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SUSCEPTIBILITY OF NATIVE AND INTRODUCED CONIFERS TO *HETEROBASIDION* SPP. IN LATVIA AND CONTROL MEASURES TO LIMIT SPREAD OF THE PATHOGENS

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SUMMARY

In coniferous forests of northern Europe, *Heterobasidion annosum* and *Heterobasidion parviporum* are the most devastating pathogens, causing stem-root rot. The aim of the thesis was to investigate the susceptibility of native and introduced conifer species to *Heterobasidion* spp., and to evaluate possible control measures against *Heterobasidion* spp. in Latvia. Growth rates of *H. annosum* and *H. parviporum* in functional sapwood were analysed. The results showed that both pathogens differ in their pathogenicity, particularly in *Pinus sylvestris*, where *H. annosum* is more pathogenic compared to *H. parviporum*. Both pathogens spread faster in living sapwood of *Picea abies* than in *P. sylvestris*. Growth rates of *Heterobasidion* spp., three Latvian isolates of *Phlebiopsis gigantea* (antagonistic fungus to *Heterobasidion*), and Rotstop® were analysed in stem sections of *Picea abies*, *Picea sitchensis*, *Pinus sylvestris*, *Pinus contorta*, *Pinus strobus*, *Pseudotsuga menziesii* and *Larix sibirica*. *P. gigantea* strains and Rotstop®, colonized wood of all tree species except for *Pseudotsuga menziesii*. Growth rate of Latvian *P. gigantea* isolates in wood of *Picea* and *Pinus* species was comparable to growth rate of the Rotstop® isolate. Stump treatment with Latvian *P. gigantea* isolates should be recommended for forest management in Latvia. The importance of mechanical injury to stems of *P. abies*, *P. sylvestris* and *P. contorta* as infection sources for *Heterobasidion* spp. and other decay causing fungi was analysed. Occasional occurrence of *H. parviporum* (but also of numerous other fungi) in *P. abies* stems bearing mechanical wounds was observed. In contrast, *Heterobasidion* infections were not detected in any of the pine species investigated. The spread of *Heterobasidion* was analyzed in *P. contorta* stands and the results indicated that freshly cut stumps of *P. contorta* are highly susceptible both to primary and secondary infections, by *H. annosum* and *H. parviporum*. Consequently, stump treatment is required to control *Heterobasidion* spp. basidiospore infections in *P. contorta* plantations in Latvia. The effect of natural colonization of *P. abies* and *P. sylvestris* stumps by *P. gigantea* on the inhibition of stump infections by *Heterobasidion* spp. was investigated. Our results demonstrated that natural colonization by *P. gigantea* was not able to restrict the pathogen. This confirms the necessity for thorough treatment of cut *P. abies* and *P. sylvestris* stumps. In *P. abies* stands after thinning, urea, Rotstop® and native Latvian *P. gigantea* isolates provided similar efficacy against *Heterobasidion* infection compared to untreated stumps. *P. abies* stump removal did not eliminate *Heterobasidion* from infested sites.

Keywords: stem and root rot, stem damage, *P. gigantea*, stump treatment and removal.

KOPSAVILKUMS

Ziemeļeiropā skuju koku audzēs ievērojamus mežsaimnieciskos zaudējumus izraisa *Heterobasidion annosum* un *Heterobasidion parviporum* stumbra-sakņu trupe. Darba mērķis ir noteikt vietējo un introducēto skuju koku uzņēmību pret *Heterobasidion* spp. infekciju un izvērtēt patogēna ierobežošanas iespējas Latvijā. *H. annosum* un *H. parviporum* micēlija augšanas ātrums tika analizēts *Picea abies* un *Pinus sylvestris* aplievas koksnē. Konstatēts, ka analizēto sēņu sugu patogenitāte atšķiras, turklāt *P. abies* koksnē micēlijs izplatās būtiski ātrāk. *Heterobasidion*, trīs Latvijas izcelsmes *Phlebiopsis gigantea* izolātu un augu aizsardzības līdzekļa Rotstop® sastāvā esošā *P. gigantea* izolāta augšanas ātrums tika novērtēts *Picea abies*, *Picea sitchensis*, *Pinus sylvestris*, *Pinus contorta*, *Pinus strobus*, *Pseudotsuga menziesii* un *Larix sibirica* koksnē. *Heterobasidion* spp. attīstījās visās koku sugās, bet *Phlebiopsis gigantea* micēlija attīstība netika konstatēta *P. menziesii* koksnē. Latvijas izcelsmes *P. gigantea* izolātu micēlija attīstība skuju koku koksnē būtiski neatšķiras no bioloģiskā preparāta Rotstop®, tādēļ vietējie izolāti ir izmantojami celmu aizsardzībai. Gan *P. sylvestris*, gan *P. contorta* uzrāda augstu rezistenci pret stumbra brūces kolonizējošām sēnēm, bet *Picea abies* raksturo uzņēmība pret *H. parviporum* un citām stumbra un sakņu trupi izraisošām sēnēm. *P. sylvestris* un *P. contorta* stumbra brūces raksturo augsta noturība pret *Heterobasidion* bazīdijsporu infekciju. Analizējot *Heterobasidion* spp. izplatību trīs *P. contorta* audzēs, konstatēts, ka *P. contorta* raksturo augsta uzņēmība pret *H. annosum* un *H. parviporum* primāro un sekundāro infekciju, lai ierobežotu *Heterobasidion* izplatību, mežizstrādes laikā nepieciešams pielietot celmu aizsardzības līdzekļus. Konstatēts, ka vietējās *P. gigantea* populācijas nenodrošina efektīvu celmu aizsardzību pret *Heterobasidion* spp. bazīdijsporu infekciju *P. abies* un *P. sylvestris* jaunaudzēs, tādēļ, veicot kopšanas cirtes, jānodrošina skuju koku celmu apstrāde. Pierādīts, ka Rotstop®, vietējās izcelsmes *P. gigantea* un urīnviela nodrošina līdzvērtīgu egļu celmu aizsardzību pret sakņu piepi. Celmu izstrāde nenodrošina pilnīgu aizsardzību pret *Heterobasidion* sekundāro infekciju, bet samazina *Heterobasidion* infekcijas potenciālu.

Atslēgas vārdi: sakņu un sumbra trupe, stumbra bojājumi, *P. gigantea*, celmu apstrāde, celmu raušana.

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INTRODUCTION

The main goal of modern forest management is to keep the balance between three main pillars: ecological, economical and socio-cultural necessities. To ensure that, it is mandatory to keep forests healthy, especially under conditions when the stands are subjected to intensive timber production combined with the pressure resulting from simultaneous wildlife management, – currently typical features of Latvian forests.

In conifer stands in the northern hemisphere, *H. annosum* and *H. parviporum* are the most devastating and economically important pathogens of pine and spruce, causing root, butt and stem rot (Gonthier and Thor 2013; Müller *et al.* 2018; Gaitnieks *et al.* 2022; Kovalchuk *et al.* 2022). Extensive logging of commercial conifer forests results in huge numbers of cut stumps over large areas: a niche which is highly suitable for *Heterobasidion* spp. (Woodward *et al.* 1998; Gonthier and Thor 2013). In the context of global warming, hypothetically we can expect suboptimal conditions for native tree species (Terhonen *et al*. 2019; Venäläinen *et al.* 2020) and more widespread distribution of a wide range of forest pathogens, including such root rot pathogens as *Heterobasidion* spp. (Müller *et al.* 2014; Terhonen *et al.* 2019) and subsequently increasing infection occurrences.

Restoration of clearcuts as well as establishment of stands in new areas (e.g., abandoned or former agricultural land), have been fundamental components of intensive forest management, including in Latvia (Klavina *et al.* 2021). In this respect, selection of the most disease resistant tree species, families, clones, is of crucial importance and applies also to root and stem rot pathogens (Hietala *et al*. 2003; Gonthier and Thor 2013). Therefore, the risks associated with the introduction of new tree species in the context of potential susceptibility against native pathogens should be evaluated (Karlman 2001) and possible inhibition methods, including biological control agents containing *P. gigantea* spores should be evaluated.

The aim of the thesis was to investigate the susceptibility of native and introduced conifer species to *Heterobasidion* spp., and to evaluate possible control measures of the pathogens in Latvia.

The objectives:

1) to conduct comparative analysis of growth rates of *H. annosum* and *H. parviporum* in the functional sapwood of *P. abies* and *P. sylvestris*;
2) to investigate growth of *H. annosum* and *H. parviporum*, Latvian isolates of *Phlebiopsis gigantea*, and the biological control Rotstop® strain in cut stems of *Picea abies*, *Picea sitchensis*, *Pinus sylvestris*, *Pinus contorta*, *Pinus strobus*, *Pseudotsuga menziesii* and *Larix sibirica*;

3) to estimate the importance of mechanical wounds on stems of *P. abies*, *P. sylvestris*, and *P. contorta* to serve as infection foci for *Heterobasidion* and other decay causing fungi;
4) to investigate host susceptibility to *Heterobasidion* spp., and modes of spread of the pathogens in *P. contorta* plantations established on forest clearcuts and former agricultural land;
5) to assess the potential of natural infections of *P. gigantea* to restrict infection and spread of *Heterobasidion* spp. in *P. abies* and *P. sylvestris* stumps;
6) to compare the efficacy of native Latvian *P. gigantea* isolates, the biological control Rotstop® agent and urea treatments against *Heterobasidion* spp. basidiospore infection;
7) to evaluate the persistence of *Heterobasidion* spp. in root fragments of *P. abies* left on clearcut sites following the removal of infected stumps.

Thesis for defense:

1) although tree species plays a major role in both pathogen and biocontrol fungus development, growth rates of *H. annosum* and *H. parviporum* differ in the same tree species;
2) mechanical stem wounds may play a role in *Heterobasidion* development in *P. abies* stands, but conversely, *P. contorta* and *P. sylvestris* wounds are decay resistant;
3) *P. contorta* is highly susceptible to both primary and secondary infections;
4) the potential of "wild" populations of *P. gigantea* to colonize and outcompete *Heterobasidion* in *P. abies* and *P. sylvestris* stumps by natural infections is insufficient to restrict infection, while stump treatment using urea, biocontrol Rotstop® or local *P. gigantea* have similar control efficacy;
5) removal of infected *P. abies* stumps may reduce infection potential in the following generation.

