. circinatum* (Durrant *et al.* 2016; Davydenko *et al.* 2018; Martín-García *et al.* 2018; Drenkhan *et al.* 2020). The pathogen is native to Central America but has since been introduced around the world, including Spain and Portugal, the only European countries where it is currently known to be established (Zamora-Ballesteros *et al.* 2019b; EPPO 2024). *Fusarium circinatum* is considered a quarantine organism and is regulated within the EU (EPPO 2021). Diseased trees characteristically show excessive resin exudation from cankers that occur in the main stem or branches, resulting in branch or tree dieback (Hepting 1946; Viljoen 1994; Storer *et al.* 2002; Bezos *et al.* 2017). Other symptoms include chlorosis and/or wilting of needles, reddening and necrotic foliage and eventual shoot dieback (Hepting 1946; Viljoen 1994; Storer *et al.* 2002; Bezos *et al.* 2017). *Fusarium circinatum* can be seed-borne (Storer *et al.* 1998) and can cause death in seedlings before or after emergence. It is an ascomycete, able to reproduce sexually and asexually, but sexual recombination appears to be extremely rare or absent in most countries (Gordon *et al.* 1996; Wikler & Gordon 2000; Gordon 2006; Iturritxa *et al.* 2011; Berbegal *et al.* 2013; Mullett *et al.* 2017). In addition, the sexual stage that involves perithecia has never been observed in the field (Wingfield *et al.* 2008), so the pathogen spreads mainly asexually through production of micro- and macro-conidia on infected host tissues (Britz *et al.* 1998; Berbegal *et al.* 2013). The conidia, or asexual spores, are readily dispersed through rain splash, wind, vectoring insects (Fernández *et al.* 2019; Zamora-Ballesteros *et al.* 2019a) and soil (Martín-Rodrigues *et al.*

2015). Long-distance spread is achieved by trade of organic material and movement of people, as previously mentioned. Although *F. circinatum* is known to cause a lethal disease on *Pinus* spp., the fungus is also known to occur as an endophyte in several species of grasses and other herbaceous species in California, South Africa, and Spain (Swett & Gordon 2012; Swett *et al.* 2014; Hernandez-Escribano *et al.* 2018) and in non-pine woody species (Martínez-Álvarez *et al.* 2014). Therefore, understory species can serve as an inoculum reservoir, enabling the fungus to survive and continue to spread in the absence of tree hosts.

The genus *Phytophthora* contains a very large number of plant pathogenic species responsible for massive economic losses and ecological damage in agriculture, horticulture and forestry (Kroon *et al.* 2012; Hansen 2015). To date, there are 326 known species of *Phytophthora* (Scott *et al.* 2019), but the true number is estimated to be about 600 (Brasier *et al.* 2022). *Phytophthora* are also known as water molds, belong to the oomycete phylum and are widespread and very diverse in their virulence and host preference (Hansen 2015). A notable adaption of *Phytophthora* is their production of motile asexual spores with a flagellum, called zoospores, enabling movement independent of vectors or environmental factors. For many *Phytophthora* species, these zoospores are the main infective agent initiating plant disease (Hardham & Blackman 2018). *Phytophthora* are very hard to eliminate once established because they produce chlamydospores that can withstand harsh conditions and contribute to their long-term survival. Evidence suggests that even some *Phytophthora* spp. have phenomenal survival abilities in dead wood. For example, viable *P. cinnamomi* and *P. lateralis* were recovered from root systems of killed trees after six and seven years, respectively (Hansen & Hamm 1996; Jung *et al.* 2013; Hardham & Blackman 2018). *Phytophthora cinnamomi* is a globally distributed species with an enormous host range, able to infect nearly 5000 host species, and therefore, is known as one of the most devastating plant pathogens (Hardham & Blackman 2018).

Many *Phytophthora* species are relevant to forestry (Hansen 2015) and are responsible for causing decline and mortality in several forest species, such as *Quercus* spp. (Jung *et al.* 2000; Jönsson 2004), *Fagus* spp. (Weiland *et al.* 2010), *Larix* spp. (King *et al.* 2015), and *Abies* spp. (Pettersson *et al.* 2017). In woody hosts, the pathogen commonly causes root rot and stem cankers, which leads to reduced growth while also increasing the trees' susceptibility

to other biotic stressors, and sensitivity to abiotic stressors (Keriö *et al.* 2020).

In the Nordic countries, the number of reported *Phytophthora* incidences has greatly increased during recent years, which is likely due to an increased number of introductions through the global trade of plants for planting, climatic change and improved capacity and methods for detection and identification (Matsiakh & Menkis 2023). Even *P. cinnamomi* has been identified in Sweden, not surprisingly from a commercial nursery (Cleary *et al.* 2021), which is the most common pathway of novel invasive forest pathogen introductions (Santini *et al.* 2013; Seebens *et al.* 2017; Pysek *et al.* 2020).

The unprecedented rise in both native and alien forest pathogen epidemics, that has been witnessed in recent years, mainly attributable to anthropogenic influences, is both alarming and worrisome since these problems are anticipated to only continue along the same trajectory. To safeguard future forests and secure their provisioning of a broad range of ecosystem services, better biosecurity infrastructure and improved surveillance techniques that will aid in the early and rapid detection of threats from known introduction pathways (*e.g.* plant consignments in the nursery trade) are needed.

1.5 Detection methods of today and the future

Forest pathogens are usually microscopic, and while some diseases produce easily recognized signs, others are more difficult to identify by visual inspection alone, requiring tools for detection and diagnosis. Traditional methods of pathogen detection and disease diagnostics are largely inadequate in terms of throughput, capacity, time-efficiency, specificity, and the necessity for specialized expertise (Goud & Termorshuizen 2003; Feng *et al.* 2014; Tremblay *et al.* 2018). For example, phytosanitary inspections at ports of entry of many countries is mainly intended as a check of compliance with the importing country's requirements by checking certificates, custom logs, and visually inspecting the goods, not to test for pests (Eschen *et al.* 2015). In the cases when further testing is performed, the inspections may range from visual scouting for symptoms characteristic of a target organism, which may be sampled for isolation, culturing, and morphological or molecular identification of the causal agent of disease. This is not always ideal, as many

species are not culturable (especially biotrophs) (Bindslev *et al.* 2002; Connon & Giovannoni 2002), are morphologically indistinguishable (Martinelli *et al.* 2015; van der Nest *et al.* 2019; Elvira-Recuenco *et al.* 2020; Luchi *et al.* 2020; Aglietti *et al.* 2021; Venbrux *et al.* 2023), or remain latent during long periods of time without symptom development in the host (Aglietti *et al.* 2019).

Other commonly used methods are targeted, such as enzyme-linked immunosorbent assay (ELISA) which utilizes antibodies that specifically bind to target antigens (Ali-Shtayeh *et al.* 1991), or molecular techniques based on polymerase chain reaction (PCR) amplification of ribosomal RNA genes (White *et al.* 1990). The latter can either be performed with targeted, species-specific primers and real-time quantification, or combined with shipment to external, commercial services for sequencing (Martinelli *et al.* 2015). However, with every method there are limitations. For example, the DNA-based methods are commonly time-demanding and expensive (Aglietti *et al.* 2019), and while well-designed species-specific PCR primers can work well for targeted detection of forest pathogens, there is a risk for cross-amplification. Additionally, because the use of species-specific primers is a highly targeted method, it requires multiple PCR runs to test for presence of different suspect organisms (Schena *et al.* 2006; Bilodeau *et al.* 2009; Capote *et al.* 2012). While the field-deployable ELISA test can quickly confirm the presence of *Phytophthora* spp. in samples, it lacks specificity as it cross-reacts with *Pythium* spp. (Avila *et al.* 2009). Furthermore, the tests cannot distinguish between *Phytophthora* species, so further testing would be needed to identify the *Phytophthora* to species level.

Nevertheless, targeted methods can offer a quick and cost effective way to determine the presence of a specific pathogen and probable causal agent of disease. Loop-mediated isothermal amplification (LAMP) is a simple, affordable, DNA-based detection method that can be performed using portable devices in the field, providing easily interpretable results within an hour (Notomi *et al.* 2000; Aglietti *et al.* 2019). It has demonstrated high specificity for in-field detection of both forest insects (Peterson *et al.* 2023) and pathogens (Aglietti *et al.* 2019; Aglietti *et al.* 2021). LAMP specifically amplifies the target DNA in a sample using several primer pairs and a polymerase with strand displacement activity (Notomi *et al.* 2000). Unlike PCR, LAMP requires no temperature cycling and products can be easily detected visually in real time by a color change upon the successful

production of amplification products on a portable, real-time fluorometer device (Aglietti *et al.* 2019; Aglietti *et al.* 2021; Peterson *et al.* 2023). Still, innovative methods that are better suited for early and rapid detection of potentially harmful pathogens are needed.

1.5.1 VOCs-based approaches for disease and pathogen detection

Volatile organic compound (VOC)-based approaches is one method that has huge potential for pathogen detection. VOCs are low-weight molecules that disperse easily across wide distances and are emitted by all living organisms, with each organism producing a unique blend (Loreto *et al.* 2014; Materić *et al.* 2015). Plants VOCs include secondary metabolites that function in intraspecific and interspecific communication (Bouwmeester *et al.* 2019). For instance, some VOCs can attract natural enemies to pests currently infesting the plant, while others can warn neighboring plants and trigger defence priming responses. The compositions and emission rates of VOCs are highly dynamic, influenced by both biotic and abiotic factors. When trees are under stress, VOC emissions can change in both quantity (Dicke & Baldwin 2010; Nordström *et al.* 2022) and quality (Brilli *et al.* 2020; Sherwood *et al.* 2024), serving as indicators of plant stress. This unique blend of VOCs, comparable to a fingerprint, can be used for identification of specific pathosystems, as each pathogen or pest may induce a unique VOC profile in its interaction with the host (Vuorinen *et al.* 2007; Johne *et al.* 2008; Ponzio *et al.* 2013). These methods offer non-invasive pathogen detection in early stages of disease development, even in asymptomatic tissues (De Lacy Costello *et al.* 2000; Martinelli *et al.* 2015; Vezzola *et al.* 2018; Brilli *et al.* 2020).

There are many examples of VOCs-based pathogen detection in agricultural systems (De Lacy Costello *et al.* 2000; Blasioli *et al.* 2010; Laothawornkitkul *et al.* 2010; Jansen *et al.* 2011; Rutolo *et al.* 2018; Cui *et al.* 2019), including in woody plants (Aksenov *et al.* 2014; Zainol Hilmi *et al.* 2019). An interesting example of a modern VOCs-utilizing application was provided in a study by Li *et al.* (2019), where a smartphone was combined with a handheld device of a colorimetric sensor array, enabling early detection of tomato late blight. In a study of VOCs in plane trees, VOCs uniquely submitted by *Ceratocystis platani*-infected trees were identified, a finding enabling the development of targeted VOCs-based approaches (Brilli *et al.* 2020).

VOC detection involves four fundamental steps: collection, separation, detection and analysis. Due to the high vapor pressure and low emission levels of VOCs, specialized devices are required for their collection and sometimes accumulation prior to analysis. The most common method for VOC collection is headspace sampling, a non-destructive approach that can be performed dynamically or statically. There are several methods for headspace sampling, ranging from simply aspirating air into a syringe to specialized materials for sorbing compounds. One common method is to use solid-phase micro-extraction (SPME) fibers. SPME fibers are inert, reusable, needle-like devices with absorbent or adsorbent coatings, the composition of which determines the kind of VOCs that are collected. They are a simple and useful tool for concentrating VOCs in a sample prior to analysis. The collected VOCs are then desorbed by heat when the SPME fiber is inserted into a gas chromatograph (GC) for subsequent separation. GCs can separate complex mixtures of analytes by passing them through a column containing a stationary phase via an inert gas. The separation of analytes is based on their different affinities for the stationary phase and their varying volatilities. Once separated, VOCs can be detected by a variety of detectors coupled to the GC. The selection of the detector depends on the needs of the user, but for more confident identifications, a mass spectrometer (MS) is used, because it provides information about the compound's molecular mass and fragmentation pattern and enables identification of the sample components. In order to be detected, compounds are first ionized (there are multiple approaches that involve adding a charge to the molecule), and then the ions are filtered based on their mass-to-charge ratio (m/z) as they pass to a detector. The end result is a list of the m/z at a given time and their relative intensities. Analysis of GC-MS data can be tailored to the specific task, but typically involves using a combination of the retention times (*i.e.* when compounds elute off the column) and the m/z values and comparing these values to databases of known standards. This process is typically extensive and complex, which is why pipelines for automation are being developed and continuously improved. For example, the open-source software MZMine 2 allows for automated mass-spectrometry (MS) data processing, streamlining the otherwise labor-intensive tasks of manual peak integration and identification. The final step is the analysis of results, which can be conducted using machine learning techniques, such as the random forest algorithm available in R (Liaw 2002). These automated analysis steps

minimize labor and eliminate human bias (although potentially introducing other bias) in the MS peak analysis process.

The use of volatile compounds as biomarkers in plant disease detection is less common than the molecular methods described below, but is gaining interest because of its potential for high throughput analysis and non-destructive sampling. Nonetheless, VOCs-based methods needs further development and optimization.

1.5.2 Sequencing for detection

DNA sequencing technologies have been progressing quickly during the last decades. The Sanger chain termination method was developed in the 1970s and is the first widely adopted and practical method of DNA sequencing that revolutionized molecular biology (Sanger *et al.* 1977). It consists of three main steps (Kircher & Kelso 2010). First, PCR is performed with chain-terminating, fluorescence labelled, nucleotides - each nucleotide with a unique fluorescent label. In the second step, all the sequences are separated and lined up by size by capillary gel electrophoresis, so that a laser beam, in the final step, can excite the nucleotide at each position and detection can occur. The Sanger method for DNA sequencing is still one of the most widely used to this day (Shendure *et al.* 2017), particularly for examining small numbers of samples. It offers highly accurate sequencing of single-species DNA, producing read-lengths of up to 1000 bases, making it the preferred method of choice for sequencing DNA from pure cultures (Hert *et al.* 2008). However, the technique only functions when the DNA in the sample has the exact same sequence in each molecule and only one part of the DNA can be examined at the same time. This limits its applicability to mixed samples and large-scale experiments.

The need for large-scale sequencing eventually, however, led to the development of next-generation sequencing platforms that offer significantly greater throughput (often dubbed high-throughput sequencing). These are high-performance technologies based on the parallelization of sequencing processes, resulting in the simultaneous reading of thousands or even millions of sequences, and have the ability to detect several types of sequences or species at once. Illumina sequencing was first released in 2005 and is now widely established as the go-to method for amplicon sequencing, offering a highly accurate method to sequence mixed samples (Bentley *et al.*

2008; Kircher & Kelso 2010). The reversible terminator technology used by Illumina employs a sequencing-by synthesis concept not unlike that used in Sanger sequencing. DNA polymerases are used to extend the sequences beyond the primer region by incorporation of fluorescently labelled and terminated nucleotides, which stops the process. After the nucleotide-specific label is read with fluorescent dyes, the sequencing reaction is continued by removal of the terminated nucleotide (Bentley *et al.* 2008; Hu *et al.* 2021). The biggest shortcoming of Illumina sequencing is the limitation in read lengths, typically less than 300 base pairs. This limitation makes sequencing of a single amplified region insufficient for accurate identification of many microorganisms, due to the high genetic similarity shared among microorganisms. This is true even in the highly variable genomic areas that are used for distinguishing species by phylogenetics, such as the ITS region commonly used for fungal identification (White *et al.* 1990).

The required machinery for Sanger and Illumina sequencing is not readily available to most laboratories, which makes utilization of external, commercial services necessary (Martinez *et al.* 2020). The time and labor required to prepare, ship samples and long queuing times to acquire data from sequencing facilities counteract the goal of establishing quick and simple detection techniques, especially for non-targeted, in-field pathogen detection.

In 2011, a long-read sequencing method called Pacific Biosciences (PacBio) was made commercially available (Karow 2011). In this sequencing method, the DNA to be sequenced is in a single-stranded circular DNA structure which is loaded onto a single-molecule real-time cell, where a polymerase replicates the DNA using fluorescently labeled nucleotides (Hu *et al.* 2021). It can be run with either a circular consensus sequencing mode to produce highly accurate reads or a continuous long-read sequencing mode to produce the longest possible read lengths (Eid *et al.* 2009), more than 135 kb base pairs (Pacific Biosciences 2020). PacBio is similarly disadvantageous because it too requires specialized sequencing facilities and has complicated library preparations (Hu *et al.* 2021).

In 2014, nanopore sequencing from Oxford Nanopore Technologies (ONT) was released. Nanopore sequencing is distinguished from the previous sequencing approaches, in that it directly sequences the nucleotides without

DNA synthesis. Instead, a long sequence of single-stranded DNA is passed through a protein nanopore, stabilized in an electrically resistant polymer membrane (Feng *et al.* 2015). By setting a voltage across this membrane, sensors detect the ionic current shift caused by the nucleotide currently occupying the nanopore in real-time; a signal that can then be translated to the corresponding base during basecalling (Loman & Watson 2015) (Figure 3). Nanopore technology enables direct sequencing of DNA or RNA (Chalupowicz *et al.* 2019); therefore there is no need for pre-sequencing PCR and PCR-associated biases can be avoided (Aird *et al.* 2011).

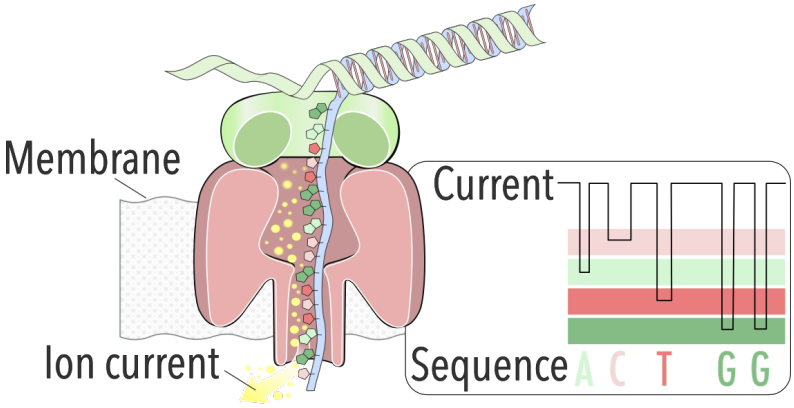

Figure 3. Schematic representation of nanopore sequencing. A single DNA strand is fed through a nanopore embedded in a membrane, resulting in characteristic shifts in the ionic current across the membrane. These current changes, corresponding to different nucleotides passing through the pore, are recorded in real-time and subsequently basecalled into the nucleotide sequence of the DNA.

Another benefit of nanopore sequencing is that it relies on a small, relatively inexpensive, sequencing device known as the MinION, which due to its portability and compact size allows for real-time *in situ*-sequencing. Its application for in-field research has already been demonstrated for plant viruses in cassava plants (Boykin *et al.* 2019), and for working in extreme locations such as the Arctic (Edwards *et al.* 2019), Antarctica (Johnson *et al.* 2017), and the International Space Station (Castro-Wallace *et al.* 2017). Thus, use of MinION in rural, peri-urban or urban forest locations seem like a challenge that could be easily overcome. Loit *et al.* (2019) found the MinION suitable for rapid diagnostics of agricultural and forest pathogens and demonstrated that the full sampling-to-results pipeline was achievable within 2.5 hours. The main disadvantage of nanopore sequencing is the high error rates in basecalling. Initially, the MinION read accuracy was barely 60% (Laver *et al.* 2015) but with the continuous development and refinement

of the MinION, flow cells and basecalling algorithms, the read accuracy has been significantly improved. It is now reported as high as 95-99% (Sanderson *et al.* 2023; Zhang *et al.* 2023), but for flow cell version R9.4.1, the read accuracy was reported in the range of 87-97%, leaning to the higher end when coupled with the super-high accuracy basecalling model of Guppy (Minei *et al.* 2018; Sereika *et al.* 2022; Ni *et al.* 2023). On the other hand, the sequencing read length capability has a significant advantage over previous sequencing platforms, with no theoretical instrument-imposed limitation at all (Laver *et al.* 2015). Read lengths of >200,000 base pairs have been documented (Santos *et al.* 2020). The ability to skip PCR amplification and the longer read lengths would be highly advantageous in the plant pathology field (Chalupowicz *et al.* 2019).

While all of the methods described above have existed for some time, they are not yet fully adjusted for implementation in practice. Continual improvements in these technologies offer hope that detection methods will evolve to become faster, more accurate, and accessible, enhancing efforts in early pathogen detection and plant biosecurity. This thesis explores the possibilities to improve biosurveillance of new and emerging threats by utilizing these maturing technologies.

