Dissertationes Forestales 379

Insights into the genomic and metabolomic adaptation of the fungal pathogens *Phacidium infestans* and *Colletotrichum salicis*

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Academic dissertation

To be presented, with the permission of the Faculty of Science, Forestry and Technology of the University of Eastern Finland, for public criticism in the auditorium N100 of the University of Eastern Finland, Yliopistokatu 7, Joensuu, on 28th of November 2025, at 12 o' clock noon.

Title of dissertation: Insights into the genomic and metabolomic adaptation of the fungal pathogens *Phacidium infestans* and *Colletotrichum salicis*
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Dissertationes Forestales 379
https://doi.org/10.14214/df.379

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ISSN 1795-7389 (online)
ISBN 978-951-651-848-3 (pdf)

Publishers:
Finnish Society of Forest Science
Faculty of Agriculture and Forestry of the University of Helsinki
School of Forest Sciences of the University of Eastern Finland

Editorial Office:
Finnish Society of Forest Science
Viikinkaari 6, FI-00790 Helsinki, Finland
http://www.dissertationesforestales.fi

Zerouki, C. (2025) Insights into the genomic and metabolomic adaptation of the fungal pathogens *Phacidium infestans* and *Colletotrichum salicis* 379. 65 p. https://doi.org/10.14214/df.379

ABSTRACT

This research investigated two fungal pathogens: *Phacidium infestans* DSM 5139, which is also known as the snow blight fungus that primarily affects conifers, and *Colletotrichum salicis* (strain SimOT6), which belongs to a significant plant pathogen group responsible for anthracnose disease. These fungi pose ecological and economic concerns due to their impact on forest and plant health. Studying their genomic and metabolomic profiles is crucial to understand their pathogenicity and adaptation to environmental conditions.

Whole genome sequencing and *in silico* analyses were performed on the selected fungi. They were investigated for their virulence effectors, secondary metabolites, carbohydrate-active enzymes and adaptation strategies. Emphasis was placed on the decomposition capabilities of *P. infestans* in its challenging ecological niche. Metabolomic analyses using mass spectrometry were conducted on *P. infestans* extracts to elucidate its ability to grow at freezing temperatures and tolerate pine needle compounds, which are known for their bioactive properties. The genomic analyses of *P. infestans* and *C. salicis* SimOT6 provide new insights into their survival strategies, nutrient acquisition and host interactions. The metabolomic study of *P. infestans* revealed its capability to decompose pine needles despite their chemical nature. On the other hand, the investigation of SimOT6 for its potential endophytic behaviour in *Salix* showed that fungi can act as latent pathogens, switching between endophytic and pathogenic lifestyles depending on the circumstances. These findings advance our understanding of fungal pathogenicity and adaptation, while also highlighting potential biotechnological applications of their enzymes and metabolites. These insights may pave the way for novel approaches in pest management and disease prevention in the context of climate change.

Keywords: snow blight, anthracnose disease, secondary metabolites, enzymes, whole genome, mass spectrometry.

ACKNOWLEDGEMENTS

This work was supported by the School of Forest Sciences, University of Eastern Finland (UEF), for two years (2020–2021). The research was further funded by the *Finnish Academy of Science and Letters* for 15 months (July 2022–September 2023). I am grateful to UEF for providing an excellent academic environment and the resources necessary for my research. I would like to thank the preliminary examiners, Docent Kristiina Hildén and Professor Benjamin Schwessinger, for reviewing this work. Their comments and suggestions greatly contributed to improving and refining the final version of this dissertation. I warmly thank Professor Frederick O. Asiegbu for kindly accepting the role of opponent in my public examination.

I would like to thank all my supervisors, Ossi Turunen, Ari Pappinen and Suvi Kuittinen for their support and for giving me the opportunity to work on this project. I met Ossi Turunen in Juva in October 2018. I arrived after sunset, carrying my Petri dishes on a bike that doesn't have brakes and lights. I think my bike intrigued him or maybe it just made him question my survival instincts. We discussed my work for a while, and then he invited me to UEF. The year after, I visited UEF as a researcher, and since then I haven't left. When I made it to Joensuu, Ossi was my unique contact, he played a major role as a mentor and a tutor and helped me settle in a new country. Ossi provided financial and scientific guidance. I can never thank him enough for being the reason behind the life that I have now.

I am thankful to have Ari Pappinen as a supervisor, his ideas, suggestions and financial support were crucial in shaping this research. When I ask Ari for material, analyses or DNA sequencing, his answer was always yes. This work would not be what it is without him. Warm thanks to Suvi Kuittinen, who has always been kind, supportive, and helpful. She gave me the opportunity to teach, and this experience only reinforced the positive impression I had of her.

I warmly thank Frank Berninger, who supported me in the last years of my PhD journey; his valuable advice and financial support greatly helped me to learn and better navigate my research. I would also like to thank Janne Jänis and Marko Mäkinen from the Department of Chemistry and Sustainable Technology, UEF, for their considerable contributions to this work, particularly their role in the metabolomic study of *Phacidium infestans* and needles decomposition (study II). I wish to acknowledge my co-authors, Omolara Mofikoya, Taskeen Badar, and Archana Yadav, for their valuable contributions and collaboration in studies II and III.

I am deeply grateful to my mother for her invaluable support. She always believed in me, even when I did not believe in myself. I am thankful for my family, especially my brother Riad, my aunts and my uncles for their unwavering support. I would like to thank my partner, Eetu, who has been a patient listener throughout my research challenges, laboratory experiments, article revisions, funding struggles and so many other things that he did not fully understand. I warmly appreciate my group of friends who made my time in Finland more enjoyable (Alicia, Shaohui, Yeray and Bradlie). I would like to thank my colleagues from the Plant Ecology group in Natura, Their joy, kindness and help have been invaluable during the latter stages of my PhD journey. Lastly, I warmly thank my Algerian and Tunisian friends, Abderrahmane, Khelifa and Monia, who have always been there for me.

Joensuu, 13th May 2025
Chahira

LIST OF ORIGINAL ARTICLES

This thesis is based on data presented in the following articles, referred to by the Roman Numerals I–III.

I Zerouki C, Chakraborty K, Kuittinen S, Pappinen A and Turunen O. Whole-genome sequence and mass spectrometry study of the snow blight fungus *Phacidium infestans* (Karsten) DSM 5139 growing at freezing temperatures (2023). Molecular Genomics and Genetics 298: 1449–1466. https://doi.org/10.1007/s00438-023-02073-7

II Zerouki C, Mofikoya O, Badar T, Mäkinen M, Turunen O and Jänis J. Metabolomic and genomic analysis of bioactive compounds of *Phacidium infestans* Karsten DSM 5139 cultivated on *Pinus sylvestris* needles (2025). Environmental Microbiology Reports 17: e70084. https://doi.org/10.1111/1758-2229.70084

III Zerouki C, Yadav A, Badar T, Kuittinen S, Pappinen A and Turunen O. Endophyte or latent pathogen? Genomic insights into *Colletotrichum salicis* SimOT6 isolated from a healthy willow cultivated in Eastern Finland. Manuscript.

Chahira Zerouki is the principal author of the three articles, with the main responsibility for experimental design and data analyses. The co-authors participated in the design of the studies and revisions of the papers. Paper II was realised in collaboration with the Department of Chemistry and Sustainable Technology, University of Eastern Finland. While all co-authors contributed to the work, Chahira Zerouki was mainly responsible for data analyses, article writing, submission and correspondence with the editors of the journals.

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ABBREVIATIONS AND DEFINITIONS

Abbreviations

aa	Amino acid
AA	Auxiliary Activity
AFP	Antifreeze Protein
APPI	Atmospheric Pressure Photoionisation
BGC	Biosynthetic Gene Cluster
bp	base pair
CAZymes	Carbohydrate-Active-Enzyme
CBM	Carbohydrate-Binding-Module
CE	Carbohydrate-Esterase
CMC	Carboxymethyl Cellulose
COG	Clusters of Orthologous Genes
DI-HRMS	Direct-Infusion High-Resolution Mass Spectrometry
DNS	3,5-Dinitrosalicylic acid
ESI	Electrospray Ionisation
FIA	Flow Injection Analysis
Gb	Gigabase
GH	Glycoside-Hydrolase
GT	Glycosyltransferase
HSTs	Host-Specific Toxins
IBP	Ice-binding Protein
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LPMOs	Lytic Polysaccharide Monooxygenases
Mb	Megabase
MEA	Malt Extract Agar
MM	Minimal Medium
PL	Polysaccharide-Lyases
UV	Ultraviolet

INTRODUCTION

General background

Microorganisms are omnipresent on Earth and can live in almost every environment. According to The United States National Science Foundation (2021), the Earth harbours approximately one trillion species of microbes. In 2016, Hawksworth and Lücking (2017) estimated that the number of fungi ranges from 2.2 to 3.8 million species, while O'Brien et al. (2005) had previously suggested a total of five million fungal species.

Fungi are the best model organisms to investigate the genomic bases of adaptive divergence in eukaryotes (Gladieux et al. 2014). To date [12 May 2025, National Centre for Biotechnology Information (NCBI)], approximately 51,486 fungal genomes are publicly available. An analysis of 325 diverse fungal genomes revealed a median genome size of 35–37 megabases (Mb), with the smallest genomes occurring in *Microsporidia* (3–6 Mb) and the largest in *Pucciniales* up to 893 Mb, with a few reported outliers exceeding one gigabase (Gb) (Stajich 2017; Fijarczyk et al. 2025). Notably, the genome of *Jafnea semitosta* has been reported at approximately 3,706 Mb, making it one of the largest fungal genomes known to date. This exceptional size, determined by flow cytometry, represents a striking outlier within the fungal kingdom (Talhinhas et al. 2021).

Fungi display different lifestyles (symbiotic, parasitic and mutualistic) or thrive independently as free-living organisms (Dutta and Paul 2012). In forest ecosystems, fungi play a leading role as decomposers and are essential for nutrient recycling (Blackwell 2011). When they colonise tree root systems, mycorrhizal fungi improve the trees' access to water and nutrients in exchange for carbon sources provided by the plant through photosynthesis. In addition, fungi can exist as endophytes, living within plant tissues without causing visible disease symptoms and conferring several benefits (Card et al. 2016). However, the nature of the endophyte-host interactions can vary and has not yet been fully elucidated. This view is further complicated by the continuum nature of these interactions that range from mutualistic to parasitic (Henriksson et al. 2023). Distinguishing between pathogenic and endophytic fungi can be difficult, especially when both exhibit similar genetic profiles (Rustamova et al. 2020). In fact, many fungal species include subspecies that can function as both endophytes and pathogens (Schouten 2019). Schulz et al. (1999) suggested that both pathogen-host and endophyte-host interactions require a mutual antagonism, often driven by secondary metabolites produced by each partner. The key difference is that pathogen-host interactions are considered unbalanced and therefore lead to disease, while endophyte-host interactions remain balanced.

Despite the ecological importance of fungi, they are also the largest group of plant pathogens. They can infect all plant parts (leaves, seeds, roots) and cause significant losses in crops (Degani 2025). Moreover, fungi are a major concern in post-harvest storage, causing significant deterioration of food and agricultural products (de Oliveira Filho et al. 2021). Fungal phytopathogens are typically divided into three categories: biotrophic pathogens, which keep the host tissue alive, and necrotrophic pathogens, which kill the host tissue to acquire nutrients. Some fungi employ a two-stage infection strategy referred to as hemibiotrophy (Doehlemann et al. 2017). For instance, *Colletotrichum higginsianum* exists as a pathogen of cruciferous plants. Initially, this fungus starts as a biotroph and then switches to a destructive phase (necrotrophy) (Kleemann et al. 2012). Given the diversity and ecological versatility of fungi, they remain a central focus in basic and applied

biological research.

Microbial adaptation

Microorganisms have evolved to withstand the pressures of their environmental conditions. This ability is referred to as microbial adaptation (Patel and Rosenthal 2007). Microorganisms can thrive in diverse environments that range from freezing to extremely hot temperatures, from land to sea, in both highly alkaline and acidic conditions, as well as in freshwater and brackish water habitats (Poli et al. 2017). Studies on microbiomes found in extreme ecosystems have shown that microorganisms possess remarkable metabolic flexibility combined with extraordinary physiological abilities that allow them to colonise and survive in harsh conditions (Viswadeepika and Bramhachari 2022). Exposure to one or more environmental stressors, including extreme temperatures, osmotic pressure, salinity, UV radiation or high atmospheric pressure can threaten microbial survival (Capozzi et al. 2009). To cope with these environmental challenges, microbes have developed a series of adaptive mechanisms. Often, the degree of tolerance to stressful conditions increases with extended exposure time (Tan et al. 2022). Therefore, fungi and bacteria employ a cascade of cellular and molecular systems to adjust to the fluctuating conditions (Wani et al. 2022). Molecular adaptation occurs through various processes, such as natural selection, amino acid substitutions, DNA repair mechanisms, horizontal gene transfer, genetic recombination, and pleiotropy-like events. This dynamic is also observed in host-fungi interactions, where intricate molecular processes drive co-evolution, thereby enabling fungi to adapt to their hosts' defences while influencing plant health and disease outcomes.

The genomic architecture of microorganisms is often reflected in their phylogenetic position and the specific lifestyle to which they are adapted (Dutta and Paul 2012; Tribelli and López 2018; Wang et al. 2017). Cold tolerance in fungi involves multiple strategies, including the storage of cold-resistant solutes such as cryoprotectants, trehalose polyols, fatty acids (lipids), and antifreeze proteins (Arai et al. 2018; Duarte et al. 2018). These solutes protect cellular structures against freezing damage and dehydration. Additionally, cold-adapted fungi produce diverse hydrolases and oxidoreductases optimised for low-temperature activity. Hydrolases such as cellulase, xylanase (and other hemicellulases), pectinase, protease, and amylase enable the breakdown of complex nutrients for metabolism under cold stress, while oxidoreductases such as superoxide dismutase help mitigate oxidative stress and maintain cellular redox balance (Abu Bakar et al. 2020; Liu et al. 2023; Wang et al. 2017). Together, these biochemical adaptations support metabolic function, nutrient acquisition, and cellular protection of fungi in their harsh environments. Research on cold-adapted fungi continues to reveal the mechanisms that underlie their adaptation to sub-zero environments. Investigations of microbial communities in Antarctica have uncovered the remarkable ability of microbes to thrive in extreme conditions, providing insights into nutrient cycling and resilience in icy ecosystems (Duarte et al. 2018; de Menezes et al. 2019; Oriana et al. 2013; Tsuji 2024). Extreme conditions drive microbes to diversify their enzymatic machinery and the chemical nature of their metabolites, leading to the production of a diverse array of bioactive compounds, such as vitamins, pigments and antibiotics. In recent years, interest in microorganisms from various extreme niches has increased (Bartholomäus et al. 2024; Zerouki et al. 2021). Moreover, understanding the molecular and biochemical mechanisms that underpin microbial adaptations can have broader implications for elucidating the limits of life and potential habitability in extreme

environments, including outer space (Mastascusa et al. 2014). These studies are relevant to astrobiological research as they offer clues as to the prerequisites of potential extraterrestrial life in similar conditions (Zucconi et al. 2025) and contribute to a large reservoir of biological features that can lead to the development of new biotechnical applications. Metabolic adaptation to extreme conditions and competition between species can be used to identify new compounds that could be utilised, for example, in pharmaceutical or other applications.

Fungal enzymatic machinery

Plant cell walls are composed of layered structures. The middle lamella is often rich in pectin, and the primary cell wall is formed of hemicellulose, cellulose, and pectin, while the secondary cell wall contains lignin and additional cellulose microfibrils. Lignin biosynthesis significantly contributes to plant and organ growth, tissue strength, lodging resistance and provides protective responses to a variety of stresses (Liu et al. 2018). However, the exact composition and structure of the cell wall can differ significantly between plant lineages (Kubicek et al. 2014).