1. LITERATURE OVERVIEW

1.1. Economic impact and distribution of *Heterobasidion* species in Europe

At the beginning of the 21st century a broad range inventory of Latvian Norway spruce *Picea abies* (L.) Karst stands revealed that approximately 22% of cut trees were colonized by rot causing fungi, and the most commonly detected fungus was *Heterobasidion parviporum* Niemelä and Korhonen (Arhipova *et al.* 2011; Arhipova 2012). It has been estimated that root, butt and stem rot pathogens may cause losses to forestry of up to 4,800 euros per hectare (Gaitnieks *et al.* 2008). Although in a wider scale, precise damages caused by *Heterobasidion* infection are almost impossible to detect, by the end of the previous century, the annual losses caused by *Heterobasidion* root-rot to forest production solely in the European Union were calculated to be between 500 and 800 million euros (Woodward *et al.* 1998; Korhonen and Holdenrieder 2005). In more recent publications, 1 billion euros in yearly loss in Europe has been estimated (Kovalchuk *et al.* 2022). As a consequence of climate change (Müller *et al.* 2014; Trishkin *et al.* 2016; Terhonen *et al.* 2019; Venäläinen *et al.* 2020) and change in land use from agriculture lands, pastures, grasslands to forest plantations (Klavina *et al.* 2021) and the increased intensity of the thinning (Vollbrecht and Agestam 1995), direct (decay caused by pathogen) and indirect (growth reduction caused by pathogen (Bendz-Hellgren and Stenlid 1997)), insect attack, wind damages to symptomatic trees etc., losses caused by root rot pathogens and pests may become even more severe (Venäläinen *et al.* 2020). Unfortunately, it has been noticed that climate change may favor plant pathogens more than mutual symbiotic organisms such as ectomycorrhizal fungi, as pathogens seem to have broader climate niches (Větrovský *et al.* 2019).

In total, forests cover around 40% of the EU's land area (Orsi *et al.* 2020, and references therein). In many European countries simple forest structures (monocultures) of conifers have been favored in the long term. Some tree species are dominant in forestry management. For example, the total share in the European Union of Scots pine (*Pinus sylvestris* L.) in commercial forests reaches almost one fifth (Mason and Alia 2000; Marčiulynas *et al.* 2019, and references therein). In Latvia, *P. sylvestris*, birch *Betula pendula* Roth. and *P. abies* are the three main tree species; moreover, conifer stands cover half of the total forest area (Jansons 2021). Conifers (*P. sylvestris* and *P. abies*) are usually used not only for regeneration but also for afforestation in Latvia (Daugaviete *et al.* 2020), despite reports that *P. abies* may be negatively affected by climate change in many regions, which could affect the forestry sector (Bolte *et al*. 2010; Schou *et al.* 2015, and references therein). However, optimal forest management in Latvian conditions

may increase vitality and resilience of *P. abies* stands (Bāders *et al*. 2020). In addition, according prediction modelling in the Nordic regions, in Finnish boreal forests in the absence of damages, conifer-oriented forestry would lead to 5–10% higher timber yields and carbon sequestration compared to mixed-stand- and broadleaf-oriented management (Pukkala 2018). Although the *Heterobasidion* species complex is distributed worldwide (Figure 1.1) and may affect both conifers and broadleaves, the most severe damages are usually observed solely in conifer stands (Gonthier and Thor 2013). The *Heterobasidion* complex consists of five species. All *Heterobasidion* complex species can survive saprophytically in woody substrates, they may switch from the role of saprophytic organism to necrotrophic, and then cause root, butt and in some tree species extensive stem rot (Garbelotto and Gonthier 2013). The biology and ecology of *Heterobasidion* has been the subjects of many studies since the pathogen was first described more than 200 years ago in 1821 (Woodward *et al*.1998; Garbelotto and Gonthier 2013). Based on morphology, mating compatibility and ecology, several species have been defined, which are intersterile within the *Heterobasidion* species complex (Korhonen 1978). The latest knowledge about genetic diversity, host range and distribution of *Heterobasidion* resolved the complex into five species (Figure 1.1). The evolution of the genus *Heterobasidion* started approx. 60 million years ago and species diverged 45–60 million years ago. Three species of this complex are well known and common in Europe *Heterobasidion annosum* (Fr.) Bref.; *Heterobasidion parviporum* Niemelä and Korhonen, *Heterobasidion abietinum* Niemelä and Korhonen, whereas *Heterobasidion irregulare* Otrosina and Garbeloto, *Heterobasidion occidentale* Otrosina and Garbelotto are native to North America (Dalman *et al*. 2010).

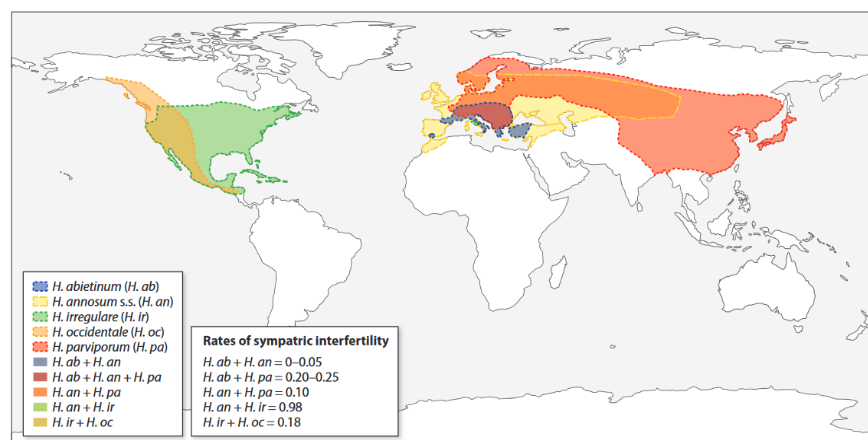


Figure 1.1. Native distribution of *Heterobasidion* species complex, different colours indicate different species; picture from Garbelotto and Gonthier 2013.

More recent research has showed that the borders of pathogen distribution are blurry, overlap or sometimes even do not exist, especially if the spread of the pathogen is facilitared by humans (Garbelotto and Gonthier 2013). *H. irregulare* (in Figure 1.1 in green colour) has been introduced to Europe from North America during World War II and currently together with *H. annosum* has become established in *Pinus* spp. stands in Italy (Gonthier *et al.* 2014). *H. irregulare* is able to hybridize with the European species *H. annosum* (Garbelotto and Gonthier 2013; Gonthier *et al.* 2014), while *H. irregulare* and *H. occidentale* hybrids appear to be extremely rare (Garbelotto and Gonthier 2013). This may be explained by evolution as *H. annosum* and *H. irregulare* are ancient "sister species" (Garbelotto and Gonthier 2013; Gonthier *et al.* 2014). The latest study in Italy also shows that *H. irregulare* basidiospore distribution is not limited by pine woodlands (main host species) and spores have also been detected in oak forests in central Italy, which implies that invasion of the pathogen is possible over long distances and is less affected by pine forest fragmentation (Garbelotto and Gonthier 2013; Gonthier *et al.* 2014).

In addition to the *Heterobasidion* species complex, the *H. insulare* species complex, includes six *Heterobasidion* species (Chen *et al.* 2015). The geographical border for the two *Heterobasidion* species complexes, as well for many animal species, developed 30– 160 M years ago between the Ural Mountains and the Turgai Straight (Sanmartin *et al.* 2001). Outside of both these *Heterobasidion* complexes is *H. araucariae* P.K. Buchanan (Chen *et al.* 2015). In Latvia, only two species have been observed (Figure 1.2) *H. annosum* and *H. parviporum* (Bruna *et al.* 2021; Gaitnieks *et al.* 2022).

A B

Figure 1.2. *Heterobasidion annosum* on *P. sylvestris* stump (A) and *Heterobasidion parviporum* on *P. abies* log (B); photos taken by A. Zaluma.

In general, *H. annosum* more frequently infects pines (*Pinus* spp.), while *H. parviporum* preferentially infects *P. abies* (Korhonen *et al.* 1998b; Gonthier and Thor 2013). However, *H. annosum* and *H. parviporum* are not strictly host specific and can develop infection centers both in pine and spruce forests (Korhonen *et al*. 1992; Vasiliauskas and Stenlid 1998; Oliva *et al.* 2011; Gonthier and Thor 2013).

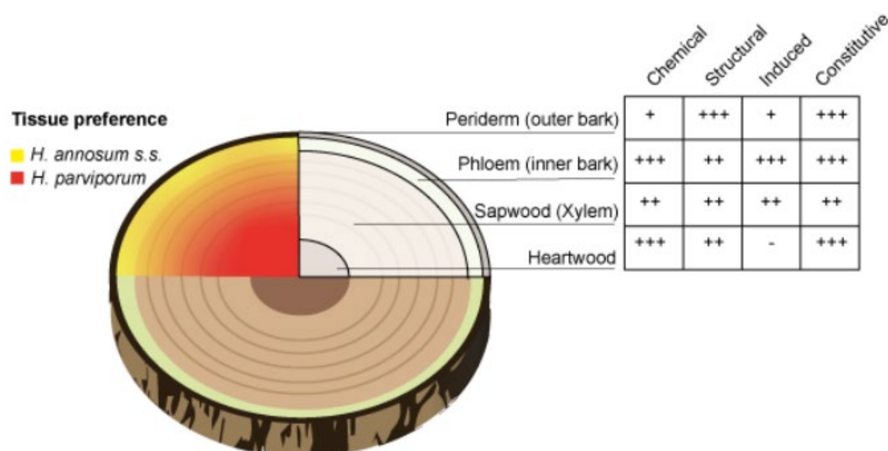
1.2. Infection biology, spread and symptoms of *Heterobasidion* species

Spore production and stump infection biology of *Heterobasidion* species have been described in the early 1950's by the British scientist John Rishbeth (Rishbeth 1951). The pathogen spreads via two pathways: by airborne basidiospores infecting freshly cut stump surfaces (primary infections), colonizing the wood, where it can survive for decades and then can spread by mycelia from colonized stumps via root contacts to uninfected adjacent trees (secondary infections) (Garbelotto and Gonthier 2013).

Primary infections of *Heterobasidion* are accomplished by dispersal of airborne basidiospores, mainly over relatively short distances. Less than 1% of spores are distributed more than hundred meters from fruit bodies (Stenlid 1994; Bruna *et al.* 2021). After landing on freshly cut stump surfaces, basidiopsores normally geminate quickly (*H. annosum* form germ tubes within 15 to 24 hours) (Asiegbu *et al.* 2005). Geminated homokaryotic mycelia may become heterokarytized relatively rapidly after compatible mating with other homokaryotic mycelia, forming ecologically stable heterokaryotic mycelia (Stenlid 1985). Success of airborne infection is dependent not only on distance from spore source to the substrate, but also *Heterobasidion* species, due to compatibility to host tree species (Vasiliauskas and Stenlid 1998; Bruna *et al.* 2021), season (Brandtberg *et al.* 1996; Morrison and Johanson 1999), stump diameter (Morrison and Johanson 1999; Gaitnieks *et al.* 2018; 2019), fungal vectors, e.g., *Hylobius abietis* L. (Viiri 2004), occurrence of root grafts (Rönnberg 2000) and wounds (Vasiliauskas *et al*. 1996), and the presence of competitive microorganisms (Hodges 1969; Holdenrieder and Greig 1998; Redfern and Stenlid 1998; Oliva *et al.* 2017) etc. Stump surfaces are susceptible to spore infection directly after cutting, but it has been estimated that in approx. two weeks, stump surfaces become less susceptible to *Heterobasidion* infection (Redfern and Stenlid 1998). For example, in *P. abies*, boundary layers of lignin and suberin are formed 7– 10 days after wounding (Rittinger *et al.* 1987). In Latvia, maximum spore load occurs from August to November, but in winter sporulation is uncommon (Donis *et al.* 2014). Only a few fungal species, including *Heterobasidion* spp., have high enzymatic activity and they can invade heartwood through wounds and root damage (Asiegbu *et al.* 2005; Vasaitis 2013; Gonthier and Thor 2013). Although *P. abies* susceptibility to wound colonizing fungi is quite

well studied (Vasaitis 2013), only one study has been published about *P. abies* stands in Latvia (McLaughlin and Šica 1996). Moreover, rather limited data is available about *Pinus* spp. susceptibility to wound colonizing fungi.

