Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2023:101
Faculty of Forest Sciences

Heterobasidion root rot infections on Scots pine: A cryptic threat to sustainable forest management in Sweden

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SWEDISH UNIVERSITY
OF AGRICULTURAL
SCIENCES

DOCTORAL THESIS
Alnarp 2023

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Cover: From left to right: *Heterobasidion* infected pine tree (late stage) showing defoliated crown; Healthy looking pine trees in stand with 20% of infection; Logs of Norway spruce with distinct advanced decay; *Heterobasidion* conidiophores detected on pine roots; Scots pine root system; Logs of Scots pine from healthy looking pine trees in stand with 20% of infection. Norway spruce stump showing advanced decay; Scots pine stump with no decay (infected). Photos: Khaled Youssef

ISSN 1652-6880
ISBN (print version) 978-91-8046-254-9
ISBN (electronic version) 978-91-8046-255-6
https://doi.org/10.54612/a.2pbp3kii82

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Print: SLU Grafisk service, Alnarp 2023

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Abstract

Root rot caused by *Heterobasidion* poses a severe threat to sustainable forestry in managed Scots pine stands in Sweden. In Scots pine, the disease remains hidden in the roots, leading to tree mortality and growth losses. Its cryptic nature often results in underestimated losses and insufficient management action. This thesis aims to highlight the issues posed by *Heterobasidion* in typical forest conditions, explores more efficient methods for disease identification, and assess the potential for future breeding of resistance in Scots pine. This work has been conducted under a combination of field and greenhouse conditions. Scots pine trees in stands designated as low-risk sites for *Heterobasidion* infection were examined for signs of infection in the crown. Subsequently, trees were uprooted, and samples were analysed for the presence of *Heterobasidion* infection. Drone imagery was used, and bud samples were retrieved for genetic analyses. In the greenhouse, the histological response to infection was analysed. A key finding of this study was the high prevalence of hidden infections in the roots of Scots pine trees on low-risk sites. Furthermore, the prevalence of these infections was positively correlated with site index, meaning that trees on higher-fertility soils were more likely to be infected. This finding calls for a change in forest management practices, specifically the application of stump treatment to Scots pine wherever *Heterobasidion annosum* is present in Sweden. Scots pine's response to infection, characterized by the formation of traumatic resin ducts, is local and impractical for effective disease detection or dating of infections. The use of drones equipped with RGB sensors demonstrated promising results, warranting further interest and development. Research is needed to explore how this technology can be applied to identifying *Heterobasidion* among other stressors. *Heterobasidion* is clearly a significant issue in Swedish forestry that deserves more attention. This thesis establishes that a promising genetic component can aid in selecting more resistant trees. In summary, this thesis underscores the cryptic nature of *Heterobasidion* disease on Scots pine, introduces available tools, and highlights the promising potential for further development.

Keywords: *Heterobasidion annosum*, root rot, Scots pine, *Pinus sylvestris*, drones, host response, traumatic resin ducts, genetic resistance, Low-risk site.

Heterobasidion -infektioner på rötterna hos tall - ett dolt hot mot hållbart skogsbruk i Sverige

Abstract

Rotröta orsakad av rotticka (*Heterobasidion*) på tall utgör ett allvarligt hot mot hållbart skogsbruk i skötta bestånd i Sverige. På tall gömmer sig sjukdomen i rötterna och orsakar död och tillväxtförluster. På grund av sitt dolda beteende underskattas ofta förlusterna och åtgärder vidtas sällan. Denna avhandling har försökt belysa problemen med rotticka på tall under normala skogsförhållanden, utvärdera mer effektiva sätt att identifiera sjukdomen och potentialen för framtida avel för resistens hos tall. Arbetet har utförts under en kombination av fält- och växthusförhållanden. Tallar i bestånd på s.k. lågriskmark för rotticke-infektioner har utvärderats för tecken på infektion i kronan, rötterna har grävts upp, och prover har analyserats för närvaro av rotticka. Bilder togs med drönare, och skottprover samlades in för genetisk analys. I växthus analyserades den histologiska responsen på infektion. En av de viktigaste upptäckterna var de frekventa infektionerna som gömmer sig i tallarnas rötter etablerade på lågriskmark. Förekomsten av dessa infektioner var dessutom positivt korrelerad med ståndortsindex, vilket innebär att träd på mer näringsrika jordar hade större sannolikhet att vara infekterade. Detta faktum kräver en förändring i skogsförvaltningen för att även tillämpa stubbbehandling på tall där den arten av rotticka som angriper bland annat tall finns närvarande i Sverige. Tallen reagerar på infektion genom bildning av traumatiska kådkanaler, men reaktionen är lokal och kan inte effektivt användas för sjukdomsdetektion eller datering av desamma. Användningen av drönare med RGB-sensorer visar lovande resultat och väcker ytterligare intresse för utveckling. Det finns ett behov av ytterligare forskning om hur denna teknik kan användas för att identifiera rotticka bland andra stressfaktorer. Rotticka på tall bör uppenbarligen vara en betydande fråga för svenskt skogsbruk och förtjänar mer uppmärksamhet. Avhandlingen visar att det finns lovande genetiska egenskaper som kan användas vid urvalet av mer motståndskraftiga trädindivider. Sammanfattningsvis belyser denna avhandling den dolda naturen hos rotticka på tall och de tillgängliga verktygen samt potentialen för ytterligare utveckling.

Keywords: *Heterobasidion annosum*, rotröta, tall, *Pinus sylvestris:*, drönare, värdrespons, traumatiska hartskanaler, genetisk resistens, lågriskområde

Dedication

My Dad Mohammad Saleh Youssef
My mother Zahra Ahmad
My siblings: Media, Manal, Awaz, Nagma, and Mustafa
My wife Amirah
My daughters: Elin and Eva

Contents

List of publications

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

I. Youssef, K*****.; Dambrauskaite, M.; Witzell, J.; Rönnberg, J (2023). Survey Study Reveals High Prevalence of *Heterobasidion* Root Rot Infection in Scots Pine (*Pinus sylvestris*) Stands Established on Seemingly Low-Risk Sites. *Forests* 14, 1018. https://doi.org/10.3390/f14051018

II. Youssef, K*. Olsson, P. O., Derebrant, P. O., and Rönnberg, J. Performance of UAV-mounted multispectral and RGB images for mapping of *Heterobasidion* root rot in pine forests – a pilot study in southern Sweden. (submitted)

III. Youssef, K*., Marttila, S., Rönnberg, J. Histological analysis of Scots pine (*Pinus sylvestris* L.) seedlings in response to root rot pathogen *Heterobasidion annosum* inoculation. (submitted)

IV. Youssef, K*., Gil Munoz, F., Hayatgheibi, H, Rönnberg, J., Elfstrand, M., and Garcia-Gil, M. R. Genetic markers associated with *Heterobasidion annosum* resistance in Scots pine under natural conditions for infection. (manuscript)

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*****Corresponding author.

The contribution of Khaled Youssef to the papers included in this thesis was as follows:

I. **K.Y**., J. W and J.R., developed the research design and methodology; **K.Y**., and M. D., conducted field and lab work; **K.Y**., performed statistical analyses; **K.Y**., interpreted results and wrote the manuscript together with co-authors; J. R., formulated the original idea.

II. **K.Y**., and J.R., developed the research design and methodology; **K.Y**., conducted field and lab work P-O, O., and P-O. D, performed image analyses, **K.Y**., interpreted results and wrote the manuscript together with co-authors; J. R., formulated the original idea.

III. **K.Y**., and S.M., developed the research design and methodology, **K.Y**., conducted greenhouse and lab work **K.Y**., performed statistical analyses; **K.Y**., interpreted results and wrote the manuscript together with co-authors; J. R., formulated the original idea.

IV. **K.Y**., M.E., M.R. G-G. and J.R., developed the research design and methodology, **K.Y**., conducted field work; **K.Y**. and F.G.M., conducted lab work; F.G.M, and H.H., performed genetic analyses; **K.Y**., interpreted results and wrote the manuscript together with co-authors; **K.Y**., M.E., M.R.G-G. and J.R formulated the original idea.

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Abbreviations

TRDs	Traumatic Resin Ducts
CRDs	Constitutive Resin Ducts
BCC	Blue Chromatic Coordinates
GCC	Green Chromatic Coordinates
RCC	Red Chromatic Coordinates
DVI	Difference Vegetation Index
NDRE	Normalized Difference Red-Edge Vegetation Index
NDVI	Normalized Difference Vegetation Index
NIR	Near-Infrared
DN	Digital Numbers
GWAS	Genome-Wide Association Study
FIS	Inbreeding Coefficient
MAF	Minor Allele Frequency
PCA	Principal Component Analysis
RGB	Red, Green, and Blue channels
SNPs	Single Nucleotide Polymorphism
DBH	Stem Diameter at Breast Height
UAVs	Unmanned Aerial Vehicles

1. Introduction

Scots pine (*Pinus sylvestris* L.) is one of the world's most widely distributed tree species (Vidakovic, 1991). It is the second most common and commercially significant tree species in Sweden, after Norway spruce (*Picea abies* [L.] Karst.), accounting for 39% of the standing volume of productive forest land (Nilsson et al., 2020). Its high-quality wood has many uses, including construction and industrial applications such as the manufacturing of pulp and sawn timber, as well as bio-energy generation. Furthermore, pine forests provide opportunities for recreation and have a high biodiversity value (Durrant et al., 2016). Sweden has a vision of converting to a bio-based economy within the first half or the 21st century. This means transitioning from an economy largely based on fossil fuels to a more resource-efficient economy built on renewable raw materials produced in a sustainable way. Scots pine, because it is so common, is one of Sweden’s most important renewable natural resources, and hence a crucial primary resource for the future bio-based economy. Recent observations have shown that growth losses due to aggressive attacks by *Heterobasidion* root rot on Scots pine may be even worse than previously suspected and can severely affect the long-term national goal of a sustainable bioeconomy.

1.1 The pathogen

1.1.1 Species and hosts

Root and butt rot caused by *Heterobasidion annosum* (Fr.) Bref. sensu lato (s.l.) is one of the most frequent and serious fungal diseases on conifers in the northern hemisphere (Asiegbu et al., 2005; Garbelotto and Gonthier, 2013). At present, the *Heterobasidion annosum* s.l. species complex comprises six recognized species including three European species, two from North America, and one from East Asia (Kovalchuk et al., 2022). The East Asian species *H. subparviporum* seems to be non-pathogenic (saprotrophic) (Yuan et al., 2021), whereas the European and North American species are pathogenic and display strong host preferences (Korhonen et al., 1992). In Sweden, two species of this complex are found: *Heterobasidion annosum* s.s. which primarily infects pines as well as other conifers such as spruce and also broad-leaved trees, and *Heterobasidion parviporum* Niemelä & Korhonen which predominantly infects Norway spruce, alongside Scots pine seedlings, larch, and silver birch (Korhonen, 1978; Werner and Lakomy, 2002). Such information and knowledge about *Heterobasidion* species and their host preferences is significant for forest management.

1.1.2 Infection routes and spread.

All pathogenic *Heterobasidion* species share the same route of infection and spreading. Understanding these routes will aid in creating sound management plans to mitigate their long-term impact. The primary *Heterobasidion* root-rot infection is initiated through the dispersal of airborne basidiospores released from fruiting bodies that form at the base of infected stumps or diseased trees (Woodward, 1998), particularly when the temperature is above 0 °C (Brandtberg et al., 1996). These basidiospores land on freshly-cut stump surfaces, created through thinning or logging operations, and produce mycelia (Isomäki and Kallio, 1974; Rishbeth, 1951). Subsequently, the mycelia colonize the host tissues, extending down into the root systems, thereby infecting neighbouring healthy trees via root-to-root contacts or grafts (Paludan, 1966; Rishbeth, 1951) (Figure 1).

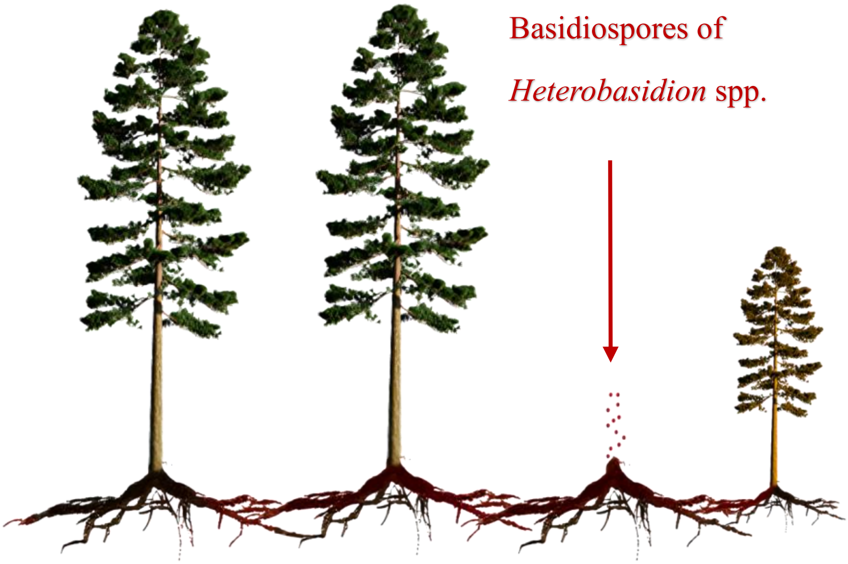

Figure 1**.** The route of *Heterobasidion* spp. infection. Basidiospores land on the surface of newly cut pine stumps, and subsequently spread from the infected stump and tree roots to neighbouring trees through root-to-root contacts. The propagation of *Heterobasidion* mycelium is visually represented in red.