Fungi employ a wide array of enzymatic systems commonly known as carbohydrate-active enzymes (CAZymes), which encompass specific array of enzymes that target the degradation of plant cell wall components. These enzymes enable fungi to decompose plant material and breach the cell walls, combining oxidative and hydrolytic enzymes tailored to break down lignocellulose and ensure effective nutrient acquisition (Drula et al. 2022). Although some fungi can infect both monocots and dicots, many exhibit host specificity. Variations in host cell wall structures have led to the adaptation of CAZyme repertoires to particular plant types and tissues.

Thus, phytopathogen CAZymes are adapted to different plant hosts and tissues. This diversity allows fungi to specifically target the wide range of polysaccharides found in plant cell walls. The expression of CAZyme genes in plant pathogenic fungi is linked to their infection strategy and lifestyle. In necrotrophic and hemibiotrophic fungi, the CAZyme genes that encode cellulases are typically induced early to facilitate initial penetration of the plant cell wall, while genes for hemicellulases and other enzymes are up-regulated later as the infection progresses (Zhao et al. 2013). In contrast, obligate biotrophic fungi show less expression and fewer CAZyme genes, as they rely on host cell viability and avoidance of triggering strong plant defence mechanisms (Jia et al. 2023). CAZyme gene expression can also be influenced by developmental cues, environmental factors and plant-derived signals (Lyu et al. 2015).

In general, glycoside hydrolase (GH) families encompass the enzymes that cleave the glycosidic bonds in oligo- or polysaccharides, which also include cellulose and various hemicelluloses. The enzymes that belong to the GH5, GH6, GH7, GH10, GH11 and GH28 families have been found to contribute to pathogenicity (Ma et al. 2019). Enzymes that cleave pectins are found in the GH28 and polysaccharide lyase (PL) families (PL1 and PL3). Since, pectin is abundant in the middle lamella and primary cell walls, these enzymes facilitate the initial penetration of the host. Carbohydrate Esterase (CE) families include cell wall degrading enzymes, particularly CE1 and CE10, which exhibit gene expression levels characteristic of particular lifestyles during infection. Hemibiotrophs exhibit the highest expression levels of CE1 and CE10. These CAZymes modify plant cell wall polysaccharides by removing acetyl and feruloyl groups, thus enhancing the action of hydrolases and lyases

(de Vries and de Vries 2020). A genomic study across 103 fungal species revealed that phytopathogenic fungi typically possess a greater abundance of GH families, as well as CE5 and PL1 enzymes (Zhao et al. 2013).

Auxiliary Activities (AA) enzyme families, such as lytic polysaccharide monooxygenases (LPMO), other oxidases, dehydrogenases, laccases and peroxidases are also important for cellulose and lignin modification, especially in woody tissues. They act by facilitating access to cellulose and hemicellulose (Ma et al. 2019). The adaptation of CAZyme repertoires to specific host lineages and tissues supports the efficiency of fungal colonisation and pathogenicity. These insights highlight the remarkable enzymatic diversity and specialisation of phytopathogenic fungi, shaped by the structure and composition of their host plants. Since many of these enzymes are of interest to the biofuel and pulp industries, comparative genomics platforms, such as the Fungal PCWDE Database have been created to mainly focus on the fungal enzymes that degrade plant cell walls. These platforms provide an important resource to study the evolution of enzymes targeting plant cell wall (Choi et al. 2013: Drula et al. 2022).

Understanding fungal enzymatic strategies opens avenues for biotechnological applications. To illustrate the practical industrial application of CAZymes, *Trichoderma reesei* stands out as a leading producer of cellulases and hemicellulases vital for biomass degradation. Discovered during World War II, it has been developed into a highly efficient enzyme producer through strain improvement and molecular biology. Its enzyme system converts lignocellulosic biomass into fermentable sugars for biofuels and supports industries such as textiles, food, feed and pulp, making *T. reesei* a cornerstone of CAZyme biotechnology and the bio-based economy (Fischer et al. 2021). While the enzymatic machinery of various fungi and other microbes has applications across a range of industries, microorganisms growing under specialised conditions are likely to provide novel tools and solutions for commercial use.

Secondary metabolites in fungi

Secondary metabolites are the compounds that are produced from the primary metabolic pathways using molecules, such as amino acids and acyl-CoAs. Amino acids are used to make non-ribosomal peptides and acyl-CoAs for building compounds, such as polyketides and terpenes. The genes implicated in secondary metabolites are grouped in biosynthetic gene clusters (BGC). Secondary metabolites from fungal phytopathogens are known for their diverse chemical structures and significant biological activities. They play central roles in plant interactions and defence mechanisms. Their possible uses in agriculture and medicine highlight the importance of these metabolites in the development of sustainable practices and novel therapeutic agents (Zhang et al. 2021). Secondary metabolites play central roles in fungal development and in interactions with other organisms. Genes in a BGC are induced based on the ecological role of the compound that they produce. For instance, virulence-related clusters, such as trichothecene biosynthetic cluster will be up-regulated in *Fusarium graminearum* during plant infection (Keller 2019). The produced mycotoxin acts by inhibiting protein synthesis in the host plant and induces wheat head blight (Amuzu et al. 2024).

Aromatic polyketides and sesquiterpenoids are among the major phytotoxic compounds produced by phytopathogenic fungi and play a pivotal role in disease development (Xu et al. 2021). Understanding the chemical and biological properties of these secondary

metabolites, including their structures and modes of action, can aid in investigating plant-pathogen interactions (Xu et al. 2021).

In the last decade (2015–2025), over 8,212 studies containing keywords such as "secondary metabolites," "bioactive compounds," "mycotoxins" and "fungal metabolites" have been recorded in the Web of Science database. The interest in secondary metabolites is diverse, with a strong focus on drug discovery, driven by advances in genome sequencing, bioinformatics, phylogenetics and the improved ability to manipulate fungal genomes (Keller 2019). The unique ability of fungi to synthesise a wide range of specialised secondary metabolites makes them valuable for agricultural applications (Zhang et al. 2021). Secondary metabolites derived from endophytic fungi have shown significant biological activities, including the ability to inhibit pathogenic strains (Rustamova et al. 2020). Furthermore, secondary metabolites such as molecules that provide protection against various physical and chemical stressors hold considerable potential for exploitation within the biotechnology sector (Sayed et al. 2020; Oriana et al. 2013; Raddadi et al. 2015). For instance, the spiromeroterpenoids, fusariumin A and B, produced by the endophytic *Fusarium* sp. YD-2, isolated from the twigs of *Santalum album* have shown significant anti-inflammatory and antibacterial properties (Yan et al. 2018).

Fungal models and knowledge gaps

The current research focuses on two fungal plant pathogens. These fungi were selected to further investigate their genomic features.

Colletotrichum salicis

Members of the *Colletotrichum* genus are economically important pathogens due to their ability to cause diseases in a wide range of hosts. These species can cause necrotic lesions on leaves, stems and fruits (Cannon et al. 2012). *C. salicis* strain was reported as a pathogenic fungus and was primarily associated with willow (*Salix* spp.). *Colletotrichum salicis* belongs to the *C. acutatum* species complex and it can also infect other woody hosts, such as *Malus*, *Acer*, *Araucaria*, *Pyrus* and *Populus* (Grammen et al. 2019). Manova et al. (2022) indicated the presence of *C. salicis* as a causal agent of fruit anthracnose in peppers and tomatoes in Bulgaria. Most of *Colletotrichum* species are considered to be hemibiotrophs (Münch et al. 2008; O'Connell et al. 2012). While many of the species are well-known as plant pathogens, research has shown that they can also colonise plants as symptomless endophytes and not cause any visible signs of disease and may indeed assist in plant growth (Newfeld et al. 2005). For instance, *C. tofieldiae* can function as either a beneficial root endophyte in *Arabidopsis thaliana* through promotion of plant growth under phosphate deficiency, or as a pathogen. The switch between mutualist (endophyte) and pathogen states depends on the fungal strain, and environmental conditions, such as phosphate levels, temperature, and how the plant responds e.g. to phosphate starvation (Hiruma et al. 2023). Since *Colletotrichum* species are known for their ecological versatility, studying *C. salicis* for its potential endophytic lifestyle under specific conditions may offer useful insights into how these fungi switch between different lifestyles. To date, this possibility has not been fully explored in this fungal species.

Phacidium infestans

Phacidium infestans Karsten (reclassified as *Gremmenia infestans* by Crous et al. 2014) is a fungal pathogen primarily known to cause snow blight in coniferous species, especially in *Pinus sylvestris* (Scots pine), in northern Europe, Asia, and North America (Hanso 2000). Previous work by Doğmuş-Lehtijärvi et al. (2016) demonstrated that *P. infestans* (*G. infestans*) can also cause disease symptoms in young seedlings of *Cedrus libani* (Cedar of Lebanon) and *Pinus nigra* subsp. *pallasiana* (Anatolian black pine). Their inoculation trials revealed crown infection rates of 48% in pine and 35% in cedar, with pines showing significantly more severe damage.

Phacidium infestans infects needles in autumn through airborne ascospores. During winter, the fungus thrives under the insulating snow layer, spreading across needles and causing characteristic reddish-brown discoloration (Roll-Hansen 1989). Mycelium growth can reach 20 to 30 cm in a single winter (Björkman 1948). In addition to needles, the fungus invades vascular tissues, leading to the death of twigs, buds and shoots located under the snow. After snowmelt, disease symptoms become most apparent in spring. The severity of infection has been linked to the duration and depth of snow cover, with prolonged snow conditions favouring fungal activity and spread (Björkman 1948). Scots pine mortality risk increases when the infected trees are between 0.35 and 1.5 m in height (Hansson et al. 2006). Furthermore, it has been shown that in managed forests, the presence of logging slash (branches and tops left after harvesting) increases the incidence of snow blight. Slash creates favourable microclimatic conditions that contribute to snow persistence, allowing *P. infestans* to spread more effectively (Hansson et al. 2006). In addition, a positive correlation of the disease incidence with the density of the host plant has been established. Greater plant densities lead to increased rates of mortality from *P. infestans* (Burdon et al. 1992). In contrast, an investigation conducted by Petäistö and Hantula et al. (2013) suggested that snow is not required for *P. infestans* to develop on Norway spruce (*Picea abies*) seedlings.

Aside from the ability of *P. infestans* to thrive in sub-zero temperatures on pine seedlings, the fungus is able to use and kill pine needles. Studies on pine needle composition have shown that cellulose accounts for approximately 52–60% of the dry weight, while hemicellulose typically contributes around 20% (Alzebdeh et al. 2019; Tolga Cogurcu 2022). Lignin content generally ranges from 13 to 15%, although a higher value (39.6%) has been reported for *Pinus taeda* needles (Gujjula et al. 2021). Extractives, including waxes, fatty acids, and resins, usually constitute about 5%, but in *P. taeda* needles, extractives have been estimated at 12% (Gujjula et al. 2021). These compounds contribute to the hydrophobic nature and slow decomposition of pine needles. Ash content is typically reported at 2.3–2.7% (Bolzon de Muñiz et al. 2014; Gujjula et al. 2021).

Beyond structural components, pine needles are rich in essential oils, particularly mono- and sesquiterpenes such as α-pinene, β-caryophyllene, camphene, and limonene. These volatiles play important roles in chemical defence against herbivores and pathogens, as well as in mediating ecological interactions (Koutsaviti et al. 2021). Pine needles bioactive compounds, for instance, terpenes, phenolic compounds, alkaloids, among others exhibit antioxidants, anti-inflammatory, and antimicrobial activities (Mahizan et al. 2019; Rana et al. 2023). Previous studies have highlighted the potential antimicrobial activity of oils and water-soluble extracts from pine needles (Mirković et al. 2024; Zeng et al. 2011). Furthermore, pine needles possess a waxy cuticle and an acidic pH, while the presence of resin acids further impedes microbial penetration and survival (Rubens et al. 2023).

However, *P. infestans* appears capable of overcoming the defences of pine needles. To date, little is known about its nutrient acquisition strategies from the needles, and more detailed molecular information remains lacking.

Rationale

The adaptation strategies of fungal pathogens are critical to deepen our understanding of their ecological roles, their interactions with hosts, and their potential impacts on forestry and agriculture. To address this, two fungal models were selected for genomic investigation: *Phacidium infestans* Karsten (DSMZ 5139) and *Colletotrichum salicis* (SimOT6).
From a forestry perspective, fungal pathogens, such as *P. infestans*, pose significant threats as they negatively impact biodiversity, disrupt the ecological balance and cause substantial economic losses. This investigation aims to explore the genomic foundations and temperature-dependent metabolic responses of this species (study **I**), with the goal to uncover how it adapts to varying environmental conditions.

From an agricultural perspective, pathogens such as *Colletotrichum* spp. are globally recognised for their ability to infect a diverse number of crops, leading to yield and economic losses. Understanding their virulence factors, environmental adaptability and host range offers valuable insights to develop sustainable pest management and disease prevention approaches. The selection of these fungal models reflects these dual priorities: *P. infestans* was studied to further investigate fungal adaptation to hostile environments (pine needles) (study **II**), while *C. salicis* SimOT6 was examined to explore the dynamics of host-fungi interactions, emphasising the endophytic continuum (study **III**).

Study objectives

The current research investigated two fungi that significantly impact forestry and agriculture (*P. infestans* DSMZ 5139 and *C. salicis* SimOT6). *P. infestans* DSMZ 5139 was initially isolated from *Pinus cembra* in Austria in 1982. However, the whole genome sequence was not available in the databases. This study aimed at:

1. Sequencing and annotating the first complete genome of the studied species (*P. infestans*), thereby establishing a foundation for future research. Additionally, mass spectrometry analysis was performed to assess cold resistance response in *P. infestans* (study **I**).
2. Since *P. infestans* thrives on needles under the snowpack, terpene tolerance and nutrient acquisition strategies from the needles were further investigated (study **II**).
3. In contrast, *C. salicis* has been identified as a pathogen in a wide variety of hosts. This research aimed to investigate the strain *C. salicis* SimOT6 as a potential endophyte of *Salix* (study **III**). Many species that belong to the *Colletotrichum* genus have been found to exist as an endophyte in one host and a pathogen in another, thereby reflecting the endophytic continuum. The genome of *C. salicis* SimOT6 was analysed *in silico* to determine whether this strain genetically stands out as an endophyte or as a latent pathogen.

The current research provides insights into the potential biotechnological applications of fungal enzymes for instance, leveraging their secondary metabolites and enzymes for innovative solutions. In this context, GH11 enzyme from *P. infestans* was cloned in competent *Escherichia coli* and partially characterised.

MATERIAL AND METHODS

Sample collection and cultivation

P. infestans Karsten DSM 5139 was originally isolated in 1982 by H. Butin in Pinzgau, Obersulzbachtal, Austria, from the needles of *Pinus cembra.* This strain was obtained from the German DSMZ collection. The *C. salicis* SimOT6 strain was earlier isolated from the bark of *Salix* × *schaburovii* I.V.Belyaeva grown in Simola, Liperi, Finland. Both strains were cultivated on Malt Extract Agar (MEA; pH 5.6) and Potato Dextrose Agar (PDA; pH 5.6) media. Fungal cultures were incubated at 22°C ± 2°C for 1–2 weeks until substantial mycelial expansion was observed across the media surface.

To purify the DNA, the fungi were cultivated in 250 mL flasks that contained 100 mL of 2% (v/w) of malt extract broth (from VWR Chemicals). The flasks containing MEA and PDA media were inoculated with a 6 mm diameter disk of mycelium and incubated for 3–7 days at 24°C with continuous shaking (150 rpm).

Cultural characterisation

The selected *P. infestans* has been previously studied by Butin and Söderholm (1984) and compared to other *Phacidium* species based on its microscopic aspect (ascomata, ascospores and asci). No further microscopic observations have been performed on *P. infestans*. In contrast, the macroscopic aspect of *C. salicis* SimOT6 has been observed on both MEA and PDA media. Here, microscopic observations were performed using the slide culture method and visualised using a Nikon Eclipse 50i Clinical Microscope (magnifications: 400× and 1000× with paraffin oil).