After colonizing stump surfaces or wounds, the mycelium grows in both directions from wound, but in stumps downwards, colonizing the root system. Adjacent tree infection differs by tree species – *Heterobasidion* spreads necrotrophically in the sapwood of living pines, but in spruces it usually spreads into the heartwood (Garbelotto and Gonthier 2013). In *P. abies*, several defense mechanisms have been described (Figure 1.3) (Capador-Barreto 2022). The formation of a reaction zone of fungistatic compounds is one of them (Oliva *et al.* 2012; Nagy *et al.* 2022). Pettersen (1984) has described that wood resistance is affected by extractive content and amount in wood. Wood can contain from 4 to 10% of extractives of the dry mass of temperate hardwood species and may reach 20% in tropical climates (Pettersen 1984), while for example living *P. sylvestris* wood contains only 5% extractives and *P. abies* even less (Whittaker and Shield 2017).



	Chemical	Structural	Induced	Constitutive
Periderm (outer bark)	+	+++	+	+++
Phloem (inner bark)	+++	++	+++	+++
Sapwood (Xylem)	++	++	++	++
Heartwood	+++	++	-	+++

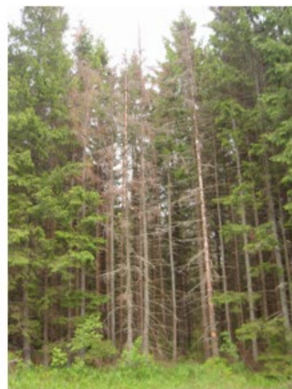
Figure 1.3. Defence strategy in the *P. abies* stem. Colours show tissue preference by *H. annosum* and *H. parviporum*; picture from Capador-Barreto (2022).

Infection symptoms of growing pines and spruces are markedly different, as a consequence of wood characteristics. Pines are characterized by resinous heartwood and initially *H. annosum* attacks sapwood, resulting in dieback of cambium and may cause tree mortality (Figure 1.4). In young trees, mortality occurs in a short period, while in mature tree dieback takes a longer time, but the decay column in stems seldom spreads higher than one meter above the root collar (Greig 1998; Korhonen and Stenlid 1998; Gonthier and Thor 2013).

A B

Figure 1.4. *Hetetrobasidion* infection in young (A) and mature (B) *P. sylvestris* stands; photos taken by T. Gaitnieks.

In tree species with non-resinous heartwood, such as *P. abies*, *Heterobasidion* infection spreads in the heartwood and can cause extensive heart rot in stem and roots, while the tree is still growing for many years or even decades, as the outer sapwood of *P. abies* remains healthy (Korhonen and Stenlid 1998; Gonthier and Thor 2013; Capador-Barreto 2022). The maximum decay column height in Sweden and Latvia has been detected as high as 11.8 m (Stenlid and Wästerlund 1986; Arhipova *et al.* 2011). Large scale monitoring revealed that in Latvia in *P. abies* stands older than 60 years, more than 12% of the stand volume are characterized as rotted, and the frequency of infected trees (Figure 1.5) increases with stand age (Arhipova *et al.* 2011).



A B

Figure 1.5. Severity of *Heterobasidion* infection increases in older *P. abies* stands (A – II class *P. abies* stand, B – III class *P. abies* stand); photos taken by A. Zaluma.

Heterobasidion spp. produces at least 10 different secondary metabolites (enzymes which are involved in wood degradation and several toxins) including fomajorins, fommanoxin,

fommanosin, fommanoxin acid, oosponol, and oospoglycol. Enzymatic activity ensures the fungus development necrotrophically in tree sapwood (living tissues) (Lind *et al.* 2014; Boddy 2016). The amount of secondary metabolites produced may differ between *Heterobasidion* species (Daniel *et al*. 1998; Asiegbu *et al.* 2005).

After *Heterobasidion* spp. invades tree root systems, infection centers may be established – causing dieback of adjacent trees (also called territorial clone formation) (Gonthier and Thor 2013). There have been many studies published regarding territorial clones of *H. parviporum* (Piri 1996; Vasiliauskas and Stenlid 1998; Piri and Korhonen 2008; Gaitnieks *et al.* 2022). It has been proved, that average growth rate of the pathogen in *P. abies* is 25 cm yr^{-1} in stump roots, whereas 9 cm yr^{-1} in tree roots (Bendz-Hellgren *et al*. 1999). Information about growth rate in pines is scarce (Gonthier and Thor 2013).

1.3. Infection prevention and stand management

Forest managers are responsible for stand vitality and *Heterobasidion* infection prevention or restriction in intensively managed forests. To prevent infection there are two accepted ways – freshly cut stem treatment with chemical or biological control agents, or planting resistant tree species. To restrict infection stump removal has been recommended (Gonthier and Thor 2013).

1.3.1. Tree species selection and improving resistance

According to the European Union Forest strategy, more than 3 billion trees are planned to be planted by 2030. In a longer perspective, regeneration of heavily infected sites with tree species resistant or at least less susceptible or tolerant to *Heterobasidion* infection would provide both environmental and economic benefits. The ability to restrict development of the pathogen is called resistance, whereas ability to reduce negative impact on tree growth in a such way, that tree can develop normally is called tolerance (Katjiua and Ward 2006; Sniezko and Koch 2017). Tree defense mechanisms against damage caused by biotic agents include also physical barriers such as lignified cell walls, waxy epidermal cuticle, bark (Franceschi *et al.* 2005) and inducible defense mechanisms, such as induction of certain biochemical pathways (responsible for secondary metabolites like phenolic acids, terpenoids, lignans) (Kovalchuk *et al.* 2013; Nagy *et al.* 2022).

The potential of genetic resistance of conifers against *Heterobasidion* spp. is widely discussed and only few conifer species have been screened using different methods for testing tree susceptibility, however breeding programs has not yet been fully implemented (Hietala *et al.* 2003; Karlsson *et al.* 2008; Skrøppa *et al.* 2015; Sniezko and Koch 2017; Elfstrand *et al.*

2020). It has been proved previously, that *P. abies* susceptibility to *Heterobasidion* infection is strongly dependent on the host and has a genetic component (Swedjemark and Karlsson 2006; Arnerup *et al.* 2010; Skrøppa *et al.* 2014; Skrøppa e*t al*. 2015; Steffenrem *et al.* 2016; Elfstrand *et al.* 2020; Capador-Barreto 2022). Moreover, analysing 466 two-year-old progenies of open pollinated *P. abies* families Chen *et al.* (2018) revealed that the genetic component, which affects fungal growth in sapwood, does not adversely affect growth and wood quality traits in late-age performance. There are several studies which suggest that there is a potential for improving *P. abies* resistance against *Heterobasidion* species infection (Swedjemark and Stenlid 1997; Swedjemark and Karlsson 2006; Chen *et al.* 2018; Elfstrand *et al.* 2020; Capador-Barreto 2022). However, data about the growth rate of the pathogen and selection of *P. sylvestris* genotypes resistant to *Heterobasidion* spp. is rather limited (Korshikov and Demkovich 2008; Gonthier and Thor 2013; Marčiulynas *et al.* 2019).

Change of tree species is a significant control method against *Heterobasidion* infection (Gonthier and Thor 2013), as rotation of resistant tree species such as broadleaved species may restrict infection. Economically, the change from conifer to broad-leaved tree species is not always profitable, and also site conditions, for example, dry, sandy soils may only be suitable for planting of pine species (Korhonen *et al.* 1998a). From the point of root rot pathogen distribution, in Lithuania it has been proved, that mixed-stand management could be a better option than pure conifer stands (Lygis *et al.* 2004a). Growing mixed stands (intercropping) has advantage over monocultures as it allows delayed thinnings for conifers and as a result lower infection risks by *Heterobasidion* and a better economical outcome could be obtained (Lygis *et al.* 2004a). Although broadleaved trees have been considered for many years to be relatively resistant to *Heterobasidion* spp. caused root rot, also broadleaved species need to be carefully evaluated as recently it has been reported that *Betula pendula* (Lygis *et al.* 2004b) and *Fagus sylvatica* L. (Łakomy and Cieślak 2008) may become severely infected on heavily infested sites.

1.3.2. Site preparation and stump removal

Stump removal as a control measure for root rot pathogens was investigated soon after *Heterobasidion* was first described, and for 150 years, knowledge about the efficacy of stump removal has been accumulated (Cleary *et al.* 2013, and references therein). Complete stump removal, especially for rotten stumps, is hard to achieve (Vasaitis *et al.* 2008). Unsuprisingly, over a 50-year period, the efficacy of stump removal on *Heterobasidion* infection in stands decreases (Cleary *et al.* 2013), however stump removal reduces *Heterobasidion* distribution to the next generation (Greig *et al*. 1980;Vasaitis *et al.* 2008; Cleary *et al*. 2013). Also more recent

research in Finland showed that stump removal is not the most efficient way to completely restrict infection, as decayed root fragments with a diameter of 1.5 cm contained living *H. parviporum* mycelium even after 74 months in soil (Piri and Hamberg 2015). Costs of stump removal can vary according to salary, fuel etc. At the beginning of 2010, cost for stump removal was reaching 1,200 US dollars per ha (Cleary *et al.* 2013), or at least 77 euros per oven-dry ton of extracted stumps (Berg 2014). Earlier observations in the United Kingdom highlighted that stump removal might be economically feasible in pine plantations with high pH (Greig 1984), or as an only option in heavily infected stands, where change of tree species is impossible.

In addition, stump removal may lead to some negative environmental effects resulting from site disturbance (Vasaitis *et al*. 2008). Evaluation of the occurrence of *Heterobasidion* after stump removal and impact of stump removal on the fungal ecology should be evaluated also in hemiboreal forests.

1.3.3. Biological and chemical control

According to Müller *et al.* (2018), in root rot infested areas it is complicated to eradicate the disease, thus a better solution is to protect stumps from primary infections by treating them with urea or biological control agents. To reduce infection by *Heterobasidion* spores, biological or chemical control agents are widely used for treatment of surfaces of freshly cut coniferous stumps. Many experiments have been conducted *in vitro*, and several biological control agents have been tested (e.g. bacteria *Pseudomonas* spp. (Gžibovska 2016), or other fungi *Trichoderma viride* Pers., *Trichoderma harzianum* Rifai, *Verticillium bulbillosum* Gams and Malla, *Hypholoma fasciculare* Kumm., *Phanerochaete velutina* Parmasto, *Vuilleminiia comedens* Maire (Rishbeth 1951; Holdenrieder and Greig 1998). However, one of the widely studied and the most promising fungus is *Phlebiopsis gigantea* (Fr.) Jülich (Figure 1.6), characterized as a saprophyte (Holdenrieder and Greig 1998; Garbelotto and Gonthier 2013). The efficacy of *P. gigantea* is mainly related to the ability of the fungus to rapidly colonize the upper parts of stumps, and to its vertical growth rate and competitive ability against *Heterobasidion* in roots (Pettersson *et al.* 2003; Berglund and Rönnberg 2004; Sun *et al.* 2009).