2. Thesis aims & objectives

A first step to effectively manage forest diseases is understanding the disease-causing agent(s) affecting host trees. For this, early, rapid, and reliable pathogen identification is crucial, and in the case of new and emerging diseases, timely interventions are critical to prevent their escalation. This thesis aimed to further develop and explore the applicability of advanced technologies for detecting and diagnosing forest pathogens. The thesis focused on two emerging technologies for disease detection, namely VOC profile fingerprinting (**Papers I and II**) and next-generation sequencing platforms (**Paper III**). The forest pathogens that are considered in the thesis are either '*new'*, *i.e.* non-native pathogens at risk of being introduced or have been recently introduced in Sweden, or '*emerging'* pathogens, *i.e.* native species that have been long-established in Sweden but are spreading to new locations or their populations are expanding. The specific objectives were:

in **Paper I**, to develop a disease detection method based on VOC emissions from pine seedlings infected by *Fusarium circinatum*, the pine pitch canker pathogen. It was hypothesized that *in vitro* VOC signatures could distinguish between different *Fusarium* species and that VOCs emitted by *F. circinatum in vitro* would reflect those emitted *in vivo*. Furthermore, VOC signatures were expected to differentiate *F. circinatum*-infected *Pinus* seedlings from mock-inoculated seedlings before visual symptoms emerged.

in **Paper II**, to identify qualitative or quantitative differences in VOCs between *Phytophthora*-infected and mock-inoculated *Quercus robur* and *Fagus sylvatica* saplings, and potential biomarkers of disease to use in future targeted methods of disease detection and diagnosis. The VOC profiles

emitted *in vitro* were hypothesized to qualitatively differ among *Phytophthora* species. These qualitative differences were expected to discriminate between *Phytophthora*-infected and mock-inoculated *F. sylvatica* and *Q. robur* saplings, as well as differentiate between *F. sylvatica* and *Q. robur* inoculated with either *P. cinnamomi* or *P. plurivora*.

in **Paper III**, to enhance our understanding of the precision and discriminatory power offered by Illumina and nanopore sequencing for detection of pine needle-associated fungi, by comparing differences in taxonomic resolution and mycobiome diversity between identical samples sequenced by the two methods. Nanopore sequencing was hypothesized to have a lower taxonomic resolution and species diversity compared to Illumina. Additionally, it was hypothesized that the microbiome taxa and diversity would vary between sampling sites, and that pathogenic species could be detected equally in both asymptomatic and symptomatic needles.

3. Materials & methods

3.1 Study material

Papers I & **II** aimed to compare VOC profiles of select forest pathogens grown *in vitro* and to explore VOC emission differences in inoculated versus healthy trees *in vivo.* Due to the quarantine status of *Fusarium circinatum* in Sweden, experiments for **Paper I** were conducted at the University of Valladolid in Palencia, Spain. Fungal cultures used in **Paper I** included *Fusarium circinatum, F. bulbicola, F. oxysporum* f.sp. *pini* and *F. graminearum*. To assess whether VOC analysis can distinguish closely related species, this set of species was selected based on their genetic proximity to *F. circinatum*; *Fusarium bulbicola* is closely related to *F. circinatum* (Herron *et al.* 2015)*, F. oxysporum* f.sp. *pini is* intermediately related (O'Donnell *et al.* 1998) and *F. graminearum* is more distantly related (Watanabe *et al.* 2011). The oomycete cultures studied in **Paper II** included eight *Phytophthora* species; *Phytophthora cambivora, P. cinnamomi, P. citricola, P. gonapodyides, P. multivora, P. plurivora, P. polonica* and *P. syringae.* These were selected because they represent important plant pathogens and a wide range of clades; five clades are represented among these eight species.

Plant material for *in vivo* experiments with *F. circinatum* were 1-year-old *Pinus sylvestris, P. radiata* and *P. pinea* seedlings (**Paper I**). The choice of *Pinus* spp. was made based on their documented susceptibility to *F. circinatum*: *Pinus radiata* has shown high susceptibility (Iturritxa *et al.* 2013), *P. sylvestris* moderate susceptibility (Drenkhan *et al.* 2020) and *P. pinea* has been considered mostly resistant or tolerant (Gordon *et al.* 2001; Iturritxa *et al.* 2013). In **Paper II**, 2-year-old *Quercus robur* and *Fagus*

sylvatica saplings were used. In both studies, the trees acclimated for a reasonable number of weeks under optimal light, watering and humidity conditions prior to the start of the experiment, in order to minimize potential impact of abiotic stressors.

In **Paper III**, asymptomatic and symptomatic pine needles were collected from a number of native and exotic *Pinus* species of varying age, namely *P. bungeana, P. cembra, P. mugo, P. parviflora, P. ponderosa, P. sylvestris* (of two varieties)*, P. x schwerinii* and some unknown *Pinus* spp. The sampled trees were found in 13 sites, 11 in Sweden and two in Spain (Figure 4), corresponding to four site types; forest, arboreta, urban and nursery environment.

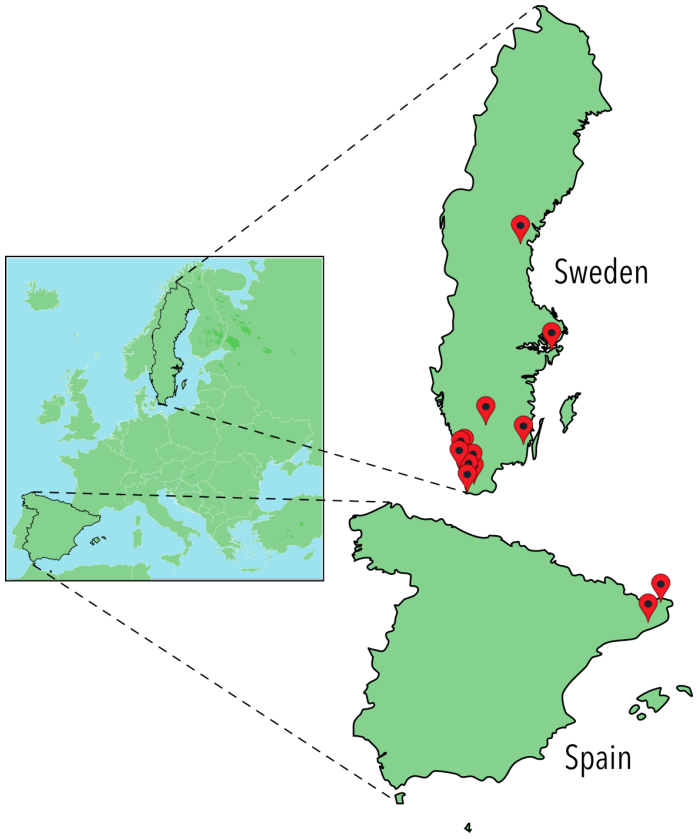

Figure 4. Map of Sweden and Spain with pins specifying the sampling sites where pine needles were collected; 11 in Sweden and two in Spain.

3.2 Experimental approach for *in vitro* VOCs studies

In **Papers I** & **II**, VOC profiles of select fungal and oomycete pathogens, respectively, were compared *in vitro* to test whether VOCs alone could

distinguish closely related fungal species. In both studies, *Fusarium* and *Phytophthora* spp. were grown by transferring mycelial plugs onto defined Elliott's medium agar (EMA) (Elliott *et al.* 1966) in glass vials capped with septa to allow insertion of SPME fibers for VOC analysis. EMA was selected as the growth medium due to its defined composition, which enhances reproducibility, and its proven suitability for the growth of *Fusarium* spp. and *Phytophthora* spp., tested in preliminary trials. Three and four replicates were analyzed per species in **Paper I** and **Paper II**, respectively. Identical vials inoculated with sterile EMA plugs served as mock-inoculated controls (MICs) to identify media-related volatiles. The vials were kept in room temperature under natural light conditions and sampled in a different time series adapted for the pathogens studied; at 7, 14 and 21 days post-inoculation (dpi) (**Paper I**) and after 14 and 30 days (**Paper II**). VOCs were sampled by inserting SPME fibers through the septa of the vial caps (Figure 5).

3.3 Experimental approach for *in vivo* VOCs studies

In **Paper I**, trees were inoculated by cutting a small slit on the seedling stems and applying spore suspension to the wound surface, then resealing the slit and securing it with parafilm until VOC sampling. MIC seedlings were inoculated with sterile potato dextrose broth, that way afflicting an identical mechanical injury without pathogen exposure. Five replicates per treatment and pine species were used. In **Paper II**, artificial inoculation was performed with either *P. cinnamomi* or *P. plurivora* by removing a section of outer bark and phloem on the main stem and replacing it with either a *Phytophthora*-colonized or sterile (for the MIC) EMA plug directly onto the exposed xylem. The wound was then sealed to protect the inoculation site from desiccation and contamination up until the day prior to VOCs sampling. Three replicates per treatment and tree species were used. The MIC trees were kept separated from the inoculated trees to avoid contamination.

Symptom development was observed and documented throughout the experiment. VOCs sampling was performed using SPME fibers by enclosing the trees in plastic bags for a fixed period of either 24 h or 48 h in **Papers I** & **II**, respectively (Figure 5). The SPME fibers had a DVB/CAR/PDMS phase, which offers a broad range of analyte compatibility, capturing both volatile and semi-volatile compounds effectively, making them ideal for

non-targeted comprehensive profiling. Different time points post inoculation in the two studies were chosen for sampling based on earlier observation of symptom development such that the sampling points would correspond to early and later stages of disease development.

Figure 5. Sampling setup *in vitro* (left) and *in vivo* (right) for **Paper I**.

3.4 GC-MS & data analysis

The SPME fibers were manually injected into a GC-MS for VOC analysis immediately after sampling. For both papers, the volatiles were separated using the same model of GC-MS and column. An ultra inert HP-5MS column was selected since its stationary phase is well suited to active compounds (such as acids) often present in plant and microbial volatiles. The same model of GC, MS and column was used for both *in vitro* and *in vivo* sampling, although the oven gradient was customized to the different sampling situations, based on preliminary tests.

For **Paper I**, GC-MS data were processed and aligned using an automated pipeline to minimize human bias and labor. Due to the absence of qualitative differences between *F. circinatum*-inoculated seedlings and MICs, the focus shifted on quantitative differences in VOC profiles rather than specific VOC identification. The Randomforest and VarSelRF R-packages were employed to reduce the vast number of VOCs to a small group that offered the highest accuracy in: i) distinguishing different *Fusarium* spp. *in vitro*, ii) distinguishing *F. circinatum*-infected *Pinus* seedlings from MICs. These selected VOC subsets were then analyzed using Permutational Multivariate Analysis of Variance (PERMANOVA) and post-hoc Holm tests. A Principal Component Analysis (PCA) and other plots were generated using the Stats R-package to simplify and visualize the multivariate data.

For **Paper II**, GC-MS data were processed manually. In this case, only qualitative differences were considered for the *in vivo* study, because such compounds represent the best candidates for biomarkers of disease. Similar statistical methods were employed after data processing, including PCA, hierarchical cluster analysis, Randomforest, analysis of variance (ANOVA) and post-hoc two-tailed Dunnett's test, were conducted. Tentative compound identifications were made by comparing the mass spectra to databases and known retention indices from the literature.

3.5 Comparison of Illumina and nanopore sequencing

A schematic illustration of the employed methodology of **Paper III** is provided in Figure 6.

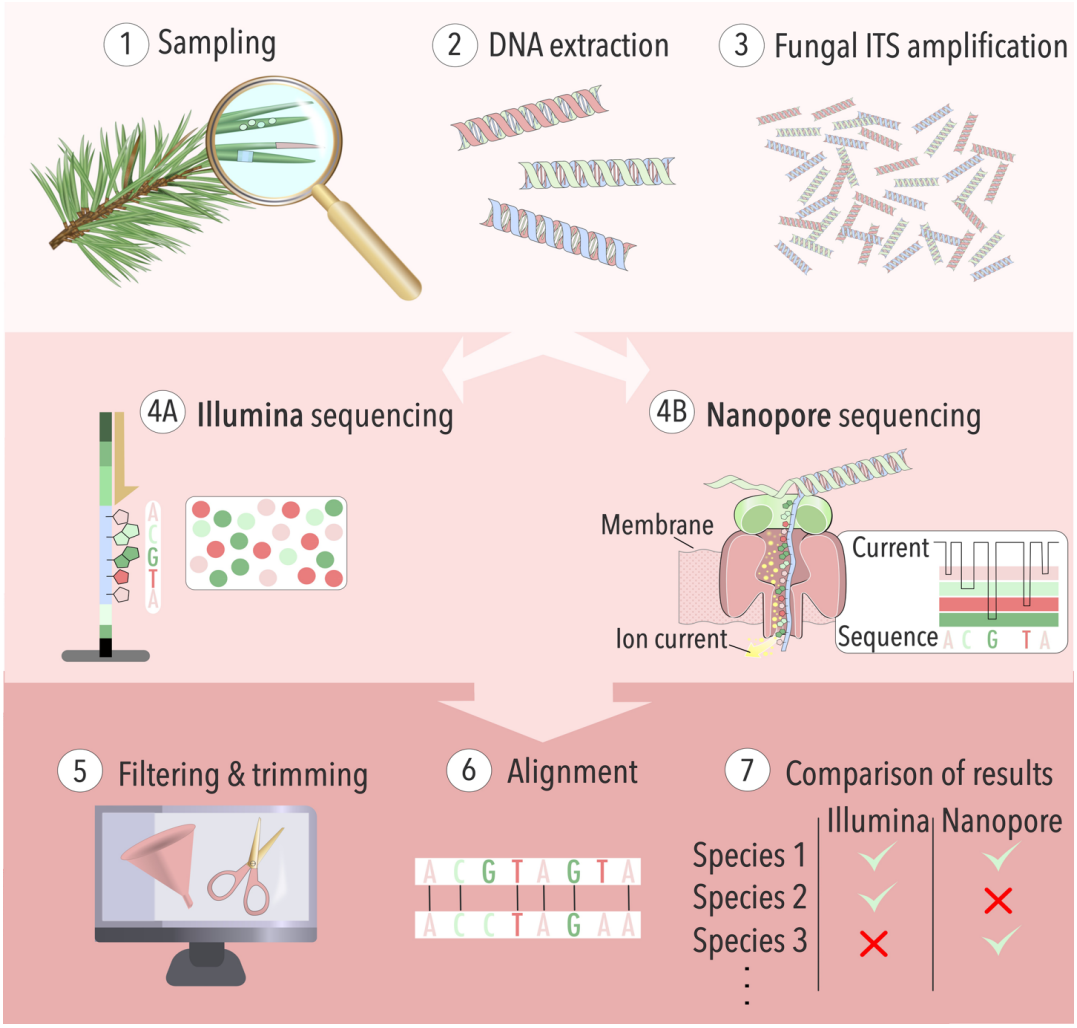

Figure 6. A visual summary of the methods for the study comparing Illumina and nanopore sequencing. Pine needles were sampled and prepared for sequencing by extracting DNA and amplifying the ITS2 (Illumina) or entire ITS region (ONT) through PCR. These amplicons were then sequenced on each platform for DNA analysis. The sequenced reads were trimmed and filtered based on read quality and length before aligning them against the UNITE database. Finally, the generated data was compared between sequencing platforms in terms of taxonomic resolution, diversity, and observed differences in asymptomatic and symptomatic needles (created by author).

3.5.1 Sample preparation for DNA analysis

The sampled needles were surface sterilized, to remove contaminants not indicative of the needles' actual mycobiome, prior to DNA extraction. The needles were then lyophilized and homogenized before DNA extraction which was performed using a standard silica column DNA extraction spin kit. Aliquots of the DNA extracts were either shipped for Illumina sequencing or processed through the nanopore sequencing pipeline, ensuring identical genetic material was used for both sequencing technologies.

3.5.2 Illumina sequencing & data analysis

Amplicon sequencing was conducted by an external service (Novogene). The ITS2 region was initially amplified by PCR using barcoded, fungal-specific primers (White *et al.* 1990). The PCR products were size-selected via electrophoresis, then pooled in equal amounts from each sample for sequencing preparation. Libraries were sequenced on a Illumina NovaSeq paired-end platform, producing 250 base pair paired-end raw reads. Reads were checked for quality, processed and sorted (without clustering) into distinct sequences called amplicon sequence variants (ASVs) that theoretically correspond to unique species.

3.5.3 Nanopore sequencing & data analysis

Nanopore sequencing was conducted following the Oxford Nanopore Technologies (ONT) protocol for PCR barcoding and multiplexed libraries. Although ONT is compatible with metagenomic sequencing approaches, a metabarcoding approach was used to improve detectability for the fungal proportion of the extracted DNA and facilitating detection of less abundant fungal taxa. In addition, most fungal database references are for the ITS region, which makes ITS amplicons a good choice for fungal microbiome analysis. Two rounds of PCR were performed. The first PCR was done in duplicate to minimize reaction-specific biases, and the PCR products were pooled. Tailed fungal-specific primers were used to amplify the entire ITS region, including ITS1, 5.8S and ITS2, while excluding the highly conserved 18S and 28S regions that add no taxonomic resolution (Lindahl *et al.* 2013). Equal moles of purified DNA were then used as templates for the second PCR, which added sequencing adapters and barcodes to each sample. The barcoded DNA samples were subsequently prepared and divided into three final multiplexed libraries, each loaded onto a separate MinION flow cell.

Each flow cell was run on the Mk1C (Figure 7) for 72 h in order to maximize the data generated.

Figure 7. The Mk1C nanopore sequencing device that allows for real-time basecalling without other computing needs.

There are three basecalling algorithms that differ in terms basecalling accuracy (*i.e.* correctly identifying the nucleotide sequences from the ionic voltage patterns) and computing power requirements. The fast basecalling model allows for near real-time analysis using only a laptop or Mk1C, but provides the lowest accuracy level of the three algorithms; the high accuracy model decreases error rates but requires more computing power and will lag behind in basecalling speed with the Mk1C and laptop; the super-high accuracy model offers the highest accuracy with reported accuracy levels of over 99% when combined with the latest MinION flow cell chemistry (Ni *et al.* 2023) but is very computationally intensive and is best achieved using supercomputing resources. The super-high accuracy model was used, coupled with supercomputing, for basecalling. The basecalled and demultiplexed samples were then trimmed to remove adapters, barcodes and primer sequences prior to assessment and filtering for sequence quality and length. Subsequent steps included dereplication and chimera removal, followed by clustering of the reads at 97% similarity, which is standard for operational taxonomic unit (OTU)-based approaches that are used when error rates prevent the use of ASVs.

3.5.4 Annotation and statistics

The sequencing data (OTUs for nanopore, ASVs for Illumina) was annotated against the UNITE database (Abarenkov 2023) including all eukaryotes to, apart from taxonomic identification of fungal species, accurately identify and remove non-fungal DNA sequences using a trained machine learning classifier via a supercomputing cluster. The taxonomic classification during alignment was done to eight levels; kingdom, phylum, class, order, family,

genus, species, and species hypothesis. Species hypothesis works as a proxy for species but sequences can also be identified to the species hypothesis level (matching a registered sequence in the database) when that specific database match has not been taxonomically identified, hence minimizing the influence of the database comprehensiveness and allowing for a more fair comparison. In addition to prior quality and length filtering, OTUs/ASVs (For ONT and Illumina, respectively) with fewer than five reads in at least one samples were removed.

RStudio was used to generate the plots and significance test results. A stacked bar plot was produced to visualize the respective taxonomic resolution between sequencing platforms. A Venn diagram visualized the proportions of species hypotheses that were shared between platforms or uniquely identified in either platform. Heatmaps were produced to visualize the abundances between platforms, per taxon, in order to examine for patterns of similarity. Abundance boxplots were produced to compare the normalized abundances (accounting for the highly variable sequencing depths) at different levels of taxonomic resolution between platforms, including the most abundant phyla/genera/species for each sequencing platform. The Shannon α-diversity measure was utilized to compare the species diversity between platforms, across all sites and per sampling site type, across all taxa and for a subset of only potential, and known, pathogenic taxa. Additionally, to compare diversity both within and between sequencing platforms for asymptomatic and symptomatic needles. The β-diversity was assessed using a Bray-Curtis dissimilarity matrix and visualized with a Principal Coordinate Analysis (PCoA) ordination plot, representing community-level diversity. The datasets were screened for a set of 60 known and potential pine pathogen, of which 15 were identified in either dataset. This subset of identified pathogens and samples in which they were found was used to compare read abundances between healthy and symptomatic needles. All differences in abundance and α-diversity were statistically compared using a paired Wilcoxon signed-rank test with a Benjamini-Hochberg correction, which was applied to control for the false discovery rate. Throughout, a more stringent significance level than the commonly used $\alpha<0.05$ was applied, because of the large sample number (n=130 per platform) and its associated higher probability for false positives.

4. Results & discussion

The main objective of the thesis was to gain new knowledge on two emerging technologies for detecting and diagnosing forest pathogens. This chapter presents and discusses the key findings of **Papers I-III**.

4.1 *In vitro* study of *Fusarium circinatum* and *Phytophthora* VOCs

In vitro VOC studies were conducted to identify compounds uniquely associated with each studied species, potentially identifying target biomarkers of disease, and to be able to compare VOCs emitted *in vitro* with those emitted *in vivo*. In **Paper I**, four *Fusarium* spp. were readily distinguished based solely on VOC analysis, irrespective of how closely they were related (Figure 8A). The automated GC-MS data analysis pipeline identified several combinations of 3-6 VOCs (out of the total 207 VOCs detected across all *Fusarium* spp.), which were sufficient to differentiate the four *Fusarium* spp. from each other.

In **paper II**, eight *Phytophthora* species were studied. Across these, 58 VOCs were emitted that were not detected in the media controls. Multivariate analysis including PCA and hierarchal cluster analysis were conducted using the complete compound list. The PCA partially differentiated the *Phytophthora* spp., but this separation was evident only at one time point and for three of the species (*P. cambivora, P. gonapodyides* and *P. polonica*), while the remaining species could not be distinguished. The cluster analysis complemented the PCA results and showed that species largely grouped together by their sampling date. All the included *Phytophthora* species except *P. cambivora* exclusively emitted at least one VOC that was not found in any of the other studied species.

These findings demonstrate that most of the *Fusarium* and *Phytophthora* species are easily discernible by VOC analysis. Moreover, the qualitative differences detected here are valuable, as they may serve as biomarkers of disease. These differences could also be utilized for chemotaxonomic identification of closely related fungal species, offering an alternative to molecular techniques that are focused on single gene regions. It is important to consider that the growth medium likely influences the VOC blend emitted by species grown *in vitro* (Blom *et al.* 2011), which suggests that more distinguishable VOC profiles might be obtained by experimenting with different growth media. Therefore, future experiments could examine how the pathogens' VOC profiles differ between nutrient media, such as media based on natural substrates derived from preferred host species (pine for **Paper I**, beech and oak for **Paper II**).