Specific media that contained 1% different substrates were used to evaluate the ability of the fungi to utilise different carbon sources and to potentially secrete extracellular enzymes. The fungi were tested on pectin, beechwood xylan, cellulose using carboxymethyl cellulose (CMC), starch and lignin media to evaluate their ability to use these substrates as a unique carbon source. The preferential carbon source tests were performed using the above-mentioned substrates with a Minimal Medium (MM) composed of (g/L): KH_2PO_4, 0.68; NH_4NO_3, 1.2; $FeSO_4 \cdot 7H_2O$, 0.03; $MgSO_4 \cdot 7H_2O$, 0.1; NaCl, 4.0; $CaCl_2 \cdot 2H_2O$, 0.02. Fungi inoculated on MEA media were used as control. The bioassays were terminated once the mycelium reached the edge of the Petri dish on any of the substrates. The fungal growth was then recorded by measuring the colony radius. In addition, extracellular enzyme screenings were performed using specific media with the pH adjusted to 5.9 (Table 1). Fungi were inoculated on the enzyme screening media and incubated at 22°C for 5–12 days. Enzyme activity was revealed by the presence of a halo (clear zone) around the colony after staining the plates with iodine or Congo red solutions.

To compare production rates between the different extracellular enzymes, the radius (and diameter) of the halos that resulted from the substrate hydrolysis around the colonies was recorded. Statistical processing of the results was performed using analysis of variance Anova one-way with IBM SPSS statistics (New York, Unites State of America).

Table 1. Carbon source utilisation and enzyme screening media.

Bioassay	Media (g/L)	Cultivation conditions	References
Growth on MEA	20g malt extract, 20g agar.	22°C for 5–12 days	Modified from Reddish 1919
Pectin utilisation	MM supplemented with 10g of pectin.	22°C for 5–12 days.	Sati and Bisht 2006
Xylan utilisation	MM supplemented with 10g of xylan.	22°C for 5–12 days	Sati and Bisht 2006
Growth on cellulose	MM supplemented with 10g of CMC.	22°C for 5–12 days	Sati and Bisht 2006
Lignin utilisation	MM supplemented with 10g of lignin.	22°C for 5–12 days	Sati and Bisht 2006
Starch utilisation	MM supplemented with 10g of starch.	22°C for 5–12 days	Sati and Bisht 2006
Extracellular pectinase screening	KCl, 1.0; $MgSO_4$, 0.5; K_2HPO_4, 1.0; $NaNO_3$, 1.0; pectin, 10; agar, 20.	22°C for 5 days, and strained using iodine solution	Kumar et al. 2012
Extracellular Cellulase screening	KCl, 0.2; $NH_4H_2PO_4$, 1.0; yeast extract, 1.0; $MgSO_4 \cdot 7H_2O$, 1.0; CMC, 10; agar, 20.	5 days, 22°C. Staining with Congo red (10 min) followed by washing with 1 M NaCl solution	Admassie et al. 2022; Florencio et al. 2012
Extracellular xylanase screening	Yeast extract, 3.0; peptone, 1.5; $MgSO_4 \cdot 7H_2O$, 0.3; beechwood xylan, 10; KH_2PO_4, 1.0; agar 20.	5 days, 22°C. Staining with Congo red (0.5 %) for 30 min, then washed using 1 M NaCl solution	Kalim and Ali 2016

DNA extraction and genome sequencing

The fungal mycelia were collected by filtration using Whatman No. 1 filter paper. The fungal pellets were disrupted using glass beads (0.5 mm) from Scientific Industries Inc. The glass beads were utilised with the Disruptor Genie® (Scientific Industries SI™) at 6°C with 2,400 rpm for 5 min. A pre-chilled material was used to avoid overheating the samples. The disrupted pellets were then collected after centrifugation and purified using a Plant/Fungi DNA isolation kit from Norgen Biotech Corp. (E5038-1KT). The DNA concentration and quality were assessed using a NanoDrop® ND-1000 (Thermo Scientific) and by electrophoresis with a Lonza FlashGel™ system. The genome sequencing of the selected fungal strains was conducted at the sequencing centre of the University of Oregon (USA) Genomics and Cell Characterisation Core Facility (GC3F) by Pacific Biosciences Sequel II technology (PacBio). The samples were further purified using the Zymo Research kit, and SMRTbell libraries were prepared with a PacBio Express Template Prep Kit 2.0 (following the manufacturer's protocol). The samples were pooled and size selected. Raw PacBio reads were then converted to FASTA format using Samtools and assembled with Flye v2.6. This protocol was used for both *P. infestans* (study **I**) and SimOT6 (study **III**).

Genome mining

After genome assembly, the sequences were assessed for their completeness using BUSCO (versions 3.0.2 and 4.0.2) and BUSCO v1 from gVolante (web server). The genome sequences were then annotated using AUGUSTUS software and COMPANION web server. Protein sequence analyses were performed using several tools, which included InterProScan (available from Galaxy Europe) and Geneious Prime package using UniProt/Swiss-Prot. Cluster of Orthologous Groups (COG) annotation was performed using WebMGA server. KofamKOALA and BlastKOALA were used for the Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotation to reconstruct pathway maps. Blast2GO was used to generate additional functional annotations.

Carbohydrate-Active Enzymes (CAZymes) were investigated using the dbCAN3 meta-server, which employed HMMER, Diamond and Hotpep tools. Analysis of BGCs was performed using the antiSMASH fungal version 7.1 (Blin et al. 2023). Several tools were employed to fully investigate the genome and conduct a comparative analysis with the closest reference strains, including Mauve software for genome alignment and OrthoVenn3 for comparisons and evolutionary analysis of COG across species.

Average Nucleotide Identity (ANI) was calculated using the ANI Calculator and FastANI (Galaxy Europe). Although ANI tools were originally developed for prokaryotic genomes, blast-based programmes such as FungANI have recently emerged for fungal genomes (Lalanne & Silar 2025). Nevertheless, a previous study on yeast, which are eukaryotes, recommended FastANI as an effective tool for species delineation (Jain et al. 2018; Cortimiglia et al. 2024). In general, ANI values above 95% indicates that both genomes might belong to the same species. However, Lalanne and Silar (2025) recommend a higher ANI threshold (above 99.5 %) for reliable species delineation.

In-silico genome-to-genome comparison, mimicking DNA–DNA hybridisation was performed to evaluate the similarity and differences between the studied fungal model and its closest relatives. Although this tool was originally developed for prokaryotic genomes, it supports large genomes and has been used for eukaryotic organisms (Chatterjee et al. 2015). A digital DNA-DNA hybridisation cutoff value above 70% indicates that the strains belong to the same species. If the observed DNA-DNA hybridisation values were above 79%, it would indicate that the strains belong to the same subspecies.

SignalP, EffectorP and SecretomeP tools were used to identify and analyse secreted proteins in *P. infestans* and SimOT6. SignalP and SecretomeP were used to generate a complete list of secreted proteins. The resulting FASTA file was submitted to EffectorP to identify potential effectors that may suppress host immunity or aid infection. Secreted proteins were also examined for the presence of CAZymes that target plant cell wall components.

Other protein web tools were used to further confirm protein identities, such as BLASTp to identify homologs of secreted proteins or CAZymes. UniProt was used to retrieve functional annotations and conserved domain information, while SWISS-MODEL was employed to predict the structures of key fungal proteins or effectors. All the bioinformatics tools utilised in this study are listed in Table 2.

Table 2. Summary of the bioinformatic tools used for the genomic studies of *P. infestans* and SimOT6.

Tool	Version	Access link	Reference
Genome completeness assessment			
gVolante	BUSCO v1	https://gvolante.riken.jp/	Simão et al. 2015
Genome annotation tools			
Augustus	3.5.0	https://github.com/Gaius-Augustus/Augustus	Stanke et al. 2004
COMPANION	2.2.11.	https://companion.gla.ac.uk/	Haese-Hill et al. 2024
Functional analyses			
AntiSMASH	Fungal v.7.1	https://fungismash.secondarymetabolites.org/#!/start	Blin et al. 2023
Blast2GO	6.0.	https://www.blast2go.com/	Conesa et al. 2005
BlastKOALA	3.1	https://www.kegg.jp/blastkoala/	Kanehisa et al. 2016
dbCAN	3	https://bcb.unl.edu/dbCAN2/index.php	Zheng et al. 2023
Galaxy Europe		https://usegalaxy.eu/	Afgan et al. 2018
Geneious Prime	2022.1.1	https://www.geneious.com/	Kearse et al. 2012
InterProScan	5.59-91.0	https://www.ebi.ac.uk/interpro/search/sequence/	Afgan et al. 2016
KofamKOALA	24-11-1	https://www.genome.jp/tools/kofamkoala/	Aramaki et al. 2019
WebMGA		https://www.weizhongli-lab.org/metagenomic-analysis/	Wu et al. 2011
Secreted effectors and virulence factors			
EffectorP	3.0.	https://effectorp.csiro.au/	Sperschneider and Dodds 2022
SecretomeP	2.0.	https://services.healthtech.dtu.dk/services/SecretomeP-2.0/	Bendtsen et al. 2004
SingalP	v. 6.0	https://dtu.biolib.com/SignalP-6	Teufel et al. 2022
Protein study tools			
BlastP		https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins	Altschul et al. 1990
Phobius	v.1.01	https://phobius.sbc.su.se/	Käll et al. 2004
SWISS MODEL		https://swissmodel.expasy.org/	Schwede et al. 2003
UniProt		https://www.uniprot.org/	The UniProt Consortium 2017
Comparative genomics and phylogeny			
ANI Calculator		http://enve-omics.ce.gatech.edu/ani/index	Rodriguez-R and Konstantinidis 2014
GGDC hybridisation	3.0	https://ggdc.dsmz.de/ggdc.php#	Meier-Kolthoff et al. 2022
Mauve		https://darlinglab.org/mauve/mauve.html	Darling et al. 2010
MEGA	11	https://www.megasoftware.net/	Tamura et al. 2021
Orthovenn3	3	https://orthovenn3.bioinfotoolkits.net/	Sun et al. 2023

Mass spectrometry analysis of *P. infestans* secondary metabolites

Mass spectrometry analyses were conducted on *P. infestans* to further investigate its adaptability to harsh climatic conditions and to obtain experimental evidence for the proteins and pathways predicted from its genome annotation. Two different experiments were performed on *P. infestans* extracts using two distinct mass spectrometry techniques.

Cold adaptation

The first mass spectrometry analysis aimed to identify the fluctuations in *P. infestans* secondary metabolites released at different temperatures. In this respect, MEA plates were inoculated by *P. infestans* and incubated at 22°C until sufficient growth was observed. The plates were then separated into two groups; some were kept at 22°C and the others were incubated at -3°C for two weeks. The experiment was conducted in five biological replicates for each treatment group. Methanol extracts were obtained by carefully scraping the fungus from the surface of MEA plates and dissolving an equal amount of fungal material in an Eppendorf tube that contained methanol. The samples were then extracted by shaking for 4 hours at 6°C to prevent the samples from overheating, followed by centrifugation to remove the fungal cells. Finally, the methanol samples were concentrated using a SpeedVac vacuum concentrator and sent for analysis to MS-Omics, Denmark (Figure 1). Controls were made using methanol without the presence of *P. infestans*. The sample preparation and quality control for mass spectrometry LC-MS/MS was conducted as detailed in study **I**. Mass spectrometry peaks were extracted using Compound Discover 3.1 from Thermo Scientific (Massachusetts, United States of America) and the annotation was performed at four levels (level 1, level 2a, level 2b and level 3). Level 1 was deemed the most confident as it used accurate mass, MS spectra and retention time. Level 2a annotation was based on retention time obtained from the standards analysed on the same system and accurate mass. Level 2b was based on MS spectra from an external library and accurate mass, while Level 3 was the least confident annotation as it was obtained using library searches with accurate mass and elemental composition. The results are expressed in relative areas (areas under the curve/ chromatographic peak).

A -3°C to 22°C ratio was calculated by dividing the average relative area of a compound obtained at -3 ºC by the average of the same compound detected at 22ºC. The resulting data was statistically processed using two-sample t-test (two tailed, type III) by assessing the significance of the mean values of the studied compounds in each treatment group (-3°C and 22°C).

Chemical tolerance

To investigate the ability of *P. infestans* to survive on pine needles, the fungus was cultivated in 250 mL Erlenmeyer flasks that contained 100 mL of distilled water with 1% (w/v) *Pinus sylvestris* needles. The needles were sterilised using gamma irradiation at Ionisos Baltics OÜ, Estonia to avoid potential heat damage. The flasks containing the fungus with the needles were incubated at 22°C for 14–28 days with continuous shaking. This experiment was conducted in triplicate. After incubation, the samples were filtrated using filter paper. The extraction of metabolites from these samples was performed using liquid-liquid extraction method. An equal volume of methanol was added. The mixture was mixed for one hour and transferred to a separation funnel. The upper layer was then

collected for further analysis. Negative controls were prepared simultaneously by following the same steps without fungus inoculation. The methanol used for this experiment was selected as a blank. The extracts were analysed by Direct Infusion High Resolution Mass Spectrometry (DI-HRMS) using a flow injection analysis (FIA) technique and two ionisation modes APPI and ESI (study **II**).

In addition, DI-HRMS analysis was conducted on *P. infestans* cultivated on malt extract liquid medium. The cultivation of the fungi and the controls, as well as the methanol extractions, were prepared simultaneously as described in the needles experiment. The aim of this experimental setup was to determine the metabolites that are secreted and metabolised specifically in the presence of the needles (Figure 1). The analysed samples and their resulting compounds from malt extract broth acted as positive controls. A CTR/SA ratio was used to compare between the samples without *P. infestans* (CTR) and the samples after *P. infestans* growth on needles (SA). Metaboscape software (Bruker, Billerica, Massachusetts, USA) was used to analyse the metabolomic data.

The obtained values (normalised absolute intensity) were further processed using a two-sample t-test (two-tailed, type III) to assess whether the mean values of each compound differed significantly between the two treatment groups (*P. infestans* samples and the control), with a *p*-value (*p*) cutoff of ≤ 0.05.

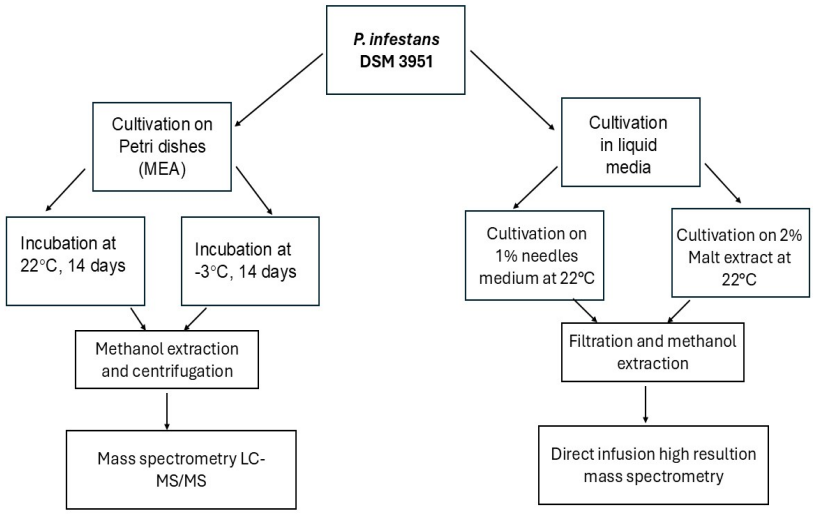

Figure 1. Sample preparation workflow for mass spectrometry analyses of *P. infestans* bioactive compounds.