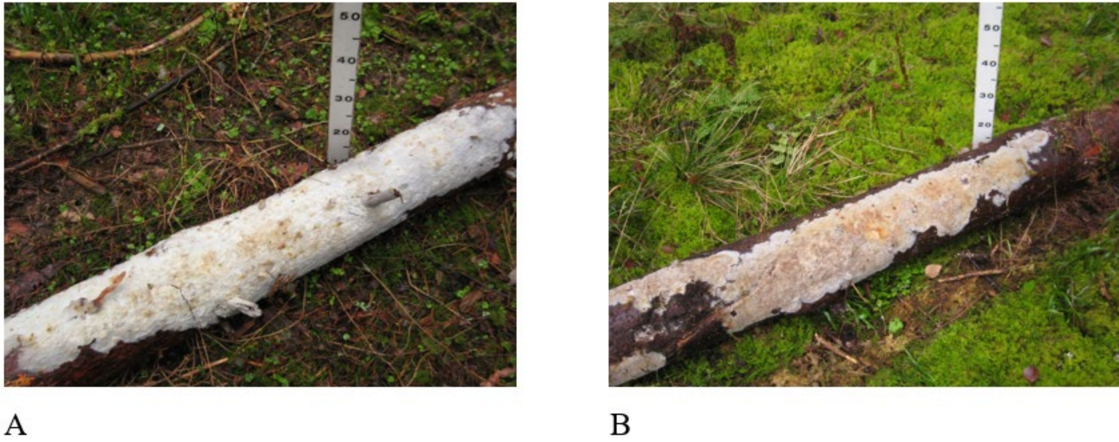


Figure 1.6. *P. gigantea* fruit bodies on *P. sylvestris* (A) and *P. abies* (B) logs; photos taken by T. Gaitnieks.

Control agents containing *P. gigantea* spores and mycelium are produced in Poland, United Kingdom and Finland (Holdenrieder and Greig 1998; Pratt 1998; Pratt *et al*. 2000). The earliest formulation of a biological control agent based on *P. gigantea* was developed in the United Kingdom by J. Risbeth in the 1960's, and is available since 1970. In 1987, a strain of *P. gigantea* almost equally effective against *Heterobasidion* in both pine and spruce stumps was isolated in Finland, and since 1991 is available as the biological control agent Rotstop® (Pratt *et al.* 2000). In the Nordic and Baltic countries one of the most frequently used biological control agent is the Finnish preparation Rotstop® (Thor 2005). Rotstop® has been used in Latvia for 16 years (Kenigsvalde u.c. 2011). Results of efficacy of Rotstop® depend on many factors, including tree species, temperature and moisture content etc. Low efficacy of Rotstop® in spruce wood has been observed in Sweden and Finnland (Berglund and Rönnberg 2004; Vasiliauskas *et al.* 2004; Berglund *et al*. 2005, Rönnberg *et al*. 2006; Piri *et al.* 2023). In Latvia, on average 36% of *P. abies* stumps treated with Rotstop® stumps were infected by *Heterobasidion* (Kenigsvalde *et al.* 2016). Previous studies showed that pine stumps are more receptive to *P. gigantea* treatment than spruce stumps (Korhonen 2003; Webber and Thorpe 2003; Kenigsvalde *et al.* 2016; Żółciak *et al.* 2020). Furthermore, more recent papers reported that *P. gigantea* efficacy might differ based not only on tree species, but also on the origin of the *P. gigantea* isolate. Studies in Latvia also have confirmed that *P. gigantea* isolated from different *P. abies* and *P. sylvestris* differ phenotypically, revealing that host-driven intraspecific phenotypic variation occurs (Kļaviņa *et al.* 2023).

Pratt *et al.* (2000) highlighted that change of the *P. gigantea* isolates used for stump treatment is crucial to maintain the competitive activity of the fungus and to avoid large scale disperse of single *P. gigantea* genotype (Figure 1.7). As some authors emphasize it is also

necessary to determine the effects of the widespread use of Rotstop® on the biological diversity of indigenous *P. gigantea* populations (Vasiliauskas *et al.* 2004), and resident bacteria communities (Sun *et al.* 2013). In earlier experiment in laboratory and forest it has been demonstrated that other isolates of *P. gigantea* can also be used as alternatives to Rotstop® at least in *Pinus pinea* L. (Annesi *et al.* 2005).

Figure 1.7. *P. gigantea* (Rotstop®) colonized pine stump surface (3 months after commercial felling and stump treatment); photo taken by A. Zaluma.

Although urea has been widely used as a chemical control agent in Europe (Thor 2003), in Sweden the use of urea was prohibited in 2015 (Blomquist *et al*. 2020, and references therein). As in previous decades, due to increasing concerns about the use of chemical pesticides and fungicides, biological agents will be required more often (Pratt *et al.* 2000). Research on biological protection methods is especially important in the context of the "EU Biodiversity Strategy for 2030" and the "EU Forest Strategy", which aims to reduce the use of pesticides by 50% by 2030. Although fungal species richness was reduced equally after treatment by urea and Rotstop® (19% and 15%), the structure of fungal communities differed markedly from each other (Vasiliauskas *et al.* 2004). Research *in vitro* has shown, that urea changes stump surface pH, and *Heterobasidion* mycelium growth was not initiated at pH above 7, which resulted in inhibition of pathogen development (Johansson *et al.* 2002). The obtained results could be affected by urea concentration (Brandtberg *et al.* 1996). Although in some reports, a 20% concentration of urea was sufficient and resulted in significantly lower colonized areas of *Heterobasidion* in comparison to untreated stumps (Vollbrecht and Jørgensen 1995; Nicolotti and Gonthier 2005), other research proves, that higher concentrations (30%) ensure better stump protection (Brandtberg *et al.* 1996). However in the latest studies, urea has been proved to be more effective than biological control in spruce precommercial thinning (Blomquist *et al.*

2020). Moreover, the development and growth rate of *P. gigantea* is more affected by biotic and abiotic factors, while use of urea results in more stable inhibition (Żółciak *et al.* 2020; Piri *et al.* 2023). Gonthier (2019) has highlighted the importance of temperature, as the efficacy of urea decreases in the autumn. It is necessary to determine whether the efficacy of urea is equal to Rotsop in Latvian conditions and which factors affect both treatment methods.

2. MATERIALS AND METHODS

2.1. Study sites and field work

2.1.1. Growth rate of *H. annosum* and *H. parviporum* in functional sapwood of *P. sylvestris* and *P. abies* (Paper I)

Four-year-old *P. sylvestris* and five-year-old *P. abies* (321 and 520 individuals, respectively, all different genotypes from Latvia) were grown under field conditions at the Forest Research station, Kalsnava, Latvia (56.6877; 25.9633). In spring 2009, saplings were dug out from the field and planted in 2 l plastic pots (MCI 17) in peat substrate KKS-M1 (70% milled peat, 30% block peat, pH approx. 4.5) (*Laflora* Ltd.). Saplings were regularly watered and also additionally fertilized with Vito-Silva (*Spodrība* JSC), in autumn 2010. Average root collar diameter of *P. sylvestris* and *P. abies* saplings was similar (1.35 ± 0.01 cm and 1.38 ± 0.01 cm (n = 520; $p > 0.05$)). Average height of *P. sylvestris* saplings was 64.36 ± 0.56 cm (n = 321), and *P. abies* – 70.71 ± 0.58 cm (n = 520; $p < 0.05$). There were slight differences in height, but no difference in root collar diameter, implying that the amount of inoculated sapwood of each tree species was about the same. Trees were inoculated with *H. annosum* and *H. parviporum* in September 2011. Saplings were incubated in field conditions for 16 weeks. In total, 201 *P. abies* and 133 *P. sylvestris* saplings were inoculated with *H. annosum*, and 199 *P. abies*, and 136 *P. sylvestris* saplings were inoculated with *H. parviporum*. For untreated controls, wounds were made, but infected wood material was replaced with sterile wood pieces instead of infected – a total 120 *P. abies* and 52 *P. sylvestris* saplings were used as controls. Diameter of both tree species was similar (1.4 ± 0.2 cm). After incubation, trees were cut, dissected and the extent of *Heterobasidion* spread measured in the laboratory.

2.1.2. Development of *P. gigantea* and *Heterobasidion* in wood of seven conifer species (Paper II)

Two visually healthy trees from seven tree species were selected (*Larix sibirica* Ledeb., *Picea abies*, *Pinus sylvestris*, *Pinus strobus* L., *Pseudotsuga menziesii* Franco, *Pinus contorta* Douglas and *Picea sitchensis* Carr.). The detailed description of tree characteristics is in **Paper II**. Each tree was dissected into 30-cm-long billets. Immediately after that six 0.5 cm deep grooves were drilled in each billet, grooves started in sapwood, ended in heartwood, each approx. 5 cm in length. Each billet was inoculated with four *P. gigantea* (three Latvian isolates

and Rotstop®) strains and *H. annosum* and *H. parviporum* (one isolate per groove plus negative control). For each groove, 0.5 ml suspensions *P. gigantea* and *Heterobasidion* (*P. gigantea* concentration: aaprox. 5000 spores/ml and the concentration of *Heterobasidion* spores to 500 spores/ml) were used (Sun *et al.* 2009). After 4-weeks incubation in field conditions, billets were cut into 2–3 cm thick discs, and fungal growth was analyzed in the Latvian State Forest Research Insitute "Silava" (LSFRI Silava).

Isolates were selected based on data acquired in laboratory by methodology developed in Finland (Sun *et al.* 2009). *P. gigantea* isolates were obtained in 2007 and 2008 from fruit bodies in Latvia, isolation and comparative analyses are described in more detail in **Paper II**.

2.1.3. Fungi inhabiting stem wounds of *P. abies*, *P. sylvestris* and *P. contorta* (Paper III, IV, V)

The study was conducted in Latvia in three stands of *P. abies*, three stands of *P. sylvestris*, and three stands of *P. contorta* (Table 2.1, Figure 2.1). In each stand, 30 living trees were randomly selected. For individual trees, the diameter at 1.3 m height (DBH) was measured two times, for perpendicular directions, estimating mean and the number of individual injuries per stem recorded. For all sampled injuries, the maximal length and width and position in relation to ground level (height of its lowest margin) were measured. (**Paper III**, **IV**, **V**). The area of exposed sapwood (wound) was estimated based on its length and width or for smaller wounds by measuring with a planimeter (**Paper III** and **V**).