1.1.3 Impact on hosts

Heterobasidion root rot poses a substantial economic and ecological menace to the forestry sector. It gives rise to annual economic losses in Europe amounting to an estimated 800 million euros (Asiegbu et al., 2005). In Sweden specifically, the annual associated losses are projected to reach up to 100 million euros (Oliva et al., 2010). The types of damage caused by this disease depend on the host species. Notably, it causes stem decay which reaches up to 4.8 m and 12 m up the trunk in spruce and larch trees, respectively (Stener and Ahlberg, 2002; Stenlid and Wästerlund, 1986). Conversely, in Scots pine the infection typically remains in the roots causing decay in root tissue and increasing the risk of windthrow. Additionally, the infection reduces stand-level volume growth and productivity by up to 10% annually (Wang et al., 2014). Furthermore, infected living pine trees were observed to have shorter needles and thinner crowns (Kurkela, 2002).

Eventually the infected trees die (Burdekin, 1972; Gibbs et al., 2002; Rönnberg et al., 2006). It is noteworthy that infected trees are likely more susceptible to other pest and disease such as bark beetles (Schmitt, 2000) and *Diplodia* tip blight (Bonello et al., 2008). Increased mortality rates can create gaps in the canopy, which alter light, moisture, and temperature regimes, thereby modifying the habitat for a diverse range of plants and animals (Kovalchuk et al., 2022).

1.2 Disease diagnosis and detection methods

In general, many symptoms and signs may indicate *Heterobasidion* root rot's presence within forest stands. These include the observation of clustered dead trees, wind-thrown trees with decayed roots, and presence of basidiocarps (fruiting bodies) on the roots and lower part of dead trees or stumps (Laine, 1976; Rennerfelt, 1952). However, these signs may not be consistently apparent and infected trees lack clear symptoms until the advanced stages of infection. Current detection methods including the use of increment borers (Stenlid and Wästerlund, 1986), the Rotfinder instrument (Oliva et al., 2011), tomography techniques (Axmon et al., 2004; Weihs et al., 1999), the Shigometer (Ostrofsky et al., 1989), and visual inspection, are based on detecting decay in standing trees or stumps. These approaches prove ineffective for *Heterobasidion*-infected pines due to the fungus' confinement to the root system without advanced decay in stems (Bendz-Hellgren et al., 1998; Wang et al., 2014). The hidden nature of pine infection, where mature trees can outwardly appear healthy despite being infected, results in delayed disease identification and underestimated severity (Rönnberg et al., 2006). Currently, the most reliable identification method involves uprooting, collecting, incubating, and examining root samples for *Heterobasidion* conidiophores under a microscope (Wang et al., 2014). However, these methods have limitations, including restricted spatial coverage, time-intensive procedures, labour intensity, high costs, and the necessity for individual tree examination. Therefore, a more cost-effective and efficient method for identifying *Heterobasidion* root rot in pine forests is imperative. Remote sensing with unmanned aerial vehicles (UAVs) is a promising approach for efficient forest disturbance monitoring. UAVs have gained popularity in forest inventories due to their versatility, high resolution, and cost-effectiveness. They have been used in a wide range of applications, including forest health monitoring (Leckie et al., 2010), monitoring regenerated forest stands, and tree species identification (Gini et al., 2014; Michez et al., 2016). Several studies have also used UAV imagery to detect bark beetle-infested trees (Junttila et al., 2022; Klouček et al., 2019; Näsi et al., 2015) (Näsi et al., 2015; Klouček et al., 2019; Junttila et al., 2022). The utilization UAVs imagery may potentially assist in early detection of *Heterobasidion* disease in pine forests.

1.3 Characteristics of High and low risk sites

The probability of *Heterobasidion* infection in Scots pine is influenced by a complex of interacting factors, including the tree's resistance, the severity of stump infection, and environmental conditions that affect both the pathogen and the host (Rishbeth, 1951).Previous studies have shown that a high incidence of *Heterobasidion* disease is linked to site conditions such as sandy soil , coarse soil texture (Alexander et al., 1975; Baker et al., 1993; Froelich et al., 1966) soil with high pH (Baker et al., 1993; Froelich et al., 1966; Rishbeth, 1951) and former agricultural soils (Rishbeth, 1951). These characteristics are therefore used to identify sites with a high risk of *Heterobasidion* infection. Conversely, their absence is taken as a sign of a low-risk site. Based on this principle, risk assessment systems have been formulated in the United Kingdom (Redfern et al., 2010) and the U.S.A (Morris and Frazier, 1966). with the aim of helping forest managers in the prevention and control this disease. However, similar risk-rating system is still lacking for Swedish forests (Wang, 2012).

1.4 Disease management

Disease transmission in a stand occurs primarily through airborne spores that infect freshly-cut stump surfaces. Thus, all protection measures focus on preventing spore infection by treating stump surfaces with biological control or chemicals immediately after thinning operations. Additionally, silvicultural measures such as planting less-susceptible species or genotypes help hinder the spread of secondary infections.

1.4.1 Biological control

Currently, stump treatment with spore suspensions of *Phlebioopsis gigantea* (Fr.) Jülich directly during thinning operations is the main effective method to prevent spore infection (Berglund and Rönnberg, 2004; Piri et al., 2023a; Rishbeth, 1963; Zaluma et al., 2021). *Phlebioopsis gigantea* (Fr.) Jülich is a saprophytic fungus naturally present in the forest, and swiftly colonizes the stump surfaces (Holdenrieder and Greig, 1998). In Sweden, the application of stump treatment with biological control is primarily conducted in commercial-thinning operations within Norway spruce forests (Thor, 2003). Despite several studies demonstrating the significant effectiveness of *P. gigantea* treatment in preventing *Heterobasidion* spp. spore infection in pine forests (Piri et al., 2023a; Rishbeth, 1963; Rönnberg et al., 2006), this approach is not currently implemented in Sweden. The utility of adopting stump treatment for pine forests warrants further investigation.

A recent study in Finland demonstrated that treating healthy pine stumps around a disease cluster with *P. gigantea* can hinder the spread of *Heterobasidion annosum* via root contacts and reduce the expansion of disease clusters in already-infected Scots pine stands. The study also found that combining *P. gigantea* treatment with inoculation of infected stumps with the *Heterobasidion* virus HetPV13-an1 can further improve control efficiency (Piri et al., 2023b).

1.4.2 Chemical treatment

Various chemicals have been evaluated as stump protectants to prevent *Heterobasidion* root rot infections, but rea is the most widely used due to its high efficacy and stability (Brandtberg et al., 1996; Piri et al., 2023a; Zaluma et al., 2021). Application of urea on stump surfaces elevates the pH, making the environment unfavourable for basidiospore germination (Johansson et al., 2002). However, its use in Sweden has been banned since 2015 (as cited in Blomquist et al., 2020).

1.4.3 Silvicultural measures

While stump treatment can effectively protect stands with little or no *Heterobasidion* infection as it is in low-risk sites from airborne spores, different silvicultural approaches are recommended to reduce primary (basidiospore infection) and secondary (mycelium spread via root-to-root contacts) spread of *Heterobasidion* spp. For instance, conducting thinning operations during wintertime, when the basidiospores are less abundant, minimises the risk of spore infections (Brandtberg et al., 1996; Kallio, 1970). Mixed forests and planting with proper spacing between trees may decrease the chance of root contacts and reduce secondary spread (Lygis et al., 2004). Shortening the rotation period in heavily infected stands is advised to minimise further economic losses (Bréda and Brunette, 2019). Furthermore, planting less-susceptible tree species or genotypes in stands already infected by *Heterobasidion* spp. is also recommended (Marčiulynas et al., 2020).

1.4.3.1 Resistant Scots pine genotypes

In forests already infected with *Heterobasidion* spp. planting less susceptible tree species or genotypes, in addition to treating stumps to prevent spore germination, is recommended to prevent new infections and hinder the fungus's secondary spread via root contact. Several studies have identified specific genetic loci in Norway spruce that are associated with resistance to *H. annosum* and *H. parviporum* using a combination of phenotypic and genotypic data in linkage mapping and genome-wide association studies (GWAS) (Capador- et al., 2021; Elfstrand et al., 2020; Lind et al., 2014). However, comparable studies have not yet been conducted for Scots pine.

1.5 Knowledge gap

Our understanding of *Heterobasidion* root rot's impact and distribution is heavily reliant on studies of Norway spruce in high-risk areas. Comparatively, less is known about this disease in Scots pine, owing to a lack of public awareness and the disease's cryptic nature in this host. Consequently, the overall impact of *Heterobasidion* root rot on Scots pine remains poorly understood, and practical control measures and concrete management recommendations have not been developed.

To formulate such recommendations and make informed decisions, forest managers require comprehensive estimates of growth loss in Scots pine caused by *Heterobasidion* infection. These calculations are currently lacking, and a critical aspect of developing them involves determining the time and level of infection.

Identifying *Heterobasidion*-infected pine trees is a challenge as the fungus resides within the root system without causing advanced decay in the stem. Traditional methods focused on stem decay are ineffective. The current approach necessitates uprooting trees and inspecting the roots for *Heterobasidion* presence. However, this method is constrained by limited coverage, time intensity, labor costs, and the need for individual tree inspection. This highlights the urgent need for a non-destructive and cost-effective detection method.

2. Thesis objectives

The primary aim of this study was to provide a scientific foundation of the impact of *Heterobasidion* root rot disease within Scots pine forests in Sweden. This foundation, in turn, may help enhance extant management strategies, which presently underestimate the profound influence of this disease. By doing so, its impact can be mitigated, improving the productivity and sustainability of pine forests. Specifically, this study pursued the following objectives:

I. Examine the current distribution of *Heterobasidion* in Scots pine forests established on ostensibly low-risk sites and its correlation with site and tree characteristics (**Paper I**).

II. Develop a non-destructive method for detecting *Heterobasidion*-infected pine trees (**Paper II**).

III. Investigate the histological response of Scots pine seedlings to *Heterobasidion* inoculation, with a particular focus on traumatic resin ducts and their feasibility for dating the infection (**Paper III**).

IV. Assess the genotypic variation of Scots pine resistance to *Heterobasidion* spp. under natural infection conditions (**Paper IV**).

3. Materials and methods

As the data in papers 1, 2, and 4 were predominantly collected from the same sites, and the identification of infected trees, calculation of tree morphological traits, and defoliation assessment were major components of all three papers, this section begins with a summary description of the methods used in each paper. Data collection for Paper 3 took place in a greenhouse on seedlings and is later addressed under a separate subheading (3.3). A summary of specific methods used in each paper will follow, but more detailed information can be found in the individual papers.

3.1 Distribution of *Heterobasidion* spp. on low-risk sites (Paper I)

3.1.1 Study sites (I, II, IV)

Data was collected from ten different Scots pine sites distributed in southern Sweden (Figure 2) with allocations across the papers as follows: Paper 1: sites 1-9; Paper 2: site 10; Paper 4: sites 1-10. These sites were selected based on three main criteria. First, the at least 80% of the tree composition must be Scots pine. Second, the stand must not have sandy soil. Third, the site should not have been previously used for agriculture or pasture. Notably, an exception was made for site 10, where the soil contained some proportion of sand deviating from the low-risk site criteria. Therefore, it was not included in Paper 1 which specifically focused on low-risk sites. The characteristics of each site are presented in Table 1.