Characterisation of GH11 from *P. infestans*

The enzymatic characteristics of *P. infestans* were assessed using the GH11 endoxylanase protein sequence as a representative example. Recombinant GH11 xylanases are valuable for pulp and paper biobleaching, animal feed improvement, biofuel processing, and food industries, due to their ability to degrade plant hemicellulose. Furthermore, the development of GH11 variants with improved properties (e.g. thermostability, altered pH profile, substrate range modifications) allow the enzyme to perform effectively in harsh industrial environments (Tian et al. 2022). The protein proseq9131 from *P. infestans* was obtained from the annotated genome sequence and was then fused with the N-terminal secretion signal pelB from *Bacillus amyloliquefaciens.* The constructed sequence was synthesised by Genscript and expressed in competent *Escherichia coli* BL21 (DE3) cells (Merck, CMC0014-4X40UL).

The recombinant *Escherichia coli* strains were cultivated on Lysogeny Broth (LB) agar, at pH7 supplemented with 100 mg ampicillin. After overnight incubation at 30°C with continuous shaking, the enzyme production was induced by 1.0 mM IPTG. After induction, the cultures were further incubated for a minimum of four hours. The crude enzyme was obtained by removing the bacterial cells by centrifugation.

The enzymatic activity of the obtained crude was assessed using 1% birchwood xylan from Carl Roth GmbH, and the dinitrosalicylic acid method (DNS) modified from Bailey et al. (1992). Absorbance was measured using a spectrophotometer at 540 nm wavelength. The pH activity profile of the enzyme was determined at 40°C with pH values that ranged from 3–8 using citrate-phosphate buffer (50 mM, pH 3–7), and potassium phosphate buffer (50 mM, pH 8). The optimal activity of the enzymes was determined at different temperatures that ranged from 25–55°C (pH 4.5). The assays were conducted in triplicate.

RESULTS

Cultural characterisation

P. infestans and SimOT6 were analysed for their ability to utilise different substrates, such as pectin cellulose, lignin and starch, as unique carbon sources. Growth was measured from the edge of the inoculation plug to the external edges of the colony (radius). SimOT6 and *P. infestans* seemed to grow actively on all substrates, however there was a distinguishable difference in their growth pattern. *P. infestans* is a slow growing fungus and thus required more time compared to SimOT6 (Figure 2).

One-way ANOVA was used to compare the effect of the tested substrates (pectin, xylan, CMC, starch, lignin and MEA) on *P. infestans* growth. The results showed significant differences in *P. infestans* growth rates ($F(5, 24) = 10.823$, $p < 0.001$). Post hoc Tukey's HSD test revealed that MEA promoted greater growth compared to lignin, cellulose and xylan, while pectin also supported significantly greater growth than xylan, cellulose and lignin ($p < 0.05$). No significant difference was found between *P. infestans* growth rates on MEA and pectin.

Statistical analysis of SimOT6 growth rates using one-way ANOVA revealed significant differences between substrates ($F(5, 24) = 77.148$, $p < 0.001$). Tukey's HSD post hoc test showed that all pairwise differences were significant, except between starch and MEA ($p = 0.974$).

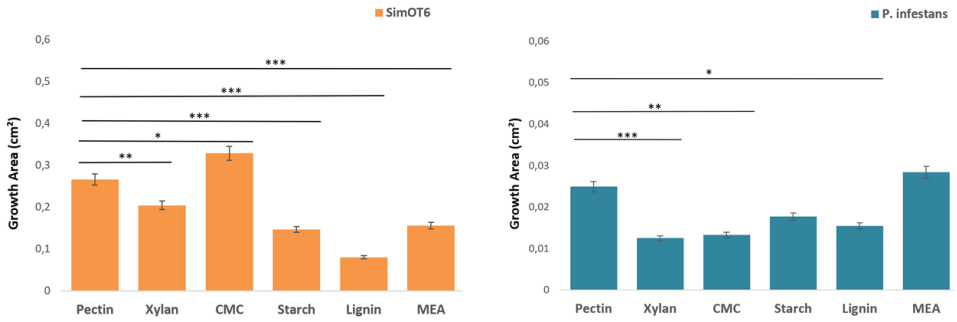

Figure 2. Growth area (cm^2) of SimOT6 and *P. infestans* on different carbon sources over a 12-day period. The asterisks indicate levels of statistical significance: $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$(***).

Table 3. Results of the extracellular enzyme screening on plates after a 12-day period. Values are expressed as the diameter of the clear zone around the fungal growth (mm).

Fungus	Pectinase	Xylanase	Cellulase	Amylase
SimOT6	3.5	-	5	-
P. infestans	29	17	33	-

The screening for secreted enzymes revealed contrasting patterns between the two fungi. *P. infestans* appeared to produce a more robust extracellular enzymatic machinery (Table 3). Under the tested conditions, SimOT6 demonstrated potential production of only two extracellular enzymes: pectinase and cellulase. Difference in the secretion (by SimOT6) of these two enzymes was found to be non-significant. *P. infestans* showed the greatest zone of clearance around the colony when cultivated on CMC followed by pectin and xylan. Extracellular amylase was undetectable under the tested conditions. The differences in extracellular enzyme production (assessed by comparing the clearance zone around the colonies) in *P. infestans* were statistically significant ($p < 0.05$). Tukey's HSD test confirmed that the potential enzyme secretion on CMC was significantly the highest, followed by pectin and xylan, respectively. Although SimOT6 grew more rapidly than *P. infestans*, the latter appeared to produce more secreted enzymes, which nevertheless did not transform into a greater growth rate.

Genome features and similarity search

PacBio sequencing followed by assembly with Flye 2.7 produced genome assemblies of 54,496,883 bp in 29 contigs for SimOT6 and 36,805,277 bp in 44 contigs for *Phacidium infestans* (Table 4). Quality control included read filtering with minimum length cut-offs of 12,005 bp (SimOT6) and 14,783 bp (*P. infestans*), and a minimum overlap of 5,000 bp. K-mer analysis used a size of 17, with erroneous and repetitive k-mers removed. Coverage-based filtering applied a minimum read coverage of 3 and a hard minimum coverage of 10. Disjointigs with low read support or excessive inner overlaps were discarded. Repeat resolution and graph simplification removed tandem repeats and flagged chimeric junctions to improve assembly accuracy. Core genome completeness, assessed with BUSCO, indicated 98.6 % completeness for *P. infestans* (BUSCO 3.0.2, fungi dataset) and 98 % for SimOT6 (BUSCO 4.0.2, eukaryota dataset). The genome sequences have been deposited in NCBI database under the accession JAFEVB000000000 for *P. infestans* and accession JAJSDM000000000 for SimOT6. COMPANION annotation provided data related to the number of genes, pseudogenes and RNA genes, among others genome features (Table 4).

Table 4. Genome statistics of SimOT6 and *P. infestans* obtained by COMPANION.

Feature	SimOT6	*P. infestans*
Chromosome size (bp)	54,496,883	36,805,277
Number of annotated regions/contigs	29	44
Number of identified genes	7884	3734
Gene density (genes/megabase)	139.07	98.98
Number of coding genes	7,579	3643
Number of pseudogenes	3,287	3,334
Number of genes with function	6,515	2,965
Number of pseudogenes with function	3,263	3,321
Number of non-coding genes	305	91
Number of genes with multiple CDSs	5,047	2,335
Overall GC%	51.64	46.39
Coding GC%	57.12	51.33
mRNA	7,579	3,643
tRNA	216	48
snRNA	11	10
rRNA	78	33

A similarity search was conducted for the sequenced strains to identify their closest relatives based on ITS (ITS1 and ITS4) and whole-genome sequences. Phylogenetic analysis of the ITS sequences from *P. infestans* showed that the fungus clustered with *P. infestans* accession U92305.1 with 99 bootstrap support. Pairwise identity analysis of ITS sequences between DSM5139 (865 bp) and the partial sequence from U92305 (479 bp) revealed a high level of conservation within the aligned region (99.37%), with near-perfect identity across 479 base pairs. Minor differences comprised a single nucleotide substitution and a small insertion/deletion near the 3′ end.

Similarly, the ITS sequences of SimOT6 clustered on the same clade as the reference strains *C. salicis* CBS 607.94 and *C. orchidophilum*. Pairwise identity analysis of ITS sequences between SimOT6 (580 bp) and *C. salicis* CBS 607.94 accession JQ948460.1 (538 bp) revealed a high level of conservation within the aligned region (99.83%), with near-perfect identity across 538 base pairs. Minor differences were limited to a single nucleotide substitution and an insertion at the 3′ end of SimOT6.

Whole-genome analysis of *P. infestans* indicated that *Lachnellula subtilissima* strain CBS 197.66 (accession QGMJ00000000), followed by *Lachnellula. hyalina* CBS 185.66, were the closest relatives available in public databases. Similarity search between *P. infestans* and *L. subtilissima* showed 92.01% ANI and 59.7% [56.9–62.5%] genome-to-genome distance (digital DNA–DNA hybridisation). SimOT6 exhibited a higher identity with the genome sequence of *C. salicis* CBS 607.94 (accession JFFI00000000.1). ANI analysis revealed 97.44% identity between the two genomes and 75.60% [72.6–78.4%] DNA–DNA hybridisation. Based on these results, and as expected, *P. infestans* and *L. subtilissima* were found to belong to different species (and genera). In contrast, the DNA–DNA hybridisation results suggest that SimOT6 and *C. salicis* belong to the same species but may represent different subspecies.

Prediction of protein-coding sequences in the genomes

Augustus annotation produced 11,357 putative protein sequences for *P. infestans* and 10,397 putative protein sequences for SimOT6. Both protein datasets contained a small number of sequences with internal stop codons. Each affected sequence was split into two parts: one preceding and one following the stop codon, with the second fragment starting at the first codon immediately after the stop codon. Therefore, the total number of protein sequences was 11,381 for *P. infestans* and 10,406 for SimOT6. The range of protein sizes was 55–8,028 amino acids in *P. infestans* and 36–8,853 amino acids in SimOT6. Size distribution of the proteins showed that the mean size was 526 (±405) amino acids in *P. infestans* (Figure 3), and 419 (±343) amino acids in SimOT6 (Figure 4). *P. infestans* had 111 protein sequences that are over 2,000 amino acids long. The corresponding number in SimOT6 was 53 protein sequences. Both strains have two large protein sequences: 6,658 amino acid- and 8,028 amino acid-long proteins in *P. infestans*, and 7,646 amino acid- and 8,853 amino acid-long proteins in SimOT6.

In comparison to *P. infestans*, *L. subtilissima* genome had 11,875 proteins (mean 472±355 amino acids) and 72 proteins with over 2,000 amino acids; the two largest proteins had 7,943 and 8,904 amino acids. In comparison with SimOT6 genome, the genome of *C. salicis* CBS 607.94 had 13,783 proteins (mean 475 ±360 amino acids) with 87 proteins over 2,000 amino acids; the two largest proteins were 8,731 and 8,109 amino acids long. On average, there were longer proteins in *P. infestans* compared to *L. subtilissima* strain CBS: 197.66 and *L. hyalina* CBS: 185.66.

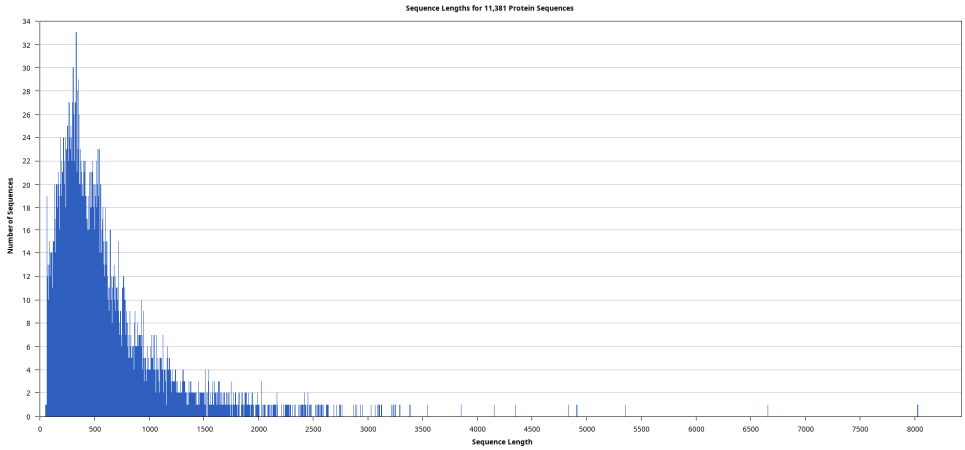

Figure 3. Size distribution analysis of *P. infestans* protein sequences using Geneious Prime.

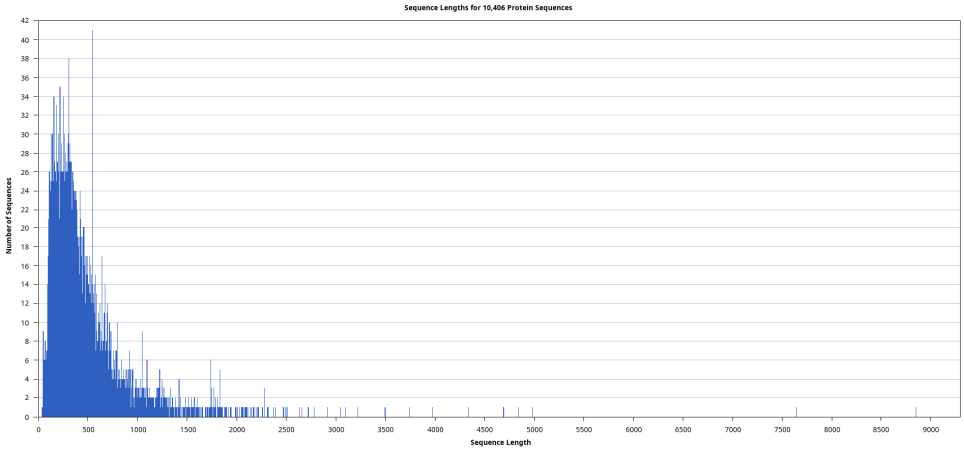

Figure 4. Size distribution analysis of SimOT6 protein sequences using Geneious Prime.

Carbohydrate-active enzyme analyses

A total of 1,692 potential CAZymes were predicted in *P. infestans*, of which 255 were predicted as secreted proteins that harboured an intact signal peptide located in their N-terminal region. Around 178 secreted CAZyme were identified by at least two dbCAN tools, and 77 by only one tool. In contrast, SimOT6 indicated 1,530 potential CAZyme entries, of which 202 entries were identified as a secreted protein (with a signal peptide). A total of 147 secreted CAZymes were identified by at least two dbCAN tools, and 55 entries by only one dbCAN tool (Table 5).

After host penetration, the fungi must be able to grow and acquire nutrients from host tissues. Therefore, the secreted CAZymes that target plant cell wall components were further investigated.

Table 5. Carbohydrate-active enzyme (CAZyme) families in SimOT6 and *P. infestans* (secreted and non-secreted proteins) predicted by at least one of the dbCAN3 tools.

CAZyme family	**SimOT6**	***P. infestans***
Glucoside-Hydrolases (GH)	848	883
Carbohydrate-Binding-Modules (CBM)	145	141
Polysaccharide-Lyases (PL)	41	30
Auxiliary-Activities (AA)	233	240
Carbohydrate-Esterases (CE)	75	84
Glycosyltransferases (GT)	306	416
Secreted proteins (with an intact signal peptide)	202	255
Total CAZyme entries	1530	1692

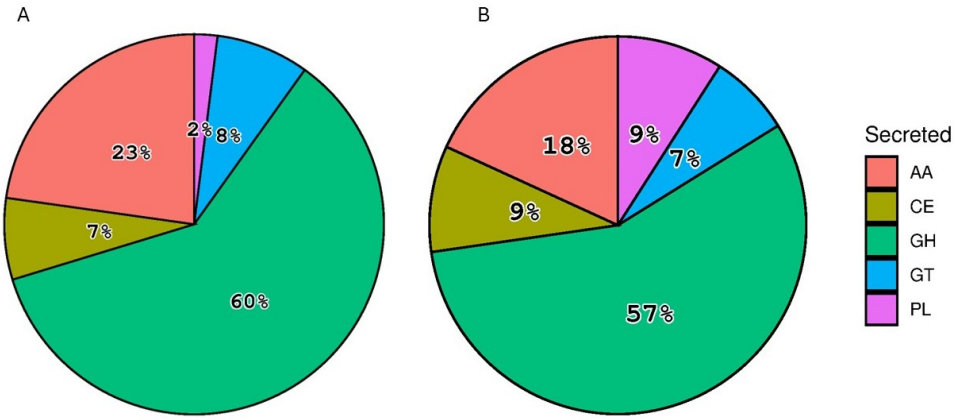

Figure 5. Secreted Carbohydrate-active enzymes (CAZymes) identified by all dbCAN3 tools. A) *P. infestans* secreted CAZymes, and B) SimOT6-secreted CAZymes.