Table 2.1. Mean parameters (mean ± SD) of analyzed *P. sylvestris*, *P. contorta*, *P. abies* stands, trees and wounds

Parameters	*P. contorta*	*P. abies*	*P. sylvestris*
Stand age min–max	31–32	32–34	140–167
No. of stems examined	90	90	90
Stem diameter at 1.3 height (cm)	16 ± 5	18 ± 5	42 ± 9
Distance of damage from ground (cm)			
lowest point	78 ± 25	88 ± 33	74 ± 44
highest point	126 ± 23	121 ± 30	219 ± 39
Examined wounds	170	157	127
No. of wounds per stem	1.8 ± 0.8	1.7 ± 0.8	1.4 ± 0.5
Wound width (cm)	7.5 ± 7	10 ± 5	25 ± 7
Wound length (cm)	27 ± 22	33 ± 20	146 ± 58
Exposed sapwood per wound (cm^2)	212 ± 469	589 ± 1,173	3818 ± 2,065
Wood discoloration or decay (%)	11	26.7	41

From each of the injuries, 1 to 2 cm below stem damage, one wood sample (bore core length half of the tree diameter, cm) was taken using an increment borer (sterilized in 70% ethanol). To avoid fungal infection from bark, it was removed from the sample immediately. In

the field each wood core was assessed for the presence of discoloration and observations were marked on sterile plastic tubes, where all samples were individually placed and transported to the laboratory for fungal isolation. In **Paper V** in total 30 trees were cut to determine the length of discoloration and decay (if present).

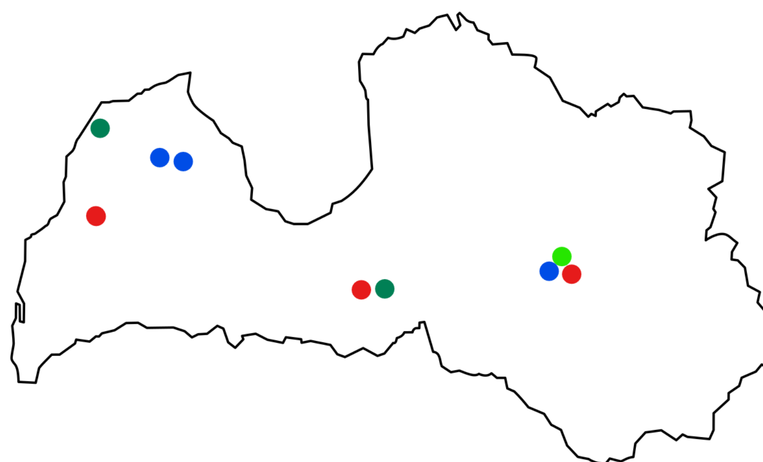


Figure 2.1. Location of study sites: green circles *P. sylvestris* sites (**Paper IV**), red circles *P. contorta* sites (**Paper V** and **Paper VI**), blue – *P. abies* sites (**Paper III**).

Additionally, wood cores were taken from 15 resin-tapped pines and from 10 control pines (**Paper IV**), to analyse the influence of resin tapping on radial increment. The trees were spatially distributed within the same stand (Kalsnava) and were of a similar age varied from 120 to 150, average 140 years old. Cores were extracted from each stem at 1.3 m height using an increment borer. Sampling was done from the opposite side of the wound (if one wound per stem), or, in cases of two wounds per stem, between the wounds. Each bore core reached at least the center of the stem.

2.1.4. Infections and clonality of *Heterobasidion* species in *P. contorta* plantations established on forest clear-cuts and agricultural land (Paper VI)

Susceptibility to *Heterobasidion* spp. and spread of the pathogen was analyzed in three *P. contorta* plantations (Figure 2.2). Plantations were established in Latvia approx. 30 years ago with a planting density for all stands of 5000 trees per ha, but each stand had different management scenario (Table 2.2). Trees and previous generation stumps were examined for *Heterobasidion* fruit bodies and tree health status was categorized in to three classes (classes described in **Paper VI**). All previous generation stumps were sampled and *Heterobasidion* spp. symptomatic trees were cut and 2–3 cm thick wood discs were taken to the laboratory. The number of sampled trees is presented in Table 2.2 and more a more detailed description presented in **Paper VI**.

Table 2.2. Charecteristics of 26–31 year-old plantations of *P. contorta* (< 5% admixture of *P. sylvestris*) in Latvia

	Stand 1	Stand 2	Stand 3
Establishment	Est. on clear-cut [a]	Est. on clear-cut [a]	Est. on agricultural land, thinned 16 years previously
Stand management	Unthinned	Unthinned before first sampling (2nd sampling done 4 years after thinning) [b]	Thinned 16 years before sampling
GPS coordinates	57.06, 21.95	56.68, 24.46	56.60, 25.49
Stand area[c], ha	0.2	0.8	0.2
Age, years	26	27 (31)	28
Tree DBH, cm	12	12 (15)	14
Stand density at sampling, ha^{-1}	2290	2986 (1471)	2350
Symptomatic and sampled trees	128	257(120)	220

[a] Represents previous forest generation. Stand 1 and Stand 2 (established on clear-cuts) consisted of approx. 100-year old *P. sylvestris*.
[b] Numbers in brackets from the second sampling in the Stand 2. The first sampling was done in an unthinned plantation, and the second sampling was done four years after thinning.
[c] Samples were taken in all stand.

Tree and stump samples from entire stands were stored in separate sterile plastic bags. All samples in the same day as sampling were transpored and analysed in the laboratory of LSFRI Silava.

2.1.5. Relative susceptibility of *P. abies* and *P. sylvestris* stumps to natural colonization by *P. gigantea* (Paper VII)

To assess the potential of natural colonization by *P. gigantea* to restrict *Heterobasidion* spore infection, a total 793 *P. abies* and 1158 *P. sylvestris* trees were cut and left for natural infection of *P. gigantea* and *Heterobasidion* spp. The experiment was established in 9 *P. abies* plots and 15 *P. sylvestris* plots, planted on former *P. sylvestris* or *B. pendula* stands. Stands at the sampling were without any dieback symptoms. Trees were cut in the 2012 and 2013 vegetation seasons. Using a chainsaw, 50 cm high stumps were made, none of them showed symptoms of discoloration or decay. Each stump was numbered. Characteristics of the sample plots, trees and number of analysed stumps per sample plot are presented in Table 2.3. After 15 to 56 weeks all stumps were examined by cutting two 2–3 cm thick disks – the first (top) disc was not used, and the second disc was marked and put in a sterile plastic bag.

Table 2.3. Characteristics of the sample plots (**Paper VII**)

Tree species	Age of cut trees (years)	Age of stumps at sampling (weeks)	Number of analysed stumps
P. abies	24	44	40
	30	20	56
	31	36	121
	39	20	95
	33	20	90
	26	27	103
	17	48	96
	34	33	45
	24	35	147
P. sylvestris	24	35	49
	15	18	84
	11	44	90
	15	56	71
	20	39	88
	19	18	68
	27	46	96
	19	44	79
	14	15	78
	15	39	104
	14	15	55
	28	39	45
	16	38	75
	16	35	95
	15	40	81

Samples were transported to LSFRI Silava for analyses.

2.1.6. Efficacy of Rotstop®, a native *P. gigantea* isolate and urea against primary infections by *Heterobasidion* species (Paper VIII)

To analyze the control efficiency of Rotstop®, a native Latvian *P. gigantea* strain and urea after pre-commercial *P. abies* thinning, 480 spruce individuals in three stands were cut in July 2018. Half of the stumps (N = 80) in each of the tree stands were cut to a height of 40 cm while the other half to 45 cm (N = 80). The high stumps were cut twice and then, after the second cut, the 5 cm thick disk cut from the top of the stump was used for the subsequent stump cover treatment.

All stumps after cutting were immediately treated with:

1) Rotstop® spore suspension;
2) *P. gigantea* 422 spore suspension;
3) 35% urea solution;
4) distilled water.

Half of the stumps were covered by the wood disks. For each treatment and untreated control (applied with distilled water), 20 stumps in each stand were used. To avoid clustering treatments, we used a randomized complete block design. More details about experiment establishment can be found in **Paper VIII**. Rotstop® and *P. gigantea* 422 spore suspensions were prepared on the same day as inoculation, using the methodology described by Kenigsvalde *et al.* (2016). Stumps were left for incubation for 14 weeks and then sampled, from each stump the first disc was cut and discarded, the second taken to the LSFRI Silava for further analysis (**Paper VIII**).

2.1.7. Persistence of *Heterobasidion* and other wood-inhabiting fungi in root remnants in forest clear-cuts six years after stump removal (Paper IX)

In total, five stump removal trials were established in cooperation with the Forest environment laboratory of LSFRI Silava during 2011–2012 in different regions of Latvia. To estimate infection frequency, stumps within sample plots were sampled in July 2012 using a Pressler increment borer, and the presence of *Heterobasidion* was estimated, 1208 stumps were sampled. After sampling in winter of 2012, stumps in all sample plots (excluding control areas) were removed using the technique described by Zimelis *et al*. (2013). In total, 1490 spruce stumps and 306 stumps of different tree species were removed from sample plots. All *Picea abies*, *Populus tremula* L., *Betula pendula* and *Pinus sylvestris* stumps smaller than 50 cm in diameter were removed. *B. pendula* and *P. sylvestris* stumps greater than 50 cm, as well as all stumps 4 m from ecological trees and deciduous tree stumps excluding aspen and birch stumps were left in the stump removal area. Samples were taken from 1008 of the removed spruce stump roots to determine the occurrence of *Heterobasidion* and other wood-inhabiting fungi.

In August 2018 (6 years after stump removal), approximately 40 root residuals were collected randomly from each sample plot where stumps were excavated (in total 203 root fragments), to detect long term *Heterobasidion* persistence. All samples were placed in separate plastic bags and transported to the LSFRI Silava, more detailed description of methods – in **Paper IX**.

2.2. Laboratory work

Microbiological analysis as well as statistics for all **Papers** were performed in the LFSRI Silava. DNA extraction, PCR for **Paper V** and **IX** were performed in the LFSRI Silava, but for **Paper III** and **IV** and for part of samples from **Paper IX** – in the Natural Resources Institute Finland (Luke) in 2013. Sanger sequencing was performed by Macrogen Europe.

2.2.1. Identification of fungi (Paper I, II, VI, VII, VIII) and somatic compatibility tests (Paper VI)

In **Paper I**, all branches from trees were removed and stems and roots were surface flame sterilized and cut into discs with surface disinfected (in 70% ethanol) secateurs. Discs were flame sterilized and incubated for seven days at room temperature in Petri dishes.

All wood discs from stumps and wood billets (**Paper II**, **VI**, **VII**, **VIII**), symptomatic trees (**Paper VI**) and samples collected from previous generation stumps and roots (**Paper VI**, **IX**) were transported to the laboratory and stored at temperature +4°C. On the same or the next day, the discs were debarked and washed and put in closed plastic bags.

After 7-day incubation at 20°C, discs were examined using a stereo microscope to inspect for *Heterobasidion* conidial stage (**Paper I**, **II**, **VI**, **VII**, **VIII**) and the presence of the antagonistic fungus *P. gigantea* (**Paper II**, **VII**, **VIII**). Presence of *P. gigantea* was detected by characteristic coloring and mycelium traits (Berglund and Rönnberg 2004; Oliva *et al.* 2015, 2017) and crystal crusted lamprocystidia (Eriksson *et al.* 1981; Breitenbach and Kränzlin 1986).