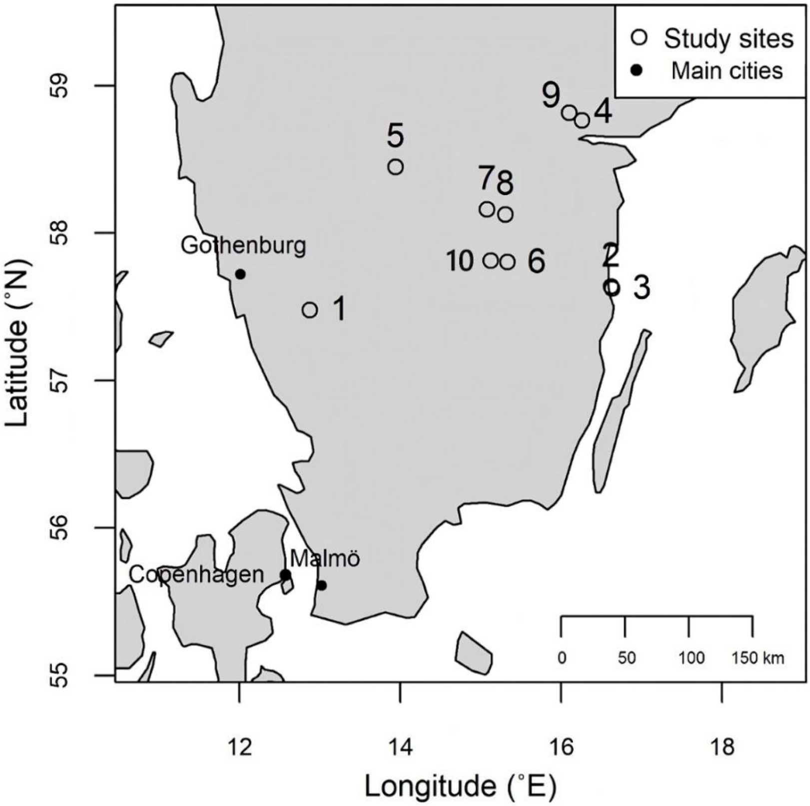

Figure 2**.** Distribution of the study sites in southern Sweden.

3.1.2 Data collection and identification of infected trees (I, II, IV)

From sites 1-9 and site 10, forest managers selected 15 and 23 trees, respectively, following normal thinning regimes. The selected trees were uprooted by a forest harvester machine. Subsequently, from each selected tree at sites (1-9) and site 10, five and eight discs (2-5 cm in thickness) were manually cut from different roots respectively, using a hand saw. Subsequently, these root discs were directly placed in labelled plastic bags and transferred to the lab. Prior to each cut, both the site of the cut and the saw were meticulously cleaned with 70% ethanol. Post-cutting, the handsaw underwent another cleaning cycle with 70% ethanol.

In the lab, all root samples were incubated for 10 days in darkness at room temperature (20 °C). Following this incubation, the diameter of each root disc was measured **(Paper I)**, and the samples were examined under stereo microscopy for the detection of *Heterobasidion* spp. conidiophores. If conidiophores were identified in one or more root discs of a tree, that particular tree was duly recorded as infected. It is noteworthy that the species of *Heterobasidion* was not identified, as Scots pine is predominantly afflicted by *Heterobasidion annosum* (Korhonen, 1978; Müller et al., 2018; Werner and Lakomy, 2002). In addition, ten bud samples from each tree were collected and stored at -20 °C for DNA extraction (**Paper IV**).

3.1.3 Calculation of tree morphological traits (I, II, IV)

When the selected trees were felled, various morphological parameters, including stem diameter at breast height (DBH), bark thickness, height, and stem length to the first living branch were measured. Tree volume was subsequently computed using the following formula:

$$V = 0.1193D^2 + 0.02574D^2H + 0.004054DH^2 + 0.007262D^2K - 0.003112DHB$$

Where V represents the tree volume above cutting in cubic decimetres (dm^3), D signifies the stem diameter at breast height (cm), H denotes the total tree height from the ground (m), K represents the stem length from ground level to the base of the first living branch (m), and B signifies the double bark thickness (mm).

3.1.4 Visual assessment of tree-crown defoliation (I, II, IV)

To evaluate crown defoliation, a reference tree was selected in each stand and compared to other sampled trees. The reference tree had ≤10% defoliation and represented typical crown morphology. Crown defoliation was visually assessed on a scale of 0 to 4, where 0 denoted healthy trees, and 4 indicated dead trees. The assessment was carried out while the trees were standing, except in stand six, where the trees had been felled prior to sample collection. To exclude defoliation caused by other foliage diseases or insects, the reference and sampled trees were checked for the presence of these stressors. No significant symptoms or signs were observed in any of the sampled stands.

composition must be Scots pine. Second, the stand must not have sandy soil. Third, the site should not have been previously used for agriculture or pasture. Notably, an exception was made for site 10, where the soil contained some proportion of sand deviating from the low-risk site criteria. Therefore, it was not included in Paper 1 which specifically focused on low-risk sites. The characteristics of each site are presented in Table 1.

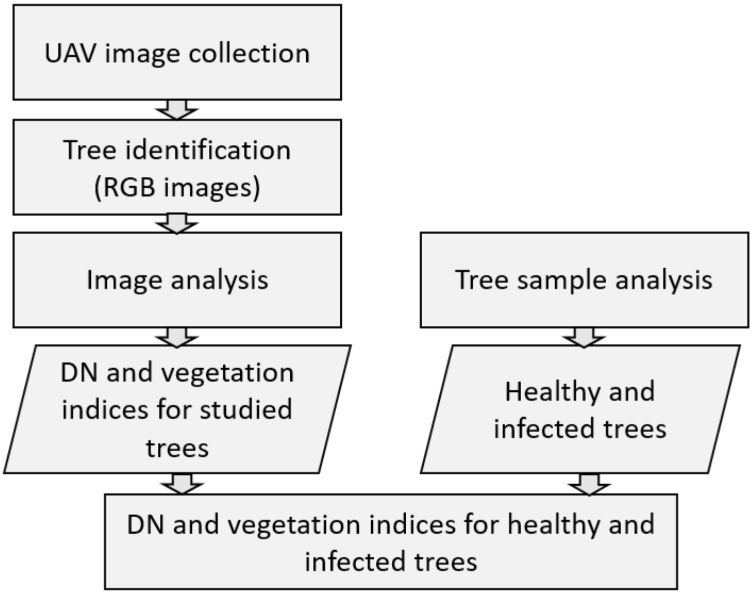

Figure 3. An illustration of the data acquisition and analysis process for Paper II

3.2.1 Aerial image collection

On May 9, 2022, a DJI Phantom 4 Pro V2 RTK drone with a multispectral camera (model FC6360) took systematic pictures of the forest while staying 80 meters above ground level (AGL) and using real-time kinematic positioning (RTK). The weather was sunny. The camera has six sensors: five multispectral sensors (blue, green, red, red-edge, and near-infrared) and one RGB sensor. Imaging operations were conducted with a nadir-oriented camera employing the DJI Ground Station Pro app, with both front and side overlaps set at 80%. During the flight, calibration panels with reflectance values of 9%, 23%, and 44% were consistently taken, which made it easier to radiometrically fix the multispectral data afterward.

3.2.2 Identification of individual trees in aerial imagery

The initial plan to use orthophotos generated from UAV images to identify 23 pine trees was unsuccessful due to blurry images and alignment issues. Instead, individual RGB images were used, but their lack of global geo-referencing and inconsistent scaling presented challenges.

The 23 trees' RTK GPS coordinates and the latitude and longitude of the RGB image centres were combined with a geo-referenced vegetation height map made from LiDAR data to find the RGB photo that was closest to each tree. The RGB images were then rotated based on metadata orientation and overlaid on the vegetation map at an approximate position and angle. Manual adjustments aligned the RGB images with the vegetation information in the map, allowing for successful identification of the selected trees. The entire tree identification process was executed using the OCAD software.

3.2.3 Image Analysis

Individual RGB and multispectral images from the 80 m flight were processed to compute reflectance images for the red, red-edge, and near-infrared bands. Due to saturated images of the reflectance-calibration panels, the spectral properties of the blue and green bands were derived from the digital numbers of the images that were corrected for vignetting and differences in exposure. Three vegetation indices (DVI, NDVI, and NDRE) and three chromatic coordinates (GCC, RCC, and BCC) were calculated for the studied trees by manually creating regions of interest (ROIs) in the RGB and multispectral images. Mean values per tree were computed for the ROIs of all healthy and infected trees that were identified in the images. However, some shorter trees were excluded since they were hidden and shaded below taller trees. Among the 23 selected trees, 13 and 18 trees were identified in the multispectral and RGB images, respectively.

3.3 Histological response (Paper III)

3.3.1 Plant materials and fungal inoculation

This experiment used three-year-old healthy bare-rooted Scots pine seedlings. At 6 cm above the soil level, the bark was removed by sterilized scalpel. Next, 5 mm plugs from actively developing *H. annosum* (isolate B11) cultures were placed on the exposed surface of the xylem and sealed with Parafilm. For the wound treatment, sterile Hagam agar plugs without fungus were employed in a similar way. All seedlings were then placed in the greenhouse on the Alnarp campus of the Swedish University of Agricultural Sciences and were subjected to ambient lighting and temperature conditions, with watering twice per week for six months until the end of the experiment.

3.3.2 Data sampling and preparation for light microscopy

All seedlings were collected, and the stem of each seedling stem was symmetrically cut into two halves. Subsequently, one half of the stem was sectioned at 1 cm intervals for 5 cm distal to the inoculation point. Thereafter, the samples were prepared for light microscopy as in Ghasemkhani et al., (2016). Thin cross sections around (1-5 µm) were produced with an ultramicrotome and put on adhesion slides (Superfrost ® Plus) for light microscopy inspection.

3.3.3 Lesion length and resin duct measurements

The vertical length of necrotic lesions within the xylem was measured with a digital ruler. Traumatic resin ducts (TRDs) are produced in the latest annual growth ring (the year of the treatment) while resin ducts in the previous year's growth ring are constitutive resin ducts (CRDs), assuming absence of any outside stimuli. The density of traumatic resin ducts indicates the number of resin ducts per unit of measured area (RD/mm2). The size of both traumatic and constitutive resin ducts including the epithelial cells and the lumen was measured using the ImageJ image processing software (Abràmoff et al., 2004).

3.3.4 Statistical analysis

A t-test for independent samples was used to compare the mean necrotic lesion length between the two treatments and to compare the mean size of traumatic and constitutive resin ducts within each treatment. A linear mixed model utilizing the “emmean” package in R was used to analyse the impact of treatments and distance from the inoculation point on the density and size of traumatic resin ducts. Treatments and distance from the inoculation point were included as fixed factors, while tree and resin-duct type were included as random factors (Vázquez-González et al., 2019).

3.4 Genotypic variations (Paper IV)

3.4.1 DNA extraction and genotyping

Total genomic DNA was extracted from the buds collected from each tree and sent to Thermo Fisher Science for genotypic analysis conducted with a custom Scots pine 50K Affymetrix Axiom SNP array.

3.4.2 Population genetic structure

The population genetic structure of the 146 trees was analysed using the "ASRgenomics" package (Gezan et al., 2022) in R. Quality refinement of called SNPs involved the removal of indels, retention of bi-allelic sites, exclusion of sites with MAF < 0.01, and elimination of SNPs deviating from the Hardy-Weinberg equilibrium. Trees were categorized based on their site of origin (one of the 10 study sites mentioned above) and infection status (coded as 1 for presence and 0 for absence of the disease).

3.4.3 Genome-wide association study (GWAS) analysis

A GWAS was conducted to investigate tree infection status using the ASReml-R package in R. The initial marker matrix included 146 individuals and 47,712 markers, but after stringent data filtering, 3,489 markers were eliminated, retaining 0.77% of initially missing SNPs. The pre-processing step used the pre.gwas" function, involving the calculation of the kinship matrix and setting MAF > 0.05. Genotype and population structure were treated as random effects. Missing markers were imputed using the mean. Next, the "gwas.asreml" function was used to fit the model using both Gaussian and binomial error distributions. Results from the two models were virtually identical, and only those based on the Gaussian method are presented in this paper. The first three principal components were considered. The significance threshold for was set at p=.0005. Then the backward elimination step was applied using threshold (P-value=0.01).

4. Main results and discussion

4.1 The current distribution of *Heterobasidion* in Scots pine forests established on ostensibly low-risk sites and its correlation with site and tree characteristics (Paper I)

4.1.1 The prevalence of *Heterobasidion* infection on low-risk sites

Heterobasidion root rot was detected in six of nine study sites, with site-level infection frequencies ranging between 0–33% (Table 1). This demonstrates the ability of *Heterobasidion* spp. to establish and persist even in sites with low-risk soil types.

None of the study stands exhibited characteristic symptoms of *Heterobasidion* disease, such as groups of dying pines, fruiting bodies, or windthrown pines with decayed roots. However, with an infection incidence exceeding 30% of trees on some sites, it is warranted to consider the risk of *Heterobasidion* spp. infection when planning silvicultural activities in all pine stands.

Furthermore, climate change is expected to increase the incidence of *Heterobasidion* spp. infection as the pathogen produces and releases more basidiospores during longer growing seasons and warmer temperatures (La Porta et al., 2008). Warmer temperatures also mean that a lower proportion of thinning operations will be conducted during the shorter cold winter period (Siev, 2014). Therefore, risk-assessment systems based solely on soil type are likely to underestimate the risk of *Heterobasidion* disease.