Glucoside-hydrolase (GH) family enzymes were the most abundant secreted proteins (Figure 5). In *P. infestans*, a total of 99 secreted GH enzymes were identified, and 79 secreted GH were found in SimOT6 (identified by at least two dbCAN3 tools).

Two non-secreted GH10 enzymes and one secreted GH11 (proseq9131) were identified in *P. infestans*. GH10 and GH11 enzymes target xylan and are implicated in cell wall penetration. In SimOT6, one secreted GH11 (seqg5620.t1) and five secreted GH10 were identified, from which, one GH10 enzyme was observed to contain cellulose-binding domain CBM1 (seqg6555.t1). In this study, two secreted GH12 enzymes in *P. infestans* and four in SimOT6 (three GH12 were found by only one prediction tool) were identified by dbCAN3.

P. infestans seemed to possess several secreted GH3, GH5, GH16 and GH131 family members, which target cellulose and β-glucans. In addition, *P. infestans* exhibited ten secreted GH28 and six secreted GH78 enzymes, which participate in pectin degradation. Only one secreted GH88 was identified in *P. infestans* (predicted by DIAMOND); this enzyme is involved in the degradation of pectin in the plant cell wall. A total of 22 CMB50 modules were identified in *P. infestans*. CBM50 can bind to various enzyme families, such as GH18, GH19, GH23, GH24, GH25 and GH73.

SimOT6 exhibited four secreted GH5 that target cellulose and hemicellulose, two GH6 (act on cellulose), eight GH16 (target hemicellulose, β-1,3 or β-1,4 glycosidic bonds in glucans, and galactans), seven GH28 (target pectin), and twelve secreted GH43 enzymes that target hemicellulose (especially arabinoxylan) and pectin. Other CAZyme families, such as GH27, GH51, and GH93, may also contribute to plant cell wall degradation, as these proteins typically target hemicellulose within plant cells. Between one and three secreted proteins from these GH families were identified in both *P. infestans* and SimOT6.

Aside from GH enzymes, other families, such as PL, CE and AA, also play a crucial role in phytopathogenesis (Lyu et al. 2015). SimOT6 exhibited over four times more secreted polysaccharide-lyase enzymes than *P. infestans* (Figure 5). In the PL family, PL1, PL3 and PL4 proteins target pectin as a substrate. SimOT6 possesses eight secreted PL1, six secreted PL3 enzymes, and only one secreted PL4. Fewer PL enzymes were found in *P. infestans* compared to SimOT6 (three PL1 enzymes, one PL3 enzyme and one non-secreted PL4).

Several secreted Auxiliary Activity (AA) family enzymes were identified, with a greater number detected in *P. infestans* than in SimOT6 (Figure 6B). In *P. infestans*, a total of 44 secreted proteins from AA1–AA10 families were observed, with the highest numbers in AA7 (16 proteins) and AA9 (8 proteins). SimOT6 exhibited fewer AA1–AA10 enzymes (32 in total), with the highest counts in AA3 (6 proteins) and AA9 (11 proteins). In addition, *P. infestans* contained five AA1 and five AA2 proteins, while SimOT6 contained four AA1 and two AA2 proteins, these enzymes target lignin.

Secreted CE families, such as CE4 (active on chitin and xylan), CE5 (targets cutin and xylan), CE8 (acts on methylated pectin) and CE12 (targets acetylated pectin as substrate) were found in *P. infestans* and SimOT6. However, SimOT6 indicated greater numbers of these protein families (Figure 6A). COMPANION (web server for genome annotations) showed the presence of three sequences predicted as putative cutinases in *P. infestans* and 18 in SimOT6.

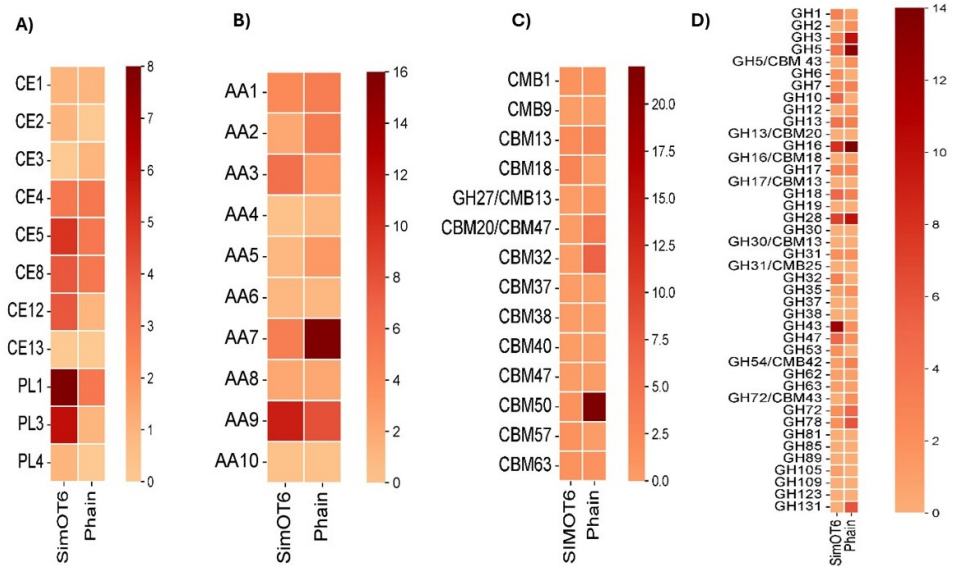

Figure 6. Heatmap of the secreted carbohydrate-active enzymes (CAZymes) that harbour a signal peptide and are potentially involved in plant cell wall degradation. The CAZyme families were identified in SimOT6 and *P. infestans* (Phain) using dbCAN3 and screened for signal peptides. A) Carbohydrate Esterase (CE) and Pectin Lyase (PL) family enzymes, B) Auxiliary Activity (AA) family enzymes, C) Carbohydrate-Binding-Module (CBM), and D) Glycoside-Hydrolase (GH) family enzymes. The heatmap colour strengths indicate the number of members in each protein group as shown in the bars with colour gradients.

The search for signal peptide and active sites in *P. infestans* cutinases indicated the presence of two secreted proteins with the presence of the catalytic triad, aspartate, serine and histidine, in their active sites. These cutinase proteins were structurally modelled (not shown) and indicated 50% sequence identity with the structure of the 4PSC cutinase from *Trichoderma reesei*. Protein structure modelling revealed the potential presence of a lid covering their active sites. A third putative cutinase was identified in *P. infestans*, however this enzyme did not have signal peptide, and only Ser was found in the conserved region (absence of His and Asp). In comparison, all the putative cutinases from SimOT6 were found to harbour a signal peptide. Their identity was further confirmed by BLASTp. The alignment of SimOT6 cutinases revealed the presence of a highly conserved region (GYSQG), as well as the presence of the Ser-His-Asp catalytic triad.

Secondary metabolite analysis

Secondary metabolite analysis using AntiSMASH (fungal version) indicated the presence of 51 regions that potentially code for BGCs in the *P. infestans* genome. Of those, only eight regions showed similarities with known BGCs. A total of 100% hits were registered with three known BGCs: Aspulvinone H/B1 (similar to *Aspergillus terreus* NIH2624), choline BGC from *Aspergillus nidulans* FGSC A4 and UNII-YC2Q1O94PT (ACRL toxin I B) from *Alternaria alternata*. A 40–66% similarity was found with BGCs that coded for the PR-toxin from *Penicillium roqueforti* FM164, trichoxide from *Trichoderma virens* Gv29-8, gregatin A from *Penicillium* sp., and squalestatin S1 from *Aspergillus* sp. Z5.

AntiSMASH indicated the presence of 55 regions in SimOT6 that potentially harboured BGCs. Thirteen potential BGCs showed similarities with known biosynthetic clusters. Four BGCs exhibited 100% similarity with known clusters. A total of 100% hits were recorded with the nectriapyrone BGC from *Pyricularia oryzae* 70–15, the choline BGC from *A. nidulans* FGSC A4, the xenolozoyenone BGC from *Glarea lozoyensis* and the 1,3,5,8-tetrahydroxynapthalene BGC from *P. oryzae* 70–15. Between 40–60% similarity hits were registered for squalestatin S1, alternapyrone, and aurofusarin synthesis genes. Between 15–31% identity hits were found for the BGC that code for the synthesis genes of mulundocandin, zearalenone, xenoacremone, monacolin K, metachelin C and cercosporin.

Virulence, pathogenicity factors and toxins

We searched for virulence and pathogenicity factors in *P. infestans* and SimOT6 in studies **I** and **III**, respectively. In *P. infestans*, three proteins were identified as pathogenesis-related proteins, one as a virulence protein and three as oxalate decarboxylases. In addition, fujikurin BGC (PKS19) were found using Geneious Prime, which would suggest the potential ability of *P. infestans* to synthesise fujikurin. Geneious Prime analysis also indicated the presence of a pyriculol pathway (incomplete in *P. infestans*) similar to *P. oryzae*. Moreover, aristolochene synthase protein was predicted in *P. infestans*; the protein structure modelling confirmed a fully conserved active site, which would suggest that the enzyme is likely functional. In addition, several clusters associated with fungal toxins were found in *P. infestans*. These clusters included aflatoxin biosynthesis proteins and aftlatoxin B synthase, as well as 22 potential satratoxin biosynthesis proteins. Furthermore, five AM-toxins, nine T-toxins, six AF-toxins, one AK-toxin and two ACT-toxins, as well as other

potential toxins (ochratoxin, gliotoxin and gamma-toxin) were predicted in *P. infestans*.

In SimOT6, 10 effector proteins were identified by InterProScan as necrosis- and ethylene-inducing peptide 1-like proteins (NLPs). Similar number of NLPs (Nep1-like protein) was also found in *C. salicis* CBS 607.94. In SimOT6, four protein sequences were missing a signal peptide and appeared shorter than *C. salicis* CBS 607.94 proteins. However, the six remaining effector proteins harboured a signal peptide, and their length was almost similar to the other *Colletotrichum* proteins. These effectors also displayed a partly conserved amino acid motif (GHRHDWE) in the central part of their domain (study **III**). None of the SimOT6 Nep1-like effectors contained a conserved motif. In contrast, *C. salicis* CBS 607.94 exhibited three Nep1-like proteins (KXH35489.1, KXH54096.1, and KXH55784.1) with a fully conserved heptapeptide motif. These results clearly highlighted the differences between SimOT6 and *C. salicis* CBS 607.94 in terms of host interaction.

A rare lipoprotein A (RlpA)-like double-psi beta-barrel protein (structurally conserved protein) associated with virulence was predicted in SimOT6 (Charova et al. 2020). In addition, a CAP20-like protein, which is a lipid droplet-coating protein found in fungi such as *Colletotrichum* and considered as a key protein for appressorium development and full virulence, was found in SimOT6 (Lin et al. 2018). This protein has a 100% amino acid sequence identity shared with the same protein in *C. salicis* CBS 607.94.

Cold adaptation of *P. infestans*

Cold adaptation proteins

The physiological mechanisms of cold tolerance in fungi are complex and involve various processes, which include the secretion of cryoprotectant, antifreeze proteins and enzymes that remain active at low temperatures. In this respect, the *P. infestans* protein list was screened for factors that potentially could provide cold tolerance capabilities. The search resulted in the identification of two secreted Ice-Binding-Proteins (IBPs). An evolutionary analysis of these IBP sequences was conducted using MEGA11. The resulting phylogenetic tree was drawn to scale, and the branch lengths represent the number of substitutions per site. This analysis involved 27 amino acid sequences from which two sequences belonged to *P. infestans*, and 25 sequences were retrieved from the NCBI GenBank (Figure 7). Positions with < 95% site coverage were removed, and ambiguous bases were allowed at any position using the partial deletion option. The tree was constructed using the Maximum Likelihood method combined with the Whelan and Goldman model (WAG+F+G).

The phylogenetic tree indicated that *P. infestans* sequences were clustered on the same clade as *Antarctomyces psychrotrophicus* and *Antarctomyces pellizariae* IBPs with 99 bootstrap support. *Antarctomyces psychrotrophicus* is a psychrotolerant (cold-adapted) fungus that possesses three IBP isoforms. The IBP protein proseq-9011and proseq-10963 from *P. infestans* were grouped with the protein sequence BBD74546.1 (isoform 2) from *A. psychrotrophicus* supported by 69 bootstrap support. Phylogenetic analysis of *P. infestans* IBPs indicated that they might function similarly to the IBPs from *A. psychrotrophicus* and *A. pellizariae*, which would hint at a potential evolutionary link between these cold-adapted fungi.

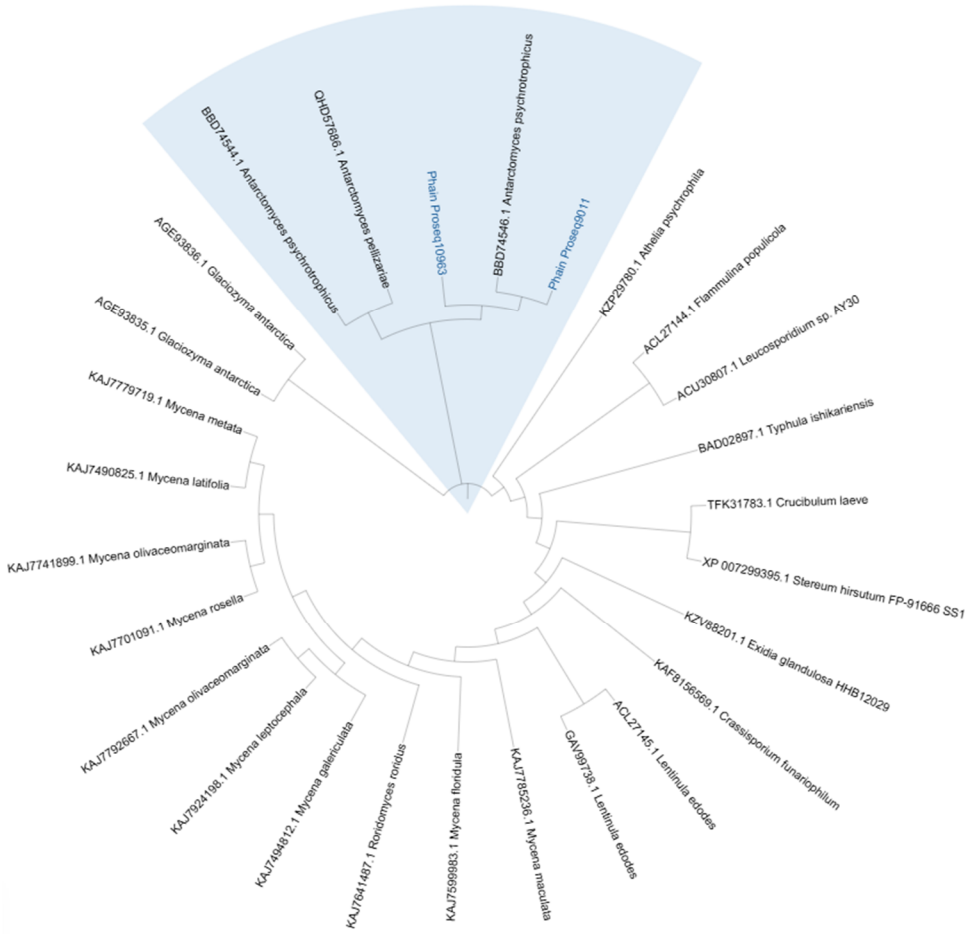

Figure 7. Phylogenetic analysis of *P. infestans* (Phain) Ice-Binding-Proteins (IBPs). The tree was constructed using MEGA 11 and the WAG+F+G model based on 1000 bootstrap replications. The clade that contains *P. infestans* sequences is shaded in blue, with the associated sequences (Phain proseq-9011 and Phain proseq-10963) marked in blue text on the phylogenetic tree.