In **Paper VI** and **IX**, samples from roots were visually inspected for presence of fungi, cut into smaller pieces approx. 0.5 × 1 cm (**Paper VI**) and 1 × 2 cm **(Paper IX)** in size. Immediately after cutting, wood samples were surface flame sterilized. Samples were incubated for 2–5 weeks on Petri dishes containing malt extract agar (15 g malt extract 12 g agar, 1000 mL H_2O) (**Paper VI**) and Hagem agar (5 g glucose, 0.5 g NH_4NO_3, 0.5 g $MGSO_4$ $7H_2O$, 5 g malt extract, 20 g agar, 1000 mL distilled H_2O at pH 5.5) (**Paper IX**). Petri dishes with samples were examined each third-fourth day to avoid infection and to isolate all emerging fungal mycelia.

Pure cultures of *Heterobasidion* were obtained to detect *Heterobasidion* clonality (**Paper VI**), all obtained isolates were subjected to somatic compatibility tests (Stenlid 1985) by pairwise somatic compatibility tests on malt extract agar in all combinations. The fungal species (**Paper VI**, **VII**, **VIII**) were determined using mating compatibility tests (Korhonen 1978).

2.2.2. Isolation of fungi (Paper III, IV, V, IX)

In **Paper III**, **IV**, **V**, **IX**, obtained wood cores were analyzed on the same or the next day after sampling. All samples were stored at +4°C to avoid further contamination. Tree samples (cores) were split into max 8 cm-long pieces and immediately after that flame sterilized. All samples were placed on malt extract agar media (**Paper III**, **IV**, **V**) or Hagem agar media (**Paper IX**); for incubation of each individual sample 9 cm Petri dishes were used. To obtain all emerging fungal mycelia, Petri dishes with wood samples were inspected every three days after

incubation at room temperature; all observed mycelia were subcultured on individual Petri dishes and divided into onse fugal species pure cultures. All obtained isolates were grouped into mycelial morphotypes by microscopic characteristics – mycelial features as described in earlier works (Arhipova 2012). The obtained fungal isolates were examined under microscope Leica DM4000B after at least one-week incubation. In **Paper IX**, eight species/genera were identified using their microscopic features (Watanabe 2002), no molecular identification was performed for *Aspergillus niger* van Tieghem, *Aureobasidium pullulans* G. Arnaud, *Botrytis cinerea* Pers., *Chaetomium globosum* Kunze, *Cladosporium* sp., *Ophiostoma piceae*, *Mucor* sp. S18, *Umbellopsis* sp. The remaining unidentified morphotypes were subjected to molecular analysis.

2.2.3. DNA extraction, amplification and sequencing (Paper III, IV, V, IX)

One to five isolates of each distinct mycelial morphotype were further subjected to molecular identification as dercribed in Arhipova *et al.* (2011) and Arhipova (2012). DNA extraction and PCR amplification were done according to protocols used in other publications (Kåren *et al.* 1997; Padutov *et al.* 2007). Fungal mycelia from different morphotypes were collected from Petri dishes using a flame-sterilized scalpel and placed into 2 ml microcentrifuge tubes. For DNA extraction, a modified CTAB method was used (Padutov *et al.* 2007). To tubes containing fugal samples 150 µl 2% CTAB extraction buffer and in flame sterilized metal beads (three) were place in 2 ml microcentrifuge tubes and samples were ground two times in the buffer using a Bead-Beater homogenizer (Mixer Mill MM 440, Germany) for 45 s at 29 r/s, followed by centrifugation at 12000 rpm for 8 s. After centrifugation, and additional 650 µl of 2% CTAB extraction buffer was added to the suspension and then samples for 5 seconds were vortexed (Bio Vortex V1, Latvia). Fungal samples were incubated in a water bath at 65°C for 60 min with intermittent shaking every 20 min during incubation period (MultiSUB Maxi, UK). After heating followed centrifugation at 12000 rpm for 20 min. After centrifugation 700 µl of the supernatant was carefully transferred to a new 2 ml microcentrifuge tube containing 700 µl of chloroform (proportion 1 : 1). The suspension of choroform and supernatant was shaken for 30 s following by 20 min centrifugation. After centrifugation solution was divided – 550 µl of the aqueous phase was transferred to a new 2 ml microcentrifuge tube, and the remaining liquid containing the chloroform phase was discarded. Prepared (preheated to 65°C in the water bath) 5% CTAB buffer (5% CTAB (w/v), 350 mM EDTA) was added to the aqueous phase (proportion 1 : 5) and carefully mixed. After samles were mixed they were incubated for 15 min in the water bath at 65°C with intermittent shaking after 7 min during incubation. An equal volume of chloroform (proportion 1 : 1) was added and immediately after that the mixture was

shaken for 30 s and then centrifuged for 20 min at 13,000 rpm speed. After centrifugation layers of solution were divided and the upper phase of solution was transferred to a new 2 ml microcentrifuge tube, and the DNA was precipitated with 2 x of isopropanol. Samples were mixed by inversion and incubated for 30 min at +4°C. After incubation, samples were centrifuged for 30 min at 13,000 rpm. As a result of centrifugation isopropanol was possible to remove and the DNA pellet was washed with 900 ml of 70% cold ethanol (stored in fridge) to eliminate salt residues adhering to the DNA. DNA samples with ethanol were centrifuged at 13,000 ppm for 5 min and then ethanol was removed from each tube. Residual ethanol was removed by placing the tubes in the fume hood for 30 min with the lid open, allowing ethanol to evaporate. DNA pellets were resuspended in 50 µl of TE buffer (1.21 g/l Tris-HCl [pH 8.0], 0.372 g/l EDTA [pH 8.0]) samples were incubated at +4°C for 24 hours. DNA concentration was determined using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, US). These method has been taken from Kåren *et al.* 1997; Padutov *et al.* 2007 protocols, detailed described in **Paper IV**, but used also in **Paper III**, **V**, **IX**.

PCR reactions were performed according to Arhipova (2012) and personal communication with N. Arhipova during the work. Used methodology described bellow is detailed described in Paper IV. In a volume of 10 µl containing 50 ng DNA, 2 µl HOT FIREPol® Blend Master Mix (Solis BioDyne, Estonia) (containing 10 mM $MgCl_2$), 0.3 µM ITS 1F (CTTG GTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) primers (Kåren *et al.* 1997) for all above mentioned piblications. For PCR thermocycler (Eppendorf Mastercycler epgradient) was used by the following protocol: initial predenaturation step at 95°C for 15 min, followed by followed by 30 cycles of 95°C for 30 s, 55°C for 35 s, and 72°C for 1 min and a final extension of 72°C for 10 min. After PCR reactions and electrophoresis on 2% agarose, gels were stained with 0.5 µg/ml ethidium bromide, and fragments visualized using an UV Transilluminator (FireReades V10 U). Description taken from **Paper IV**, but used also in **Paper III**, **V**, **IX**.

PCR products were purified using FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I (EF0651 and EN0581, ThermoFisher Scientific, US) and sent to Macrogen Europe (Amsterdam, the Netherlands) for further Sanger sequencing (Arhipova 2012). Sequencing was conducted in one direction using the universal primer ITS4 for all samples. All sequences were manually edited using the Lasergene software package SeqMan (DNASTAR, Madison, Wisconsin) (Arhipova 2012 and personal communication with author). BLAST searches were performed using sequence database GenBank **(Paper III**, **IV**, **V**, **IX)**. The Internal Transcribed Spacer (ITS) sequence homology was set at 98–100% for delimiting fungal

taxon and varied from 94 to 98% (differed between **Paper III**, **IV**, **V**, **IX)** for delimiting at the genus level (Arhipova 2012). All ITS sequences obtained were deposited in GenBank.

2.3. Calculations, tree-ring width measurements and statistical analyses

Standard characteristics such as species composition, age and site type were acquired from the stand inventory database in the Forest State Register and the Forest Research Station database.

Mann-Whitney Tests (**Paper I**, **II**, **VII**), t-tests (**Paper I**, **VII**, **IX**), Wilcoxon tests (**Paper IV**, **VI**), ANOVA (**Paper IX**), Kruskal-Wallis Tests (**Paper VIII**), Chi-squared tests (χ^2; **Paper VII**), GLM (**Paper I**, **VII**, **VIII**), Spearman`s rank correlation analysis (**Paper V**, **VII**), Pearson's correlation (**Paper IX**) were performed in R v. 3.6.1 (R Core Team 2019). For analysis of similarity between fungal communities (**Paper IX**), the Sorrensen similarity and Shannon diversity index was calculated (Magurran 1988) using the software EstimateS (Chao *et al.* 2005). Statistical analyses are described in more detail in the publications.

Resin tapped *P. sylvestris* wood cores (**Paper IV**) was measured using LINTAB 5, with the precision of 0.01 mm (RinnTECH, Germany). Tree ring measurements were made by WinCELL 2007 software (Regent Instruments, Canada). An EPSON GT 15000 scanner was used to acquire sample images with 24-bit colour depth and 1200 dpi resolution. Additional to 25 sampled trees to tree ring measurments one tree was rip-cut (Figure 2.2).

A B

Figure 2.2. Rip-cut of resin tapped *P. sylvestris* (A) and wood discoloration (B); photos taken by A. Zaluma.

Length of sampled tree discoloration (Figure 2.2) was measured in vertical and horizontal directions.

3. RESULTS

3.1. Development of *Heterobasidion* spp. in native and introduced conifers (Paper I, II)

To investigate development of *Heterobasidion* spp. in native and introduced conifers:

a) growth rate of *H. annosum* and *H. parviporum* in the functional sapwood of *P. abies* and *P. sylvestris* was determined (**Paper I**);
b) growth rate of H. annosum and H. parviporum, Latvian isolates of Phlebiopsis gigantea, and the biological control agent Rotstop® strain in cut stems of Picea abies, Picea sitchensis, Pinus sylvestris, Pinus contorta, Pinus strobus, Pseudotsuga menziesii, and Larix sibirica was evaluated (**Paper II**).

3.1.1. Growth rates of *H. annosum* and *H. parviporum* in functional sapwood of *P. sylvestris* and *P. abies* (Paper I)

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SHORT COMMUNICATION

Growth rates of *Heterobasidion annosum* s.s. and *H. parviporum* in functional sapwood of *Pinus sylvestris* and *Picea abies*

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Summary

Growth rates of *H. annosum* s.s. and *H. parviporum* were investigated in the functional sapwood of young *Pinus sylvestris* and *Picea abies* plants as an indicator of the relative susceptibilities of the hosts to these pathogens. The stems of 520 five-year-old *P. abies* and 321 four-year-old *P. sylvestris* plants were inoculated and the extent of infection determined 16 weeks later. *H. annosum* s.s. grew further than *H. parviporum* in *P. sylvestris* sapwood, while in *P. abies,* no differences between the two *Heterobasidion* spp. were found. Both *H. annosum* s.s. and *H. parviporum* spread faster in the sapwood of *P. abies* than in *P. sylvestris.* There was high within-host species variation in growth rates for both *P. sylvestris* and *P. abies* suggesting it may be possible to identify tree genotypes with lower susceptibility.