Our results indicate that a hazard risk assessment system for *Heterobasidion* should consider soil type, stand management history (e.g., first generation or previous agricultural or pasture areas), and the presence of *Heterobasidion* spp infection in the current and previous plantations. If one or more of these factors is present in a pine stand, prevention methods such as stump treatment and winter cutting must be implemented promptly. This is especially important given the expected increase in planting of Scots pine as an adaptation to climate change.

Table 1. *. Detailed overview of the characteristics of the ten study sites, including the infection frequency for each site.

Stand ID	**Coordinates**	**Stand age (yr.)**	**Site index**	**Soil type**	**Previous tree species**	**No. sampled trees**	**No. infected trees**	**Infection frequency (%)**
1	57°28'42.0"N 12°52'27.0"E	38	T25	Moraine	Scots pine / Norway spruce	15	0	0
2	57°38'15.3"N 16°37'00.1"E	52	T18	Moraine	-**	15	0	0
3	57°37'24.2"N 16°38'03.8"E	47	T21	Moraine	-**	15	0	0
4	58°45'48.3"N 16°15'22.4"E	42	T29	Silty clay	-**	15	5	33.33
5	58°26'53.2"N 13°56'35.3"E	37	T24	Glacial sediments	Scots pine	15	2	13.33
6	57°48'05.4"N 15°19'48.4"E	62	T25	Moraine	Scots pine	15	2	13.33
7	58°09'40.4"N 15°04'27.3"E	45	T27	Moraine	Norway spruce	15	3	20
8	58°07'29.8"N 15°18'09.8"E	58	T26	Moraine	Scots pine	15	3	20
9	58°48'51.4"N 16°06'05.7"E	36	T28	Moraine	Scots pine	15	5	33.33
10	57°48'45.7"N 15°17'52.9"E	45	T28	Sandy moraine	Norway spruce / Scots pine	23	12	52.17
total						158	32	20.25

* Data from site ten was excluded from Paper 1 because the soil type did not meet the criteria for low-risk site.
** Information about previous plantation tree species is unavailable, but forest managers have confirmed that these three stands are old forest site

4.1.2 Correlation between site index and infection frequency

A significant positive correlation was observed between site index and infection frequency ($p=0.004$), indicating that pine stands on more fertile sites were more susceptible to *Heterobasidion* spp. infection. This may be explained by the fact that pine trees on more fertile soils prioritize growth over defence, making them more vulnerable to infection. Rishbeth (1957) reported that fast-growing trees are more prone to *Heterobasidion* damage compared to slow-growing trees. While there seems to be a relationship between site index and infection frequency, it is difficult to define clear thresholds for low or high risk. Regardless of site index, the probability of *Heterobasidion* root-rot infection in pine stands can be reduced by implementing stump treatment and thinning during winter.

4.1.3 *Heterobasidion* infection and crown defoliation

Approximately 40% of trees had healthy crowns, of which 4.2% were infected with *Heterobasidion* spp. Among trees showing defoliation, 40% were classified as defoliation class 1, and 34.6% of these trees were found to be infected. This suggests a weak trend towards slight or moderate defoliation levels in the crowns of infected trees. However, these findings indicate that visual assessment of crown condition or defoliation alone is not a reliable indicator of infection, leading to underestimation of infection rates, which is consistent with previous studies from high-risk sites (Kurkela, 2002; Rönnberg et al., 2006; Wang et al., 2014).

4.2 Development of a non-destructive method for detecting *Heterobasidion*-infected pine trees (Paper II)

Mean digital number (DN) values for healthy and infected trees were calculated for each wavelength band in the multispectral data to determine the spectral characteristics of the two tree states. However, the results revealed a significant overlap in DN values between healthy and infected trees across all wavelength bands, making it challenging to accurately discriminate between the two states (Figure 4). While multispectral data has been used successfully to detect other plant diseases (De Silva and Brown, 2023), it was not effective for detecting trees infected with *Heterobasidion* under the circumstances of our study.

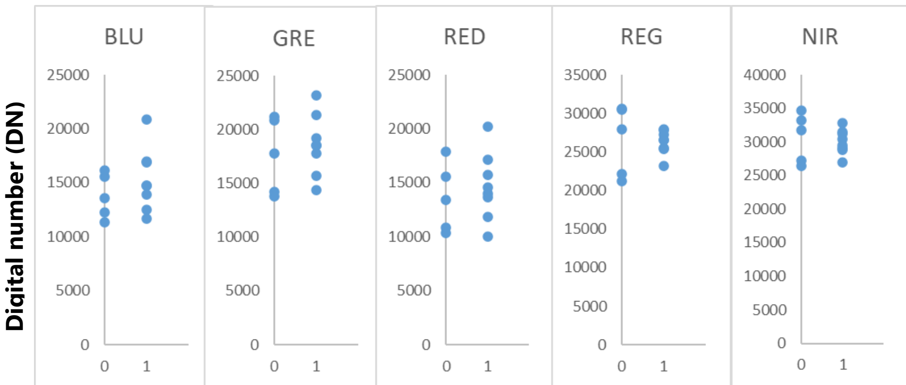

Figure 4. Mean digital number (DN) values for healthy and infected trees across the five wavelength bands of the multispectral camera. Each data point represents an individual tree. Vignetting correction and compensation for varying exposure was applied to individual images. DN values are unitless. Key to band names: BLU=blue, GRE=green, RED=red, REG=red edge, NIR=near infrared.

Unlike the spectral data, the blue chromatic coordinates (BCC) showed better distinguishability between healthy and infected trees (Figure 5). When classifying trees into healthy and infected categories using 0.21 as a BCC threshold, 73% (8 out of 11) of the infected trees were correctly identified as infected (true positive), and 71% (5 out of 7) of healthy trees were accurately classified as uninfected (true negative). However, it is important to note that we only examined eight areas within each tree's root system and did not inspect the entire system. That two healthy trees were falsely identified as infected might be due to these trees having infections in their root systems outside the sampled areas. Kankaanhuhta et al., (2000), using multispectral imagery on *Heterobasidion* root rot-infected trees, found distinct differences in the visible spectrum, particularly the red spectral range, but not in the near-infrared region. In contrast, our study observed that infected pine trees reflected more blue light, suggesting potential defoliation, as woody parts reflect more blue light than needles (Moore et al., 2016).

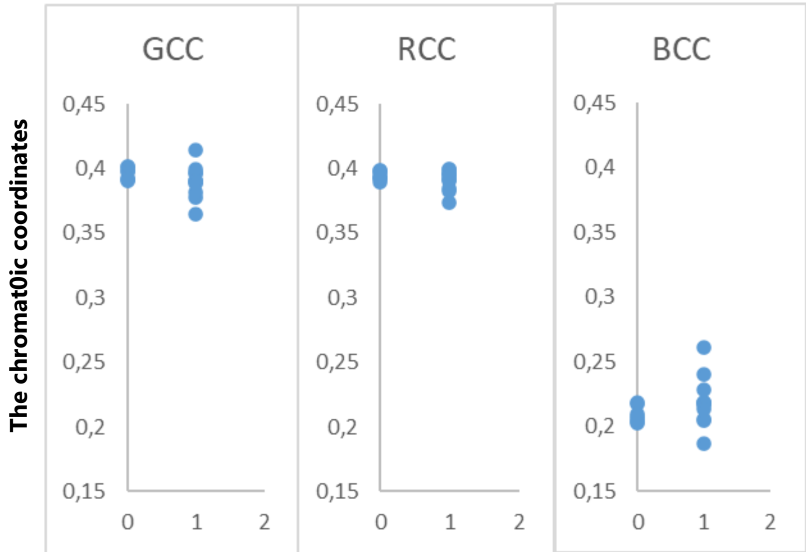

Figure 5. Mean values of the blue (BCC), green (GCC), and red (RCC) chromatic coordinates obtained using an RGB camera. The chromatic coordinates are unitless.

Various stressors other than *Heterobasidion* induce similar symptoms in trees, such as slight defoliation or changes in needle reflectance across different wavelengths. Distinguishing *Heterobasidion* from other stressors might be challenging. Nevertheless, there is an interest in developing a method to identify stressed trees using drones, aiming to establish a comprehensive database for multiple stands. This could eventually enhance the ability to differentiate among various stressors, potentially in conjunction with weather, soil, or management data specific to a site. It is crucial to note that this study used only a limited number of trees and did not validate its results on an independent dataset, so extrapolating defoliation classes to other stands would have questionable accuracy.

One limitation of this study is that radiometric correction of the multispectral images is challenging in sunny conditions due to the mixture of sunlit and shaded canopies and the strong influence of viewing direction. Additionally, the reflectance calibration panels for the blue and green bands were saturated, making it impossible to compute reflectance images for these bands. Cloudy conditions are more favourable for radiometric correction, but it is still challenging if the cloud cover is not homogeneous.

Another limitation was the identification of relatively short and partially concealed trees beneath taller canopies in aerial imagery, leading to the exclusion of some trees from the study. The shading of shorter trees further complicated the comparison of multispectral data, necessitating the analysis of more RGB-images than multispectral images. To enhance future studies, it is recommended to select trees that are easily visible in UAV images. Additionally, conducting flights at a higher altitude above the canopy is advised to minimize the impact of leaning trees and facilitate orthophoto generation. While prioritizing dominant trees is practical, future evaluations should extend to smaller and lower trees once the methodology is appropriately calibrated and validated on visibly distinct trees, recognizing the importance of identifying the infection in all trees, not solely the tallest ones. While our findings indicate that using chromatic coordinates can enhance the identification of *Heterobasidion* root rot disease, additional research is essential to validate these observations in bigger and more diverse datasets.

4.3 The histological response of Scots pine seedlings to *Heterobasidion* inoculation, with a particular focus on traumatic resin ducts and their feasibility for dating the infection (Paper III)

4.3.1 Necrotic lesion length

Scots pine seedlings produced significantly longer browning necrotic lesions in response to *Heterobasidion annosum* inoculation in comparison to wound treatment (p=0.000002, n=9; Figure 6).

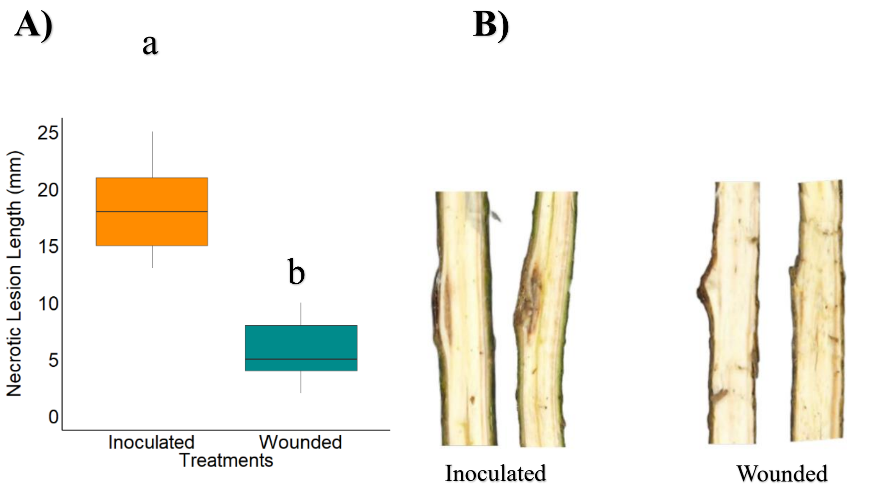

Figure 6. A) Necrotic lesion length (mm) in the xylem of Scots pine seedlings following inoculation with *Heterobasidion annosum* or a wounding treatment. Distinct letters signify statistically significant differences ($p<0.05$) between the two treatments. B) Photo of longitudinal sections of necrotic lesions in the xylem of two representative Scots pine seedlings in response to each treatment.

The observed variation can be linked to the initial induced responses of pine seedlings to the invasion of the pathogen. This response serves to impede pathogen spread through the conveyance of potentially toxic compounds such as terpenoids, lignin, and phenolics to the vicinity of the inoculation site

(Johansson et al., 2004; Liu et al., 2022; Mukrimin et al., 2019; Raffa and Smalley, 1995).

4.3.2 Characteristics of traumatic resin ducts

The results of a mixed-effects model indicated a significantly higher density of traumatic resin ducts in *H. annosum*-inoculated seedlings compared to those subjected to wounding (p=0.036, n=3; Figures 7, 8). The most pronounced differences were observed closest the inoculation point, specifically at distances of 1-3 cm. However, with increasing distance from the inoculation point, the density of traumatic resin ducts declined, and the observed differences were no longer statistically significant at distances of 4 and 5 cm (Figure 8).