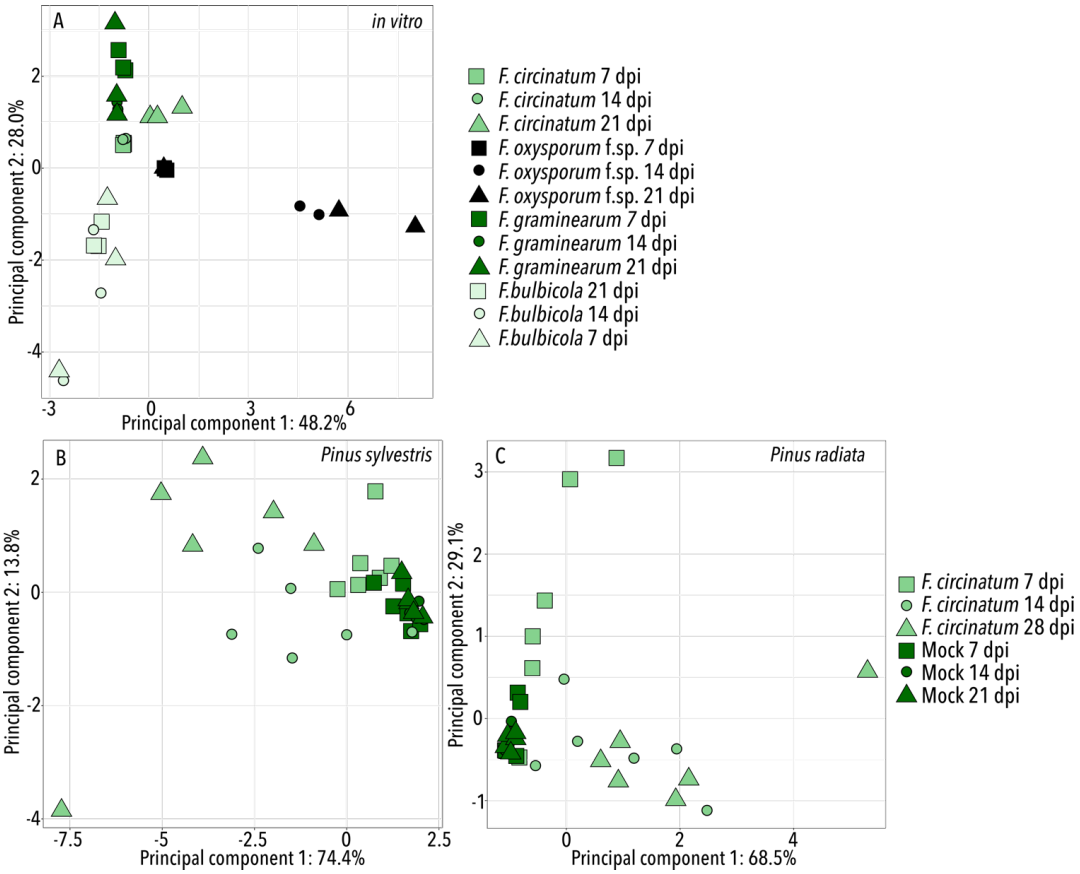

Figure 8. Principal component analyses for (A) *in vitro* studies of *Fusarium* spp., n=3 for each species and time point, (B) *in vivo* studies of *F. circinatum*- or mock-inoculated *Pinus sylvestris*, n=5 for each inoculation type and time point, and (C) *in vivo* studies of *F. circinatum*- or mock-inoculated *Pinus radiata*, n=5 for each inoculation type and time point.

4.2 *In vivo* VOC studies of *Fusarium circinatum* in pine and *Phytophthora* spp. in beech and oak

In vivo experiments were conducted to study VOC emissions of *F. circinatum*-inoculated *P. sylvestris, P. radiata,* and *P. pinea* (**Paper I**), and *F. sylvatica* and *Q. robur* trees inoculated with *P. cinnamomi* or *P. plurivora* (**Paper II)**. The VOCs data analysis primarily focused on identifying qualitative differences between inoculated trees and MIC, as compounds emitted exclusively from inoculated trees can be directly targeted and are therefore especially desired in the pursuit of developing new detection tools.

In total, five VOCs were detected in the *P. cinnamomi*- and *P. plurivora*-inoculated *Q. robur* and *F. sylvatica* trees that were absent in the MIC trees (**Paper II**). Notably, an unknown VOC was constitutively emitted by *P. cinnamomi*-inoculated *Q. robur* at both the 9 and 21 dpi time points, yet this compound was not detected in *F. sylvatica* nor when *P. cinnamomi* was cultured *in vitro*. At the later time point, another unknown VOC was emitted by both *P. cinnamomi*- and *P. plurivora*-inoculated *Q. robur*, and the same was observed for the tentatively identified compound anisole *F. sylvatica*. None of the VOCs detected *in vitro* were uniquely present in inoculated trees *in vivo*, which does not support the hypothesis that the VOCs identified *in vitro* can serve as biomarkers for detecting infected trees. However, the qualitative differences observed *in vivo* may still hold potential as biomarkers. For example, the tentatively identified compound anisole, detected in *F. sylvatica* inoculated with either *Phytophthora* species but absent in MICs, presents a promising candidate. This compound has not been reported in previous studies (Tollsten & Müller 1996; Joó *et al.* 2010; van Meeningen *et al.* 2016), suggesting it may be emitted specifically during certain stress events, and could serve as a potential indicator specifically for *Phytophthora* infection in beech trees.

In **Paper I**, a total of 307 unique VOCs were detected between the three *Pinus* spp. and the majority of these were detected in all three species. However, none of the detected VOCs were exclusively emitted from the *Fusarium*-inoculated pines; therefore, the VOC analysis was focused on quantitative differences using an automated pipeline. A subset of VOCs, that were constitutively emitted across all time points, were identified in each of the pine species as reliable indicators of *F. circinatum* infection. These subsets could readily distinguish between *F. circinatum*-inoculated and

healthy seedlings, as shown by statistical analyses. Comparison of VOC profiles between MIC (control) and inoculated plants indicated disease already at 7 dpi for *P. radiata* (Figure 8C), whereas for *P. sylvestris*, differences in the VOC profiles were evident at 14 and 28 dpi (Figure 8B). The results further underlined the documented relative resistance of *P. pinea* to *F. circinatum* (Iturritxa *et al.* 2013), as no visual symptoms nor differences in VOC profiles were seen between the *F. circinatum*-infected and MIC seedlings throughout the experimental period.

The VOC subsets selected via machine learning in **Paper I** were tailored to each *Pinus* species, chosen based on their low error rates as indicators of *F. circinatum* infection across all the time points. While other combinations of VOCs might have yielded better discriminatory power at specific time points – potentially distinguishing *F. circinatum*-infected *P. sylvestris* from MIC as early as 7 dpi – the pursuit of robust detection methods necessitates VOC biomarkers that are consistently applicable throughout all phases of the disease development. Likewise, when developing a targeted, qualitative VOC-based detection method, it is essential that potential biomarker compounds are constitutively emitted, and at least one such compound was found for each tree species in **Paper II**.

Qualitative differences between inoculated and healthy trees are particularly valuable and enhances the prospects for commercially viable and user-friendly detection methods, such as the e-nose, which is designed to detect specific VOCs. Especially when pathogens emit the same VOCs both *in vitro* and *in vivo*, as observed in *Ceratocystis platani* by Brilli *et al.* (2020) in plane trees, there is a strong opportunity to seize in the quest for robust detection methods. It is possible that pathogen-derived VOCs could have been captured in either study if the sampling and data analysis had been more targeted. Future studies should consider exploring a more targeted sampling strategy and selective MS analysis approach focusing on VOCs previously detected *in vitro*, to enhance the detection of pathogen-specific VOCs. However, not all pathosystems are amenable to such targeted approaches. In cases where no potential biomarkers are detected, a multivariate approach focusing on quantitative VOC differences may be more appropriate. Such approaches provide more extensive analyses in the sense that they account for all present VOCs, can offer more extensive information, and can be applied similarly to any pathosystem. Coupling a quantitative analysis with an automated analysis pipeline, machine learning models and an established

library for training these models would make a widely applicable detection tool.

In **Paper I**, symptom development in *P. radiata* and *P. sylvestris* was similar. Both species displayed shoot wilting and needle chlorosis, with the first signs of symptoms appearing around 14 dpi. By 28 dpi, symptoms had become pronounced and visually distinct (Figure 9). Over the experimental period of more than 5 months, *P. pinea* never developed symptoms. At 50 dpi, the beech and oak trees examined in **Paper II** remained asymptomatic. However, lesion lengths differed in both *P. cinnamomi*- and *P. plurivora*-inoculated beech and oak trees when compared to MIC.

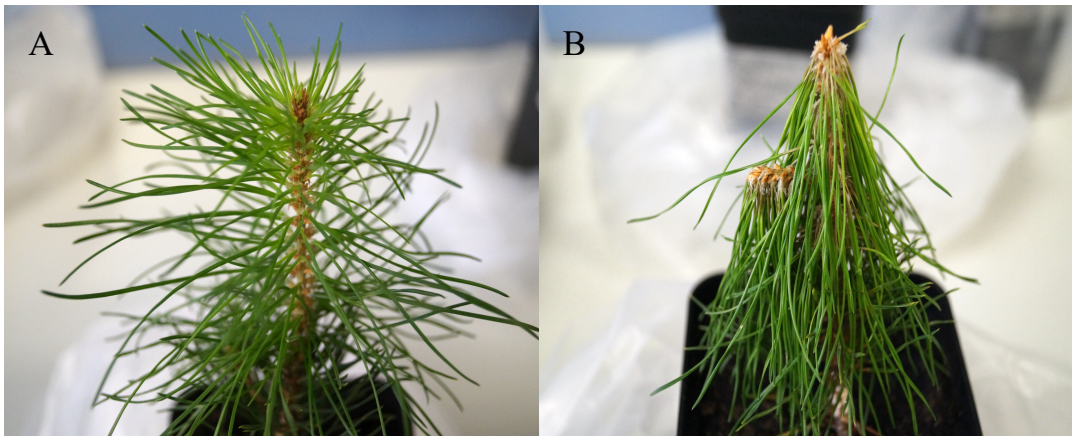

Figure 9. Visual comparison of *Pinus sylvestris* seedlings at 28 dpi: A) mock-inoculated seedling as control, B) *Fusarium circinatum*-inoculated seedling displaying shoot wilting and needle chlorosis.

These findings in **Papers I and II** suggest that VOC analysis has potential to enhance biosurveillance, making further efforts in developing the applicability likely worthwhile. Specifically, up-scaling the technique to analyze larger plant consignments, the reproducibility of SPME-sampling, and integrating portable machinery like handheld GC-MS instruments should be explored.

4.3 Illumina versus Nanopore sequencing: Comparison of two technologies for detection of pine needle fungi (Paper III)

In **Paper III**, the Illumina and ONT sequencing platforms were compared for detection of pine needle associated fungi in terms of taxonomic

resolution, species diversity, and feasibility to detect pathogenic species in asymptomatic needles. Despite using an identical sample set for each platform, this study revealed that the choice of sequencing technology influences the data generated. Firstly, nanopore identified 70% of the total OTUs to the species hypothesis level, versus 60% for the Illumina-generated ASVs (Figure 10). For disease diagnostics, this is highly relevant as pathogens commonly have close relatives that are harmless, so misidentification could lead to unnecessary intervention, or worse: harmful species can be missed, ruining chances of eradication or correct management strategies, which can be even more costly. The longer read length of the nanopore data may offer an explanation to its higher taxonomic resolution, an advantage that compensates well for its higher error rates.

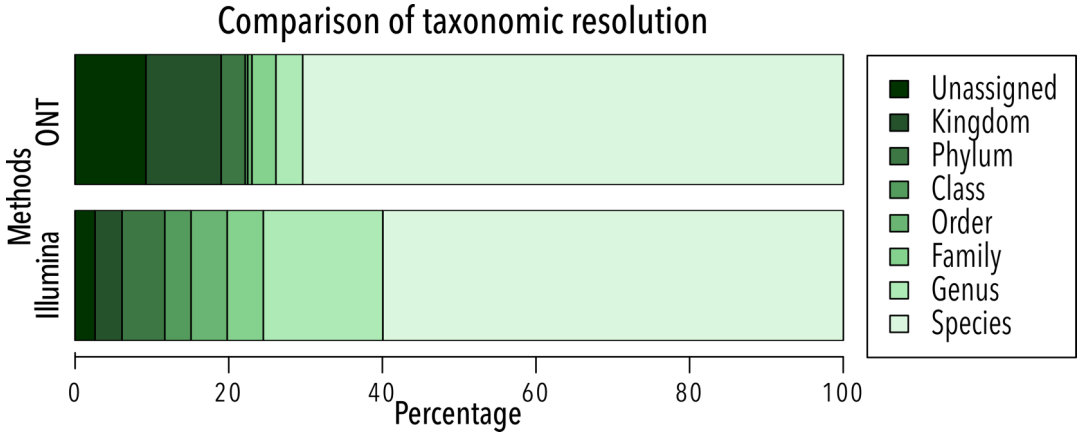

Figure 10. Stacked bar plot visualizing the proportions of Illumina ASVs and nanopore OTUs identified to the respective taxonomic ranks between platforms. The total number of Illumina ASVs and nanopore OTUs included in the analysis were 4,866 and 76,070, respectively. The "Species" rank includes all ASVs/OTUs that were identified to the species hypothesis level.

In this thesis, a metabarcoding approach was chosen with the MinION sequencer to make sure the fungal DNA was not lost among the abundant host DNA. This way, the proportion of fungal DNA was amplified using fungal-specific primers. In future studies, the long-read compatibility of ONT could be further leveraged by combining it with a metagenomic approach, ensuring distinction even for species complexes where sequencing the entire ITS often falls short for species-level identification. Additionally, the ITS region is known to harbor homopolymers exceeding 10 bases (Tedersoo *et al.* 2022), which is problematic for nanopore as a large proportion of its sequencing errors relate to regions of low complexity (Delahaye & Nicolas 2021). This is due to the inconsistent DNA

translocation speed and the similar disruptions in electrical current caused by identical bases, making it difficult to determine exact homopolymer lengths (Sarkozy *et al.* 2018).

The results further revealed that the Illumina platform detected an additional 861 species hypotheses than nanopore and generally higher species diversity (Figure 11). The choice of sequencing platform had a greater impact on the resulting species diversity than the sampling site type or the visual health status of the needles. This suggests that Illumina might be more suitable in broader screening scenarios, for detection of a wide range of pathogens. When needed, it could be complemented with additional analysis for confident identification to the species level. The reason why Illumina captures a larger proportion of species hypotheses may be attributable to its short reads that are more easily matched to sequences in the reference database, and may therefore lead to false matches. The fact that Illumina data is compatible with ASV-based approaches may also help for keeping the captured diversity, part of which might be lost during the clustering step of Nanopore's OTU-based pipeline. A less likely explanation to Illumina's higher diversity is arguably the highly variable sequencing depth, because both platforms varied rather equally and rarefaction curves suggested adequate sequencing depths in both cases.

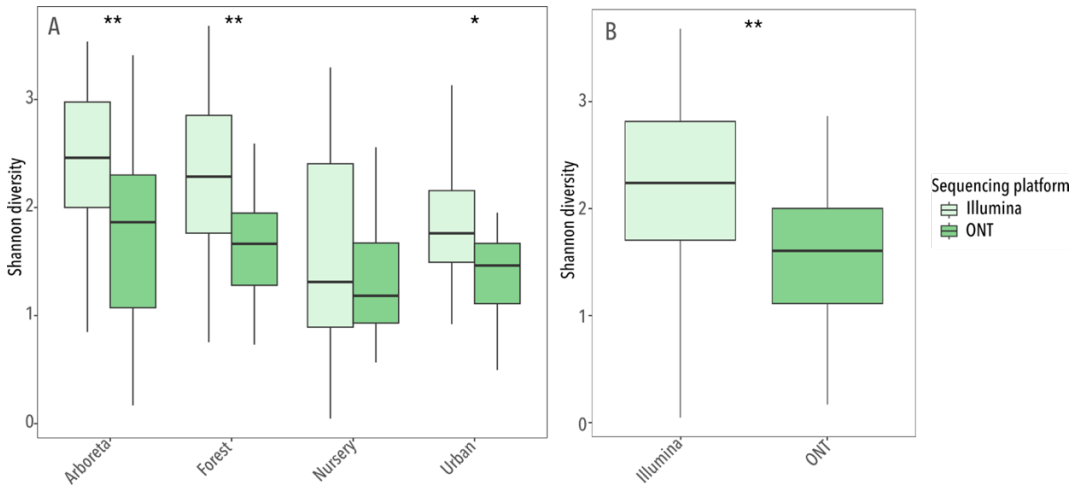

Figure 11. Shannon α-diversity measure, comparing the species diversity between sequencing platforms by sampling site type (A) and globally across the datasets (B). Sample sizes were n= 54 for arboreta, n= 43 for forests, n=16 for nursery, and n=17 for urban, a total of n=130 each sequencing platform. * indicates significant differences between sequencing platforms for that site type at the $\alpha<0.01$ level, and ** at the $\alpha<0.001$ level.

The sequencing datasets were screened for a set of 60 known or potential pine pathogens, of which 15 were identified to species level in either dataset. The pathogen diversity was greater in Illumina compared to nanopore, and the lowest diversity for both sequencing platforms was observed in nursery samples, reflecting the overall pattern seen across all taxa in Figure 11. Statistical comparisons of normalized read counts for pathogens in asymptomatic and symptomatic needles identified three combinations with significant differences; Illumina's mean read count was higher for *Cyclaneusma minus* in symptomatic needles and for *L. seditiosum* in asymptomatic needles, while ONT's mean read count for *F. oxysporum* was higher in asymptomatic needles (Figure 12). Finding higher levels of *L. seditiosum* in asymptomatic needles showed that the pathogen could be detected in an early phase.

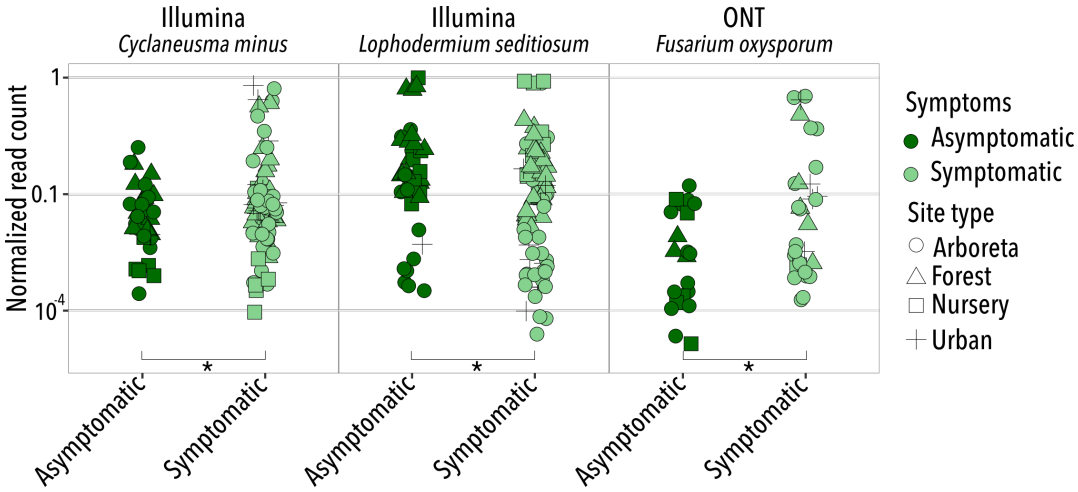

Figure 12. Normalized read counts for the comparisons in which the pathogen abundance differed significantly between asymptomatic and symptomatic needles, for either sequencing platform.

5. Conclusion and future perspectives

This thesis provides new insights into emerging technologies that can advance the biosurveillance of tree pathogens using VOC profile fingerprinting (**Papers I and II**) and next-generation sequencing platforms (**Paper III**). The main findings are:

- Pathogenic *Fusarium* spp. can be readily distinguished based on their VOC profiles *in vitro*
- Most *Phytophthora* spp. exclusively emit at least one VOC
- Quantitative differences in VOCs profiles are capable of distinguishing *F. circinatum*-inoculated *Pinus sylvestris* and *Pinus radiata* trees from mock-inoculated trees
- Qualitative differences in VOCs profiles can distinguish *Fagus sylvatica* and *Quercus robur* saplings inoculated with either *P. cinnamomi, P. plurivora* or sterile medium
- VOCs emitted by *Fusarium* and *Phytophthora* spp. *in vitro* are not detected exclusively in inoculated trees *in vivo*
- Nanopore sequencing provides a proportionally higher taxonomic resolution at the species level compared to Illumina
- Illumina identifies more taxa and captures greater species diversity compared to nanopore
- Both sequencing platforms can identify pathogenic species in asymptomatic needles
- All VOC- and DNA-based approaches studied in this thesis can be useful for early detection, *i.e.* before the onset of visual symptoms

Early detection and rapid response are the most cost-effective means for timely interventions to control new and emerging threats. So far, a lack of high-throughput and accurate tools hinders applicability of available technologies into biosurveillance efforts. Simplicity, operability by non-expert personnel, and short analysis times are essential features of effective and applicable detection tools. The lack of qualitative VOC differences and consequent need for multivariate analysis seen *in vivo* in **Paper I** means that automated data analysis is required to make the detection method feasible for future use in plant protection infrastructures. While manual GC-MS data analysis of distinct biomarkers is manageable, the data from a multivariate approach is much too time-consuming and prone to errors (due to the large number of peak integrations) unless an automated pipeline is utilized. In contrast, using a targeted detection tool to confirm or exclude the presence of a certain chemical is straightforward and requires minimal training for biosecurity personnel. However, this method depends on the presence of qualitative differences in VOCs emission between infected and healthy hosts, which were seen in the pathosystem studied in **Paper II**.