A total of eight putative stress proteins were identified in *P. infestans*, and these could play a potential role in stress response. *P. infestans* protein annotation indicated the presence of several desaturases. Mannitol-, glycerol- and trehalose-related proteins and pathways were examined using the UniProt (Swiss-Prot) database. In total, five mannitol dehydrogenases were identified (proseq-3048, proseq-8770, proseq-68, proseq-4152 and proseq-65), two polyol dehydrogenases (proseq-1401, proseq-8533) and two polyol transporters (proseq-7722, proseq-9414). Many putative glycerol-related proteins were found in *P. infestans*, which included glycerol dehydrogenases (proseq-7612, proseq-7710, proseq-10150, proseq-10999 and proseq-7121). One phospholipid, diacylglycerol acyltransferase, was identified and attributed to the protein sequence proseq-378.

In fungi, trehalose synthesis involves two enzymatic steps. The first enzyme implicated is trehalose-6-phosphate-synthase; in *P. infestans*, this enzyme was attributed to the protein sequences proseq-10883 (97.9% shared identity with *L. hyalina*) and proseq-343 (96.1 % shared identity with *L. hyalina*). The second reaction is mediated by trehalose-6-phosphate phosphatase identified as the proseq-547 sequence in *P. infestans* (98.9% shared identity with *L. subtilissima*).

Aside from these, five α-trehalose glucohydrolases were identified in *P. infestans* and attributed to the protein sequences proseq-609, proseq-810, proseq-10883, proseq-8057 and proseq-922. In addition, one trehalose synthase (proseq-2150) was found in *P. infestans.* Based on the identified protein sequences, *P. infestans* seems to have a complete and functional trehalose pathway, and this might play a significant role in its cold tolerance.

Mass spectrometry analysis of P. infestans metabolites in freezing temperatures

The ability of *P. infestans* to withstand cold conditions was investigated by incubating the fungus at 22ºC and -3ºC for 14 days. The cultures were extracted using cold methanol and analysed by LC-MS/MS, which detected thousands of compounds. The difference in the compounds detected at -22ºC and -3ºC was statistically evaluated by a t-test.

Sixteen compounds showed a statistically significant difference between the samples cultivated at -3ºC and 22ºC (*p*-values cutoff ≤ 0.05); however, the majority of the detected compounds showed non-significant differences between the two treatment groups (Table 6). Annotation of the mass spectrometry compounds was conducted at four levels (level 1, 2a, 2b and 3) and the calculated ratio (-3ºC/22ºC) was used to provide a comparative measure of how temperature affected the consumption or production of the detected compounds.

Annotation level 1 detected numerous compounds; however, none presented a significant *p*-value. Three compounds detected by the annotation level 2a showed statistically significant differences between the two treatment groups (-3ºC and 22ºC). Acetylmuramic acid was over three times greater at -3ºC compared to the 22ºC samples, which would suggest possible production or accumulation of this compound in freezing temperatures.

A total of six compounds detected by the annotation level 2b indicated a statistically significant difference between the samples cultivated at -3ºC and 22ºC (*p*-values≤ 0.05). Carbidopa exhibited one of the highest ratios and was over four times more abundant at -3ºC. Annotation level 3 detected a total of seven compounds that showed a significant difference between the two treatment groups (-3ºC and 22ºC). The highest ratio was attributed to g-butyrobetaine, followed by dihydrolipoamide, and AC-D-PRO-OH which is a modified proline derivative (Table 6). The compound annotated as 1-methyl-8-methylene-3-oxogibb-4-ene-1,10-dicarboxylate seemed to be a gibberellin derivative.

Table 6. Mass Spectrometry results (LC-MS/MS) of the compounds detected in *P. infestans* samples cultivated at -3ºC and 22ºC, presenting a significant difference between the two treatment groups.

Annotation	Compound	Formula	-3ºC/22ºC[a]	*p*-value[b]
Level 2a	Ferulic acid	$C_8H_{12}O_4$	0.93	0.03
Level 2a	Dihydrothymine	$C_5H_8N_2O_2$	1.68	0.04
Level 2a	Acetylmuramic acid	$C_{11}H_{19}NO_8$	3.9	0.05
Level 2b	2'-Deoxyadenosine	$C_{10}H_{13}N5O_3$	1.96	0.04
Level 2b	Sepiapterin	$C_9\ H_{11}\ N_5\ O_3$	2.9	0.003
Level 2b	Carbidopa	$C_{10}H_{14}N_2O_4$	4.47	0.04
Level 2b	Toliprolol	$C_{13}H_{21}NO_2$	2.77	0.02
Level 2b	10b-methyl-1,5,6,10b-tetrahydroimidazo [2,1-a] isoquinolin-2(3H)-one	$C_{12}H_{14}N_2O$	0.38	0.009
Level 2b	5-Methyl-2-(4-methylphenyl)-2,4-dihydro-3H-pyrazol-3-one	$C_{11}H_{12}N_2O$	0.29	0.056
Level 3	acrylic acid	$C_3H_4O_2$	1.86	0.04
Level 3	Pyrrolidone	C_4H_7NO	0.77	0.009
Level 3	5-Methyl-2,5-cyclohexadiene-1,4-diol	$C_7H_{10}O_2$	0.58	0.056
Level 3	g-butyrobetaine	$C_7H_{15}NO_2$	26.57	0.04
Level 3	(2S,3S)-2,3-dihydro-3-hydroxyanthranilic acid zwitterion	$C_7H_9NO_3$	3.5	0.059
Level 3	AC-D-PRO-OH	$C_7H_{11}NO_3$	3.01	0.009
Level 3	Dihydrolipoamide	$C_8H_{17}NOS_2$	5.11	0.0003
Level 3	1-methyl-8-methylene-3-oxogibb-4-ene-1,10-dicarboxylate	$C_{19}H_{20}O5$	0.93	0.05

[a] Ratio of the average value of the area (chromatographic peak) obtained at -3ºC divided by the average relative area obtained at 22ºC.
[b] Calculated using two-tailed t-test, type III between the values obtained at -3ºC and 22ºC samples

Adaptation of *P. infestans* to chemical environment

The ability of *P. infestans* to survive on pine needles was investigated in study **II** with the FIA technique using DI-HRMS and both APPI and ESI ionisation modes. In this study, whole-genome sequence analysis, proteomic profiling and mass spectrometry-based metabolomics were combined to reveal the genetic potential and metabolic adaptations of *P. infestans* during colonisation of Scots pine needles. Focus was placed on the phenylpropanoid pathway linked to detected metabolites, as well as the utilisation of phenolic compounds and carbohydrates present in the pine needles.

Over 500 features were detected in the needle extract using the ESI mode. The annotation of these compounds resulted in 66 being identified, with their formulas and compound names using MetaboScape software (Table 7). Two main patterns were observed. The first comprised a group of compounds that decreased significantly in the needle extracts after *P.*

infestans growth. This group included carbohydrates, such as D-glucose, D-xylose, octose, disaccharide, hexose (unidentified) and cellobiose (p-value ≤ 0.05). Similarly, a statistically significant decrease was observed in some sugar acids that included glucuronic acid, gluconolactone, glucoheptonic acid, 4-O-Methyl-D-glucaric acid and 5-Dehydro-4-deoxy-D-glucaric acid (p-value ≤ 0.05). The methylated glucose derivative methyl-D-glucoside, along with the sugar conjugates galactosyl pinitol and galactosylglycerol, were significantly decreased in the *P. infestans* samples. Benzoic acid, coumaric acid, salicylic acid, shikimic acid, vanillic acid and the phenolic glycoside tachioside decreased in the needle extract after *P. infestans* growth, in comparison to the controls (needles without *P. infestans*).

The second observed pattern involved resin acids. These compounds showed a different pattern compared to the previous compound group and were present in larger amounts on the needles post- *P. infestans* growth. The identified resin acids included dehydroabietic acid, dehydropinifolic acid, hydroxyoxodehydroabietic acid, imbricatolic acid, isocupressic acid, lambertianic acid, pinifolic acid, pinifolic acid derivatives, pinonic acid, pinusolidic acid and ß-caryophyllonic acid (Table 7).

Table 7. Mass spectrometry results of some of the compounds detected in the needle extracts using the Electrospray Ionisation (ESI) mode.

Compounds	**Formula**	**CTR/SA ratio** [a]	***p*-value**[b]
Benzoic acid	$C_7H_6O_2$	7.46	0.015
Coumaric acid	$C_9H_8O_3$	16.81	0.009
Dehydroabietic acid	$C_{20}H_{28}O_2$	0.34	0.002
Dehydropinifolic acid	$C_{20}H_{30}O_4$	0.47	0.008
Galactosyl pinitol	$C_{13}H_{24}O_{12}$	60.46	0.015
Galactosylglycerol	$C_9H_{18}O_8$	36.6	0.038
Hydroxyoxodehydroabietic acid	$C_{20}H_{26}O_4$	0.33	0.0004
Imbricatolic acid	$C_{20}H_{34}O_3$	0.06	< 0.0001
Isocupressic acid	$C_{20}H_{32}O_3$	0.46	0.01
Lambertianic acid	$C_{20}H_{28}O_3$	0.31	< 0.0001
Methyl-D-glucoside	$C_7H_{14}O_6$	13.68	0.095
Pinifolic acid	$C_{20}H_{32}O_4$	0.45	0.01
Pinifolic acid derivatives	$C_{20}H_{34}O_4$	0.09	< 0.0001
Pinonic acid	$C_{10}H_{16}O_3$	0.39	0.002
Pinusolidic acid	$C_{20}H_{28}O_4$	0.15	< 0.0001
Salicylic acid	$C_7H_6O_3$	47.24	0.0003
Shikimic acid	$C_7H_{10}O_5$	1.36	0.217
ß-caryophyllonic acid	$C_{15}H_{24}O_3$	0.47	0.0004
Tachioside	$C_{13}H_{18}O_8$	50.3	0.023
Vanillic acid	$C_8H_8O_4$	4.63	0.006

[a] Control-to-sample ratio (CTR/SA) calculated using the average of the absolute intensity of the normalised and scaled data.

[b] Calculated using t-test, tails 2, type III between the control values and the samples.

Table 8. Mass spectrometry results of some of the compounds detected in the needle extracts using the Atmospheric Pressure Photoionisation (APPI) mode.

Compound	Formula	CTR/SA ratio [a]	*p*-value[b]
Allohydroxymatairesinol	$C_{19}H_{20}O_6$	16.85	0.0007
Demethoxylpinoresinol	$C_{19}H_{20}O_5$	199.16	< 0.0001
Ligballinol	$C_{18}H_{18}O_4$	38.45	0.0002
Taxiresinol	$C_{19}H_{22}O_6$	289.76	0.0009

[a] Control-to-sample ratio (CTR/SA) calculated using the average of the absolute intensity of the normalised and scaled data.
[b] Calculated using t-test, tails 2, type III between the control values and the samples.

Analysis of the samples using the APPI mode detected over 350 features, among those 40 features were annotated by providing the formula and compound name. Several resin acids were detected, similarly to the ESI mode. The APPI mode analysis showed the presence of several lignans. Two unspecified lignans with the formula $C_{20}H_{22}O_5$ and $C_{19}H_{22}O5$ were completely consumed and reduced to zero. Similar observations were noted for pinoresinol and lariciresinol ($p < 0.05$). Other lignans, such as demethoxylpinoresinol, taxiresinol, ligballinol, and allohydroxymatairesinol, significantly decreased in the *P. infestans* (in presence of needles) samples compared to the controls (needles only) (Table 8).

To confirm that the detected compounds originated exclusively from the degradation and/or the assimilation of the needles content, a similar experimental design and extraction steps were conducted on *P. infestans* cultivated on malt extract broth. Malt extract broth (without the fungus) was also incubated and extracted similarly to serve as a control. The DI-HRMS analysis of the malt extract samples detected around 340 features with the ESI mode and over 1400 features with APPI. However, only a few were annotated with the formula and compound name. The ESI features of the malt extract samples mostly included monosaccharides, disaccharides, trisaccharides and oligosaccharides. The APPI mode indicated the presence of features that included phenolic compounds, such as 5-hydroxyferulic acid, acetyleugenol, ferulic acid, dihydroresveratrol and menadiol. These phenolic compounds presented CTR/SA ratios that ranged from 2.52 to 7.69 with $p \leq 0.001$ (excluding menadiol, $p = 0.12$), which would suggest their consumption by *P. infestans*.

Furthermore, malt extract derivatives and byproducts were identified and included maltosan, maltol galactoside, maltol 6'-O-beta-D-apiofuranosyl-beta-D-glucopyranoside and maltol. These compounds indicated CTR/SA ratios that ranged from 1.59 to 4.54 ($p < 0.05$, excluding maltosan). No resin acids nor lignans were detected in the malt extract samples. Malt extract compounds analysis highlighted its sugar-rich composition, which is supportive of rapid fungal growth.

Host-adaptation

Phacidium infestans exhibited genomic features that would suggest host-adaptation, which encompasses a wide range of CAZymes that are used to degrade cell wall components and acquire nutrients from pine needles. In addition, the presence of secreted cutinase enzymes enable the fungus to penetrate its host by breaking down the outer protective layer of the needles (cuticule). Moreover, lipases are involved in needle wax degradation. In total, 49 entries were associated with lipase functions, which included 13 GDSL-like lipases, six patatin-like phospholipases, three phospholipase D-like domains that contained proteins and

four lipases, with one secreted. Furthermore, two pisatin demethylases were identified in *P. infestans*, these enzymes are part of the cytochrome P-450 monooxygenase enzyme system.

In contrast, SimOT6 seemed to adapt to its host by harbouring less CAZymes and enzymes that target the cell wall components, such as GH family and CBM domains, compared to the reference *C. salicis* CBS 607.94 strain (study **III**). COG analysis showed that SimOT6 contained less protein entries compared to its closest relative in class G, which is attributed to carbohydrate transport and metabolism. In addition, it appeared that the smaller number of proteins in SimOT6 was also caused mainly by differences outside of basic metabolism. Othovenn3 analysis showed that there were many clusters related to pathogenicity and virulence that were present in *C. salicis* CBS 607.94 but missing in SimOT6. Analysis of SimOT6 adaptation to its host was fully investigated in study **III**. SimOT6 was studied for the presence of secondary metabolites. The results showed that this fugus exhibited the greatest number of terpenes biosynthetic gene clusters. A total of 16 terpene clusters were found in SimOT6 compared to 13 terpene clusters in *C. salicis* CBS 607.94. Eight NRPS-TPKS were found in SimOT6 and four in *C. salicis* CBS 607.94. SimOT6 also showed a greater number of BGCs compared to its closest relatives.

The comparative analysis of effector repertoires between SimOT6 and its closest relative *C. salicis* CBS 607.94 revealed notable differences (study **III**). SimOT6 encoded a total of 4,134 predicted effectors, which included 3,731 cytoplasmic effectors and 408 apoplastic effectors, while *C. salicis* CBS 607.94 encoded 3,402 predicted effectors, with 2,879 cytoplasmic and 505 apoplastic effectors. In addition, *C. salicis* CBS 607.94 had a greater proportion of dual-localised effectors compared to SimOT6, which likely reflect distinct infection strategies and ecological adaptations. In contrast, the greater number of apoplastic and dual-localised effectors noted in *C. salicis* CBS 607.94 indicated possible specialisation in extracellular interactions and adaptation to specific host environments or defence mechanisms.