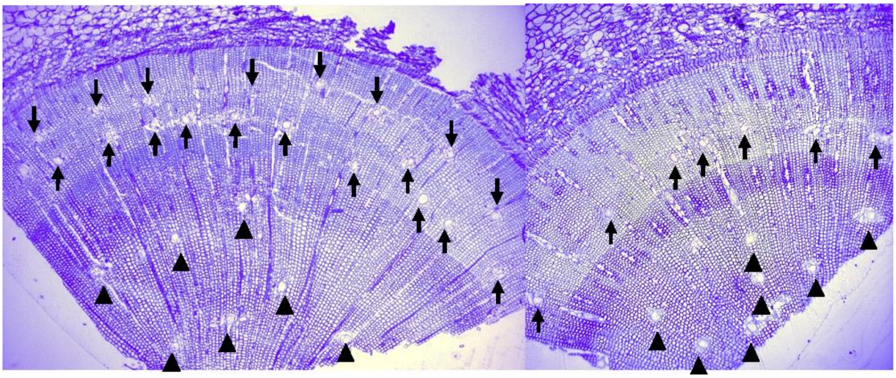

Figure 7. The resin-based defence response of three-year-old Scots pine seedlings to (A) *Heterobasidion annosum* and (B) wounding treatments. In the last annual growth ring, there are single or multiple series of traumatic resin ducts (arrows), while in the previous year growth ring, only constitutive resin ducts are present (triangles).

The increased density of traumatic resin ducts in pine seedlings may be a defence strategy to minimise unprotected gaps at the attack site, hindering the advancement of fungal hyphae and preventing its further spread (Nagy et al., 2000). Higher duct density correlates with increased resin flow (Ayres and Lombardero, 2000; Blanche et al., 1992). Similar patterns were observed in Norway spruce, where higher duct density occurred in response to *Heterobasidion annosum* compared to sterile inoculation (Krekling et al., 2004). In addition, Gibbs, (1968) reported that pine tree resistance to *Heterobasidion* root rot is correlated with the ability to mobilize resin.

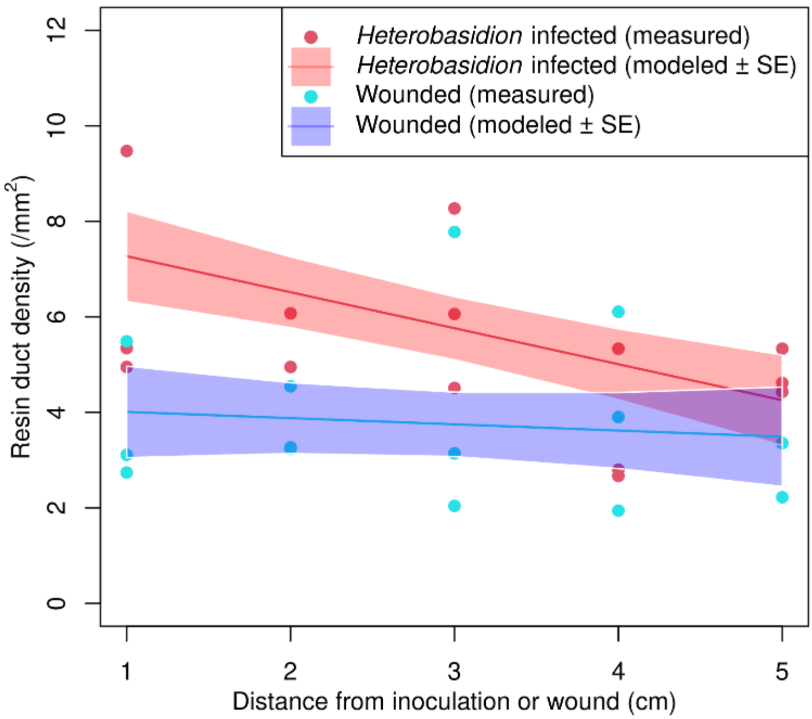

Figure 8. The density of resin ducts in Scots pine seedlings that were subjected to *Heterobasidion annosum* inoculation and wound treatment at different distances from the inoculation point. Points show measured values for individual seedlings, and the trend lines show estimated mean responses according to the model described in Paper 3. The shaded areas show the standard error of these mean estimates. The lack of overlap in these standard errors up to 3 cm indicates significant differences at p<.05 within these distances from the inoculation point.

Accordingly, our results confirm the significant role of both resin and resin ducts in enhancing pine resistance to *Heterobasidion* disease.

The intensity of this response progressively decreased with increasing distance from the inoculation point for both treatments but decreased more strongly for the infected treatment. This means that the response to *H.*

annosum is indistinguishable from the wound response after 3 cm. Traumatic resin ducts were not identified as a dependable marker for dating *H. annosum* infection in Scots pine. The localized response of Scots pine to both treatments is likely attributed to the prompt containment of fungal colonization and a greater dependence on constitutive anatomical defences. Furthermore, there was no significant difference in the mean size of traumatic and constitutive resin ducts between the *H. annosum*-inoculated and wounding treatments. However, within the wounding treatment, the mean size of traumatic resin ducts was significantly smaller than that of constitutive resin ducts. This suggests that Scots pine seedlings can adjust their defence responses based on the severity of the encountered challenges.

4.4 The genotypic variation of Scots pine resistance to *Heterobasidion* spp. under natural infection conditions (Paper IV)

4.4.1 Genetic diversity and population structure

Except for stands four, six, and seven, the analysis did not reveal distinct genetic differentiation among trees from different stands (Figure 9A). Similarly, the PCA did not detect any apparent segregation between infected and healthy trees; there is no observable separation based on infection status in the PCA (Figure 9B). Scots pine trees infected with *H. annosum* are not genetically distinct from the rest of the sampled Scots pine population.

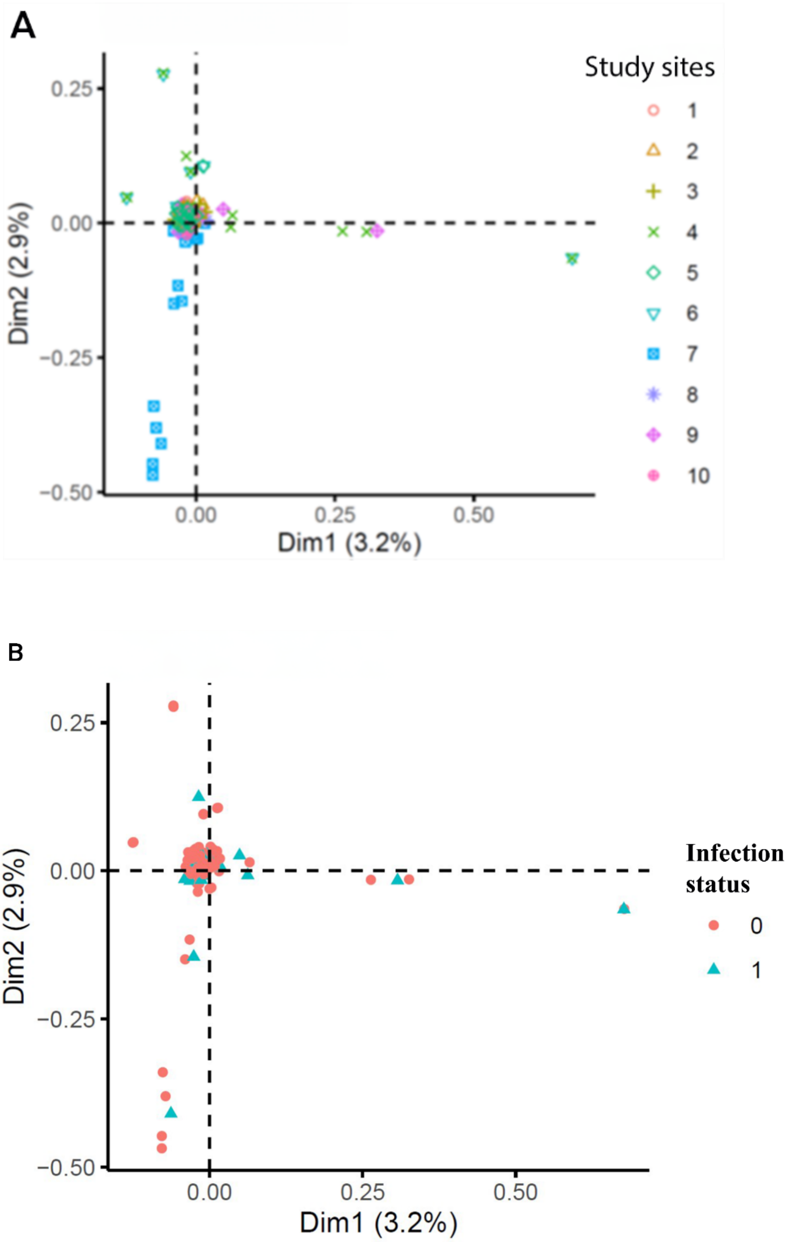

Figure 9. PCA scatterplots of ten populations reveal little genetic variation between populations and infection status. A) Genetic variation among sites. B) Genetic variation based on infection status.

4.4.2 Specific loci are associated with the presence of *Heterobasidion annosum* in the root system.

The genome-wide association studies (GWAS) revealed that resistance to *Heterobasidion* spp. is likely under polygenic control in Scots pine, with multiple significant SNPs having small effects on the trait. The GWAS identified seven SNPs that are significantly associated with the infection status of the trees, with the most significant SNP explaining about 7% of the variation (Table 2, Figure 10).

Table 2. Details of the 7 markers identified through the genome-wide association study (GWAS) as significantly associated with tree infection status. The information includes position, minor allele frequency (MAF), effect size, standard error, Z ratio, p-value, and the percentage of explained variance (Expl. var%).

Marker	**Position**	**MAF**	**Effect size**	**Std. error**	**Z ratio**	**P-value**	**Expl. var %**
AX_117434139	4796	0.382	-0.135	0.0374	-3.595	2.729 e-04	5.2
AX_388252429	16338	0.309	0.138	0.0390	3.526	3.531e-04	4.9
AX_389009589	29489	0.077	-0.279	0.0677	-4.116	3.572e-05	6.7
AX_389219315	33908	0.158	0.167	0.0503	3.318	7.391e-04	4.5
AX_389220706	33944	0.382	0.144	0.0378	3.817	1.150e-04	6.0
AX_389302104	35112	0.129	-0.141	0.0560	-2.514	9.784 e-03	2.7
AX_389450590	36999	0.169	-0.152	0.0522	-2.903	2.980 e-03	3.9

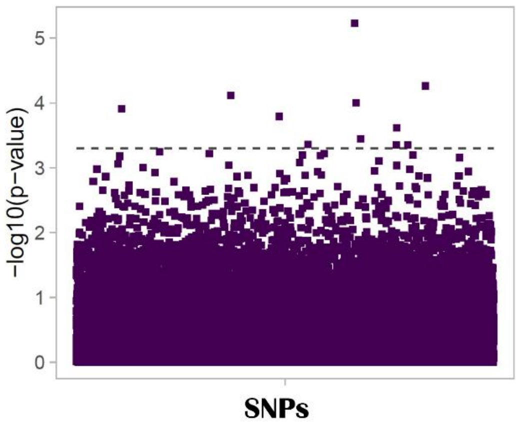

Figure 10.A Manhattan plot visualizing the distribution of SNPs’ significance of association with infection status.

The analysis of SNP AX_389009589 indicates that trees homozygous for this SNP are significantly less prone to *Heterobasidion annosum* infections in the root system compared to trees heterozygous at this specific position (Table 3).

Table 3. Heterozygous and homozygous status of the most significant SNP “AX_117434139” within all sampled populations.

Status	**Heterozygous**	**Homozygous**	**Total**
no infection	9	99	108
infection	12	16	28
Total	21	115	136

Despite our small dataset of only 144 trees, we identified 7 loci in the Scots pine genome that are significantly associated with the presence of the pathogen in the root system. This suggests that there is a strong genetic component to resistance to *H. annosum* in Scots pine.

Our results are consistent with other studies that have shown that there is a large genetic variation in resistance to *H. annosum* between Scots pine families. This suggests that it is possible to breed for resistance to *H. annosum* in Scots pine. However, resistance breeding is difficult because the disease develops slowly. We also found that specific loci in the Scots pine genome are significantly associated with resistance to *H. annosum*. This is encouraging because it means that it may be possible to identify and select for more resistant genotypes using molecular methods. This would be a faster and more efficient way to breed for resistance to *H. annosum* than traditional breeding methods.

An assembled genome of Scots pine is not currently available. However, when a genome becomes available, we will be able to annotate and map the significant SNPs that we identified. This will provide new insights into the cellular mechanisms that control resistance to *H. annosum* in Scots pine.