A key advantage of a multivariate VOC detection approach is its applicability to any pathosystem, regardless of whether it presents identifiable biomarkers compared to healthy hosts. Establishing databases with species-specific VOC libraries could offer training datasets to customize and enhance machine learning tools for screening different shipments. For instance, a shipment of *P. sylvestris* could be analyzed against a customized library specific to *P. sylvestris*, optimizing the recognition of known stress-induced VOCs emitted by the host and associated pathogens. With further method development, VOC detection has the potential to be scaled-up, offering a cost-efficient method for large-scale plant shipment screening.

When pathogen or pest-induced VOCs emission changes in quality, meaning a change in the type of emitted molecules, a targeted approach could be the most efficient choice for identifying a specific pathogen. One such tool is the e-nose (Cui *et al.* 2018; Rutolo *et al.* 2018), a device equipped with sensors that can be customized to target compound groups of interest. These targeted approaches are viable options for in-field use, when there are suspicions of what pathogen possibly present. For broader surveillance purposes, a VOC detection method that captures all emitted VOCs is more appropriate, like

GC-MS approaches used in this thesis. Handheld GC-MS devices offering comparable sensitivities as the benchtop ones are also available (Beck *et al.* 2016; Sharma *et al.* 2019). For example, a fully automated pipeline based on a portable GC-MS, machine learning and statistical analysis was able to successfully distinguish between control and aphid-infested milkweed plants (Sharma *et al.* 2019). This pipeline, completed in only an hour, could identify infested individuals within 48-72 hours of an attack with >90% accuracy. This example shows great promise for adaptation to other pest- and pathosystems.

A potential future port-of-entry biosecurity testing scenario could involve an established VOC-based analysis system for shipments, using a VOC-collecting device, such as the SPME fiber, placed inside the shipment container. The fiber would be analyzed immediately upon arrival at a port-of-entry to assess the health of the shipment's plants before they are allowed to be further transported or dispersed into parks, forests or commercial settings. The need for staff expertise could be minimized by implementing an analysis pipeline in which the GC-MS results are processed through a machine learning system, providing simplified and easily interpretable results for the staff. This concept is analogous to the established ion mobility screening for explosives at airports, which is also performed by non-experts with minimal training in chemistry.

The use of dogs for VOCs detection presents an intriguing prospect for future in-field detection methods. A few case studies have explored this approach for detection of forest pathogens such as *Phytophthora* spp. (Carter *et al.* 2023) and *Heterobasidion* spp. (Wysocka 2021). While the dogs detected the pathogen more often than chance, their accuracy remained relatively low (69% and 70%, respectively, for each study), which in the latter case was not surprising given that the study involved amateur dogs and trainers. In contrast, professional dogs and trainers, such as those used in law enforcement for narcotics detection, the accuracy for detection is 98-100% (Lee Rice & Velasco 2023). Admittedly, the VOCs blend from any given pathosystem will likely be more complex than the narcotics-emitted VOCs, so the accuracies may never be comparable, but sniffer dogs may offer a cost-efficient alternative as a first screening for pathogens. Recent experiments in Australia have demonstrated that dogs trained to sniff out *P. cinnamomi* can detect the pathogen with high accuracy from the soil of infected plants in avocado orchards, in native forests of *Xanthorea* species (grasstrees), and in

likely scenarios where the pathogen could be spread, *i.e.* on wheels of vehicles harboring soil (Cleary 2024). This suggests that sniffer dogs have great potential to be used as a first step in biosurveillance programmes in support of detection and identification even in low-density pathogen populations where detection may be difficult and cryptic (*e.g. Phytophthora* spp.).

When fully developed, VOC based screening methods, can also be of good use for high-throughput inspections of high-risk shipments where they can serve as an initial first warning system. Plant consignments identified as high-risk could be further tested using either a targeted method such as LAMP, if there is a clear suspicion of what the pathogenic agent is, or a non-targeted sequencing method that could identify all the species in a sample. For biosurveillance, high-throughput sequencing would make a better option than LAMP, since the former can detect multiple pathogens simultaneously, including those for which there is no targeted approach available.

High-throughput sequencing is an option to be employed in national biosurveillance programmes that could increase efficacy in detecting harmful species. As seen in the studies presented here, Illumina is likely a better fit for large-scale screening as it captures a wider species diversity compared to nanopore. For diagnostic purposes, nanopore sequencing offers a higher taxonomic resolution at the species level, which is important for identifying closely related species correctly.

Nanopore sequencing not only has the advantage of multiplexing samples, but it can also be employed to detect an endless number of organisms in any DNA or eDNA sample from soil, spore traps or plant tissue DNA. Furthermore, it allows for detection of low-abundance species that may remain undetected when using conventional detection methods (Lindahl *et al.* 2013). Lastly, ONT data analysis pipelines could be established and optimized to automate data analysis, which would save time, labor, and circumvent the need for specialized expertise. The sequencing results would then be directly aligned and provided in real-time, a process which is made faster by using a host-specific database.

When gazing into the future, there are numerous exciting prospects for improving detection methods further. Firstly, ONT sequencing could benefit from the advent of solid state nanopores, which have greater stability and durability compared to the protein based nanopores (He *et al.* 2021). This

could lead to improved single base pair resolution, greater customization of pore properties and easier integration into electronics (Goto *et al.* 2020; Mahajan-Mallakmir *et al.* 2023). ONT is also developing automated "sample to sequence" devices such as the TraxION that serves as a portable and easily operated work station that can extract DNA, prepare the sequencing library, and sequence all in one. Secondly, improvements in MS also make VOC-based pathogen detection more feasible. Proton-transfer-reaction MS is a relatively new method that offers real time results of volatiles, is highly sensitive, with non-invasive sampling, and has already been used successfully to monitor plant VOCs under natural conditions including pathogen attack (Steeghs *et al.* 2004; Harren & Cristescu 2013).

Other new technologies, not utilized in these projects, may also prove invaluable for plant biosecurity in the future. For example, the CRISPR/Cas system is being used for plant pathogen detection (Wheatley & Yang 2021) and artificial intelligence and machine learning can be used for leaf image analysis for disease prediction and potentially assist landowners to apply the right mitigation strategies at the right times (Anand *et al.* 2022).

This thesis provide valuable insights that can help guide biosecurity organizations to improve screening and diagnostics infrastructures for safeguarding our forests and urban landscapes. In the quest for applicable and accurate detection methods, it is key to consider the trade-offs and respective advantages with each method in order to optimize testing in different scenarios. The results herein provide an increased understanding for utilizing VOC analysis and high-throughput sequencing as detection or diagnostic tools for forest pathogens.

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Popular science summary

Forests and urban trees are increasingly at risk from harmful fungi and other pathogens, both foreign and native. History shows that these harmful species can wipe out entire tree species and be extremely costly in several ways. Climate change makes it easier for these species to grow, while simultaneously weakening trees. Forest disease outbreaks are happening more often than ever, but we lack good tools for biosecurity agencies to detect these threats early. This research looked at existing technologies to see how effective they are for recognizing tree diseases. It focused on studying chemical odors (volatiles), that trees and the disease-causing agent release during infection and modern DNA-based methods to identify fungi on pine needles. When grown in culture, different pathogens could be told apart by comparing the different odors that they produced. When testing on real trees; pine, beech and oak, the odors from infected trees differed from those of healthy trees. However, none of the compounds that the pathogens emitted when grown in culture were emitted when grown on trees. Two DNA sequencers (*i.e*. devices that can read and decipher the genetic code of a sample), the portable nanopore sequencer and the more established sequencer Illumina, were tested for detection of fungal DNA in pine needles to see whether nanopore can improve testing procedures for early detection and monitoring. We found that nanopore had a higher precision in identifying fungal species, while Illumina detected more species overall. Both techniques detected harmful species also in healthy-looking pine needles, which suggests that they can be used to detect disease before visible symptoms appear. Since each method has its own strengths, it's important to consider these trade-offs when choosing the most suitable approach for a specific situation. Illumina is likely better for broad screening, such as at border control, while nanopore is more suited for diagnostic purposes, helping to distinguish dangerous species from harmless relatives. Overall, this research highlights the potential of using existing technologies to enhance how we protect forests from future disease threats.

Populärvetenskaplig sammanfattning

Skogar och stadsträd utsätts allt oftare för skadliga svampar och andra skadegörare från när och fjärran. Historiska exempel visar att dessa skadliga arter kan utrota hela trädpopulationer och vara extremt kostsamma både ekonomiskt och ekologiskt. Klimatförändringar förvärrar problemet ytterligare genom att gynna dessa skadegörare och samtidigt göra träd mer infektionskänsliga. Utbrott av skogssjukdomar sker oftare än någonsin, men vi saknar bra verktyg för att kunna upptäcka dessa hot i tidigt skede. Denna forskning utvärderade befintliga teknologier för att se hur effektiva och användbara de är för att upptäcka sjukdomar hos träd. Fokuset låg på att studera dofter (flyktiga ämnen) som värdträd och skadegörare avger, samt modern DNA-sekvensering för att identifiera svampar i tallbarr. När skadegörarna växte på agarplattor kunde olika arter skiljas åt genom att jämföra de olika dofter som producerades. Vid tester på riktiga träd; tall, bok och ek, var dofterna från infekterade träd annorlunda än de från friska träd. De specifika ämnena som skadegörarna avgav när de odlades på agarplatta var dock inte samma som de som avgavs när de växte på träd. Två olika metoder för DNA-sekvensering, portabel nanopore-sekvensering och den mer etablerade Illumina, jämfördes med avseende på detektion av svamp-DNA i tallbar för att utvärdera om nanopore kan förbättra testprocedurer för tidig detektion och övervakning inom biosäkerhet. Vi fann att nanopore identifierade svampar med högre precision, medan Illumina upptäckte fler arter totalt. Båda teknikerna upptäckte skadliga arter även i friska tallbarr, vilket tyder på att de kan användas för att upptäcka sjukdomar innan symptom utvecklas. Eftersom varje metod har egna styrkor är det viktigt att överväga dessa för- och nackdelar för att välja den bäst lämpade metoden vid varje enskild situation. Illumina passar troligen bättre för bred screening, till exempel vid gränskontroller, medan nanopore lämpar sig bättre för diagnostik för att särskilja skadliga arter från närbesläktade men ofarliga arter. Denna forskning belyser potentialen i att använda befintliga teknologier för att skydda framtida skogar mot skadegörare.

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Appendix I: Supplementary information for **Paper III**

Table S3(a). Sequencing depth for Illumina. Total read counts per sample and sequencing platform post filtering by quality and length.

Illumina Reads	Illumina samples	Illumina Reads	Illumina samples	Illumina Reads	Illumina samples	Illumina Reads
74689	Q5H_Ill	65331	OE1_Ill	36554	X2C_Ill	71334
57820	Q6D_Ill	47742	OE2_Ill	69786	X3A_Ill	64959
30715	Q6H_Ill	49710	ES2_Ill	71541	X3B_Ill	67805
46032	Q7D_Ill	74314	ES3_Ill	75292	X3C_Ill	72818
15490	Q7H_Ill	12219	ES4_Ill	65715	X4A_Ill	62120
37636	Q8D_Ill	15538	ES6_Ill	34484	X4B_Ill	70629
78053	Q8H_Ill	42021	ES7_Ill	50377	X4C_Ill	37306
49017	Mi2_Ill	16501	ES8_Ill	70815	X5A_Ill	39307
60569	Mi3_Ill	77085	ES9_Ill	26357	X5B_Ill	48680
37728	Mi4_Ill	77589	ES10_Ill	59152	X5C_Ill	69604
84568	Mi5_Ill	55050	R1_Ill	42940	X6A_Ill	72696
44761	Mi6_Ill	79135	R2_Ill	54578	X6B_Ill	75852
8076	T1B_Ill	47023	R3_Ill	32382	X6C_Ill	78353
27264	T2A_Ill	54766	P1_Ill	76120	X7A_Ill	64740
49413	T2B_Ill	27914	P2_Ill	75187	X7B_Ill	70313
29876	T3A_Ill	65908	P3_Ill	76721	X7C_Ill	75012
81181	T3B_Ill	39957	P4_Ill	82034	X8A_Ill	26284
38906	T4A_Ill	74119	P5_Ill	64991	X8B_Ill	76015
70029	T4B_Ill	27817	P6_Ill	35430	X8C_Ill	67100
47280	T5A_Ill	59731	P7_Ill	14079	X9A_Ill	54662
22624	T5B_Ill	34065	P8_Ill	39535	X9B_Ill	66389
57700	T6A_Ill	69812	P10_Ill	68325	X9C_Ill	68706
21172	T6B_Ill	58940	P11_Ill	74241	X10A_Ill	62385
5630	T7A_Ill	60532	P12_Ill	75947	X10B_Ill	74253
70211	T7B_Ill	42508	P13_Ill	71211	X10C_Ill	68700
5506	T8A_Ill	79083	P14_Ill	70250		
46219	T8B_Ill	49089	P15_Ill	74070		
40882	T9A_Ill	28828	P16_Ill	26436		
72035	T9B_Ill	34789	P17_Ill	74052		
28885	T10A_Ill	64559	P18_Ill	80645		
69039	T10B_Ill	29695	X1A_Ill	20733		
35150	T11A_Ill	68013	X1B_Ill	36638		
66362	T11B_Ill	69916	X1C_Ill	49860		
11605	T12A_Ill	48720	X2A_Ill	78444		
60372	T12B_Ill	43532	X2B_Ill	81764		

Table S3(b). Sequencing depth for ONT. Total read counts per sample and sequencing platform post filtering by quality and length.

Nanopore samples	Nanopore Reads	Nanopore samples	Nanopore Reads	Nanopore samples	Nanopore Reads	Nanopore samples	Nanopore Reads
S1A_Nano	44699	Q5H_Nano	45218	OE1_Nano	7908	X2C_Nano	33924
S1B_Nano	6564	Q6D_Nano	15470	OE2_Nano	79406	X3A_Nano	11704
S1C_Nano	4270	Q6H_Nano	58938	ES2_Nano	21438	X3B_Nano	50569
S2A_Nano	40963	Q7D_Nano	11579	ES3_Nano	10645	X3C_Nano	76250
S2B_Nano	24603	Q7H_Nano	9791	ES4_Nano	37183	X4A_Nano	16687
S3B_Nano	17566	Q8D_Nano	11247	ES6_Nano	3435	X4B_Nano	16296
S4A_Nano	21103	Q8H_Nano	111948	ES7_Nano	4166	X4C_Nano	10836
S4B_Nano	45245	Mi2_Nano	12389	ES8_Nano	13568	X5A_Nano	16612
S5A_Nano	7253	Mi3_Nano	63172	ES9_Nano	11805	X5B_Nano	5829
S5B_Nano	6895	Mi4_Nano	8860	ES10_Nano	9356	X5C_Nano	17408
A1_Nano	6554	Mi5_Nano	15829	R1_Nano	16396	X6A_Nano	27557
A2_Nano	11148	Mi6_Nano	6700	R2_Nano	11739	X6B_Nano	40745
A3_Nano	8768	T1B_Nano	19126	R3_Nano	17632	X6C_Nano	25572
C1_Nano	36414	T2A_Nano	27935	P1_Nano	6657	X7A_Nano	6009
C2_Nano	14639	T2B_Nano	7118	P2_Nano	78271	X7B_Nano	11353
C3_Nano	56719	T3A_Nano	4955	P3_Nano	20720	X7C_Nano	44418
D1_Nano	92641	T3B_Nano	53476	P4_Nano	54839	X8A_Nano	9307
D2_Nano	37889	T4A_Nano	6370	P5_Nano	22148	X8B_Nano	23285
E1_Nano	26006	T4B_Nano	24034	P6_Nano	14195	X8C_Nano	25820
F1_Nano	65599	T5A_Nano	11874	P7_Nano	12284	X9A_Nano	17338
F2_Nano	20275	T5B_Nano	9157	P8_Nano	15337	X9B_Nano	10992
G1_Nano	68164	T6A_Nano	6359	P10_Nano	13783	X9C_Nano	19520
G2_Nano	19689	T6B_Nano	11586	P11_Nano	94384	X10A_Nano	28759
H2_Nano	10954	T7A_Nano	12460	P12_Nano	6507	X10B_Nano	25302
I1_Nano	56517	T7B_Nano	6693	P13_Nano	23108	X10C_Nano	13742
I2_Nano	6618	T8A_Nano	9714	P14_Nano	48665		
J1_Nano	13976	T8B_Nano	8720	P15_Nano	46717		
J2_Nano	17916	T9A_Nano	10542	P16_Nano	31917		
Q1D_Nano	15506	T9B_Nano	6984	P17_Nano	17038		
Q1H_Nano	73399	T10A_Nano	8837	P18_Nano	57586		
Q2D_Nano	28771	T10B_Nano	9692	X1A_Nano	11600		
Q2H_Nano	13134	T11A_Nano	32581	X1B_Nano	23902		
Q3D_Nano	20956	T11B_Nano	9237	X1C_Nano	22486		
Q4H_Nano	57149	T12A_Nano	12972	X2A_Nano	40054		
Q5D_Nano	35307	T12B_Nano	7953	X2B_Nano	16780		

Table S5(a). P-values for comparisons of normalized abundances between platforms at three taxonomic ranks: Phylum, genus and species.

Main phyla: Ascomycota & Basidiomycota	Phylum	Adjusted p-value (Benjamini-Hochberg Correction)
	Ascomycota	2.61047817111595e-22
	Basidiomycota	0.00287033770578654

Top 5 genera per sequencing platform	Genus	Adjusted p-value (Benjamini-Hochberg Correction)
	Lophodermium	3.680440829771e-15
	Neocatenulostroma	5.01528097167807e-10
	Hormonema	7.70066876304859e-22
	Cyclaneusma	1.04928009472732e-20
	Cladosporium	3.3493330849134e-19
	Heterotruncatella	3.23345817686462e-11
	Vishniacozyma	0.000976139560251945
	Fusarium	3.99421003027087e-08

Top 10 species per sequencing platform	Species	Adjusted p-value (Benjamini-Hochberg Correction)
	Fusarium_oxysporum	4.44536267944193e-10
	Lichenostigmatales_sp	3.62896984682411e-11
	Lophodermium_seditiosum	1.38554712781221e-20
	Lophodermium_conigenum	6.05863594408919e-18
	Neocatenulostroma_microsporum	4.44536267944193e-10
	Hormonema_macrosporum	6.25679336997698e-22
	unknown.Lophodermium	9.26940122775458e-22
	unknown.Cladosporium	6.25679336997698e-22
	unknown.Ascomycota	1.43836162081688e-16
	unknown.Didymellaceae	1.93408816223745e-10
	Fungi_sp	2.2853958930363e-20
	Vishniacozyma_victoriae	0.00780659296047221
	Lophodermium_pinastri	0.0138932453214299

Table S5(b). P-values of comparisons between alpha-diversities, visual health status, site types and platforms.

Alpha-diversity plots

Comparing Illumina and nanopore data globally (paired)

	Adjusted p-value
Illumina vs nanopore	8.894896e-11

Comparing Illumina and nanopore data by site type (paired)

Site type	Adjusted p-value
Arboreta	0.0000000235
Forest	0.000000114
Nursery	0.141
Urban	0.00445

Comparing Illumina and nanopore data by visual health status, including only known

Visual health statu	Adjusted p-value
Healthy	4.84e- 5
Symptomatic	1.17e-17

Comparing diversity differences in visual health status by sequencing platform, inclu

Sequencing platfo	Adjusted p-value
Illumina	0.692
ONT	0.00384

Comparing Illumina and nanopore data by site type, including only known pine path

Site type	Adjusted p-value
Arboreta	2.06e-10
Forest	2.06e-10
Nursery	8.28e- 1
Urban	4.42e- 4

Comparing Illumina and nanopore data in different scenarios

Native and exotic pine species in Alnarp arboreta

Visual health statu	Adjusted p-value	Sequencing platform	Adjusted p-va
Healthy	0.000460	Illumina	0.0135
Symptomatic	0.0000168	ONT	0.0191

Pinus sylvestris in forest sites

Visual health statu	Adjusted p-value	Sequencing platform	Adjusted p-va
Healthy	0.000727	Illumina	0.737
Symptomatic	0.000727	ONT	0.706

Pinus mugo in arboreta and urban sites

Visual health statu	Adjusted p-value	Sequencing platform	Adjusted p-va
Healthy	0.0935	Illumina	0.00225
Symptomatic	0.00193	ONT	0.0166

Table S1. Sampling site attributes and number of samples per site, *Pinus* species and visual health status.

Location	Site Type	Site characteristics	*Pinus species*	No. of trees sampled	Total samples	Asymptomatic	Symptomatic
Huljen	forest	Mixed forest (pine, spruce, birch, alder) of mixed ages	*P. sylvestris*	5	10	5	5
Nybro	forest	Monoculture (pine); ~60 years	*P. sylvestris*	2	2	-	2
Tagel	forest	Shelterwood; planted and natural regeneration (~5 years)	*P. sylvetsris*	12	23	11	12
Ekebo	nursery	Mixed forest surrounding	*P. sylvestris*	10	16	7	9
Lidingö	urban	solitary tree at residence	*P. mugo*	1	1	-	1
Malmö	urban	solitary tree at residence	*Pinus spp.*	1	3	1	2
Torekov	urban	solitary tree at residence	*P. mugo*	1	4	-	4
Båstad	urban	solitary tree at residence	*P. mugo*	1	4	-	4
Lund	urban	solitary tree at residence	*Pinus spp.*	1	1	-	1
Höganäs	urban	solitary tree at residence	*P. mugo*	1	4	-	4
Alnarp	arboreta	botanical collection of native and exotic species	*P. sylvestris* 'beuvronensis'	1	6	2	4
			Pinus x schwerini	1	6	2	4
			P. ponderosa	1	6	2	4
			Pinus spp.	4	9	3	6
			P. mugo	7	15	6	9
			P. bungeana	1	4	2	2
			P. parviflora	1	1	1	1
			P. cembra	1	2	-	2
			P. sylvestris	2	4	1	3
Fogars de Montclus	forest	Mixed forest of mixed ages	*Pinus* spp.	3	3	-	3
La Jonquera	forest	Mixed forest of mixed ages	*Pinus* spp.	5	5	-	5

Table S3. Pathogen-host associations displaying the sample counts per host species, sampling site type and visual health status for each pathogen that was found among the datasets.