1 Introduction

Root and butt rot caused by species of *Heterobasidion* is the most economically important disease of coniferous forests in the Northern Hemisphere (Garbelotto and Gonthier 2013). Two species occur in Northern Europe: *Heterobasidion annosum* (Fr.) Bref. sensu stricto and *Heterobasidion parviporum* Niemelä & Korhonen (Garbelotto and Gonthier 2013). *H. annosum* s.s. typically infects pine, particularly *Pinus sylvestris* L., but the pathogen also attacks other tree species, while *H. parviporum* is mainly pathogenic on *Picea abies* (L.) H. Karst. (Korhonen et al. 1998). In pine species, *Heterobasidion* species attack the sapwood and kill the vascular cambium, leading to mortality. In contrast, in *P. abies, Heterobasidion* species cause extensive heart-rot, allowing infected trees to retain vigour for decades (Garbelotto and Gonthier 2013, and references therein). However, in inoculation experiments, infection readily colonizes and spreads within the functional sapwood of young *P. abies* plants with the potential for causing mortality (Swedjemark et al. 1999). There are several studies showing direct evidence that *Heterobasidion* species infect *P. abies* sapwood under natural conditions (Bendz-Hellgren and Stenlid 1997, and references therein). To date, numerous isolates have been used to investigate resistance of *P. abies* and *P. sylvestris* seedlings to *Heterobasidion* spp., but the number of analysed seedlings has been rather limited (Swedjemark et al. 1999, and references therein). The aim of this study was to investigate differences in growth rate of *H. annosum* s.s. and *H. parviporum* in functional sapwood of *P. abies* and *P. sylvestris* on a broad range of genotypes of the two host species.

2 Materials and methods

2.1 Plant material

A total of 520 five-year-old *P. abies* and 321 four-year-old *P. sylvestris* plants were used; all different genotypes from Latvia. Plants were grown under standard field conditions in the forest nursery of the Jaunkalsnava Forest Research Station, Latvia (56.6°N, 26.0°E). In 2009, plants were individually re-potted in a peat substrate (KKS-M1; 70% milled peat, 30% block peat, pH approx. 4.5; produced by '*Laflora*') and distributed randomly in the field. Plants were fertilized with NPK fertilizer Vito-Silva 'Spodriba' and watered regularly. When sampled, diameters at soil level of *P. sylvestris* (1.4 ± 0.2 cm, mean and SD) and *P. abies* (1.4 ± 0.2 cm) were similar ($p > 0.05$), but *P. sylvestris* plants were significantly shorter (64.4 ± 10.1 cm), than those of *P. abies* (70.7 ± 13.3) ($p < 0.05$).

2.2 Inoculum

Heterokaryotic isolates of *H. parviporum* (S37) and *H. annosum* s.s. (VStr2821aP) used were isolated in Latvia from living *P. abies* and *P. sylvestris.* The species of *Heterobasidion* were determined by pairing the heterokaryotic isolates with homokaryotic tester strains. Inoculum was prepared using a procedure modified from Swedjemark et al. (1999). *P. abies* sapwood pieces, 0.8 cm long and 0.3–0.4 cm in diameter, were sterilized and placed onto pathogen mycelium growing on malt agar and incubated at 20°C for 6 weeks.

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2.3 Inoculation

Inoculations were made in September 2011. A total of 201 plants of *P. abies* and 133 *P. sylvestris* were inoculated with *H. annosum* s.s., whereas 199 *P. abies* and 136 *P. sylvestris* were inoculated with *H. parviporum*. One hundred and twenty *P. abies* and 52 *P. sylvestris* were used as controls. Each stem was wiped with 70% ethanol, and a circular wound (0.4 cm diam.; 0.5 cm deep) was made 1 2 cm above the soil surface using a surface disinfected (70% ethanol) drill bit positioned at an angle 45 degrees to the stem. Colonized inoculum was inserted and the wound covered and sealed with gardening wax. Controls were treated similarly, using sterile wood pieces. Plants were maintained under normal field conditions and watered regularly (1 2 times per week) until harvested.

2.4 Sampling

After 16 weeks, plants were removed from pots and height and diameter at soil level measured. Plants were severed from the root systems at root collar level. Branches were removed, and the stem surface was flamed to sterilize. Lengths of stem between 10 and 15 cm above and 4 and 8 cm below the inoculation point (down deep till the root collar, in some cases situated 7 cm below the peat surface) were cut into 0.2- to 1.1-cm-thick discs using secateurs surface disinfected in 70% ethanol between each cut. Each disc surface was flame surface sterilized and incubated for 7 days at room temperature on sterile filter paper moistened with sterile deionized water in Petri dishes. Discs were examined under a low-power stereo microscope for conidiophores of *Heterobasidion* spp. The longitudinal fungal growth was estimated for each stem. To comply with Koch's postulates and confirm re-isolation of inoculated isolate, re-isolations were made from 10 *P. sylvestris* and 10 *P. abies*, and five plants of each inoculated with *H. annosum* s.s. and five plants with *H. parviporum*. Isolates were paired on malt agar with the original strain used for inoculation to test for compatibility (Stenlid 1985).

2.5 Statistical analysis

A Mann Whitney Test was used to compare mean longitudinal fungal growth and GLM to compare infection frequency at the inoculation point. Mean diameters and heights of plants were compared using *t*-tests for unequal sample size. The coefficient of fungal growth variation (ratio of the standard deviation to the mean, expressed as a percentage) was calculated. For all tests, comparisons were made between host and between pathogen species. R software environment (R Foundation for Statistical Computing, Vienna, Austria) was used.

3 Results and discussion

All re-isolations were vegetatively compatible with each respective inoculated strain. No *Heterobasidion* spp. were observed in uninoculated control plants, and no contamination or necrosis was detected in the plants. Although infection occurred in most inoculated plants (Table 1), no mortality was observed.

The results of the work provide additional evidence that *H. annosum* s.s. and *H. parviporum* differ in pathogenicity, particularly in *P. sylvestris*. Spread of *H. annosum* s.s. in the sapwood of *P. sylvestris* was significantly greater than that of *H. parviporum* ($p < 0.001$), whereas in *P. abies*, the differences in spread between the two fungi were not significant ($p = 0.332$). The greater susceptibility of *P. abies* compared with *P. sylvestris* regarding growth rate of *Heterobasidion* spp. mycelium within sapwood ($p < 0.001$) was also confirmed. *P. abies* was more frequently infected by both fungi ($p < 0.05$). These results are in agreement with related studies (e.g. Swedjemark et al. 1999, and references therein) and also with observations made in the field. According to Korhonen et al. (1998), *H. parviporum* causes limited damage to *P. sylvestris* in forest stands. In this work, there was also significant within-host species (among individual plants) variation in pathogen

Table 1. Mean longitudinal fungal growth, infection frequency and coefficient of fungal growth variation in functional sapwood of *Pinus sylvestris* and *Picea abies.*

Pathogen	*P. sylvestris*[1]	*P. abies*[1]
H. annosum s.s		
Mean longitudinal fungal growth (max./min.)	1.8 ± 1.3 a (7.0/0.2)	7.8 ± 3.6 b (18.2/1.6)
Coefficient of fungal growth variation (%)	70.6	46.4
Infection frequency (%)	94.0 a	99.5 b
Infection frequency (%) at root collar	1.5	31.3
H. parviporum		
Mean longitudinal fungal growth (max./min.)	1.2 ± 0.8 c (3.0/0.2)	8.0 ± 3.4 b (18.0/1.8)
Coefficient of fungal growth variation (%)	63.3	42.0
Infection frequency (%)	86.7 c	100 b
Infection frequency (%) at root collar	0	28.1

[1]For each variable, means with different letter subscripts indicate significant differences ($p < 0.05$), in both rows and columns.

growth within the sapwood, particularly for *P. sylvestris* (Table 1). This result provides further encouragement for work to select *P. sylvestris* genotypes showing lower susceptibility to *H. parviporum*, as has been demonstrated with *P. abies* (Swedjemark and Karlsson 2006, and references therein). *P. abies* as small as 6 mm in diameter can harbour *Heterobasidion* infections (Piri and Korhonen 2001). The work presented here showed that both *Heterobasidion* spp. were able to infect nearly one-third of *P. abies* plants of approx. 1.4 cm diameter at the root collar level (Table 1), implying that small plants might be important for spread of the disease. The results of this work may help in the development of improved methods for managing this disease, for example identification of the most resistant genotypes of *P. abies* and *P. sylvestris* for breeding to improve resistance against *Heterobasidion* spp. and by afforestation with *P. sylvestris* on (suitable) *P. abies* sites infested by *H. parviporum.*

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3.1.2. Development of *P. gigantea*, *H. annosum* and *H. parviporum* in wood of seven conifer species (Paper II)

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ORIGINAL ARTICLE

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Growth of *Phlebiopsis gigantea* in wood of seven conifer species

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Abstract

Heterobasidion parviporum and *Heterobasidion annosum* are widely distributed root-rot fungi that infect conifers throughout Europe. Infection of conifer stumps by spores of these pathogens can be controlled by treating fresh stumps with a competing non-pathogenic fungus, *Phlebiopsis gigantea*. In this study, growth of three Latvian strains of *P. gigantea* and the biological control agent 'Rotstop' strain was evaluated in stem pieces of Norway spruce, Scots pine, lodgepole pine, Douglas-fir, Weymouth pine, Siberian larch and Sitka spruce. The growth rates of one *H. parviporum* and one *H. annosum* isolate were also measured in the same stem pieces. The growth rate of *P. gigantea* varied greatly in wood of different conifer species. It was higher in the three pine species, lower in Norway spruce and lowest in Sitka spruce and Siberian larch, and in Douglas-fir, this fungus did not grow. The largest area of wood occupied by *P. gigantea* was in lodgepole pine. Growth of Latvian isolates of *P. gigantea* in the wood of *Pinus* and *Picea* species was comparable to that of the Rotstop isolate. Consequently, stump treatment with local *P. gigantea* isolates should be recommended. However, our results suggest that Douglas-fir stump treatment against *Heterobasidion* by *P. gigantea* may be ineffective and other stump treatment methods should be considered.

KEYWORDS
Abies < host genus, Larix < host genus, Picea < host genus, Pinus < host genus, root disease – primary < disease type, stem decay < disease type

1 | INTRODUCTION

Root and stem rot caused by different species of *Heterobasidion* is a serious threat to conifers and causes significant economic losses (Redfern & Stenlid, 1998). *Heterobasidion annosum* (Fr.) Bref. and *Heterobasidion parviporum* Niemelä & Korhonen are widely distributed species in Europe. The former infects mainly Scots pine (*Pinus sylvestris* L.) and other *Pinus* species, and the latter, mostly Norway spruce (*Picea abies* (L.) Karst.) (Korhonen, Capretti, Karjalainen, & Stenlid, 1998). In southern Sweden, the incidence of Heterobasidion butt rot in Norway spruce stumps at final felling is 30%–47% (Rönnberg, Berglund, Johansson, & Cleary, 2013). In mature Norway spruce stands in Latvia and Lithuania, root rot is present in ca. 22% and 28% of trees, respectively (Arhipova, Gaitnieks, Donis, Stenlid, & Vasaitis, 2011; Vasiliauskas, Juska, Vasiliauskas, & Stenlid, 2002).