5. Major limitations

Several factors limited our sample sizes, including the cost and time associated with using heavy machinery to uproot trees, the severe drought in summer 2018 that prohibited the use of heavy machinery in forests due to fire risk, and a subsequent outbreak of spruce bark beetle in Sweden which occupied all available machinery in cleanup operations and reduced its availability for pine forests. Additionally, it was difficult to find suitable pine forests that met the low-risk site criteria.

The current version of Paper IV was constrained by the recent acquisition of genotypic data analysis and the unavailability of the annotated Scots pine genome. This limited the depth and breadth of our analysis, but we provide a succinct analysis of the obtained results, acknowledging the limitations imposed by the restricted timeframe and data availability.

6. Conclusions

The key finding of this thesis is that Scots pine trees can be infected with *Heterobasidion* root rot disease without showing distinct outward symptoms even when they are growing on low-risk sites where the perceived probability of infection is low. Additionally, there was a positive correlation between site index and infection frequency meaning that pines growing on more fertile soils have a higher risk of infection. Collectively, these findings call for a change in forest-management practices. Logging during winter and stump treatment should be applied to Scots pine in all types of sites, similarly to recommendations for Norway spruce. Currently, stump treatment to prevent *Heterobasidion* infection in Scots pine forests is not applied even on high-risk sites. It is also noteworthy to mention that supposed high-risk sites may not always have a high incidence of *Heterobasidion* disease (Wang, 2012). Therefore, the justification for stump treatment will vary from site to site depending on several factors including site condition (soil type, site index), thinning timing (winter vs. warmer seasons), infection frequency in the current and previous plantation, and the type of *Heterobasidion* species present (**Paper I**).

Early detection of *Heterobasidion* is vital, and drones equipped with a normal RGB camera show promise as an affordable method for detection, achieving an accuracy rate of 73%. Although the optimal threshold of blue chromatic coordinates (BCC) found here may not be all-encompassing for *Heterobasidion*, drones with RGB cameras can serve multiple purposes, providing a general overview of tree vitality in the stand (**Paper II**).

Determining the infection date is crucial for accurately calculating growth losses. Traumatic resin ducts did not prove to be a reliable marker for dating *Heterobasidion* infection, as Scots pine relies more on constitutive defence mechanisms. Additionally, Scots pine exhibited significantly longer lesions in response to *Heterobasidion* inoculation compared to the wounding treatment. As necrotic lesion length varied within the *Heterobasidion* treatment, measuring this variable could potentially serve as a marker in the early stages of evaluating and selecting Scots pine genotypes resistant to *Heterobasidion* (**Paper III**).

The genetic analysis of Scots pine trees under natural conditions revealed potential genetic components associated with *Heterobasidion* infection. Seven single nucleotide polymorphisms (SNPs) were significantly correlated with infection status, providing a promising foundation for breeding programs aimed at producing less susceptible pine plants. It is important to note that further validation will be possible once the Scots pine genome is annotated (**Paper IV**).

7. Future research

- Additional investigations are warranted to quantitatively assess the incidence of *Heterobasidion* infections within young pine plantations. Such research will enhance our understanding of the consequences of existing management strategies on the impact of *Heterobasidion* on both current and subsequent plantations.

- Long-term experiments are imperative to analyze growth losses in pine forests attributable to *Heterobasidion* infections. This prolonged observational approach will provide comprehensive insights into the sustained effects of the pathogen on forest growth over time.

- Further research is essential to validate the effectiveness of employing blue chromatic coordinates (BCC) for *Heterobasidion* detection, concurrently exploring the application of satellite imagery. This validation process is integral to establishing the robustness and reliability of these methodologies in disease detection.

- Given the reliance of Scots pine on constitutive resin defense, there is a pressing need for in-depth investigations to elucidate the intricate details of constitutive defense mechanisms in Scots pine in response to *Heterobasidion* infections. This inquiry will contribute to a deeper understanding of the innate defense strategies employed by Scots pine.

- Subsequent to the annotation of the Scots pine genome, further validation is imperative for the seven Single Nucleotide Polymorphisms (SNPs) identified in this study. This validation process should extend to the identification of associated genes and their functions, offering a more comprehensive understanding of the genetic factors governing susceptibility to *Heterobasidion* infections.

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Acknowledgements

During the period of my Ph.D. Studies in Southern Swedish Forest Research Centre, I received generous support from many people. For this, in the following lines I will try to do my best to express my sincere gratitude to everyone who played a role in completing my thesis:

Jonas Rönnberg words fall short in conveying the depth of my appreciation and gratitude to you for your unconditional support throughout this journey. Your guidance, expertise and encouragement have been invaluable to me at every stage of this project. I am particularly grateful for your patience and understanding, especially during the challenging times. I could not have completed this thesis without you. Beyond the professional realm, I want to express my heartfelt thanks for not only being a supervisor but also a close friend in whom I place complete trust. Your influence has not only contributed to my academic growth but has also played a significant role in shaping me into a better person. Stort tack!

Johanna Witzell and Michelle Cleary, I express my deepest gratitude for your belief in me at the commencement of my academic journey and for affording me the invaluable opportunity to learn under your guidance. Without both of you, I wouldn't be where I am today. I am sincerely grateful for you!

Matts Karlsson, I extend my gratitude for simplifying the complexities of forestry especially during our engaging field day. It was truly an enlightening experience learning from you.

Forest pathology group: **Iryna**, **Patrick, Dusan, Donnie, Ida, Sezer, Noelia, Bea, Mimmi, Maja, Diana, Mohammed, Delnia, Iva**, thank you all for establishing an inspiring working environment and a friendly atmosphere.

Malin Elfstrand and Rosario Garcia Gil, I would like to express my sincere gratitude for your exceptional scientific support and assistance, even during the weekends, throughout the finalization of my thesis. Your dedication and expertise played a crucial role in shaping the outcome of our genotypic study.

Francisco Gil Munoz and Haleh Hayatgheibi thank you so much for your incredible contribution to finalize the genotypic study.

Carl Salk, I extend my sincere thanks for your invaluable support in both linguistics and R. Your assistance has been greatly appreciated.

Salla Marttila, I appreciate your fervor for microscopy. Your meticulous attention to detail has been instrumental in enhancing my understanding. Thank you sincerely for your valuable comments and contributions to our Histology study.

Henrik Böhlenius, I extend my gratitude for the enjoyable conversations we've shared. A special thank you for providing me the opportunity to test my Swedish skills in teaching the forest health lecture. Your support is truly appreciated.

Per-Ola Olsson, I express my gratitude for your contribution to analysing the drone data. Thank you sincerely for your valuable input.

Benjamin Forsmark, I am truly grateful for your advice and support. Your wealth of knowledge and analytical approach to results has been highly valuable and appreciated.

Jan-Eric Englund and Adam Flöhr, thank you so much for your outstanding support in the statistical analysis. Your expertise and contributions have been invaluable, and I appreciate your genius in navigating the complexities of the analysis. Thank you sincerely for your assistance.

People in Horticum: **Ramish**, **Mulatu**, **Waleed**, **Faraz**, **Mahboobeh**, **Sajeevan**, **Mahbubjon**, thank you all for the friendly moments and engaging scientific conversations.

Milda Dambrauskaite, it was indeed my pleasure to work with you during your master's project. I extend my gratitude for your dedicated fieldwork.

Igor Drobyshev, thank you for all the enjoyable conversation we have shared. Because of you, my interest in Thank you so much for your generous time in listening to me whenever I dropped by your office. I appreciate how both of you consistently greeted me with a welcoming smile.

Violeta, **Zhanna**, and **Klas**, thank you for all your help! Very appreciated!

I extend my sincere gratitude to all **my current and former colleagues at the Southern Swedish Forest Research Centre**. thanks to everyone for contributing to such a great work atmosphere.

To all **my PhD fellows,** both current and former, I extend my heartfelt thanks for the wonderful conversations and moments we have shared. Wishing each of you all the best in your future endeavors.

To all our collaborative forest managers: **Rikard Wevel**, **Olov Norgren** (Holmen Skog), **Thomas Höijer**, **Niklas Fogdestam** (Sydved), **Claes Berg** (Mossöja AB), **Fredrik Gustafsson**, **Peter Samuelsson**, **Peter Wallin** (Boxholms Skogar AB), I extend my sincere gratitude for allowing me access to your forests and providing the necessary machinery and resources. Your support and willingness to advance our knowledge for improved forest productivity and the future are truly appreciated. Thank you. A special thank you to **Peter** for our friendly conversations. Your extensive knowledge and vast experience in forest management are admirable. I appreciate your enthusiasm for testing new technology and methods to advance forest management practices.

Jaber, **Hozan**, **Hussain**, **Abdulghani**, **Sewalem** I express my heartfelt gratitude for your unconditional support and assistance. You are not just friends; you are brothers. Thank you so much.

Dear **friends**, **relatives**, and **former students**, I extend my sincere gratitude for all your support. Thank you so much.

Dad, **Mohammad Saleh Youssef**, and Mother, **Zahra Ahmed**, your extraordinary tolerance, and unwavering support throughout my entire life have been a source of immense strength. The value of your encouragement and guidance is immeasurable. Thank you for everything you have done for me. Even if I dedicated my entire life to returning the favor for every small thing you have done, I couldn't fully express my gratitude. I wish you both good health and a long life filled with happiness. With deepest appreciation. "Win her habên yabo û yadê".

My sisters, **Media**, **Manal**, **Awaz**, **Nagma**, and my brother, **Mustafa** and your families, I would like to express my deepest appreciation to each of you. Thank you for all your efforts in supporting me and for bringing happiness into my life. I wish each of you a life filled with success and fulfilment.

Warm regards.

To my father, **Naeif**, my mother, **Khalsa**, my sisters, **Amina** and **Maream**, and my brothers, **Ahmad**, **Hamodi**, **Yusuf**, and **Jan**, along with my in-laws, I want to express my heartfelt gratitude for your constant help, encouragement, and support. Everything you have done for me has not gone unnoticed and is deeply appreciated. Thank you all.

My wife, **Amirah**, I want to express my deepest gratitude for your indispensable help and unwavering support without which my thesis would not have been possible. You are truly a gift from God, and I thank you for your endless support, for taking care of our daughters, for creating a positive and happy environment. I wish you a very long life filled with happiness, health, and great achievements. With all my love.

My sweet daughters, **Elin** and **Eva**, you are the source of my happiness and inspiration. Thank you for every moment you make me happy. I wish each of you a life filled with happiness and success. With love.

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Article

Survey Study Reveals High Prevalence of Heterobasidion Root Rot Infection in Scots Pine (*Pinus sylvestris*) Stands Established on Seemingly Low-Risk Sites

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Abstract: *Heterobasidion* spp. are among the most destructive root rot pathogens, causing severe economic losses to conifer forestry. High infection frequency has been observed in Scots pine stands growing on dry sandy soils with low organic matter or former agricultural soils. In this study, we investigated the incidence of *Heterobasidion* spp. infection in Scots pine forests established on low-risk sites where the trees looked healthy and unlikely to be infected. In total, 135 healthy-looking pine trees from nine different stands were examined for *Heterobasidion* spp. presence. *Heterobasidion* spp. was detected in six stands and infection frequency was 13%–33%. There was a significant correlation between site index and infection frequency, which was higher in pine stands established on more fertile soils. There was no correlation between disease incidence and defoliation level, diameter of tree at breast height, root diameter, tree volume, or stand age. Overall, our results showed that, regardless of the soil type, Scots pine can be intensively infected by Heterobasidion pathogens while showing no outward signs. Therefore, the risk of Heterobasidion disease should be taken into consideration in management of pine forests growing on both low- and high-risk sites for more productive and sustainable forests.

Keywords: root rot disease; *Heterobasidion annosum*; Scots pine; low-risk site; pine forest management; crown defoliation; stump treatment; thinning

Citation: Youssef, K.; Dambrauskaite, M.; Witzell, J.; Rönnberg, J. Survey Study Reveals High Prevalence of Heterobasidion Root Rot Infection in Scots Pine (*Pinus sylvestris*) Stands Established on Seemingly Low-Risk Sites. *Forests* **2023**, *14*, 1018. https://doi.org/10.3390/f14051018

Academic Editor: Simon Francis Shamoun

Received: 22 March 2023
Revised: 7 May 2023
Accepted: 9 May 2023
Published: 15 May 2023

1. Introduction

Scots pine (*Pinus sylvestris* L.) is one of the most distributed pine species in the world [1]. In Sweden, Scots pine is the second most common and economically important tree species after spruce with 39% of the standing volume on productive forest land, and it is present all over the country [2]. The wood is used for pulp, sawn timber, and bio-energy and the forests offer recreational as well as invaluable biodiversity values [3]. However, these forests are increasingly threatened by root and butt rot caused by the fungal pathogen *Heterobasidion annosum*, which is among the most frequent and severe fungal diseases of conifers in the northern hemisphere. In Europe, losses due to Heterobasidion root rot have been estimated at EUR 800 million per year [4]. In Sweden, two species of *Heterobasidion* are present: (1) *H. annosum* sensu stricto (Fr.), which mainly attacks pines, but also infects other conifers such as Norway spruce and broadleaved trees [5–7], and (2) *H. parviporum* Niemelä & Korhonen, which mainly attacks Norway spruce, but can also infect Scots pine seedlings, Siberian larch, and silver birch [5,6,8].