		P.s								P.m				P.s.b.		PxS		P.po		P.b		P.pa		P.c		P.							
		F		U		N		Ar		U		Ar		Ar		Ar		Ar		Ar		Ar		Ar		F		U		N		Ar	
Platform	Pathogen	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S
Illum.	Biscogniauxia nummularia	-	-	-	-	2	2	-	-	-	1	3	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	1	-	1
ONT	Biscogniauxia nummularia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Illum.	Cenangium ferruginosum	1	3	-	-	1	1	1	3	-	7	3	8	2	4	2	4	2	3	1	-	1	-	-	2	-	-	-	1	-	-	1	6
ONT	Cenangium ferruginosum	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-	-	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	2
Illum.	Cladosporium herbarum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ONT	Cladosporium herbarum	1	1	-	-	2	5	1	1	-	5	3	7	2	2	2	2	1	2	-	1	1	-	-	-	-	-	-	2	-	-	2	4
Illum.	Colpoma quercinum	-	-	-	-	2	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ONT	Colpoma quercinum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Illum.	Cyclaneusma minus	1	3	-	-	5	7	1	3	-	8	6	1-	2	4	2	4	2	3	1	2	1	1	-	2	-	8	1	4	-	2	3	6
ONT	Cyclaneusma minus	-	-	-	-	-	2	-	-	-	1	3	7	1	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	2
Illum.	Diplodia scrobiculata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ONT	Diplodia scrobiculata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Illum.	Fusarium lateritium	1	1	-	-	-	-	1	1	-	4	2	7	-	2	1	2	1	3	-	-	1	1	-	2	-	7	1	3	-	-	2	4
ONT	Fusarium lateritium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Illum.	Fusarium oxysporum	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ONT	Fusarium oxysporum	-	-	-	-	5	2	-	-	-	3	4	4	1	3	1	4	2	2	2	1	1	-	-	1	-	5	-	2	-	-	2	6

Illum.	Lecanosticta acicola	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ONT	Lecanosticta acicola	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Illum.	Lophodermium conigenum	-	-	-	-	7	7	-	-	-	4	5	7	1	4	2	3	2	2	2	2	1	1	-	-	-	8	1	3	-	2	2	4
ONT	Lophodermium conigenum	-	1	-	-	5	5	-	1	-	3	1	1	1	-	-	-	-	1	-	1	-	-	-	-	-	1	-	2	-	-	2	2
Illum.	Lophodermium macci	1	3	-	-	-	-	1	3	-	6	3	7	1	2	1	2	1	2	1	2	1	1	-	2	-	8	1	3	-	-	2	3
ONT	Lophodermium macci	-	1	-	-	-	-	-	1	-	-	-	1	1	2	-	-	-	1	-	-	1	1	-	1	-	1	-	-	-	-	-	-
Illum.	Lophodermium pinastri	1	3	-	-	5	3	1	3	-	6	6	1-	2	4	2	4	2	3	2	2	1	1	-	2	-	8	1	4	-	2	3	6
ONT	Lophodermium pinastri	-	2	-	-	2	1	-	2	-	6	2	8	-	1	1	2	1	2	1	-	-	-	-	2	-	1	-	2	-	1	2	4
Illum.	Lophodermium seditiosum	1	2	-	-	7	7	1	2	-	2	5	9	2	4	2	4	2	3	2	1	1	1	-	2	-	8	1	3	-	2	2	5
ONT	Lophodermium seditiosum	-	-	-	-	6	4	-	-	-	-	1	-	1	3	1	2	1	1	1	-	-	-	-	-	-	1	-	-	-	2	-	-
Illum.	Nothophaeocryptopus gaeu	-	2	-	-	-	-	-	2	-	7	2	5	1	2	1	1	1	2	1	-	-	-	-	2	-	-	-	1	-	-	1	3
ONT	Nothophaeocryptopus gaeu	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Illum.	Xenopolyscytalum pinea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ONT	Xenopolyscytalum pinea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-

Illum.= Illumina, ONT=Oxford Nanopore Technology. P.s=*Pinus sylvestris*, P.m=*Pinus mugo*, P.s.b=*Pinus sylvestris 'beuvronensis'*, PxS=*Pinus x Schwerinii*, P.o= *Pinus ponderosa*, P.b=*Pinus bungeana*, P.pa= *Pinus parviflora*, P.c=*Pinus cembra*, P=*Pinus* spp. F=Forest site, U=Urban site, N=Nursery site, Ar=Arboteta site. A=asymptomatic, S=Symptomatic.

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Utilizing volatile organic compounds for early detection of *Fusarium circinatum*

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***Fusarium circinatum*, a fungal pathogen deadly to many *Pinus* species, can cause significant economic and ecological losses, especially if it were to become more widely established in Europe. Early detection tools with high-throughput capacity can increase our readiness to implement mitigation actions against new incursions. This study sought to develop a disease detection method based on volatile organic compound (VOC) emissions to detect *F. circinatum* on different *Pinus* species. The complete pipeline applied here, entailing gas chromatography—mass spectrometry of VOCs, automated data analysis and machine learning, distinguished diseased from healthy seedlings of *Pinus sylvestris* and *Pinus radiata*. In *P. radiata*, this distinction was possible even before the seedlings became visibly symptomatic, suggesting the possibility for this method to identify latently infected, yet healthy looking plants. *Pinus pinea*, which is known to be relatively resistant to *F. circinatum*, remained asymptomatic and showed no changes in VOCs over 28 days. In a separate analysis of in vitro VOCs collected from different species of *Fusarium*, we showed that even closely related *Fusarium* spp. can be readily distinguished based on their VOC profiles. The results further substantiate the potential for volatilomics to be used for early disease detection and diagnostic recognition.**

Forests globally are increasingly threatened by alien invasive pathogens and pests. Globalization is primarily responsible for the increasing rate of establishment of invasive alien species (IAS) and no saturation point is yet predictable[1]. Climate change also compounds the spread of IAS through the elimination of environmental barriers, allowing IAS to establish and survive in new geographic locations. There are many potential pathways of introduction of alien pests and pathogens affecting trees in urban and forested landscapes, *e.g.*, trade of plant-derived commodities[2–5] including seeds[6], potting substrates and other plant products valuable for other human activities. Preventing new introductions of IAS is achievable through better biosecurity measures at, for example, border entry locations. However, biosecurity in the plant trade is often curtailed by a lack of resources and necessary skills to recognize problems during plant inspections and a lack of modernized tools with high throughput capacity for detection of alien species in plant shipments[7–10]. Countries with stricter border control have fewer established quarantine alien insects[11] and fungal plant pathogens[12]. According to Santini et al.[2] approximately 50 invasive forest pathogens currently found in Europe are accidentally introduced alien species, of those approximately 26% attack gymnosperms mainly causing dieback, death and/or reduced growth. Of all invasive forest pathogens in Europe, only 1% have been successfully eradicated by sanitary measures[2], a likely result of missing the critical window where early detection and rapid response could lead to effective eradication of the founding population.

To combine recent technological advances with knowledge about specific metabolic responses in pests, pathogens and the trees that they infect is a challenge that calls for interdisciplinary competence. Traditional approaches of disease detection are unsatisfactory for largescale plant screening; usually shipments are only spot-checked if at all, and apart from visual scouting for symptoms, testing is generally targeted and uses tedious and expensive DNA-based or serological detection assays[13]. Innovative methods that are better suited for early and rapid detection are needed[13]. Detecting volatile organic compounds (VOCs) released by pathogens

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and during disease is one such method that could be utilized as an early warning system, facilitating the choice of plant material to be processed for more specific DNA-based diagnosis. As stated by Materić et al.[14], VOCs are secondary metabolites produced by all living organisms, the composition of which is unique to every species and presumably also all specific plant-pathogen interactions, comparable to a chemical fingerprint. Emission rates and composition of VOCs are highly dynamic, influenced by biotic and abiotic stresses, and can serve as an indicator of plant health status[14]. Sampling of VOCs can be done in non-destructive ways from many plants simultaneously, and could serve as a high-throughput detection tool for plant diseases[13,15–17].

Detection of plant pathogens by analysis of VOCs emitted during infection has been reported in multiple studies, for example, for the early detection of spoilage diseases in crops and grains[15,18–22] and the general understanding is that VOC emissions reflect the specific plant-pathogen combination[23]. Similar methods have also been developed for woody plants with importance for the food industry, like palm[24] and lemon trees[25]. An example of a VOCs application currently near commercial use is the in-field detection of *Candidatus* Liberibacter asiaticus, a bacterium that is the causal agent of citrus greening disease, commonly known as Huanglongbing, that has devastated the global citrus fruit industry[26,27]. Pathogen detection methods based on VOCs in a forestry context is far less developed. However, Vuorinen et al.[28] could distinguish birch trees exposed to pathogens or herbivores on the basis of VOC profiles, and similarly Johne et al.[29] could differentiate between two pathogenic fungi in horse chestnut trees (*Aesculus* spp.). It has been shown that VOCs may serve as an indicator of fungal infection in asymptomatic spruce[30] and recently, Brilli et al.[31] found a few VOCs to be uniquely emitted from *Ceratocystis platani*-infected asymptomatic *Platanus* trees, highlighting the potential for targeted VOCs analysis for disease detection. Further method development is needed for VOCs applications in the forestry field.

A method utilizing VOCs requires strategies for collection, separation, detection and analysis of the VOCs. Plant VOC collection is most extensively performed by headspace (HS) sampling, a non-destructive approach offering a more realistic picture of the plant VOC profile as compared to alternatives such as extractions of VOCs from plant tissues in organic solvents. Sampling of HS can be achieved using dynamic methods or by static solid phase micro-extraction (SPME)[16]. SPME fibers are inert and reusable sampling devices having absorbant or adsorbent coatings to which the targeted compounds are sorbed. The chemical properties of the coating determines what type of compounds can be sampled successfully. SPME is easy to use, and once equilibrium with the surrounding HS is reached, the SPME fiber can be thermally desorbed in a gas chromatograph (GC) for subsequent separation of the components in the sample[16]. Analytes separated by GC are most commonly analysed by a flame ionization detector or a mass spectrometer (MS)[15]. The final challenge to complete a detection method pipeline lies in the analysis of the big data sets generated from GC–MS analysis, which can be done utilizing for example MZMine 2, an open-source software for MS data processing[32]. This makes the pipeline fully machine based and, correctly implemented, this approach has potential to be as easily applied as the ion mobility mass spectrometry routinely used in airport security.

Fusarium is a large genus of (mostly) plant-associated filamentous fungi, consisting of 23 defined species complexes and almost 300 distinct species[33,34]. *Fusarium circinatum*, the causal agent of pine pitch canker (PPC) disease[35], poses a serious threat to pine forests across the globe[36]. The *Fusarium fujikuroi* species complex, to which *F. circinatum* belongs, includes several clades of species with a wide plant host range and varying host specificity[37]. The American-clade species *F. circinatum* causes a serious disease on a variety of pine (*Pinus*) species and on Douglas fir (*Pseudotsuga menziesii*)[38]. Early symptoms of *F. circinatum* infection on pine include resinous cankers, chlorosis and/or wilting of needles while late symptoms appear as shoot dieback, reddening and dead foliage[39]. This pathogen originates from the south-eastern USA but has now been recorded in 14 countries across Africa, Asia, South America, and south-western Europe[35,38]. In countries with significant coniferous timber production, preventing the introduction of *F. circinatum* is crucial. Models of the potential spread and damage caused by *F. circinatum* suggest that currently, the pathogen may cause limited damage in pine forests and plantations in Northern Europe, but the potential distribution is expected to expand northward in all climate change scenarios[40]. Even in currently unfavorable geographic regions, *F. circinatum* can thrive in nurseries where it acts as a damping off disease and causes considerable financial consequences also in regions where field conditions are generally not considered suitable for PPC[38]. Plants infected in nurseries will be the origin of future outbreaks in the forest when planted. Once established, *F. circinatum* spreads readily by rain splash, wind and vectoring insects[10,41] but is also soil-borne[39]. Asymptomatic infection has been reported[42,43] even in non-pine[44], grass[45] and herb species[46], making visual detection impossible, emphasizing the need for reliable high-throughput diagnostic protocols. Furthermore, *Fusarium* spp. are morphologically very similar, can sometimes be difficult to distinguish by culture morphology, and therefore require more detailed molecular analysis to identify to a species level[37].

Host susceptibility to PPC varies among pine species. *Pinus radiata* is the most planted conifer globally[47] and has a large economic and societal value. The species is known to be highly susceptible to PPC and is the main host in northern Spain where PPC is established and causing significant damage[42]. *Pinus sylvestris,* a dominant tree species in northern European forests, and the most widely distributed pine species in the world[48], is also shown to be susceptible to PPC based on greenhouse and field inoculation trials on young trees of Spanish, Scottish and Czech origin[38,44,49]. *Pinus pinea* is distributed all around the Mediterranean basin including northern Spain, which could enable rapid spread of this plant disease. However, *P. pinea* has remarkable phenotypic plasticity in functional traits that may explain its relatively higher resistance to *F. circinatum*-infection[50] compared to other pine species.

The aim of this study was to develop a disease detection method based on VOC emissions from pine seedlings. By establishing a library of chemical fingerprints characterizing specific emission profiles, it should prove possible to non-destructively scan plant consignments in ports of entry or plant nurseries to detect the presence of disease, and rapidly respond with further measures to limit its establishment and potential losses. The study sought to: (1) test whether in vitro VOC signatures can distinguish between different *Fusarium* spp. (2) examine

Retention time (min)	Retention index (RI)	Tentative ID	*F. circinatum**	*F. oxysporum* f.sp. *pini**	*F. bulbicola**	*F. graminearum**
25.34	1417	Unknown sesquiterpene	1.85 ± 0.983	103 ± 24.7	ND	ND
27.09	1515	Unknown oxygenated sesquiterpene 1	ND	391 ± 138	223 ± 67.4	ND
30.24	1778	Unknown oxygenated sesquiterpene 2	7.00 ± 0.745	ND	ND	ND

Table 1. Randomforest selection of VOCs to distinguish between Fusarium species. PERMANOVA analysis with pairwise interspecific comparisons based on three VOCs, selected by Randomforest from MZMine 2-processed data, resulted in significant differences ($p = 0.006$). Tentative compound IDs for the VOCs and their respective retention indices are given, as well as average base peak area ± standard error ($n = 3$) for each VOC and species. ND indicates that no peaks above the applied threshold were detected. *All values are to be multiplied by 10^4.

whether in vitro *F. circinatum* VOCs are present in in vivo, and (3) test whether infection of *F. circinatum* on pine seedlings can be detected on the basis of VOCs prior to expression of visible symptoms of disease.

Results and discussion

Fusarium spp. cultured on defined media are readily distinguished by VOC profiles. As an initial proof-of-concept pilot study, VOC profiles of four *Fusarium* spp. (*F. circinatum*, *F. oxysporum* f.sp. *pini*, *F. bulbicola* and *F. graminearum*) grown in vitro were compared to test whether analysis of VOCs alone could distinguish closely related species. The selection of the three *Fusarium* spp. included here in addition to *F. circinatum* was based on their genetic proximity to *F. circinatum*[51,52]. The VOCs were collected using SPME, analyzed by GC–MS before the output data was processed through an objective pipeline. Several combinations consisting of 3–6 VOCs fulfilled the criteria to distinguish the four species with a significant accuracy ($p \leq 0.05$) for every pairwise interspecific comparison, explained further below. An example of a VOC combination utilizable to distinguish the *Fusarium* spp. regardless of timepoint, *i.e.* 7–21 days post inoculation (dpi), with a 0% confusion matrix error rate and $p = 0.006$, is shown in Table 1. A total of 211 different VOCs were detected from the four *Fusarium* spp. A visualized principal component analysis (PCA) of the *Fusarium* spp. separation based on the 11 VOCs identified by ten repeated Randomforest runs demonstrated the unambiguous groupings irrespective of time point (Fig. 1). A larger study with more replicates would be required to draw confident conclusions regarding the VOC emission characteristics by each species. The results presented here do point to the potential for VOCs analysis as a novel fungus identification method to replace current inadequate or challenging morphological or time-consuming DNA-based approaches that often fall short due to the high morphological and genetic similarity of these species.

The Randomforest selected VOCs observed in Table 1 could not be identified further than to chemical classes, as none of their respective MS data matched any compound in the MS databases, see methods. All three of the compounds were, however, sesquiterpenes, a chemical class previously reported to be emitted from species in the *Fusarium fujikuroi* species complex[53]. It is known that plant emitted monoterpenes such as limonene and linalool can inhibit germination of fungal spores[54,55], which makes it interesting to find that hyphae of plant pathogenic fungi emit similar compounds, such as the sesquiterpenes found here, emitted by *Fusarium* spp. There were a number of compounds found to be exclusively detected in just one of the four *Fusarium* spp. despite the close genetic proximity of the species, for example oxygenated sesquiterpene 2 exclusively emitted by *F. circinatum* (Table 1). This finding demonstrated the ease with which a VOCs-based detection method could distinguish between morphologically and genetically similar *Fusarium* spp.

VOC profiles can distinguish between _F. circinatum_-inoculated and mock-inoculated seedlings. VOCs were sampled from stem-inoculated seedlings of *P. sylvestris, P. radiata* and *P. pinea*. *Fusarium circinatum*-inoculated seedlings were compared to control (mock-inoculated) seedlings, hereafter referred to as "inoculation types", at 7, 14 and 28 dpi. The same pipeline used for the in vitro studied *Fusarium* spp. was applied to these in vivo samples, including MZMine 2, Randomforest and PERMANOVA for GC–MS data analysis, which resulted in a number of significant distinctions (Table 2). There were significant ($p \leq 0.05$) differences in VOC profiles between the two different inoculation types of *P. radiata* at all time points, including the earliest time point at 7 dpi when no symptoms were yet visible. In terms of detection tool development for biosecurity, the ability to detect disease earlier than the point of symptom appearance is an important detail. This enables identification of infected, yet apparently healthy seedlings that could otherwise slip through ports of entry and plant nurseries unnoticed, an introduction pathway that remains difficult to address. For *P. sylvestris,* significant differences were seen at 14 and 28 dpi, and for *P. pinea,* considered to have very low susceptibility to *F. circinatum*, no symptoms developed and no significant differences in VOCs emissions were observed between the inoculation types at any time point. These results are visualized by a principal component analysis (Fig. 2). None of the VOCs detected were exclusively detected in the *F. circinatum* inoculated seedlings, therefore the analysis was based on relative quantitative comparisons between samples.

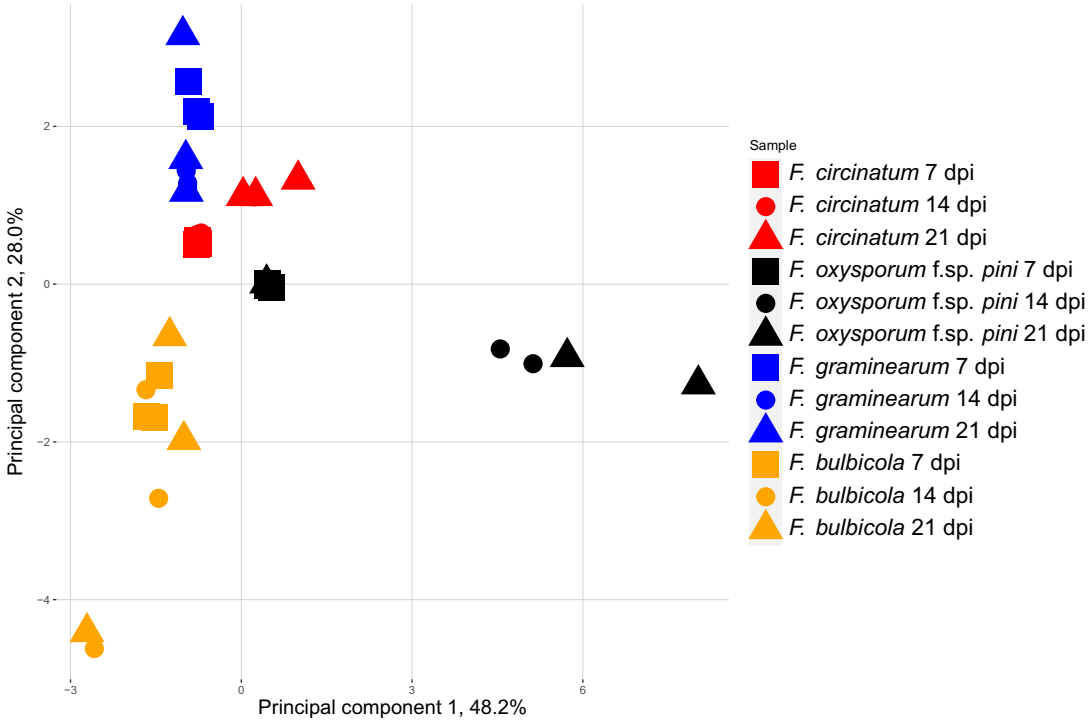

Figure 1. Principal component analysis (PCA) of VOCs emitted by four Fusarium spp. The PCA was computed based on a subset of the 11 VOCs identified by ten consecutive runs of Randomforest. n = 3, each of which were repeatedly sampled at three time points; 7, 14 and 21 days post inoculation (dpi).