Evaluation of GH11 endo-xylanase

Investigation of an example secreted enzyme may provide some direction with regard to the general temperature and pH properties of the fungus in question. GH11 xylanase is part of the enzymatic machinery that degrades plant cell wall materials, and therefore, its properties are likely to inform the behaviour of the fungus in these conditions. GH11 endo-xylanase from *P. infestans* was expressed in *E. coli*, this enzyme was then induced and secreted into the cultivation medium. The crude enzyme was collected and used for enzymatic characterisation by measuring the activity with the DNS method.

The enzyme exhibited mesophilic properties, with optimal activity at 45°C, and retained activity up to 55°C. These findings suggest that the *P. infestans* GH11 enzyme may function beyond the previously described growth temperature limits. Optimal growth of *P. infestans* has been reported to range from 10°C to 15°C, with growth ceasing at 25°C (Björkman 1948). GH11 showed optimal activity at pH 5.0 and remained active within a pH range of 3.4 to 4.5, becoming inactive at pH 8. The amino acid composition at the enzyme's active site suggests that this xylanase has a mildly acidic pH profile, indicating that the fungus may thrive under such conditions.

DISCUSSION

This thesis investigated two fungal phytopathogens that can be used as models to study molecular behaviour and fungus-host interactions, especially in forestry and agricultural ecosystems. *Phacidium infestans* DSM 5139 and *Colletotrichum salicis* strain SimOT6 were first studied for their phenotypes and cultural characteristics, with growth observed on different carbon sources. A preliminary agar-plate screening was then carried out to assess the secretion of carbohydrate-active enzymes, including pectinase, cellulase and xylanase. The cultural characterisation was compared with data from whole-genome sequence analysis. For example, the ability of the SimOT6 and *P. infestans* to grow on lignin was supported by the presence of potential lignin-modifying enzymes (e.g. AA1 and AA2) identified in the genome sequences.

Each fungus was investigated at the genomic level based on CAZymes, secondary metabolites, adaptation traits and virulence factors. Each genome was also compared to its closest relative using a wide array of tools, such as COG, KEGG and Orthovenn3.
Temperatures below zero under the snowpack on pine needles rich with chemical compounds is a challenging combination of conditions for microbial growth. To our knowledge, the whole-genome sequence of *P. infestans* DSM 5139 is the first genomic sequence available on any database for this species, and it is expected to shed light on microbial life at the molecular level under these extreme conditions.

The genome mining of *P. infestans* revealed functionally important genetic and molecular features. Whole-genome annotation of *P. infestans* using COMPANION revealed a relatively low gene count (3,734 genes). Gene numbers in fungi can be influenced by evolutionary streamlining, niche specialisation, genome-defence mechanisms, and sometimes by technical limitations of genome annotation. Such low counts often reflect adaptation to a highly specialised lifestyle (Fijarczyk et al. 2025; Stajich 2017). Analysis of its BGCs indicated the potential presence of several clusters that encode the enzyme groups that are needed to synthesise each secondary metabolite. Mycotoxins are among the secondary metabolites secreted by fungi and have several ecological roles. They are used to compete with other fungi, bacteria and predators, but they are also used to attack their hosts by suppressing their defences and disrupt their cellular integrity (Künzler 2018; Wang and Kuzyakov 2024). Some of the identified gene clusters were attributed to various toxins, for instance, PR-toxin, UNII-YC2Q1O94PT (ACRL Toxin I B), aflatoxin biosynthesis proteins, aflatoxin B synthase and satratoxin biosynthesis proteins. Satratoxin is a macrocyclic trichothecene mycotoxin that has been found to be produced by *Stachybotrys chartarum* (Islam et al. 2006). In total, 15 putative cercosporin biosynthetic proteins were identified in *P. infestans*. Studies on the photoactivated cercosporin toxins produced by *Cercospora* species have shown that this toxin plays a major role in pathogenesis of host plants by damaging the plant cell membranes and inducing leakage of nutrients, thereby supporting the growth of pathogenic fungi (Daub and Ehrenshaft 2000). Aflatoxins are carcinogenic mycotoxins known for their high toxicity, with aflatoxin B1 the most dangerous. They are produced by *Aspergillus* species and are able to contaminate a large variety of crops. Aflatoxins are known for their stability and difficult elimination (Rushing and Selim 2019). Aflatoxins in *A. flavus* and *A. parasiticus* help outcompete other microorganisms by inhibiting their growth (Mamo et al. 2021). They are also implicated in pathogenesis and host interactions (Lee et al. 2016). Five AM-toxins were predicted by Geneious Prime (UniProt).

These phytotoxins are classified into several types (types I, II and III) and interact directly with the host. In *A. alternata* f. sp. *mali*, AM-toxins are crucial as they facilitate the infection process by damaging the host plant's cells. Host-specific toxins (HSTs) characterised in *Alternaria* species include AAL-toxins, AK-toxins, AM-toxins, AF-toxins, ACR-toxins and ACT-toxins (Meena et al. 2017). Biosynthetic genes for most of these toxins (excluding AAL-toxins) were also found in *P. infestans*. The HSTs act by targeting different plant cell organelles and induce cell death in hosts (Meena et al. 2017). Furthermore, Fujikurin and aristolochene synthase enzyme were identified in *P. infestans*. Fujikurin is a secondary metabolite usually found in *Fusarium* species and has been linked to plant-fungal interactions, acting as a virulence factor (von Bargen et al. 2015). Aristolochene synthase enzyme plays a key role in the production of sesquiterpene-derived toxins, such as PR-toxins, which are implicated in host colonisation and disease progression (Proctor and Hohn 1993).

Secondary metabolite analysis of *P. infestans* using AntiSMASH indicated the presence of aspulvinone BGCs. This metabolite is usually produced by *A. terreus* and appears to be related to general stress responses. Aspulvinone is implicated in the growth, survival and metabolic processes of fungi (Guo et al. 2015). In addition, aspulvinones, including aspulvinone H, exhibit α-glucosidase inhibitor activity, which may play a role in the fungus' interaction with its environment or other organisms (Wu et al. 2021). A choline BGC was also identified in *P. infestans* and has been shown to help maintain glycine betaine homeostasis in fungi by supporting the conversion of glycine betaine to choline with a reversible choline to glycine betaine metabolic pathway (Hai et al. 2019). This pathway prevents toxic accumulation, provides metabolic flexibility and supports the growth and survival of fungi under varying environmental and nutritional conditions (Lambou et al. 2013). The genetic analysis of BGC in *P. infestans* and SimOT6 was indicative that these fungi could synthesise the corresponding secondary metabolites. Previous studies on *Colletotrichum* species have shown that specific enzymes, such polyketide synthases, might facilitate host adaptation, thereby enabling genetic plasticity (Jayawardena et al. 2021). Experimental evidence is required to confirm the production of these compounds and enzymes, while genetic analyses can provide guidance for this search.

The study of CAZymes in the selected fungal models (*P. infestans* and SimOT6) showed that *P. infestans* exhibited a much large number of CAZymes compared to its closest relatives (*Lachnellula* species). In contrast, SimOT6 exhibited a smaller number of CAZymes compared to the reference *C. salicis* CBS 607.94 strain. A growing number of studies has demonstrated that whether a fungus is pathogenic, latent pathogen or non-pathogenic is linked to the diversity and abundance of its CAZymes, especially those that target plant cell walls. Previous studies have shown that hemibiotrophic and necrotrophic fungi often secrete larger amounts of cell wall degrading enzymes compared to biotrophic fungi (Jia et al. 2023). These include cellulases, hemicellulases and pectinases (Lyu et al. 2015). In contrast, saprophytic and endophytic fungi seem to possess fewer CAZymes compared to pathogens, which would reflect their adaptation to decomposing dead organic matter or their ability to live within a plant without causing harm (Zhao et al. 2013). In this respect, the ability of *P. infestans* to degrade the plant's cell wall compounds confirms its necrophiliac lifestyle.

While some AA enzymes facilitate polysaccharide accessibility by modifying or degrading lignin in the plant cell wall. Others, such as AA7 and AA9, act directly on carbohydrates or participate in redox processes that enhance access to polysaccharides. Both *P. infestans* and SimOT6 exhibited a high number of AA secreted enzymes, with AA7

and AA9 being among the most abundant. AA7 enzymes are important in the redox network as they allow fungal pathogens to degrade plant cell walls and evade plant immune responses by modifying cell wall-derived oligosaccharide (Turella et al. 2025). Moreover, AA9 are LPMOs that are active on cellulose and hemicellulose (e.g. xylan, glucomannan). Previous research has demonstrated that during fungal biomass degradation, certain AA7 dehydrogenases can directly transfer electrons to AA9 LPMOs through the redox network, thereby enhancing cellulose degradation by AA9 LPMOs without the need for external reductants (Haddad Momeni et al. 2021).

Further metabolomic investigations were conducted on *P. infestans* using two experimental setups. The first experiment employed LC-MS/MS to compare extracts obtained from *P. infestans* incubated at -3°C and 22°C. The second experiment was conducted using DI-HRMS and aimed to understand *P. infestans* nutrient acquisition from needles and to evaluate its ability to tolerate, or even utilise, the needle compounds that are often considered toxic to microorganisms. The LC-MS/MS analysis indicated compounds present at significantly higher levels in the -3 ºC samples compared with those cultivated at 22°C. These included dihydrolipoamide, acetylmuramic acid, 2'-deoxyadenosine, carbidopa, toliprolol, sepiapterin, acrylic acid, g-butyrobetaine, AC-D-PRO-OH and dihydrothymine. The latter is part of pyrimidine metabolism and is associated with valine, leucine and isoleucine metabolism (Nyhan 2005). Pyrimidine metabolism is also implicated in fungal stress responses, which include temperature stress and/or nutrient-limited condition (Bouwknegt et al. 2022).

G-butyrobetaine is a carnitine precursor that is involved in fatty acid metabolism. Fungi are known to modify their membrane composition and fluidity in response to cold temperatures, often involving changes in lipid metabolism (Abu Bakar et al. 2020). In addition, carnitine is an important osmoprotectant that can enhance tolerance to heat (thermotolerance), cold (cryotolerance) and pressure (barotolerance). In the cells, carnitine can act as a compatible solute by protecting against stress or be used through specific pathways as a nutrient source (Meadows and Wargo 2015). It can be taken from the environment or synthesised from metabolic precursors (Meadows and Wargo 2015). In this study, carnitine levels were higher in the -3°C samples compared to those cultivated at 22°C, however, this increase was statistically non-significant under the tested conditions.
A previous study investigated the growth and metabolism of *Beauveria bassiana* (spores and mycelia) using HPLC-MS and KEGG database. The results showed a decrease in several compounds in spores, including dihydrothymine, acetylcarnitine and fructose-1, among others, and an increase in betaine, carnitine and glycerophospholipids. These metabolic changes were interpreted as mechanisms to maintain spore dormancy, protect against adverse conditions and enhance long-term survival (Liu et al. 2015). Some of these compounds, such as carnitine and betaine, exhibited a similar trend, with increased levels observed in the *P. infestans* samples incubated at -3°C. Compounds, such as 10b-methyl-1,5,6,10b-tetrahydroimidazo [2,1-a] isoquinolin-2(3H)-one, 2-pyrrolidone, 2-methyl-2,5-cyclohexadiene-1,4-diol and 1-methyl-8-methylene-3-oxogibb-4-ene-1,10-dicarboxylate were significantly decreased in the -3ºC samples.

Aside from the increase in potential cryoprotectants, *P. infestans* genomic annotation indicated the presence of two putative IBPs, a complete trehalose pathway and several desaturases. Apart from membrane fluidity, cold-adapted fungi must also increase the intracellular concentrations of trehalose and polyols, which act as cryoprotectants (Robinson 2001). Research on *Mrakia psychrophila*, an obligate psychrophilic fungus, has shown that desaturase and glycerol 3-phosphate dehydrogenase involved in the biosynthesis

of unsaturated fatty acid and glycerol were upregulated when the microbes were exposed to cold. The cold adaptation of *M. psychrophila* was found to be mediated by the synthesis of unsaturated fatty acids that maintain membrane fluidity and the accumulation of glycerol as a cryoprotectant (Su et al. 2013). Desaturases introduce double bonds into fatty acids and play a key role in maintaining membrane fluidity at low temperatures (Rodríguez-Vargas et al. 2007). Our LC-MS/MS results strongly suggest that *P. infestans* adjusts its metabolism to its environmental conditions (cold).

The DI-HRMS analysis showed that *P. infestans* uses sugars, such as hexose, xylose and disaccharides, and the cell wall polymers available in pine needles. Many of these compounds significantly decreased after *P. infestans* growth on the needles. Many lignans were also completely metabolised. Lignans have many biological properties, such as functioning as anti-inflammatory agents and as antioxidants. They also play a major role in protecting plants from pathogens (Rodríguez-García et al. 2019).

Previous *in vivo* and *in vitro* research on lignans in *Myristica fragrans* Houttyn (nutmeg) seeds demonstrated antifungal properties of these compounds against plant pathogenic fungi. The identified lignans included erythro-austrobailignan-6, meso-dihydroguaiaretic acid and nectandrin-B, which conferred protection against barley powdery mildew, tomato late blight and moderate inhibition of rice sheath blight (Cho et al. 2007). Few studies have been conducted on the use of lignans as a carbon source by fungi (Rugolo et al. 2022), and the metabolic pathways and enzymatic mechanisms involved in lignan degradation remain largely unexplored. However, our results strongly suggest that *P. infestans* is capable of utilising lignans.

Resin acid compounds are derived from coniferous tree resins and are structurally related to diterpenes. They typically have hydrophobic properties and play key roles in plant defence (Geisler et al. 2016). Most of the resin acids identified in the needle extracts increased after *P. infestans* growth (compared to the controls). Some of these resin acids can display antimicrobial properties, including dehydroabietic acid and β-caryophyllonic acid (Li et al. 2017; Ríos-López et al. 2024; Selestino Neta et al. 2017), although the abundance of these two compounds did not appear to have a strong impact on *P. infestans* growth.

Benzoic acid and salicylic acid are known to exhibit antimicrobial properties (Krátký et al. 2012). These compounds seemed to be metabolised by *P. infestans* since they significantly decreased in its samples. Benzoic acid and salicylic acid are metabolised through specific enzymatic pathways involving aromatic hydroxylation and ring-cleavage. In KEGG pathways, these reactions are classified under the degradation of aromatic compounds. Benzoic acid is typically hydroxylated to p-hydroxybenzoic acid and further degraded via ring-cleavage pathways, while salicylic acid undergoes hydroxylation to catechol or related compounds. Enzymes involved in the degradation of these two compounds were identified in study II, supporting the capacity of *P. infestans* to fully utilise them.

While the intracellular metabolic pathways used by fungi to detoxify and assimilate aromatic compounds from plant biomass are not fully understood, it is well established that fungi are the main degraders of plant-derived aromatics (Mäkelä et al. 2015). *P. infestans* genome mining indicated the presence of two putative proteins that permit the uptake and transport of acyclic terpenes and terpene detoxification (study **II**). In addition, several trichothecene efflux pumps, drug/metabolite transporters and pleiotropic drug resistance proteins were found. These transporters help maintain cell integrity, virulence, and confer resistance against environmental and plant-related stresses (Popiel et al. 2019).