The primary Heterobasidion root rot infection is caused by basidiospores dispersed from fruiting bodies that are formed at the base of infected stumps or diseased trees [9] when the temperature is above 0 °C [10]. Basidiospores land on newly created stump surfaces or wounds of living trees and produce mycelia [11,12] which colonize the host tissues and grow into the root systems, subsequently infecting neighboring healthy trees via root contacts or grafts [9,13].

The types of damage caused by *Heterobasidion* depend on the host species. For instance, it causes stem decay which grows up to 4.8 m and 12 m in spruce and larch trees, respectively [14,15]. However, in Scots pine, the infection typically remains in the roots, causing root tissue decay and increasing the risk of wind throw. Additionally, the infection can reduce the volume growth and productivity of a stand by up to 10% annually [16]. Further, infected pine trees have been observed to have shorter needles and increased crown transparency [17]. The infection eventually leads to trees' mortality [18–20].

The probability of *Heterobasidion* infection in Scots pine is affected by complex and interacting factors, such as host resistance, intensity of stump infection, and environmental conditions affecting both the pathogen and the host [9]. Previous studies have shown that high incidence of *Heterobasidion* disease is associated with site conditions, such as sandy soil with low organic matter content [9,21–23], coarse soil texture [21–23], soil with high pH [9,22,23], and former agricultural soils [9]. These characteristics are therefore used in identifying sites with a high risk of trees becoming infected; conversely, their absence is taken as a sign of a low-risk site. A soil risk rating scale developed by Morris and Frazier (1966) [24] for potential *H. annosum* infection in loblolly pine stands classifies sands, loamy sands, and sandy loams as high-risk soils; loams and silt loams as intermediate-risk soils; and clays and clay loams as low-risk soils. A similar risk rating system was later developed in the United Kingdom [25]. Because these systems rely only on soil type to distinguish high and low risk sites, they may not be directly applicable for evaluation of risk levels in other countries and regions where other biological and management-related factors influence *Heterobasidion* infection. Such a risk-rating system is still lacking for Swedish forests [26].

In pine forests, *Heterobasidion* infection is usually difficult to detect because of the lack of clear symptoms, especially in the early stages of the infection. Wang et al. (2014) [16] found that 87.5% of trees in a high-risk Scots pine forest in southern Sweden were infected by *Heterobasidion* without above-ground symptoms. Rönnberg et al. (2006) [20] assessed trees' crown condition and *Heterobasidion* incidence in pine forests established on former agricultural soils. They found *Heterobasidion* spp. to be present in fourteen of fifteen sites. Although 73% of sampled trees were infected, 45% of infected trees were assessed as healthy. In addition, infection was more frequent among trees with defoliated crowns. In another study, Kurkela (2002) [17] found that the average crown condition of Scots pine was correlated with *Heterobasidion* disease incidence when dead trees were included, whereas the correlation was not significant without dead trees. In other words, based on crown condition, infected trees could not be distinguished from healthy trees. The difficulty of identifying clear visual signs of infection hinders efforts to manage the disease in pine stands [27]. All of these studies were performed on sites considered to be high risk. The external signs of infection on trees on low-risk sites may be different; the risk may not actually be lower, but the disease expression may be different. Regardless, the use of external symptoms such as crown defoliation may not be very useful for assessing infection levels in a stand. The situation on low-risk sites is not well investigated and may be different due to different soil and other conditions.

In Sweden, biological control using an antagonistic fungus, *Phlebiopsis gigantea* (Fr.) Jülich, is done mainly during the thinning of Norway spruce (*Picea abies* (L.)) stands. However, it is possible that *Heterobasidion* can also become established in Scots pine stands thinned during the summer if stump treatment is not applied. Several earlier studies have also shown the efficacy of stump treatment with *P. gigantea* for preventing *Heterobasidion* infection in pines. Rishbeth (1963) [28] inoculated pine stumps with *P. gigantea* spores and found a good result against *Heterobasidion*. More recently, Pellicciaro et al. (2021) [29] showed that treatment of Scots pine stumps with *P. gigantea* MUT 6212 completely prevented colonization by *H. annosum*. To support informed management decisions regarding stump treatments in pine stands, more evidence about infection risks is needed, especially on sites where the risk has traditionally been assumed to be low.

The aim of this study was to produce new knowledge about *Heterobasidion* root rot prevalence on low-risk sites by: (1) investigating the present prevalence of *Heterobasidion* root rot infection in Scots pine forests growing on low-risk sites, (2) analyzing the relationship between infection prevalence and stand characteristics (age, site index (the total height to which dominant trees of a given species will grow on a given site at 100 years), and tree growth measurements (volume, tree root diameters)), and (3) assessing the correlation between *Heterobasidion* infection and visually estimated crown defoliation of Scots pine trees on the same sites.

2. Materials and Methods

2.1. Study Sites

Data for this study was collected from nine Scots pine stands in southern Sweden between 2018 and 2021 (Figure 1, Table 1). The stands were selected based on three main criteria. First, the stands should not be on sandy soils and should not have previously been used as pasture or for agriculture. Second, the trees had to be ≥80% Scots pine. Third, the stand must have been thinned at least once, preferably during the summer or late spring, i.e., when spores of *Heterobasidion* spp. are expected to be abundant.

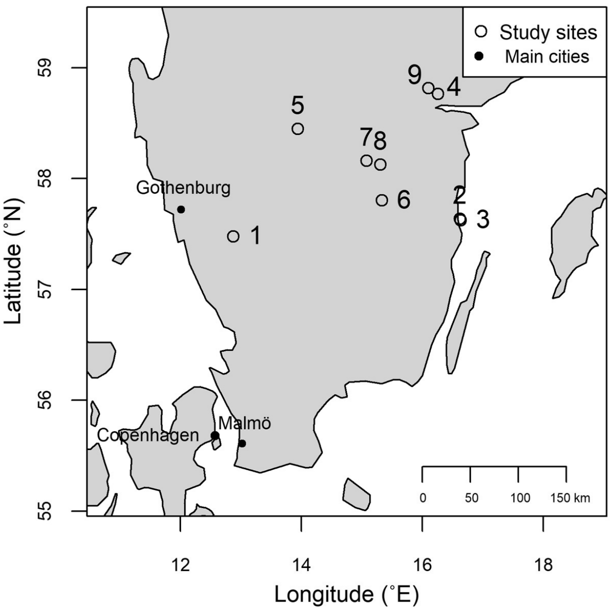

Figure 1. Location of the study sites in southern Sweden. The figures adjacent to open circles on the map correspond to the stand id, as indicated in Table 1.

2.2. Sample Collection and Identification of Infected Trees

On each site, fifteen trees were selected by the respective forest manager following their regular thinning regime. To collect samples from pine roots, the selected trees were uprooted using a single-grip forest harvester machine. When the trees were down, stem diameter at breast height (DBH), bark thickness, height, and stem length to the first living branch were measured. Diameter was measured by cross-calipering at 1.30 m above ground level. Bark thickness was measured at DBH height at three points around the stem using a bark gauge (Haglof Barktax Bark Gauge, Hammerdal, Sweden). A measuring tape was used to measure stem length (height) from ground level to the base of the first living branch as well as the total height of the tree. Tree volume was calculated using the following formula [30]:

$$V = 0.1193D^2 + 0.02574D^2H + 0.004054DH^2 + 0.007262D^2K - 0.003112DHB$$

where V = tree volume above cutting (dm^3), D = stem diameter at breast height (cm), H = total tree height from the ground (m), K = stem length (height) from ground level to the base of the first living branch (m), and B = double bark thickness (mm).

The root system of each tree was cleaned using a garden spade. From each tree, five roots were randomly selected and haphazardly sampled at a 0–75 cm distance from the root collar [16]. The sampling area was cleaned with a brush and sprayed with 70% ethanol. Discs 2–5 cm thick were cut with a Japanese hand saw and put immediately into a labelled plastic bag and transferred to the lab for microscopy analysis. To prevent cross-contamination between samples, the saw was thoroughly cleaned by removing any dust and then sprayed with 70% ethanol between cuts. The diameter of root samples was measured by calculating the average of the perpendicular diameters of the root discs, and the samples were assigned to five classes according to their diameters.

2.3. Microscopy Analysis

From nine different Scots pine stands, a total of 135 Scots pine trees and 675 root samples were examined for the presence of *Heterobasidion*. The root samples were first incubated at room temperature (20 °C) in darkness for 10 days. After that, samples were checked in a random order for presence of *Heterobasidion* spp. conidiophores by using a stereo microscope. When conidiophores were found, the sample was recorded as infected. The species of Heterobasidion were not identified because Scots pine is primarily infected by *Heterobasidion annosum* [5–7].

2.4. Defoliation Assessment

A local reference tree was selected in each stand to compare with other selected trees. The reference tree was representative of typical crown morphology in the stand and had $\leq$10% defoliation. Crown defoliation level was visually evaluated on a scale of 0 to 4 (0—healthy trees, defoliation 0%–10%; 1—slightly defoliated trees, defoliation 11%–25%; 2—moderately damaged trees, defoliation 26%–60%; 3—severely damaged trees, defoliation 61%–99%; and 4—dead trees, defoliation 100%) [31]. The defoliation assessment was conducted while the trees were standing. Since the trees in stand six were felled prior sample collection, the defoliation assessment is not provided. To exclude defoliation caused by other foliage diseases or insects attack, the reference and sampled trees were checked for the presence of these stressors; no marked symptoms or signs of them were observed in any of the sampled stands.

2.5. Statistical Analysis

The correlation between Heterobasidion root rot disease incidence and defoliation level, root diameter, and stand characteristics was tested using linear mixed models in the R package "lme4" with stand as a random effect. The null hypothesis was lack of an association between defoliation level, root size, and Heterobasidion root rot disease incidence. All analyses were performed using R version 4.1.3 (R Core Team, 2022).

3. Results

3.1. The Prevalence of Heterobasidion Infection on Low-Risk Sites

Heterobasidion root rot infection was recorded in six out of nine sites. In total, 20 trees (14.8%) and 28 root samples were infected. The frequency of infection across all sites was 0%–33% (Table 1).

Table 1. Details of nine study sites with expected low risk of infection of *Heterobasidion* spp. in southern Sweden including actual infection rates.

Stand Id	Site Name	Location	Stand Age (yr)	Site Index	Soil Type	Previous Tree Species	Number of Thinnings	Number of Uprooted Trees	Number of Infected Trees	Infection Frequency (%)
1	Svenljunga	57°28′42.0″ N 12°52′27.0″ E	38	T25	Moraine	Scots pine + Norway spruce	3	15	0	0
2	Västervik	57°38′15.3″ N 16°37′00.1″ E	52	T18	Moraine	- *	- **	15	0	0
3	Västervik	57°37′24.2″ N 16°38′03.8″ E	47	T21	Moraine	- *	- **	15	0	0
4	Norrköping	58°45′48.3″ N 16°15′22.4″ E	42	T29	Silty clay	- *	1	15	5	33.33
5	Skövde	58°26′53.2″ N 13°56′35.3″ E	37	T24	Glacial sediments	Scots pine	1	15	2	13.33
6	Österbymo	57°48′05.4″ N 15°19′48.4″ E	62	T25	Moraine	Scots pine	2	15	2	13.33
7	Boxholm	58°09′40.4″ N 15°04′27.3″ E	45	T27	Moraine	Norway spruce	1	15	3	20
8	London Grytfall	58°07′29.8″ N 15°18′09.8″ E	58	T26	Moraine	Scots pine	2	15	3	20
9	Simonstorp	58°48′51.4″ N 16°06′05.7″ E	36	T28	Moraine	Scots pine	1	15	5	33.33
total								135	20	14.8

* Documentation about previous tree species is not available. ** Although thinnings have been carried out, there is a lack of documentation containing historic data, but they are confirmed by forest managers to be old forest sites.

3.2. Relation between Infection Frequency and Stand Characters (Site Index, Stand Age)

A significant positive correlation was found between site index and infection frequency (p = 0.004, t = 4.1946, df = 7; Table 1). The average site index of infected stands (i.e., stands with presence of *Heterobasidion* spp. infection) (T26.2) was higher than for healthy stands (T21.3) There was no significant correlation between infection frequency and age of the stand (p = 0.072). (Table 1).