Time point	*P. sylvestris*	*P. radiata*	*P. pinea*
7 dpi	0.071	**0.015**	0.537
14 dpi	**0.003**	**0.007**	0.839
28 dpi	**0.003**	**0.043**	0.465

Table 2. Summary of PERMANOVA comparison p-values of *Pinus* seedlings. P-values represent intraspecific differences between *F. circinatum*- and mock- inoculated seedlings at each time point; significant p-values ($p \leq 0.05$) in bold. VOCs were recorded at three time points; 7, 14 and 28 days post inoculation (dpi), n = 5–6.

Randomforest produced a subset of eight compounds for *P. sylvestris,* three for *P. radiata* and five for *P. pinea*, which were subsequently utilized in the statistical models (Table 3). These compounds were detected through machine learning, within the complete VOC profiles, as important because of their low error rate as indicators of the seedling inoculation type irrespective of time point (see methods). A total of 307 unique VOCs were found between the three *Pinus* spp., most of which were present in all three species. It is possible that VOCs other than the subset found here by Randomforest could strengthen the outcomes, for example the distinction between *P. sylvestris* inoculation types specifically at 7 dpi, if Randomforest had been set to examine each timepoint separately. However, the objective here was to find VOCs that allow for a robust distinction irrespective of time post infection, as a detection method must be applicable regardless of the (often unknown) infection age.

Observations of symptom development were carefully documented throughout the experiment. All *Pinus* spp. had resinous wounds at the inoculation site at 7 dpi, but were otherwise asymptomatic at this time, regardless of inoculation type. *Fusarium circinatum*-inoculated *P. sylvestris* and *P. radiata* were consistently symptomatic at 14 dpi, with light chlorosis and/or slight wilting of needles, which was described as grade 1 symptoms in the scale used by Martín-Rodrigues et al.[39]. At 28 dpi, symptoms on *P. sylvestris* and *P. radiata* had progressed to grade 3, with severe wilting (Fig. 3). *Pinus pinea* seedlings remained asymptomatic at all time points. Symptom development on *P. sylvestris* and *P. radiata* were consistent with previous reports of inoculations on 2-year-old *P. radiata*[59]. The *P. sylvestris* used in this study had similar susceptibility as *P. radiata* to *F. circinatum*, underlining the potentially serious threat posed by PPC to forests of northern Europe dominated by *P. sylvestris*.

No VOCs detected were uniquely, and consistently, emitted from *F. circinatum*-inoculated seedlings. Therefore, no single VOC detected here can independently be used as a reliable indicator of disease, which also rules out the idea of identifying a *F. circinatum*-specific VOC emitted regardless of growth medium, as could be done

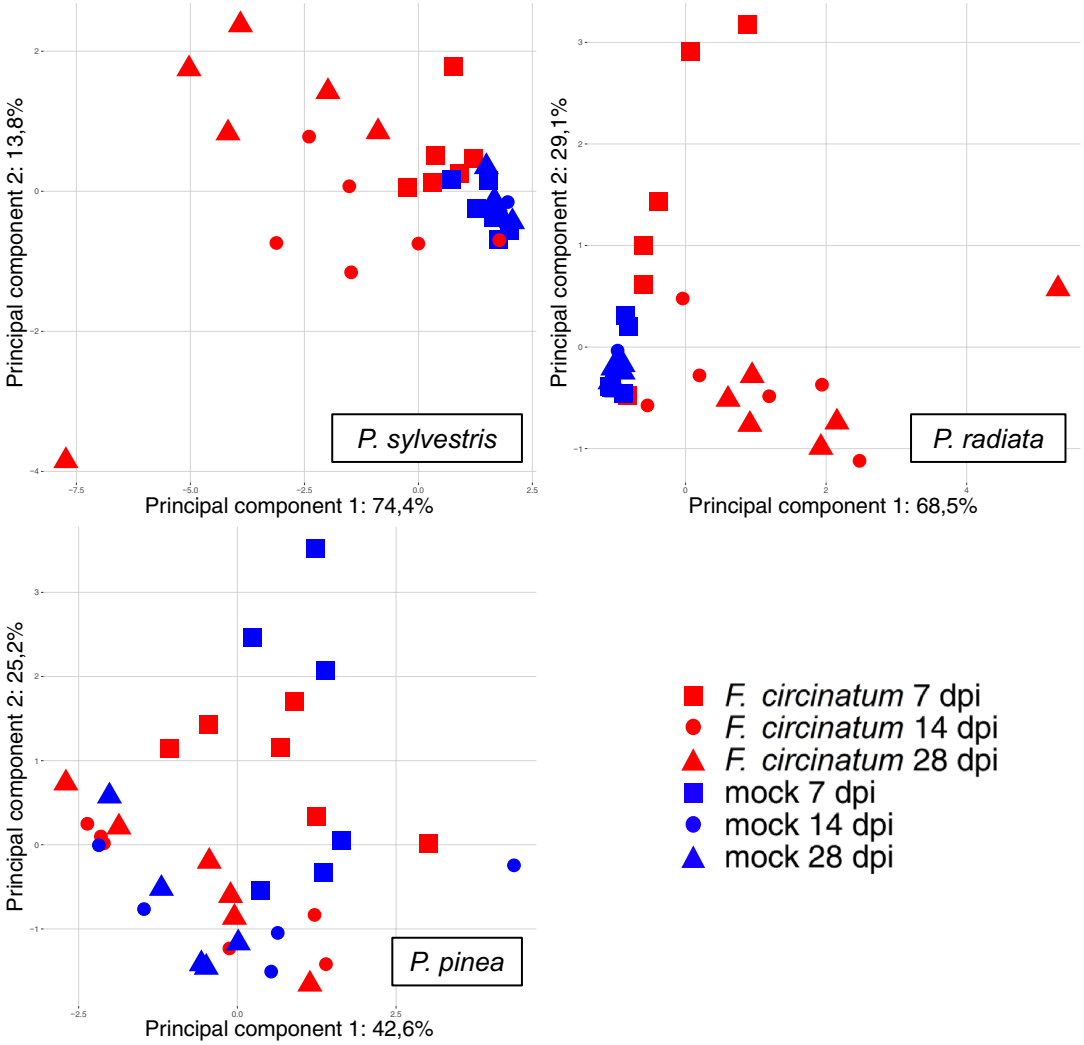

Figure 2. Principal component analysis of VOC subsets from *Pinus* spp. seedlings inoculated with *F. circinatum* or mock inoculated. n = 5–6 seedlings per inoculation type, sampled at three time points: 7, 14 and 28 days post inoculation (dpi). Percentages given on each axis of the plots show the total variance explained by that principal component.

for example in the study by Brilli et al.[31]. This means that multivariate data analysis, preferably using a machine learning-based pipeline as presented in this study, is required.

The use of a fully automatic pipeline entailing automated data analysis and machine learning instead of manual processing, beyond being immensely time saving, eliminates the risks of introducing human errors, arbitrariness and need for GC–MS expertise. Advanced competence is needed to manually process GC–MS data, identifying a few hundred VOCs per run sample, and peaks (corresponding to VOCs in the samples) often coincide, making manual peak integration impossible. By elimination of manual processing, the detection method can be performed by nonexperts, overcoming barriers of entry to use this kind of detection method. Machine learning can, in addition, allow for detection of multivariate patterns that are difficult or impossible to detect manually, increasing the detection accuracy. Its accuracy can be further improved by calibrating the models using much bigger data sets than for example the ones available in this study.

The VOCs identified as predictors of the *Pinus* seedling inoculation type were tentatively structurally identified based on retention indices and comparison with mass spectral data from libraries and previous literature (Table 3). These VOCs were predominantly terpenoids, chemicals strongly associated with VOC emission from pine trees. One example is verbenone, a monoterpene found important in the distinction of mock- and *F. circinatum*-inoculated *P. sylvestris* as well as *P. radiata*, as emission levels were higher in infected trees. Verbenone is known to be emitted from a variety of plants, and also functions as an insect pheromone with important roles, for example, as a repellent to mountain pine beetles[60]. A list of the Randomforest-selected VOCs and some of their known functions is found in the supplementary information (Table S1).

Species	Retention time	RI	RI ref	Tentative ID
P. sylvestris	12.15	958	952[1]	camphene
	12.26	963	957[2]	verbenene
	14.13	1032	1026[2]	*p*-cymene
	15.98	1098	–	propenyl toluene isomer
	17.74	1155	1143[2]	trans-verbenol
	18.78	1190	1173[2]	isopinocamphone
	19.46	1212	1212[3]	homomyrtenol
	19.85	1224	1204[2]	verbenone
P. radiata	12.26	963	957[2]	verbenene
	15.98	1098	–	propenyl toluene isomer
	19.85	1224	1204[2]	verbenone
P. pinea	11.72	941	939[2]	α-pinene
	14.86	1058	–	unknown
	16.52	1115	–	spiro[4,5]decane
	25.34	1416	–	dimethoxy-propenyl benzene isomer
	26.54	1479	–	unknown

Table 3. Tentative IDs of VOCs identified by Randomforest as suitable indicators for distinguishing between mock- and *F. circinatum*-inoculated pine seedlings. [1]:[56] [2]:[57] [3]:[58]. RI = retention index.

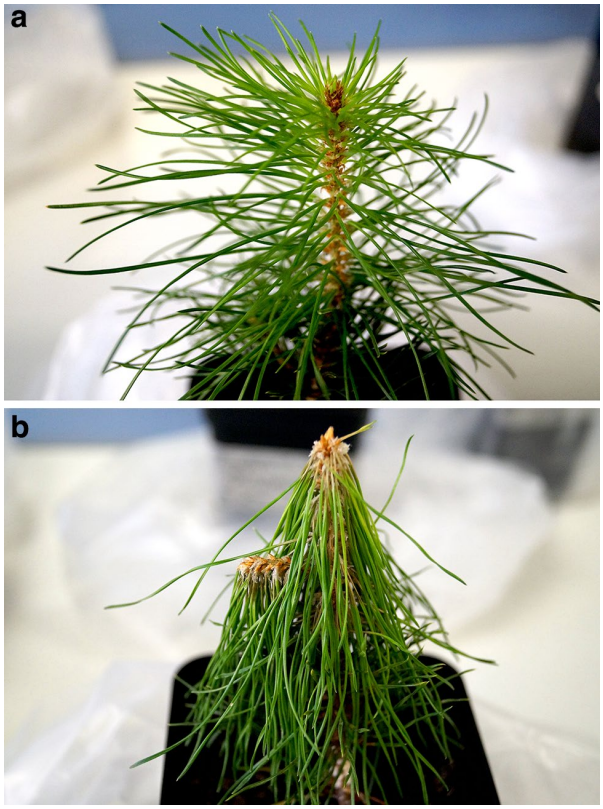

Figure 3. Symptom development in *P. sylvestris* shoots following stem inoculations. (**a**) Mock inoculated *P. sylvestris* seedling at 28 dpi, a healthy shoot with no signs of disease; (**b**) *Fusarium circinatum* inoculated *P. sylvestris* seedling at 28 dpi, displaying characteristic symptoms of shoot wilting and needle chlorosis.

Our study showed that VOCs analysis can distinguish *F. circinatum*-infected *P. radiata* seedlings before visible symptom development, suggesting the potential to scale up this detection tool for in-field use. In this study, a benchtop GC–MS instrument was used, but other options include the electronic nose (E-nose) and portable GC–MS instruments. In contrast to the E-nose that only detects specifically targeted VOCs classes, GC–MS theoretically can detect all VOCs present in a sample. Additionally, the E-nose detection limits are typically in the μg L^{-1} range, as compared to pg-ng for FID and MS[15], suggesting that the portable GC–MS may be a better option for use in an up-scaled and in-field scenario. When combined with SPME HS sampling, it has potential as an application for high-throughput detection of problems in large plant shipments. Portable GC–MS instruments are commercially available, and for example are currently employed by Homeland Security in the U.S., with similar sensitivity to a basic benchtop instrument[61]. Portable GC–MS has been used to distinguish between healthy and pest-infested milkweed (*Asclepias* spp.)[62] as well as to readily identify potential fungal biomarkers when coupled with SPME[63]. This would make an interesting alternative for testing in future work with forest pathogens, especially *F. circinatum* based on our results, for detecting the pathogen in asymptomatic seedlings in nursery consignments, but also in soil, another known pathway of introduction for this pathogen.

Comparing the VOCs profiles of pine seedlings inoculated with different pine pathogens would be an important next step to this work. Such a comparison could determine whether VOCs profiles of pine seedlings inoculated with different pathogens can be distinguished from one another (as described in horse chestnut trees by Johne et al.[29]), or whether pine seedlings' VOCs responses to fungal pathogens are non-specific, yet further investigations are warranted.

Methods

***Fusarium* spp. cultured on defined media.** For examination and comparison of VOCs produced by *Fusarium*, four *Fusarium* spp. were grown on defined Elliott's medium agar (EMA) without sterol[64]: *Fusarium circinatum*, the closely related *F. bulbicola*, the intermediately related *F. oxysporum* f.sp. *pini*[51] and the more distantly related *F. graminearum*[52]. For strain information, see Table S2. EMA was dispensed in slanted 20 mL clear glass vials (Merck KGaA, Darmstadt, Germany), capped with permeable magnetic screw caps with polytetrafluoreten/silicone 1.33 mm septa (Merck KGaA, Darmstadt, Germany). The capped vials were incubated at room temperature under natural light conditions and sampled for 24 h at days 7, 14 and 21 after sub-culture by inserting divinylbenzene/carboxen/polydimethylsiloxane SPME fibers through the septa. The SPME needle size was 24 ga, 2 cm long and coated with 30 μm (CAR/PDMS layer), 50 μm (DVB layer) (Merck KGaA, Darmstadt, Germany).

***Fusarium circinatum* inoculated *Pinus* spp.** *Fusarium circinatum* strain *FcCa6* (obtained from the laboratory of Prof. Julio Javier Díez) was stem-inoculated on 1 year-old *P. sylvestris, P. radiata* and *P. pinea* (for information on sources, see Table S3). The seedlings were obtained from Viveros y Servicios Forestales Caselas, S.L., a nursery in Mondoñedo Lugo, Spain, and transported by express courier in December 2020 to the forest pathology laboratory of the Universidad de Valladolid, Palencia, Spain. The plant material used in this study complies to relevant guidelines and all necessary permissions were in place. Seedlings were transplanted to 0.77 L pots into black peat moss previously autoclaved twice at 121 °C for 20 min. Plants were acclimated for 3 months in a climate chamber at 21.5 °C under a 16/8 h day/night regime and approximately 68% relative humidity. Throughout the acclimation and experimental period, seedlings were watered twice a week.

Stem inoculations were performed, using a method described elsewhere[59,65], by cutting a small wound on the stem, approximately 7 cm above the root collar and applying 20 μL of a potato dextrose broth (PDB) (Sharlau Microbiology, Barcelona, Spain) based spore suspension containing 10^6 *F. circinatum* spores mL^{-1}, directly to the surface of the wound. Wounds were covered with Parafilm (Bemis Company Inc., Neenah, USA) until the start of the SPME sampling. Mock inoculations were identical but without spores in the PDB. During the experiment, symptom development was observed and documented on the seedlings. To confirm that the mycelial growth seen on stems was *F. circinatum*, the mycelia were harvested and sub-cultured to EMA[64] before examination under the microscope, where coiled sterile hyphae characteristic of *F. circinatum* were seen. VOCs sampling was performed using SPME fibers for static HS sampling: each seedling, including pot, and SPME fiber was wrapped in 38 L high-density polyethylene bags (Labbox labware, Barcelona, Spain), maintained at room temperature for 24 h and thereafter the SPME samples were immediately analyzed using GC–MS.

GC–MS and data analysis. Immediately after sampling, the SPME fibers were manually injected through an ultra-inert, splitless, straight, 2 mm liner (Agilent, Santa Clara, USA) on a 6890 N GC (Agilent Technologies, Santa Clara, USA) coupled with a 5973 MS (Agilent Technologies, Santa Clara, USA). The column was a HP-5 ms ultra inert 60 m GC column, 0.25 mm, 0.25 μm, 7 inch cage (Agilent, Santa Clara, USA). A C8-C20 hexane mix (Merck KGaA, Darmstadt, Germany) was used as an assurance that there was no shift in retention time over the project time span. GC–MS was performed through MSD ChemStation version E.02.02.1431 (Agilent Technologies, Santa Clara, USA) with an initial oven temperature of 50 °C, followed by an 8 °C/min increase to 100 °C, subsequently increasing by 4 °C/min to 160 °C, a final ramp of 16 °C/min to 280 °C and hold for 2.5 min (Table S4). GC–MS data were transformed to .cdf files and processed (ADAP chromatogram builder, chromatogram deconvolution, multivariate curve resolution) and aligned (ADAP aligner) with MZMine 2 (v 2.53)[32]. The Randomforest compound selection (see below) for distinguishing between mock- or *F. circinatum*-inoculated seedlings (in vivo), or *Fusarium* species (in vitro), were tentatively identified by matching mass spectrometry data and back-calculated retention indices[66] with literature values from authentic standards found in Nist20 and Wiley12 MS databases.

Programming, machine learning and statistical tests. Randomforest and VarSelRF[67] are two packages in R[68] that were used to select a reduced model to a set of VOCs that were predictive of *Fusarium* spp. in the in vitro and inoculation type in the in vivo experiments[69]. Randomforest is used to tune and reduce the model error and VarSelRF chooses a model of VOCs with the lowest error rate. VarSelRF uses the confusion matrix testing parameter "out-of-bag" error as a criterion to remove variables (*i.e.* individual VOCs) in a backward elimination starting with the least important VOCs. The least important variables are those defined by Randomforest from the mean decrease in accuracy[70]. The model selection stops when current out-of-bag error rate becomes larger than the previous iteration. The selected subsets of *Fusarium* spp. and *Pinus* spp. VOCs were then run through Permutational Multivariate Analysis of Variance (PERMANOVA)[71], to determine relative differences in VOCs between *Fusarium* spp., or inoculation type in *Pinus* spp. Posthoc Holm tests[72] were thereafter applied. Data for PERMANOVA were Hellinger transformed. The Stats package, prcomp function was used to generate the PCA analysis and plot, scaling the input data to visually display differences among the compared groups (RStudio, version 1.1.456).

Additional information. All necessary permissions were obtained to complete this study, no ethics considerations are applicable. Supplementary data is publicly available through the Swedish National Data Service (SND), doi: https://doi.org/10.5878/hc9w-7694. The voucher specimens of the three *Pinus* species included in this study were provided by Viveros y Servicios Forestales Caselas, S.L., a nursery in Mondoñedo Lugo, Spain, but have not been deposited to any publicly available herbarium. The *Fusarium circinatum* isolate *FcCa6* used in this study was identified in previous work by Martínez-Álvarez[73], provided and maintained by the laboratory of Prof. Díez, available in lab collections in several countries but yet no public herbarium.

Data availability

The data that support the findings of this study are openly available in doi: https://doi.org/10.5878/hc9w-7694.

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Author contributions
I.N., P.S., M.C., J.J.D., B.B. and S.W. conceived, designed and supervised the project. I.N. conducted experiments and wrote the manuscript. B.B. analyzed the GC–MS data. D.L.P. performed statistical analyses. J.N.S. and T.S.G. performed the post-hoc PCR and culturomic assays. All authors commented on the final version.

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Detecting Pathogenic *Phytophthora* Species Using Volatile Organic Compounds

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Abstract: There are several highly damaging *Phytophthora* species pathogenic to forest trees, many of which have been spread beyond their native range by the international trade of live plants and infested materials. Such introductions can be reduced through the development of better tools capable of the early, rapid, and high-throughput detection of contaminated plants. This study utilized a volatilomics approach (solid-phase microextraction coupled to gas chromatography–mass spectrometry) to differentiate between several *Phytophthora* species in culture and discriminate between healthy and *Phytophthora*-inoculated European beech and pedunculate oak trees. We tentatively identified 14 compounds that could differentiate eight *Phytophthora* species from each other in vitro. All of the *Phytophthora* species examined, except *Phytophthora cambivora*, uniquely produced at least one compound not observed in the other species; however, most detected compounds were shared between multiple species. *Phytophthora polonica* had the most unique compounds and was the least similar of all the species examined. The inoculated seedlings had qualitatively different volatile profiles and could be distinguished from the healthy controls by the presence of isokaurene, anisole, and a mix of three unknown compounds. This study supports the notion that volatiles are suitable for screening plant material, detecting tree pathogens, and differentiating between healthy and diseased material.

Keywords: gas chromatography–mass spectrometry; tree disease; volatilomics; *Fagus sylvatica*; *Quercus robur*

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1. Introduction

Phytophthora is an extremely important genus of plant pathogens responsible for massive economic losses and ecological damage in agriculture, horticulture, and forestry [1,2]. Currently, approximately 200 species of *Phytophthora* are known, but it has been estimated that the total number of species globally is likely to be 600 or more [3]. The host ranges of *Phytophthora* spp. vary greatly, but as an example, *Phytophthora cinnamomi* has a host range close to 5000 species of plants, including many of importance in agriculture, forestry, and horticulture [4,5]. As tree pathogens, *Phytophthora* are most damaging as root rots and stem cankers, but they can also cause foliar blights. Infection can lead to reduced growth, plus an increased sensitivity to drought, herbivores, and other stresses [4–6], the decline and death of individual trees, and even widespread mortality in the landscape [1]. Their diverse host ranges, persistence in soil once introduced, and potential for highly damaging outbreaks have made *Phytophthora* species some of the most important plant pathogens regarding plant health and management practices.