In this work, the relationships between the fungus and its environment, as well as between the fungus and its host, were explored. In SimOT6, several features of the genome data suggest a lower pathogenicity potential compared to its closest relative *C. salicis* CBS 607.94. Endophytes have obligate or facultative biotrophic lifestyles (Schulz and Boyle 2005) and exhibit a reduced CAZyme portfolio to minimise host damage and avoid triggering strong plant defence responses, which allows them to maintain their benign or latent colonisation (Lyu et al. 2015). The analysis and comparison of SimOT6 and *C. salicis* CBS 607.94 (study **III**) suggests that SimOT6 might belong to this category. However, it is not possible to determine with certainty whether a microorganism is an endophyte, or a latent pathogen based solely on genomic mining. In fact, fungal endophytes have been defined by mycologists as fungi living within the plant without causing visible symptoms (Schulz and Boyle 2005). Schulz and Boyle (2005) presented a hypothesis that neutral interactions do not happen. Instead, endophyte-host relationships are a balance of antagonistic forces. Fungal infection always requires some level of virulence, regardless of the infected plant organ. Host defence mechanisms restrict fungal growth and disease progression. If this interaction becomes unbalanced, disease might appear, and the host defence will actively fight the fungus (Kogel and Hückelhoven 2006). These hypotheses align with the results obtained from the SimOT6 genome mining in this thesis.

The presence of both conserved and less conserved virulence factors, including proteins such as secreted cutinases (found in CE5 family), and pectinases which are among the first enzymes employed by the fungi to invade their hosts (Arya and Cohen 2022). While direct studies on pectinase production specifically in *C. salicis* are limited, related *Colletotrichum* species such as *C. lindemuthianum* have well-characterised pectinolytic enzyme repertoires. These enzymes play important roles in host infection by degrading the pectin-rich middle lamella and primary cell walls, facilitating tissue invasion (da Silva et al. 2022). Therefore, it is reasonable to infer that pectinases are relevant to *C. salicis*, aiding the fungus in colonising and infecting its host plant. These findings suggest that under suitable conditions, SimOT6 may be capable of switching its lifestyle and adopting a virulent state.

Within the *Colletotrichum* genus, many fungi known as parasites in one host were found to exist as endophytes in another (Redman et al. 2001). Among those, *C. magna*, the causative agent of anthracnose in cucurbit plants, can grow asymptomatically in non-cucurbit species (Kogel and Hückelhoven 2006). As such, additional elements must be considered. A previous study by Manzotti et al. (2020) that assessed the endophytic communities of asymptomatic tomato roots found that root pathogens were the most abundant taxa. When these isolated fungi were inoculated onto tomato seedlings, the plants developed visible disease symptoms. This research suggests that the interactions among members of the microbiome community may play a key role in maintaining community equilibrium and could help prevent pathogens from causing disease (Manzotti et al. 2020). Fungi-plant interactions are a continuum from pathogenic to beneficial (Collinge et al. 2022). In addition, some studies have indicated that the distinction between endophytes and pathogens within a plant may be linked to their abundance or concentration. Microorganisms that behave as endophytes are usually present in lower quantities than pathogens, which may prevent them from triggering a plant response (Mishra et al. 2021). Numerous studies have highlighted the importance of secretomes, which also contain effector proteins. A comparison of effectors from pathogenic and endophytic *Fusarium oxysporum* strains by de Lamo and Takken (2020) showed that they possess homologous effectors, but the endophytic strains possessed fewer effector candidates compared to pathogenic ones. Many studies established the investigation of what they referred to as

‘effectorome’ (effectome), which might effectively predict the strains lifestyle (Arroyo-Velez et al. 2020). Previous research on effectors have shown that pathogenicity proteins evolve rapidly in phytopathogens to avoid host recognition. Effectors are subject to positive selection, which suggests that they evolve as a response to different environmental conditions (Lu et al. 2022).

In this thesis, the comparison of SimOT6 and *C. salicis* CBS 607.94 revealed clear differences in their effector profiles. SimOT6 contains more cytoplasmic effectors, which would suggest a greater ability to manipulate host cells, while *C. salicis* CBS 607.94 has more apoplastic and dual-localised effectors, which would indicate a focus on extracellular interactions. Differences in effector distribution often indicate a possible evolutionary divergence between species and highlight the central role of effectors in shaping the pathogenic potential and host adaptation of *Colletotrichum* fungi (Ma et al. 2025). The differences between SimOT6 and *C. salicis* CBS 607.94 likely reflect distinct infection strategies.

SimOT6 exhibited a similar number of NLPs (Nep1-like proteins) as the reference *C. salicis* CBS 607.94 strain. However, none of the SimOT6 effectors contained the conserved heptapeptide motif (GHRHDWE). NLPs are secreted effectors with dual roles in plant-pathogen interactions and can induce necrosis and ethylene production in eudicot plants, promote pathogen invasion and accelerate disease progression. At the same time, NLPs act as pathogen-associated molecular patterns (PAMPs) and trigger plant immune responses and activate defence mechanisms (Feng et al. 2014; Pirc et al. 2023). The presence of substitutions in the motif (GHRHDWE) alter the interactions with the plant receptors resulting in an absence of toxicity (Lenarčič et al. 2019). In this respect, the absence of conserved motifs suggests that SimOT6 may exhibit reduced toxicity or possibly an endophytic lifestyle, although further studies are needed to fully assess its pathogenic potential.

P. infestans also indicated features of host adaptation and appears to possess the enzymatic machinery necessary to metabolise the fatty acids present in the needles wax layer. Furthermore, two pisatin demethylases were identified in *P. infestans*; these enzymes, part of the cytochrome P450 monooxygenase system, are primarily involved in the detoxification of the phytoalexin pisatin (Matthews and Van Etten 1983). This mechanism may allow the fungus to neutralize pisatin or structurally similar phytoalexins encountered in its environment (Delserone et al. 1999). Additionally, three proteins in *P. infestans* were identified as oxalate decarboxylases, which degrade and regulate oxalic acid during infection. These enzymes contribute to the virulence of necrotrophic fungi that rely on oxalic acid as a pathogenicity factor (Lv et al. 2025).

Carbohydrate-active enzymes from cold-adapted fungi are of particular interest for biotechnology. Our findings indicate that *P. infestans* is a promising source of xylanases and other glycoside hydrolase enzymes, due to its unique ecological and physiological characteristics. As a cold-adapted fungus that thrives on snow-covered coniferous trees, it is likely to produce enzymes that are active at low temperatures.

Xylanase enzymes catalyse the hydrolysis of β-1,4-glycosidic bonds in xylan, a renewable polysaccharide and a major hemicellulose component of many plant cell walls, although its abundance and complexity differ between plant types (Zhang et al. 2024). Xylanase is regarded as an environmentally friendly enzyme with wide industrial applications, contributing to reduced processing costs and environmental impact (Verma 2021). In the paper industry, xylanase improves bleaching efficiency, while in food production it enhances bread texture and facilitates the clarification of juices and wine. It is

also employed as an additive to animal feed (Abena and Simachew 2024). In biorefinery applications, xylanase plays a key role in the pretreatment of agricultural residues and the saccharification of carbohydrate polymers, thereby enhancing sugar yield and boosting bioethanol production (Kumar et al. 2018). However, its broader commercial use remains constrained by factors such as low thermal stability, pH sensitivity, the structural complexity of xylan, and high production costs. Recent advances in enzyme engineering are beginning to overcome these limitations, increasing the industrial relevance and application potential of xylanase (Phuyal et al. 2023; Qeshmi et al. 2020).

Cold-adapted xylanases present promising alternatives for low-temperature processes and offer energy-saving benefits across industries such as food, textiles, pulp, and paper (Feller, 2003; Kuddus et al. 2024; Kim et al. 2021; Littlechild 2015). Furthermore, *P. infestans* infects plant tissues that contain xylan, suggesting that xylan-degrading enzymes may contribute to its pathogenicity. Enzymes from *P. infestans* are expected to function efficiently under mildly acidic and cold conditions, which aligns with the natural habitat of the fungus, as pine needles typically have a pH ranging from 3.7 to 4.9 depending on species, age, and environmental factors (Parzych et al. 2017). Enzymes from psychrotolerant fungi often display a high catalytic efficiency at low temperatures, a broad pH activity range and ease of inactivation by heat features. In addition, fungal xylanases often remain active at temperatures above the growth temperature of the fungi, due to the intrinsic thermostability of the enzyme protein itself (Mendicuti Castro et al. 1997). Previous reports on fungal strains isolated from Antarctica have shown their ability to produce cold-adapted enzymes that can function at low and mild temperatures. These enzymes include several extracellular hydrolases and oxidoreductases. Their cold-adapted enzymes have shown a wide range of optimal pH (1–9) and temperature (10–70°C) values. Their production has been conducted at the laboratory-scale using submerged culture conditions (Duerte et al. 2018). As a relatively underexplored species, *P. infestans* offers potentially novel enzymes with unique properties that could outperform those currently used in industrial applications at very low temperatures and potentially function at temperatures even below zero. The GH11 xylanase from *P. infestans*, in tandem with its other enzymes, presents an opportunity for engineering to meet industrial needs.

Ice-binding proteins (IBPs) that includes antifreeze proteins, found in organisms such as fungi, plants, and fish, are increasingly recognised for their importance in biotechnology. These proteins bind to ice crystals, inhibiting ice growth and recrystallisation, thereby reducing freezing-induced damage in cells and tissues. Their applications extend to improving the viability and quality of plant and animal cells, tissues, and organs in cryopreservation protocols, and they are also employed in the food industry to enhance the texture and stability of frozen products by preventing undesirable ice crystal formation (Fu et al. 2025). In addition, metabolic genetic engineering has enabled the introduction of AFP and IBP genes into crops (e.g. *Arabidopsis*, tobacco, tomato, and wheat), conferring improved freezing tolerance and thereby extending the potential range and resilience of agricultural species (Delesky et al. 2022; Naing and Kim 2019).

Interestingly, *P. infestans* was found to encode two putative IBPs, highlighting its potential to withstand freezing stress. Such findings may also contribute to the broader exploitation of fungal IBPs in biotechnology. Nonetheless, challenges remain in achieving scalable production and minimising cytotoxicity associated with certain protein types (Fu et al. 2025).

SYNTHESIS AND SCIENFIC POSITIONING

Forest pathogens in boreal regions face many environmental challenges, including host chemical defences and the cold temperatures typical of northern ecosystems. This study investigated how these pressures influence the genomic and metabolomic traits of two ecologically different fungal species: *P. infestans* and *C. salicis*. While *P. infestans* (Phain) is primarily known as a snow blight pathogen of conifers, *C. salicis* SimOT6 was isolated from a healthy willow (*Salix* sp.) tree cultivated in Finland and may exhibit both pathogenic and endophytic potential.

P. infestans displays features linked to cold adaptation, such as IBPs, potential cryoprotectants and cold-active enzymes that enable the fungus to survive and function efficiently at low temperatures. Furthermore, *P. infestans* can break down tough pine needle components by producing enzymes that degrade the waxy surface, plant cell walls and neutralise harmful compounds. These abilities allow the fungus to access nutrients inside the needles and thrive even under challenging environmental conditions.

In contrast, SimOT6 displays characteristics of both pathogenicity and endophytism. Genomic analyses revealed the presence of virulence-related genes and conserved effectors, such as CAP20-like proteins. However, the absence of a conserved heptapeptide motif in its Nep1-like proteins suggests reduced toxicity, accompanied by an enzymatic profile with fewer CAZymes compared to the *C. salicis* CBS 607.94 strain. These findings highlight the ecological plasticity of SimOT6, though further functional studies are necessary to clarify whether it primarily functions as a mild pathogen or as an endophyte.

Fungal adaptation to stressors is not limited to single traits but involves a combination of genomic and metabolic features. The results presented in this thesis enhance our understanding of fungal resilience, which enables survival under changing and challenging conditions. To further consolidate the positioning of this dissertation within the broader scientific context, a thematic synthesis table is presented below (Table 9).

Table 9. Thematic synthesis and positioning of this dissertation in the scientific context.

Phenomenon	General view in the literature	Findings in this research	Contribution and novelty	References
Cold tolerance mechanisms in fungi	Describes cold tolerance in psychrophilic fungi, such as *Mrakia psychrophila.*	*P. infestans* produces cryoprotectants, and processes enzymes associated with cold tolerance (IBPs).	First detailed analysis of cold tolerance of *P. infestans* at the molecular level.	Su et al. 2013; Study **I**
Tolerance to host defence compounds	Set of detoxification efflux pumps and protective mechanisms in e.g. *Botrytis cinerea* resistance to plant secondary metabolites.	*P. infestans* tolerates resin acids and metabolises the phenolic compounds, and lignans present in conifer needles.	Demonstrates fungal resistance to conifer defences in a boreal context.	You et al. 2024; Study **I**
Host-specific virulence	Production of specialised effectors and toxins, and the genes that encode virulence proteins.	Presence of toxins, cutinase and lipases that target the needles waxes.	First annotated genome and virulence trait profile for *P. infestans.*	Li et al. 2020; Amuzu et al. 2024; Wilson et al. 2024; Study **II**
Ecological role of CAZymes	Plant cell wall degradation, host invasion and colonisation, nutrient acquisition, fungal cell remodelling.	Both fungi show adaptive gene sets that match their environments.	Suggests enzyme adaptation tied to ecological niche specialisation.	Lyu et al. 2015; Prasanth et al. 2022; Study **II;** Study **III**
Secondary metabolites	Fungi produce diverse secondary metabolites, including toxins (e.g. polyketides, non-ribosomal peptides, terpenoids) that can contribute to virulence and competition.	Identification of gene clusters for polyketide synthases, non-ribosomal peptide synthases, and terpenoid synthases.	Revealed the genetic potential for BGCs and mycotoxins.	Keller 2019; Study **II;** Study **III**
Efflux-mediated detoxification	ATP-binding cassette (ABC), MFS transporters.	Potential phytoalexin detoxification encoded by P-450s, pleiotropic drug resistance (PDR) proteins.	Highlights the role of efflux pumps and detoxification enzymes in overcoming host defences and drug resistance.	Harris et al. 2021; Study **II**
Dual lifestyle potential	*Colletotrichum* species can switch between endophytic and pathogenic phases, influenced by host, environment and genetics.	Partial differences in the number of effectors and conserved virulence motifs in SimOT6, conserved CAP20-like CAZyme profile.	Comparison between SimOT6 and the closest species highlighted the difference in effectors, CAZymes and secondary metabolites.	O'Connell et al. 2012; Study **III**

CONCLUSION

This dissertation aimed to elucidate how the plant-associated fungi *Phacidium infestans* and *Colletotrichum salicis* adapt to their ecological niches from a genomic perspective. Genome sequencing revealed a substantial number of predicted proteins, with both species yielding over 10,000 protein sequences. The vast volume of protein data generated from fungal whole-genome sequences represents a valuable resource for novel discoveries, particularly in biotechnology and pharmaceutical research. Furthermore, it contributes to a deeper understanding of the ecological roles of fungi within forest and agricultural ecosystems. Our findings support this potential and further demonstrate that the unique conditions of boreal ecosystems such as low temperatures and specific interactions with host plants are reflected in the genomic architecture and metabolic traits of these fungi.

To confirm the ecological importance of the identified genes and to verify the production of secondary metabolites and toxins, additional functional studies are needed. A critical evaluation of these predictions is essential, as their accuracy depends on the quality of the underlying annotations and may be influenced by incomplete genome coverage. Methods such as gene expression analysis, protein detection and targeted gene knockouts will be necessary to fully investigate the roles of these genetic pathways in adaptation and pathogenicity. A better understanding of molecular features and behavioural patterns can be achieved through genomic and metabolomic approaches, which reveal large-scale characteristics that may be further analysed to uncover more specific insights. The growing scientific interest in fungal genomics reflects the significant ecological, biotechnological and medical roles that fungi play. Large-scale initiatives, such as the 1000 Fungal Genomes Project (https://1000.fungalgenomes.org/), have greatly contributed to mapping genomic diversity across many underexplored fungal lineages.

A comprehensive knowledge platform on fungi is a valuable resource to advance research on their ecological dynamics, biotechnological and pharmaceutical potential, and responses to climate change.

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