3.3. Relationship between Infection Frequency and Tree Characteristics (Tree Volume, Root Diameter, Crown Defoliation)

There was no correlation between infection incidence and volume of the trees (p = 0.45). At the stand level, the differences in volume between infected and non-infected (healthy) trees were also not significant in all cases (Figure 2A).

In total, 28 of the 675 examined root disc samples were infected by *Heterobasidion* spp. (i.e., 4%) The diameter of sampled roots ranged from 1.85 to 12.55 cm (Figure 2B). A linear mixed model showed no correlation between root diameter and infection (p = 0.81, chi-square = 0.059, df = 1).

The visual assessment of crown status showed that about 62% of the investigated trees showed signs of defoliation while 38% had healthy crowns. Among the trees showing signs of defoliation, 18.5% were infected and 81.5% were not infected (Figure 3A).

In infected stands, 40% of trees had healthy crowns and 4.2% of them were infected. About 40% of trees showing defoliation belonged to defoliation class 1 and 34.6% of these were infected (Figure 3B). Defoliation level trended higher in trees infected by *Heterobasidion* (p = 0.072).

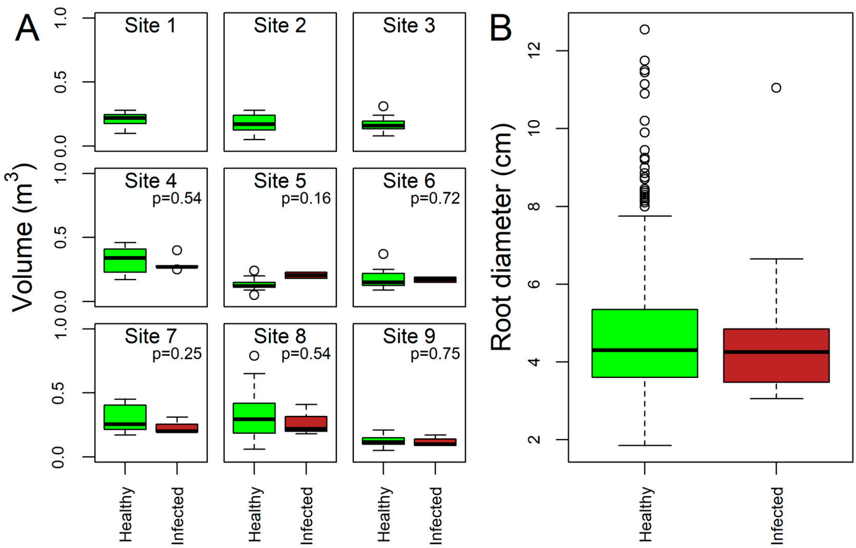

Figure 2. *Heterobasidion* spp. infection frequency and tree characteristics (tree volume, root diameter) in the analyzed stands. (**A**) Volume of infected and non-infected trees in each sampled stand). (**B**) Diameter of infected and non-infected (healthy) root discs. The boxes show the 25% and 75% quantiles, with the thick central line at 50%. The whiskers show the range of values no more than 1.5 times the interquartile range from the extremes of the boxes; the open points are observed values beyond this range.

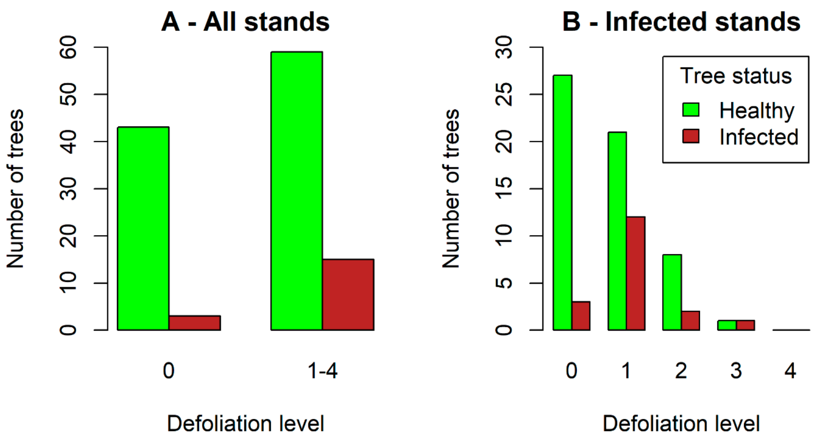

Figure 3. (**A**) Tree defoliation status in all stands. (**B**) Defoliation level of healthy and infected trees in infected stands.

4. Discussion

Although the incidence and distribution of *Heterobasidion* root rot infection has been studied intensively in Scots pine forests growing on high-risk sites, this is the first study to investigate the prevalence of *Heterobasidion* infection in Scots pine forests established on sites thought to have a low risk of *Heterobasidion* infection. Six of nine stands had at least one tree infected by *Heterobasidion* spp., which demonstrates the ability of *Heterobasidion* spp. to establish and persist even in low-risk sites. None of the study stands showed any characteristic symptoms for *Heterobasidion* spp. such as groups of dying pines, fruiting bodies, and windthrown pines with decayed roots. With an infection incidence above 30% of trees on some sites, it seems warranted to consider the risk for *Heterobasidion* infection when planning silvicultural activities on all sites. Furthermore, due to climate change, higher infection incidence by *Heterobasidion* spp. may be expected as the pathogen produces and releases more basidiospores during longer growing seasons and warmer temperatures [32]. Warmer temperatures also mean a lower proportion of thinning operations will be conducted during a shorter cold winter period [33]. Therefore, risk assessment systems based only on soil type are likely to underestimate the risk of *Heterobasidion* disease. Our results indicate that a hazard risk assessment system should consider soil type, stand management history (first generation or previous agricultural or pasture areas), and presence of *Heterobasidion* infection in the current and previous plantations. If one or more of the above-mentioned factors is present in a pine stand, prevention methods such as stump treatment and winter cutting must be implemented promptly, especially considering the potential increased planting of Scots pine as an adaptation to climate change.

In the Swedish classification system, higher site index reflects higher productivity, i.e., higher soil fertility [34,35]. We found infection incidence to be higher in more fertile stands (Table 1), which agrees with results of earlier studies [36,37]. Soil fertility may have a positive influence on pine trees' growth, but Rishbeth (1957) [38] reported a tendency for fast-growing trees to suffer more serious *Heterobasidion* damage than slow-growing ones. Even if there seems to be a relationship between the site index and the infection frequency, it is challenging to define clear thresholds for low or high risk. Nevertheless, stump treatment or winter cutting are likely to reduce the risk of *Heterobasidion* root rot infection in pine stands regardless of site index.

Once a tree is infected by *Heterobasidion* spp., some portion of the nutrients that are necessary for tree growth will be allocated to induce different defence mechanisms [39,40]. Over time, the fungus colonizes more root tissues, leading to dysfunction of roots and their capacity for water and nutrient uptake [41,42] and, consequently, declining tree growth. Wang et al. (2014) [16] found that the annual reduction in volume production of Scots pine stands infected by *Heterobasidion* spp. was approximately 10%. Likewise, Burdekin (1972) [19] has shown that the total loss of volume of Scots pine stands infected by *Heterobasidion annosum* was represented not only by volume of dead trees but also by a reduction in volume of the live, infected trees. However, our comparison between volumes of infected and non-infected trees in each stand did not reveal significant differences. This may have two main causes: first, the lower number of infected trees compared to non-infected ones (Figure 2A), and second, trees were sampled as part of ordinary thinning operations that first aimed to remove weak, malformed, or slow-growing trees; thus, the thinned non-infected trees may be of lower quality than the remaining non-thinned trees. Nevertheless, while the trees infected by *Heterobasidion* may survive for a long time without showing any symptoms, their overall productivity is likely to decline, which can significantly reduce the revenues from a plantation [26].

Heterobasidion infection spreads vegetatively from the roots of infected stumps to neighboring healthy trees via root contacts or grafting. The infection usually develops from smaller roots up into the primary root and then the stem of the tree [43]. Despite the lack of strong correlation between root diameter and disease incidence in this study, the infection was mainly observed in roots of diameter $\leq$6 cm; small-diameter roots have thinner bark which may become infected more easily. This result is in agreement with Wang et al. (2014) [16], who found that Scots pine roots with a diameter between 1–4 cm had the largest percentage of detected *Heterobasidion* infections. Morrison and Redfern (1994) [44] found that the diameter of Sitka spruce roots infected with *H. annosum* was less than 6 cm, and the site of the infection was at least 1 m from the root collar. Taken together, these findings suggest that in early stages of infection, the smaller roots are infected, leading to growth reduction and no immediate increase in windthrow risk as the infected trees reallocate energy to block fungal growth. This result should also be considered when planning sampling strategies for *Heterobasidion* infections; detection frequency could be improved by focusing sampling efforts on smaller roots.

Crowns of infected trees in this study had a weak trend toward slight or moderate defoliation levels, but no significant change in crown length. This indicates that visual assessment of crown condition or defoliation alone are not reliable infection indicators, in agreement with previous studies from high-risk sites [16,17,20,45]. Thus, foresters lack a practical method for detecting infection. New aerial monitoring methods based on multispectral or hyperspectral bands from UAV imagery [46] may provide efficient and affordable detection of cryptic *Heterobasidion* infections in the near future.

Our sample sizes were limited for several reasons. The most important is the cost of using heavy machinery to uproot trees. This problem was compounded by the severe drought in summer 2018 when no such machinery was allowed in forests due to fire risk. Further, a subsequent outbreak of spruce bark beetle in Sweden meant all machinery was occupied in cleanup operations, further limiting access to suitable pine forests. However, even with such limitations, the overall distribution of sampled stands was appropriate.

This study's results are important from a practical point of view, since stands on assumed low-risk sites are currently not managed to prevent *Heterobasidion* spp. infection, such as through application of stump treatment during summer thinnings. Treatment with *P. gigantea* combats primary infection by *Heterobasidion* spp. on Scots pine stumps [47,48] and may improve site productivity. However, this treatment is costly. Applying *P. gigantea* [49] to Scots pine stands in central Sweden with a site index of 25 during a typical rotation (two thinnings at 33 and 48 years and a final felling at 73 years) is estimated to cost SEK 2738 ha^{-1} (ca. EUR 267), a value equivalent to 10 m^3 of pulpwood (SEK 270 m^{-3} SUB, solid under bark) [50]. If calculated using a 3% discount rate back to the first thinning [16], the corresponding cost would be SEK 1341 ha^{-1} (ca. EUR 131) at age 33. This value corresponds to 5.0 m^3 of timber. The estimated annual volume growth reduction due to *Heterobasidion* infection in the Wang et al. (2014) [16] study site cannot be compared to our results. The 5.0 m^3 figure can, however, be compared with the number of trees this volume would correspond to at this site index. At final felling, this is about ten trees, or around 1.4% of the trees in a hectare. In this study the overall infection frequency is 14.8%. It is therefore very likely that stump treatment would yield economic benefits.

5. Conclusions

The results of the present study confirm that *Heterobasidion* root rot disease can be present in pine roots, even in areas previously thought to pose a low risk. These findings suggest that the problem of *Heterobasidion* disease may be underestimated in Sweden and elsewhere, and that there is a significant risk of its spread and prevalence in pine forests due to climate warming and intensifying thinning actions, which are increasingly being conducted during the spore deposition period. To ensure sustainable forest management, prevention measures such as stump treatment are recommended to control *Heterobasidion* root rot in Scots pine forests.

Author Contributions: Conceptualization, J.R.; Data curation, K.Y. and M.D.; Formal analysis, K.Y.; Funding acquisition, J.R.; Methodology, K.Y. and J.R.; Supervision, J.W. and J.R.; Validation, K.Y. and J.R.; Writing—original draft, K.Y.; Writing—review and editing, K.Y., M.D., J.W. and J.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by FORMAS, grant number 2015-01413; and the Rattsjö Foundation.

Data Availability Statement: Data is contained within the article and available on request from the corresponding author.

Acknowledgments: We would like to thank Thomas Höijer (Sydved skog), Rikard Engdahl Wevel and Olov Norgren (Holmen skog), Peter Samuelsson and Peter Wallin (Boxholms Skogar) for giving access to their forests and allowing sampling. Adam Flöhr provided statistical support. Matts Karlsson provided valuable comments.

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

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Acta Universitatis agriculturae Sueciae
Doctoral Thesis No. 2023:101

This thesis investigated the cryptic root rot caused by *Heterobasidion* in Scots pine, revealing infections even on ostensibly low-risk sites. The integration of drones and physiological markers for disease detection was explored, while also investigating the potential for breeding resistant pines in Sweden. The results underscore the importance of implementing preventive measures against *Heterobasidion* infections on Scots pine. Emphasizing the imperative for swift and efficacious action is crucial to moderate the impact on pine forest productivity.

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ISSN 1652-6880
ISBN (print version) 978-91-8046-254-9
ISBN (electronic version) 978-91-8046-255-6