Many of the most problematic *Phytophthora* spp. are so because they are invasive alien species (IASs) and the native flora lack sufficient and evolved defenses against them [7]. Economic and ecological losses stemming from the introduction of IASs have been recognized as an increasingly difficult challenge worldwide in agriculture, horticulture, and forestry [8,9]. The spread of plant pathogens that can potentially become invasive is enabled foremost by the international plant trade, which has increased substantially in recent decades [10], with no signs of slowing down [11]. *Phytophthora* spp. are common IASs found in the plant trade [12], often in the soil and compost used in the production of hardy woody nursery stock [13], making them difficult to detect by traditional methods in shipments of asymptomatic potted plants. Moreover, in nurseries, large numbers of plants are raised in restricted spaces, promoting the survival and proliferation of *Phytophthora* spp. due to the high host plant availability, regular irrigation, and favorable temperatures, with the consequence of the pathogens accompanying the plants to their final planting positions. Numerous introductions of *Phytophthora* spp. into forest environments have occurred over many years in this way [12]. This problem can be reduced by the implementation of suitable management and prevention practices and novel detection techniques in trade and at ports of entries [14,15]. Proactive strategies and the development of advanced pathogen detection methods could greatly improve our capacity to mitigate the infiltration of invasive alien pathogens in international and national trade and limit their introduction to new areas.

Nucleic-acid-based techniques for detecting plant pathogens are improving rapidly [16]. For example, loop-mediated isothermal amplification and nanopore sequencing can be used on-site for point-of-need detection [17,18], while the long-read sequencing capabilities of PacBio and Oxford Nanopore Technologies grant a better taxonomic resolution for regions like the fungal internal transcribed spacer [19]. Despite these improvements, molecular methods still have limited utility in plant biosecurity due to (i) the extensive volume of plants traded internationally, which overwhelms the staffing levels at entry ports, limiting the ability to conduct comprehensive inspections on a significant portion of the units in transit [15,20], (ii) limitations in detection, in that DNA analyses require the destructive sampling of the correct tissues on the plants or the infested compost, and (iii) the need for molecular and bioinformatics proficiencies in ensuring the correct sample processing. Hence, novel non-DNA-based approaches better tailored for early and high-throughput detection are needed. Such approaches could also serve as initial screening tools when combined with more targeted molecular techniques.

Plants release a multitude of volatile organic compounds (VOCs) into their immediate environment, which fulfill critical roles in growth, intra- and interspecific communication, defense, and survival [21]. The composition of emitted VOCs, akin to distinct chemical fingerprints, dynamically varies among plant species and may differ in each plant–pathogen interaction, offering a potential utility for VOCs as indicators of plant health [21,22]. For example, VOCs produced by plant pathogens are already targeted for the detection of foodstuff spoilage in agriculture [22,23]. In forestry, VOCs-based detection methods are less researched and have not yet been implemented commercially. Recently, Nordström et al. [24] successfully distinguished *Fusarium circinatum*-infected *Pinus* spp. seedlings from healthy ones, and this study also revealed that each included *Fusarium* spp. showed discernible VOC blends, even when cultivated on the same substrate. Vuorinen et al. [25] pointed to the potential of VOCs as pathogen-specific disease indicators, as *Betula* spp. trees exposed to pathogens could be distinguished from those affected by herbivores. In addition, Johne et al. [26] could distinguish between pathogenic fungi in *Aesculus* spp. in infected oak acorns, and Borowik et al. [27] were able to distinguish between *Phytophthora plurivora* and *Pythium intermedium* using a VOCs-based detection method.

This project was devised as two separate but complementary experiments. The first experiment examined the in vitro VOCs of multiple *Phytophthora* spp., with the aim of determining whether an analysis of VOCs can be used to distinguish species and generate potential VOC biomarkers for the tested species. The second experiment examined the in vivo VOCs of stem-inoculated trees to ascertain whether infected trees could be

distinguished from healthy controls. For this work, we used solid-phase microextraction (SPME) fibers in conjunction with gas chromatography–mass spectrometry (GC-MS) to examine the VOCs from eight *Phytophthora* species when grown in vitro, and differences in *P. cinnamomi*- and *P. plurivora*-infected pedunculate oak (*Quercus robur*) and European beech (*Fagus sylvatica*) were compared to mock-inoculated controls (MIC). Volatilomics using SPME is a versatile technique commonly used for the static capture of VOCs in biological systems, because it is an economical, simple, and non-destructive sampling strategy that can capture a large fraction of the full volatilome [28]. Combining SPME and GC-MS is, therefore, useful and germane in a screening study like this, where the objective is to discover biomarkers of disease that can be used in targeted methods for disease detection and diagnosis in the future.

2. Results

2.1. In Vitro Study

The objective of the in vitro study was to discover *Phytophthora*-related volatiles that were qualitatively different from the media controls. In total, we found 58 compounds from the *Phytophthora* species (isolates listed in Table S1) that were not in the media-only control vials; a list of these compounds is presented in Table 1. There was a similar number of *Phytophthora* compounds detected at both collection time points, 14 days post-inoculation (dpi) and 30 dpi. In total, 43 compounds were detected at 14 dpi and 46 compounds at 30 dpi. Of the 58 total compounds, 31 were observed at both time points, while 12 were only detected at 14 dpi and 15 were only detected at 30 dpi. There was considerable variability in the number of compounds observed between the *Phytophthora* species examined (Table 1). *Phytophthora gonapodyides* and *P. polonica* had the most compounds detected with 25 each, *P. cambivora* was next with 22, followed by *P. multivora* with 18. Meanwhile, nine compounds were detected from *P. plurivora*, eight from *P. cinnamomi*, five from *P. citricola*, and only three from *P. syringae*. *Phytophthora plurivora* had all nine of its detected compounds occurring at both the 14 and 30 dpi time points. *Phytophthora cinnamomi* had all but one of its eight compounds occurring at both time points. *Phytophthora cambivora*, *P. gonapodyides*, and *P. polonica* had the most differences in the number of compounds between time points. In *Phytophthora cambivora* and *P. gonapodyides*, 13 and 17 compounds, respectively, were uniquely present at the 30 dpi time point, while *P. polonica* had 14 compounds present only at the 14 dpi time point.

All *Phytophthora* species, except *P. cambivora*, had at least one exclusive VOC. *Phytophthora polonica* had the highest number of compounds only found in a single species, with 12 compounds, *P. multivora* had 7, *P. gonapodyides* had 6, and *P. cinnamomi* had 2, while *P. citricola*, *P. plurivora*, and *P. syringae* each had 1. Since most of the examined species had only a few unique compounds (many of which were specific to a certain time point), multivariate analyses were run on the full compound list in Table 1. The PCA demonstrated that some *Phytophthora* species can be distinguished based on VOCs (Figure 1). At 30 dpi, *P. cambivora* and *P. gonapodyides* were well separated from the other species via PC1, but did not separate well from each other. *Phytophthora polonica* was separated from the other species at the 14 dpi time point, predominantly via PC2. The remaining species and time points had a poor resolution, with only *P. multivora* at 30 dpi showing some separation. The top five loadings for PC1 and PC2 are listed in Table 2.

A cluster analysis largely corroborated the PCA, as *P. polonica* (at 14 dpi), *P. gonapodyides*, *P. cambivora*, and *P. multivora* (all at 30 dpi) tended to form distinct clusters with greater separation from the other species based on node height (Figure 2). The other species had shorter branch lengths and lower node branching points, indicating that they were more similar. For all species, except *P. citricola*, replicates did generally cluster by species and sampling time.

Table 1. Occurrence of volatiles, denoted by x, after 14 and 30 days of growth for the eight *Phytophthora* species when grown in vitro.

Peak No.	MS Library Match	RT [1] (min)	KI (KI lit.)	*P. cambivora*		*P. cinnamomi*		*P. citricola*		*P. gonapodyides*		*P. multivora*		*P. plurivora*		*P. polonica*		*P. syringae*	
				D14	D30	D14	D30	D14	D30	D14	D30	D14	D30	D14	D30	D14	D30	D14	D30
1	2,3-Butanediol	6.19	777 (785) [1]									x	x						
2	Hexanal	6.53	803 (801) [2]		x	x	x				x					x	x		
3	2-Furanmethanol	7.48	856 (867) [1]															x	
4	Unknown 1	9.53	957														x		
5	1-Heptanol	9.83	971 (959) [2]									x	x						
6	1-Octen-3-ol	10.03	980 (980) [3]						x							x	x		
7	Unknown 2	10.45	998								x								
8	2,4-Heptadienal #	10.73	1013 (1013) [2a]		x						x			x	x				
9	Unknown 3	11.23	1039													x			
10	Unknown 4	11.44	1050													x			
11	Unknown 5	11.52	1054	x										x	x				
12	Unknown 6	11.94	1074		x						x								
13	Unknown 7	12.16	1084													x			
14	3,5-Octadien-2-one #	12.4	1095 (1098) [4]		x						x								
15	2-Nonanol	12.5	1100 (1097) [2]									x		x	x				
16	3-Nonen-1-ol #	13.57	1157 (1157) [2b]													x			
17	2,6-Nonadienal #	13.58	1158 (1154) [2c]		x						x								
18	4-Ethylphenol	13.8	1169 (1178) [5]											x	x				
19	Unknown 8	13.81	1169													x			
20	1-Nonanol	13.86	1172 (1165) [2]		x						x		x			x			
21	Unknown 9	14.34	1195		x						x					x			
22	2,4-Nonadienal #	14.74	1218 (1212) [2a]		x						x					x			
23	Unknown 10	15.06	1236											x	x				
24	4-Decen-1-ol #	15.48	1259 (1259) [2d]													x			
25	Unknown 11	15.66	1269													x			
26	1-Decanol	15.71	1272 (1266) [2]	x	x						x	x	x	x	x	x			
27	6-Undecen-2-one #	15.86	1279 (N/A)			x	x		x								x	x	x
28	4-Ethylguaiacol	15.98	1286 (1282) [6]	x		x	x	x				x		x	x	x	x		
29	2,4-Decadienal (*E*,*Z*)- *	16.17	1296 (1292) [2]		x						x		x			x	x		
30	2,4-Decadienal (*E*,*E*)- *	16.58	1320 (1319) [2]		x						x					x	x		
31	3-Undecen-2-one #	16.97	1344 (1344) [7a]	x	x	x	x			x	x	x	x	x	x	x	x		
32	Methyl 2,4,6-trimethyl benzoate	17.15	1354 (1349) [8]			x	x												
33	Decanoic acid	17.28	1362 (1366) [2]	x	x					x	x			x	x				
34	2-Undecenal #	17.36	1367 (1366) [7b]													x	x		
35	2,4-Undecadien-1-ol #	17.4	1369 (N/A)													x			
36	Unknown 12	17.75	1389		x	x	x			x						x			
37	Unknown 13	18.17	1414							x	x								
38	Unknown 14	18.37	1428							x									
39	1-Phenyl-2-hexanone	18.7	1449 (N/A)									x	x						
40	2,6-Dodecadienal #	18.77	1454 (1445) [2c]								x								

Table 1. *Cont.*

Peak No.	MS Library Match	RT [1] (min)	KI (KI lit.)	*P. cambivora*		*P. cinnamomi*		*P. citricola*		*P. gonapodyides*		*P. multivora*		*P. plurivora*		*P. polonica*		*P. syringae*	
				D14	D30	D14	D30	D14	D30	D14	D30	D14	D30	D14	D30	D14	D30	D14	D30
41	Unknown 15	19.25	1483					x	x									x	
42	Unknown 16	19.29	1486	x						x		x	x						
43	2-Tridecanol	19.56	1503 (1510) [9]										x						
44	Aristolochene	19.62	1507 (1487) [10]			x	x												
45	Unknown 17	19.84	1522		x						x								
46	Unknown 18	20.24	1549					x											
47	Dodecanoic acid	20.43	1562 (1565) [2]		x						x		x						
48	1-Tridecanol	20.67	1578 (1570) [2]										x						
49	Unknown 19	20.82	1587													x			
50	Unknown 20	20.92	1594	x								x	x						
51	Unknown 21	21.33	1622														x		
52	1-Tetradecanol	22.17	1681 (1671) [2]	x								x	x						
53	6-Pentadecen-2-one [#]	22.21	1684 (1667) [2b]										x						
54	γ-Dodecalactone	22.31	1691 (1676) [2]			x				x	x		x			x	x		
55	δ-Dodecalactone	22.76	1724 (1704) [2]	x						x	x								
56	1-Hexadecanol	24.86	1845 (1874) [2]										x						
57	Unknown 22	25.55	1874								x								
58	Unknown 23	25.62	1876								x								

RT = retention time; KI = Kovâts index; lit. = KI from literature that used authentic standards for KI calculations. D14 = 14 days post-inoculation; D30 = 30 days post-inoculation. x = present in the species at that given time point, absent in all controls. * = suggested isomer based on KI values and MS match. [#] = cis-trans isomerism cannot be confirmed. [1] Ames et al. [29]; [2] Adams [30] [a = (2*E*, 4*E*)-isomer; b = (*Z*)-isomer; c = (2*E*, 6*Z*)-isomer]; d = (*E*)-isomer KI 1259 or (*Z*)-isomer KI 1262; [3] Beaulieu et al. [31]; [4] Lozano et al. [32]; [5] Martí et al. [33]; [6] Steinhaus and Schieberle [34]; [7] Lazarević et al. [35] [a =isomer unspecified; b = (2*E*)-isomer]; [8] Rostad and Pereira [36]; [9] Ohnishi and Shibamoto [37]; [10] Retta et al. [38].

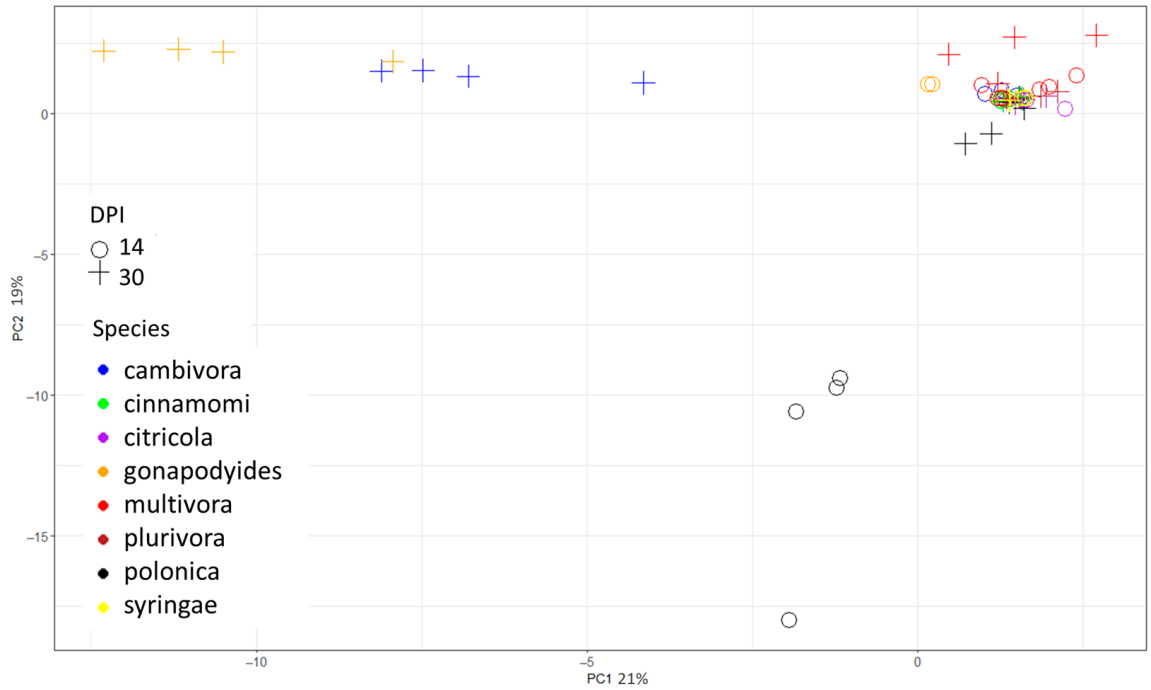

Figure 1. Principal component analysis (PCA) components plot of volatile compounds detected at 14 and 30 days post-inoculation (DPI) from eight *Phytophthora* species grown in vitro. N = 4 for each species by time point.

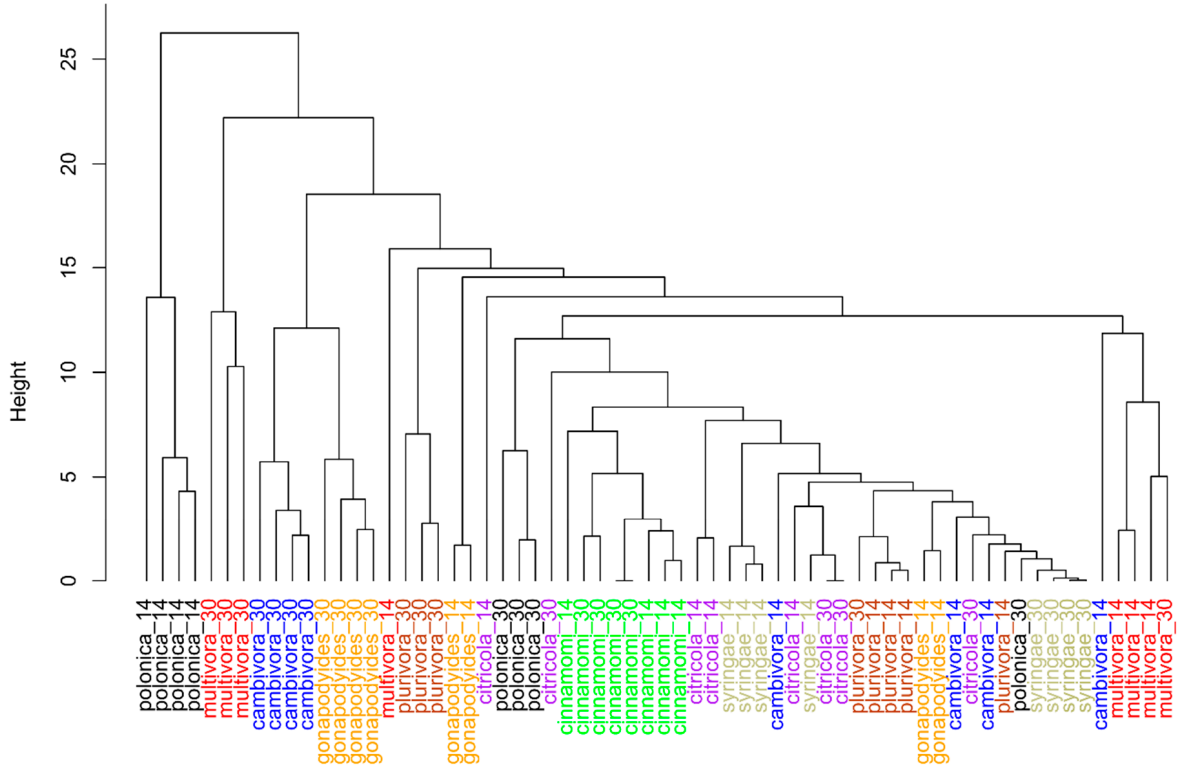

Figure 2. Scaled hierarchical cluster analysis of *Phytophthora* species based on the volatile compounds detected at 14 and 30 days post-inoculation. Species names are abbreviated to the specific epithet and are followed by the numeral for the sampling date post inoculation.

Table 2. The five most important compounds for distinguishing *Phytophthora* species from each other based on principal components (PC) 1 and 2 and the random forest (RF) analysis in the full set of in vitro produced compounds and in which species they were detected.

Peak No.	Tentative ID	Selection Criteria	Species Detected in
2	Hexanal	PC1	camb, cinn, gona, polo
6	1-Octen-3-ol	RF	citr, polo
10	Unknown 4	PC2	polo
13	Unknown 7	PC2	polo
16	3-Nonen-1-ol	PC2	polo
17	2,6-Nonadienal	PC1	camb, gona
18	4-Ethylphenol	RF	plur
22	2,4-Nonadienal	PC1	camb, gona, polo
25	Unknown 11	PC2	polo
31	3-Undecen-2-one	PC1, RF	camb, cinn, gona, mult, plur, polo
33	Decanoic acid	RF	camb, gona, plur
34	2-Undecenal	PC2	polo
44	Aristolochene	RF	cinn
45	Unknown 17	PC1	camb, gona

Phytophthora species abbreviations: camb = *P. cambivora*; cinn = *P. cinnamomi*; gona = *P. gonapodyides*; mult = *P. multivora*; plur = *P. plurivora*; polo = *P. polonica*.

The five most important compounds for discriminating between *Phytophthora* species, according to the random forest analysis based on mean Gini scores, are listed in Table 2 (see Table S2 for the full random forest analysis results and Figure S1 for mass spectra for unknown compounds in Table 2) and were tentatively identified as 1-octen-3-ol, 4-ethylphenol, 3-undecen-2-one, decanoic acid, and α-selinene.

2.2. In Vivo Study

All *Phytophthora*-inoculated trees developed lesions that were significantly larger than those on the MIC trees (Figure 3; see Tables S3 and S4 for statistical analyses). Across both tree species, five compounds in total were detected in the inoculated trees that were not present in the MIC trees (Table 3). Two of these compounds, tentatively identified as anisole and isokaurene, occurred only in the beech trees. Anisole was detected in beech trees infected with either *P. cinnamomi* or *P. plurivora*, but only at 21 dpi. Isokaurene and an unknown compound were detected only in *P. plurivora*-inoculated beech trees at 9 dpi.

Table 3. Tentatively identified in vivo compounds uniquely present in *Phytophthora*-inoculated trees.

Tree	Pathogen	Compound	MF	RT (min)	KI (KI lit.)	9 dpi	21 dpi
Quercus robur	*P. cinnamomi*	Unknown 1	$C_{14}H_{20}O_2$	25.10	1445	x	x
		Unknown 3	$C_{15}H_{24}$	30.25	1661		x
	P. plurivora	Unknown 3	$C_{15}H_{24}$	30.25	1661		x
Fagus sylvatica	*P. cinnamomi*	Anisole	C_7H_8O	10.00	922 (913) [1]		x
	P. plurivora	Anisole	C_7H_8O	10.00	922 (913) [1]		x
		Unknown 2	$C_{15}H_{24}$	29.48	1627	x	
		Isokaurene	$C_{20}H_{32}$	38.21	1964 (1988) [2]	x	

MF = suggested molecular formula; RT = retention time; dpi = days post-infection; KI = Kovâts index; lit. = KI from literature that used authentic standards for KI determinations. x = present in inoculated trees, not detected in controls. [1] Adams [30]; [2] Skaltsa et al. [39].

In oak trees, an unidentified sesquiterpene was detected at 21 dpi in trees inoculated with either *Phytophthora* species. An unknown compound was also detected at both 9 and 21 dpi, but only in trees inoculated with *P. cinnamomi*.

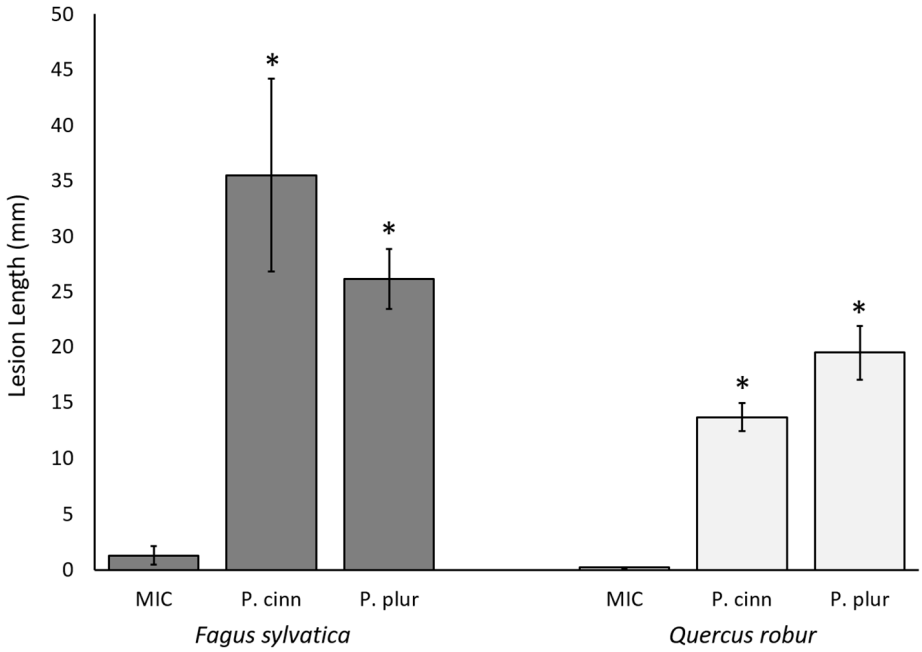

Figure 3. Average stem lesion lengths on *Fagus sylvatica* and *Quercus robur* saplings artificially inoculated with *Phytophthora cinnamomi* (P. cinn) or *Phytophthora plurivora* (P. plur) compared to mock-inoculated controls (MIC). Lesion lengths were recorded 50 days post inoculation. N = 3 for each bar; error bars are ± SD. Asterisks denote significant differences in lesion length compared to the MIC trees of the same species as determined by Dunnett's test at the $\alpha = 0.05$ level.

3. Discussion

This work reports diagnostic volatiles from several known *Phytophthora* pathogens of trees in urban and forest landscapes. Many of these pathogens are introduced to new locations via the global trade of live plants, and due to their cryptic nature, are difficult to detect. Discerning VOCs indicative of the presence of *Phytophthora* species may allow for fast and in vivo detection in traded plants. While the in vitro VOC profiles from most of the *Phytophthora* species in our analysis were similar, some species were still easily discernable, and all but *P. cambivora* produced at least one volatile compound that was not present in the other species. Such qualitative differences between species are desirable, because unique compounds could serve as biomarkers of disease and indicate which *Phytophthora* species are present in an unknown sample. These differences would also be useful for chemotaxonomy, particularly for discriminating between closely related species [40,41] and the species complexes that are common in the genus *Phytophthora* [42]. Obtaining a richer blend of in vitro volatiles for biomarker generation could be achieved by using different media with more complex substrates for metabolism [43], something observed by Qiu et al. [44] with *P. cinnamomi*. EMA is a basic medium with only one carbon source and one nitrogen source, not including the amendment β-sitosterol, so there may be a limited capacity for variable VOC production. Future studies comparing species should consider using a blend of nutrients and media constituents, potentially derived from host material to maximize variation.

We hypothesized that in vitro compounds could be useful as biomarkers for detecting infected plants, but none of the in vitro volatiles were observed to differ qualitatively between the infected and mock-inoculated control (MIC) plants. In fact, only five compounds were observed in the *Phytophthora*-infected trees that were not present in the MIC trees. Of these five compounds, two were tentatively identified, anisole and isokaurene; both occurred only in beech. Anisole was detected in beech trees inoculated with *P. cinnamomi* and *P. plurivora*, but other studies examining European beech VOCs have not reported anisole [45,46], including a study looking at VOCs from trees infested with aphids [47]. If

anisole is produced only during certain stress events, it may be a useful marker of *Phytophthora* infection in beech trees. Anisole was reported in the roots of hybrid oak (*Quercus petraea* × *Q. robur*) after *Melolontha hippocastani* feeding [48], indicating that damage-induced anisole production might be tissue-specific, pest-specific, or both, since it was not seen after stem infection in this study. It is also possible that anisole was produced by the *Phytophthora* species. Anisole is known to be produced by at least one *Penicillium* sp. [49], but its occurrence in *Phytophthora* is unknown. Nonetheless, its occurrence only in the infected beech makes it a potential target for disease diagnosis. Isokaurene has not previously been reported as occurring in European beech either, but it has been induced in maize tissue when infected by different fungi [50]. Isokaurene is a diterpene, and thus, considerably less volatile than most other compounds in this study, so passive sampling methods such as SPME, especially when short sampling times are applied, may not consistently be able to detect it. Isokaurene does, however, have a distinct mass spectrum, meaning it can be unambiguously identified in a sample should it be captured, making it an excellent biomarker in that regard. The other beech-specific volatile was an unknown sesquiterpene, which was only present at the 9 dpi time point. Sesquiterpenes are generally difficult to identify due to their ambiguous mass spectra, and if this compound is only ephemerally present in the early stages of disease, it may not be a suitable biomarker of disease, while if it is consistently present at later time points not examined here, it may still be of value as a biomarker.

In the infected oak trees, two unknowns were detected, an unknown sesquiterpene and an unknown compound with the suggested molecular formula $C_{14}H_{20}O_2$ (Table 3). Of all the in vivo compounds detected for both tree species, the latter unknown compound was the only one present at both sampling time points, and was specific to the *P. cinnamomi*-inoculated trees. The specificity of this unknown to the *P. cinnamomi* treatment and its consistency at both time points make it an ideal candidate for biomarker selection and worthy of further structural elucidation.

In this study, we chose to analyze only compounds that were qualitatively different from the controls in order to increase the likelihood of identifying a viable biomarker that could later be used in targeted and more commercial approaches, such as e-nose devices or ion-mobility spectrometry. In the quest for robust solutions to the burgeoning challenges posed by IASs, and specifically *Phytophthora* spp., finding such "silver bullets" of qualitative differences would present an opportunity for them to be exploited by future VOCs-based tree disease detection methods, marking a new era in plant biosecurity and ecosystem protection. Plants produce and alter their volatile profiles in response to a plethora of different stimuli. Many of these VOCs are shared between different stimuli, are transient, and differ quantitatively depending on the intensity of the stimuli [21,51,52]. Therefore, using compounds that differ quantitatively to differentiate between healthy and diseased plants may lead to erroneous classifications when environmental conditions and other biotic stressors are variable and sampling methods are inconsistent. Brilli et al. [53] successfully used a targeted approach, where plane trees infected with *Ceratocystis platani* were readily distinguishable from healthy controls using a few compounds that were uniquely present in the infected trees. We similarly saw disease-exclusive compounds, but unlike Brilli et al. [53], our unique compounds were likely not from the pathogens themselves. A targeted method may be of limited value in systems with no prominent pathogen-derived VOCs or in pathosystems that do not have any qualitative differences. For example, pine species inoculated with *Fusarium circinatum* could be distinguished from their healthy control plants using SPME-collected VOCs, despite there being no qualitative differences in volatiles between treatments [24]. Whether these quantitative differences are still present under non-laboratory conditions is unknown. Other studies were able to distinguish different disease and insect damage treatments based on differences in volatiles in a variety of tree species [54–57].

Different tree organs can have different VOC profiles [58,59]. Since some pathogens only attack certain plant organs and substructures with different chemical compositions,

volatile profiles associated with damage to a given structure may be sufficiently different for disease diagnosis. Tissue-targeted analyses should increase the sensitivity and specificity of VOCs-based detection methods [60]. Our in vivo sampling method sampled all of the above-ground tissue, but if we had excluded the leaves and only collected VOCs around the inoculation sites, we may have obtained more disease-associated volatiles, and perhaps even some pathogen-derived VOCs. Future studies should consider using a more targeted sampling method that is focused on symptomatic tissues or organs of interest for a certain pathosystem. The extent to which different pests attacking the same tissues can be differentiated is less clear, but some studies have shown that different pests attacking the same tissues emit different VOC profiles [25,26,61–63]. In this study, trees infected with *P. cinnamomi* were discernable by VOCs from trees infected with *P. plurivora*, for both oak and beech, even without tissue-targeted sampling. These results further support the contention that pathogens of the same tissue can be differentiated in planta by using volatiles.

Although none of the in vitro *Phytophthora* compounds were found in the in vivo study, some have been reported in other *Phytophthora* pathosystems. For example, 1-octen-3-ol, which was found in *P. polonica* and *P. cambivora* and was an important determinant of *Phytophthora* species from the random forest analysis, was the only compound found at higher levels in the volatiles from solvent extracts of *Phytophthora ramorum*-inoculated *Rhododendron* plants compared to mock-inoculated controls [64]. The C-8 alcohol 1-octen-3-ol is one of the most common fungal volatiles [65]. Its occurrence in oomycetes is less reported, but it was produced by *P. cinnamomi* in culture [66,67].

Hexanal is another common volatile that has been recorded in a number of microbial volatile studies. Interestingly, in Qiu et al. [44], hexanal was observed only in the blank media controls (V8 agar and potato dextrose agar), but not in *P. cinnamomi* colonized media. We observed the opposite, where hexanal was produced by *P. cinnamomi* but was not observed in the control EMA. Hexanal was also detected in *P. cambivora*, *P. gonapodyides*, and *P. polonica* and was identified as an important discriminating compound by PCA. In the in vitro study, this compound was only detected as a minor peak. As hexanal is also prevalent in the environment, its value as a biomarker of disease is limited in practice. Furthermore, our results are in agreement with Qiu et al. [44], in that *Phytophthora cinnamomi* did not produce 4-ethylphenol in culture. However, Qiu et al. [44] did detect 4-ethylphenol from *P. cinnamomi*-infected plants and infested soil, whereas we did not. We did, however, detect 4-ethylphenol from *P. plurivora* cultures in vitro, where it was an important compound for discriminating between species according to the random forest analysis. *Phytophthora plurivora* and *P. cinnamomi* volatiles were also reported by Loulier et al. [66], but none of the compounds they detected for either *P. cinnamomi* or *P. plurivora* were observed by us for the same species. Their methods used a different in vitro growth medium and SPME fiber chemistries, so these differences are not completely surprising, but do demonstrate that volatiles may vary considerably between different setups.

In a study examining the effects of *Phytophthora cactorum* and *P. plurivora* infections on the physiology of hybrid poplar, Ďurkovič et al. [67] found that infected trees emitted germacrene D and α-cubebene from detached leaves, while control trees did not. In this study, neither of these compounds were found solely in the *Phytophthora*-infected trees, but both compounds are known to be emitted by pedunculate oak [68,69] and germacrene D by European beech [70]. Neither germacrene D nor α-cubebene were evident in this study when manually searching for them in the chromatograms of the MIC trees of either species. Since these sesquiterpenes were not found and are known to be emitted by the host trees, they are not considered as suitable biomarkers by our a priori criterion regarding qualitative differences. Furthermore, neither germacrene D nor α-cubebene match either of the unknown $C_{15}H_{24}$ compounds in Table 2, despite all being sesquiterpenes (based on tentative molecular formulas and fragmentations), because their respective retention indices are considerably different from those reported for the unknowns with similar mass spectra [71,72].

4. Materials and Methods

4.1. In Vitro Phytophthora VOC Study

Eight *Phytophthora* species were chosen for the in vitro volatile analysis: *Phytophthora cambivora*, *Phytophthora cinnamomi*, *Phytophthora citricola*, *Phytophthora gonapodyides*, *Phytophthora multivora*, *Phytophthora plurivora*, *Phytophthora polonica*, and *Phytophthora syringae* (see Table S1 for isolate information). All *Phytophthora* isolates were cultivated on Elliott's medium agar—EMA [73]. Three-millimeter plugs containing hyphae were excised from the margins of actively growing cultures using a sterilized cork borer and transferred to the center of agar slants of EMA amended with β-sitosterol. To make the EMA slants, concentrated β-sitosterol in ethyl acetate (30 mg mL^{-1}) was added to cooling but not solidified EMA to reach a final concentration of 10 mg L^{-1} [73], then 5 mL of the amended medium was pipetted into 20 mL glass headspace vials (SU860097, Merck, Darmstadt, Germany). The slants were allowed to cool and solidify before inoculation. The inoculated slants were sealed with headspace vial caps (SU860101, Merck). EMA slants inoculated with sterile EMA plugs were used as non-inoculated controls for identifying background non-Phytophthora-derived volatiles. The vials were incubated at room temperature for 14 or 30 days prior to VOC sampling; no cultures or control vials were sampled at more than one time point. Four replicate vials for each species by time point were used. The sampling time points were chosen based largely on the growth rates of the different *Phytophthora* species and preliminary tests. Most species examined had nearly overgrown the agar slant by day 14, so it was used as an active-growth time point, while day 30 represented a more stagnate-growth metabolism. Volatiles from earlier time points were found to be very similar to those at 14 dpi in preliminary tests, so earlier time points were not used.

Culture volatiles were sampled using divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 24 ga SPME fibers (57348-U, Merck) with a 50 μm DVB layer and a 30 μm CAR and PDMS layer. All fibers were conditioned at 260 °C for 5 min before sampling. The fibers were inserted into the vials through the pre-pierced cap septa, and the vials were placed in an incubator maintained at 35 °C and sampled for 24 h. The fibers were manually injected into a 6890 N gas chromatograph (GC) coupled with a 5975 inert mass selective detector (MS, Agilent Technologies, Santa Clara, USA). The injection inlet conditions were splitless, with a temperature of 260 °C and a purge flow of 30 mL min^{-1} for 0.5 min, with an ultra-inert, straight, 2 mm liner. The column was a HP-5 ms ultra inert 60 m, 0.25 mm, 0.25 μm, 7-inch cage (19091S-436UI, Agilent Technologies) with an initial oven temperature of 50 °C. The initial oven temperature of 50 °C was held for 2 min, followed by an 8 °C min^{-1} ramp to 280 °C and a 2.5 min hold. The transfer line temperature was 150 °C. The MS was operated in positive ion mode with a scanning range of 29–500 m/z and an ion source temperature of 230 °C run at 70 eV. The quadrupole temperature was 150 °C and the detector voltage was 1906 V. An alkane standard mixture C8–C20 (04070, Merck) was also sampled by SPME for 2 h at room temperature in the same headspace vials and injected into the GC-MS using the same parameters for calculating the retention indices.

4.2. In Vivo VOC Study

Phytophthora cinnamomi and *P. plurivora* were selected for the in vivo inoculation experiments on pedunculate oak (*Quercus robur*) and European beech (*Fagus sylvatica*). The trees were approximately 2 years old, potted in 3 L pots, and maintained in greenhouse conditions with a 16 h light cycle, an average day temperature of 25 °C, and regular watering to runoff for 3 weeks prior to experimentation. The trees were artificially inoculated on either side of the main stem by removing a 1 cm × 0.5 cm piece of bark to expose the xylem. The inoculation points were approximately 5 and 10 cm above the soil line. The excised tissue was replaced by an EMA plug of the same size, taken from the margin of actively growing *Phytophthora* cultures. The mock-inoculated control (MIC) trees were treated with a sterile plug of EMA instead of colonized agar. The inoculation sites were sealed with Parafilm to

limit desiccation and contamination. Every treatment and control was run in triplicate for a total of 18 trees.

Volatiles from the inoculated and control trees were analyzed at 9 and 21 days post-inoculation (dpi). These time points were chosen based on lesion development in a pilot inoculation test. Lesions were still small at around 9 dpi, but had grown considerably by 21 dpi. The selected time points attempted to capture an early stage and later stage of symptom development. Parafilm was removed 1 day prior to the day 9 sampling. In preparation for the VOC sampling, a cut Sterilin autoclave bag (11329103, Thermo Fisher Scientific Inc., Waltham, USA) was placed around the stem and soil line to cover the potting mix to limit soil volatiles. The volatiles were collected using the same SPME fibers detailed above, placed in empty, uncapped headspace vials, and secured to the tree near the inoculation point. Immediately after, conditioned fibers were placed in the vials, and the above-ground parts of the trees were encased in another autoclave bag, which was taped shut at the base of the stem and above the cut autoclave bag. The fibers were left for 48 h before being removed and analyzed by GC-MS with the same inlet settings, liner, and column as above. An initial oven temperature of 50 °C was held for 2 min followed by a 5 °C min^{-1} ramp to 200 °C and a 2.5 min hold, followed by a 10 °C min^{-1} ramp to 280 °C with a 2 min hold.

To ensure the inoculations were successful, the bark was gently peeled back using a scalpel to expose the lesions at 50 dpi. The lesion lengths were measured and averaged to obtain a single lesion length per tree.

4.3. Data Analysis

For both in vitro and in vivo data sets, GC peaks present in the *Phytophthora*-inoculated treatments but not present in the controls were of greatest interest and further analyzed. This was conducted because we posit that qualitative differences are more relevant than quantitative differences for biomarker selection, particularly given the non-quantitative nature of SPME fibers. To be included, a peak had to be present in at least three of the four replicates for a *Phytophthora* species in the in vitro experiment, and in two of the three treatment replicates in the in vivo experiment, while not being present in any of the MICs. All peak integrations were performed with MSD ChemStation version E.02.02.1431 (Agilent) and the peaks were deconvoluted using AMDIS 32 (NIST). Based on preliminary analyses, a minimum peak area of 11,000 was used for peak calling. The peaks of interest observed in the treatments had their key ions manually searched for in the control specimens to verify their absence. The retention indices for the peaks were calculated based on the retention time of the alkane standards using the calculator from [74]. Tentative identifications were made by matching the mass spectra to compounds in the NIST20 and Wiley12 MS databases and a comparison of known retention indices from verified standards from the literature.

Statistical analyses were performed using RStudio 2023.06.1+524 (Posit). A principal component analysis (PCA) was run on the in vitro compound data sets using the prcomp function to observe the data trends and similarity of the *Phytophthora* VOC profiles. A hierarchical cluster analysis was run with the hclust function on autoscaled data. A random forest analysis was used to determine the in vitro compounds most important for predicting *Phytophthora* species, using the randomForest (with ntree = 500) and caTools packages. A one-way analysis of variance (ANOVA) and a two-tailed Dunnett's post hoc test were used to determine if the lesion lengths of the *Phytophthora*-inoculated trees differed from those of their respective MICs.

5. Conclusions

We demonstrated that *Phytophthora*-infected trees can be distinguished from MIC trees based on the presence of anisole, isokaurene, and a few unidentified VOCs. We also showed that several *Phytophthora* species can be differentiated from each other based on their in vitro volatiles. These compounds have the potential to be used as biomarkers for the development of faster, simpler, and cheaper methods of disease detection, such as

e/bio-noses and proton transfer reaction-mass spectroscopy, which offer near real-time analysis. New, higher-throughput methods for identifying diseased plants, particularly those that are still in the asymptomatic phase, are key to limiting the spread of diseased material and safeguarding forests. Volatilomics approaches for disease detection like those used here are one step towards a more secure future in plant health.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules29081749/s1, Figure S1: Mass spectra of important unidentified compounds 4, 7, 11, and 17 in Table 2 from the in vitro study; Figure S2: Mass spectra of unidentified compounds 1–3 in Table 3 from the in vivo study; Table S1: Phytophthora species used in the study; Table S2: Random forest output; Table S3: R outputs of ANOVA tables and Dunnett's test for lesion lengths in pedunculate oak trees; Table S4: R outputs of ANOVA tables and Dunnett's test for lesion lengths in European beech trees.

Author Contributions: Conceptualization, P.S., M.C. and S.W.; methodology, P.S. and I.N.; writing, P.S., I.N., B.B., S.W. and M.C.; funding acquisition, M.C. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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This thesis presents new insights to the field of biosurveillance by demonstrating the potential of innovative detection tools. My research shows that VOC analysis can effectively distinguish between healthy and infected trees, capturing both quantitative and qualitative differences. Additionally, nanopore sequencing was found to offer a greater taxonomic resolution but lower species diversity when compared to Illumina. All studied techniques detected pathogens in asymptomatic plant tissue. My findings can help guide biosecurity agencies in protecting future forests and urban landscapes.

Ida Nordström received her PhD education at the Southern Swedish Forest Research Centre, Swedish University of Agricultural Sciences, Alnarp, Sweden. She holds a Master's degree in Bioresource Technology Engineering, specializing in Plant and Forest Biotechnology (2014-2019), from Umeå University, and a Bachelor's degree in Nursing (2009-2012) from Mid Sweden University.

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