In Latvia, Scots pine forests cover ca. 27% and Norway spruce forests ca. 19% of the total forest area (Jansons, 2017). In the future, however, the proportion of introduced conifer species might increase due to management plans aimed at planting tree species with higher productivity in a warmer climate (Jansons, Matisons, Šēnhofa, Katrevičs, & Jansons, 2016; Puriņa, Matisons, Jansons, & Šēnhofa, 2016). In this regard, research conducted in Latvia has

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shown that *Pinus contorta* Dougl. has higher productivity than *P. sylvestris* (Sisenis, 2013). As shown by Greig (1976), susceptibility to root rot should be taken into account when attempting to increase productivity by changing target species in silviculture, for example *Pinus* sp. to *Picea sitchensis* (Bong.) Carr. or *Pseudotsuga menziesii* (Mirb.) Franco.

Heterobasidion spreads aerially by spores to freshly cut stumps and injured parts of growing trees (primary infection), and by mycelial growth along roots to adjacent trees and to the next tree generation (secondary infection) (Cleary et al., 2013; Gonthier, Garbelotto, Varese, & Nicolotti, 2001; Korhonen, Bobko, Hanso, Piri, & Vasiliauskas, 1992; Möykkynen & Pukkala, 2010; Vasaitis, Stenlid, Thomsen, Barklund, & Dahlberg, 2008). Primary infection by *Heterobasidion* can be reduced by treating freshly cut stumps with biological preparations containing spores of the fungus *Phlebiopsis gigantea* (Fr.) Jülich (Holdenrieder & Greig, 1998). Efficacy of these preparations is based on competition between *P. gigantea* and *Heterobasidion* and is strongly related to the growth rate of *P. gigantea* in wood (Sun, Korhonen, Hantula, Asiegbu, & Kasanen, 2009b; Sun, Korhonen, Hantula, & Kasanen, 2009a). Fast growth of *P. gigantea* into deeper parts of stumps can limit the spread of *Heterobasidion* into stump roots and further spread via root systems within tree stands. The development and colonization of *P. gigantea* mycelium in wood have been extensively investigated in stumps of Norway spruce and Scots pine, but almost only in the upper parts of stumps. Less is known about the growth of *P. gigantea* in basal part of the stumps and in stumps of other tree species. Detailed investigations of *Heterobasidion* mycelial growth have been conducted mostly in stumps and roots of Norway spruce, Scots pine and Sitka spruce (Dimitri, Zycha, & Kliefoth, 1971; Kallio, 1971; Pettersson, Rönnberg, Vollbrecht, & Gemmel, 2003; Redfern, 1998; Redfern, Pratt, Gregory, & MacAskill, 2001; Rönnberg, Sidorov, & Petrylaite, 2006).

Development of *Heterobasidion* and its competitor *P. gigantea* in the same stump has been reported in only a few studies (Berglund & Rönnberg, 2004; Holdenrieder, 1984; Sicoli, Trigona, Luisi, & Mannerucci, 2003). In Latvia, both *P. gigantea* and *Heterobasidion* sp. were present in 12% of analysed pine stumps and in 37% of spruce stumps. When natural *P. gigantea* infection covered less than 10% of the stump surface area of Norway spruce, the area occupied by *Heterobasidion* was significantly higher (Kenigsvalde et al., 2016). A relationship between *P. gigantea* stump surface coverage and efficacy of control against *Heterobasidion* infection was also observed in other studies (Berglund & Rönnberg, 2004; Korhonen, 2003; Tubby, Scott, & Webber, 2008).

To develop efficient protection strategies for reducing the risk of *Heterobasidion* infection, additional information about the development of *P. gigantea* in different conifer species is needed. The aim of this study was to compare the growth of four *P. gigantea* strains in wood of *Larix sibirica* Ledeb, *P. abies*, *P. sitchensis*, *P. contorta*, *P. sylvestris*, *P. strobus* L. and *P. menziesii*.

2 | MATERIALS AND METHODS

2.1 | Isolates of *P. gigantea* and *Heterobasidion* sp

In 2007 and 2008, search was made for fruit bodies of *P. gigantea* growing on spruce and pine stumps, logs and windthrown trees in different parts of Latvia. Pure cultures of *P. gigantea* were isolated from wood under fruit bodies. Fifty-six Latvian *P. gigantea* isolates were obtained. Using the methodology of Sun, Korhonen, Hantula, and Kasanen (2009a), all *P. gigantea* isolates and the Rotstop® isolate (Verdera Oy, Finland) were evaluated on malt extract agar medium in Petri dishes for (a) growth rate, (b) growth rate over mycelial colonies of *H. parviporum* (isolate 'Rb 175', isolated from spruce in Sweden (Swedjemark & Stenlid, 1995)) and *H. annosum* ('385 Rv' obtained from birch in Lithuania [Lygis, Vasiliauskas, & Stenlid, 2004]) and (c) the number of asexual spores in 3-week-old cultures. Three Latvian isolates ('Gi107P', 'K4', 'G1') showing fast growth on agar medium ('Gi107P', 'K4'), and fast growth over *Heterobasidion* colonies ('G1', 'K4') and high spore production ('G1', 'Gi107P'), were selected for further tests (Sun, Korhonen, Hantula, Asiegbu, et al., 2009b). To evaluate the development of *Heterobasidion* in stem pieces of coniferous trees, two isolates were used: *H. parviporum* (isolated from spruce 'Ērgļi', Latvia) and *H. annosum* ('385 Rv', Sweden). The former isolate was selected because it showed long growth columns in spruce stems, and the latter because it was previously used in several experiments in Sweden and Latvia to infect growing *P. sylvestris*, *P. abies* and *Larix* sp. trees (unpublished data).

2.2 | Growth rate in coniferous wood

The experiment was established on 10 July 2008. Two trees of each of the seven tree species were cut. Five of the analysed tree species were obtained in the eastern region of Latvia from the territory of the Forest Research Station (Kalsnava) within a radius of 3 km from each other: *L. sibirica* (age 32 years), *P. abies* (age 44 years), *P. sylvestris* (age 34 years), *P. menziesii* (age 23 years) and *Pinus strobus* (age 30 years). Wood samples of two species, *P. contorta* (age 29 years) and *P. sitchensis* (age 29 years), were obtained in the central region of Latvia from the territory of experimental plantations in Zvirgzde (the distance between tree species was approximately 400 m). In either case, the lower part of the stem, with as few branches as possible, was cut into one-metre-long pieces and transported to the location of the experiment. Immediately before the experiment, the stem pieces were cut into 30-cm-long pieces (billets). The diameter of billets varied between 11 and 17 cm. The billets were numbered starting from the root collar. The upper surface of the billet was divided into six sectors and a 0.5-cm-deep oval groove was drilled in each sector, so that the groove extended over sapwood and heartwood, approx. 5 cm in length (Figure 1).

To determine the growth rate of *P. gigantea* and *Heterobasidion* in wood, each billet was inoculated with asexual spores of the three selected Latvian *P. gigantea* isolates, the Rotstop isolate and two *Heterobasidion* isolates. The treatment suspensions were prepared

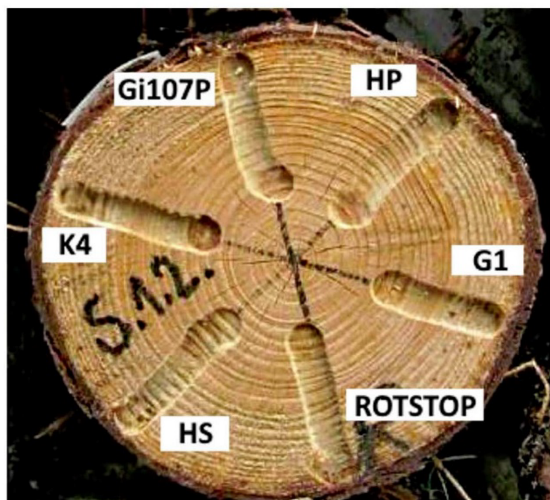

FIGURE 1 Inoculation grooves on the billet surface and the order of four *Phlebiopsis gigantea* isolates and two *Heterobasidion* isolates (HS = *H. parviporum*; HP = *H. annosum*)

two hours before the start of the experiment according to Sun, Korhonen, Hantula, and Kasanen (2009a). Spores were collected from Petri dish cultures and suspended in water. The concentration of *P. gigantea* spores in the treatment suspension was adjusted to ca. 5,000 spores/ml and the concentration of *Heterobasidion* spores to ca. 500 spores/ml. Using an automatic pipette, the grooves were filled with 0.5 ml of spore suspension. The isolates were added to each billet in the same order, and the two isolates of *Heterobasidion* were placed between *P. gigantea* isolates diagonally to each other. Each isolate was applied to six billets from each tree species, 2–4 billets from each tree individual, at different stem heights. Billets were incubated in field conditions in the Forest Research Station (Kalsnava) in a partly shaded location for 4 weeks in July. They were placed on folded garden fabric (to avoid direct contact with soil), and moistened occasionally to avoid excessive drying. The weather during the incubation period was mostly rainy; day temperatures varied between 20 and 25°C and night temperatures between 7 and 15°C.

After incubation, the billets were cut into 2- to 3-cm-thick discs, obtaining 6–8 discs from each billet. Before cutting, two V-shaped lines were cut using surface-disinfected chainsaw on the lateral surface of each billet, to indicate the correct order and position of discs. The precise thickness of each disc was measured. Discs were debarked, washed with a brush in running tap water and incubated at ca. 18°C in a vertical position in loosely closed plastic bags. After 7 days, both sides of the discs were examined. The presence of *P. gigantea* was identified by the characteristic orange brown colour of wood on disc surfaces (Berglund & Rönnberg, 2004). A transparent sheet, divided into square centimetres, was placed on the disc surface, and each square was examined under a stereomicroscope for presence of *Heterobasidion* conidiophores. Borders of the area of *P. gigantea* occupation on the disc surface were redrawn on transparent sheets and measured using a planimeter (PLANIX 10S 'Marble', Tamaya). The amount of sapwood and heartwood was also measured.

2.3 | Calculations and statistics

Axial fungal growth in the billets was calculated by determining the presence of each fungus in the sample discs. The percentage area of wood coloured by *P. gigantea* strains on disc surfaces (including sapwood and heartwood) was used as a measure of wood occupation. All sample discs were examined. Mean values were calculated for each fungal strain on each tree species and at each billet height. The non-parametric Mann–Whitney test was used to determine significant differences in growth rates and occupation of fungal isolates among the seven tree species. Proportions were arcsine-transformed before analyses. Analyses were performed in R 2.15.0 software (R Development Core Team, 2012).

3 | RESULTS

3.1 | Screening of *P. gigantea* strains

The results from the screening of 56 Latvian *P. gigantea* isolates and the Rotstop isolate are shown in Table 1. Growth rate of *P. gigantea* isolates on malt extract agar medium varied from 4.1 to 8.6 mm/day (mean 6.7 ± 0.13 mm/day), and growth rate over colonies of *H. parviporum* varied from 0.6 to 1.4 (mean 0.8 ± 0.02) mm/day and over *H. annosum* from 0.5 to 0.9 (mean 0.7 ± 0.01) mm/day. Differences between the growth rate of *P. gigantea* isolates over colonies of *H. parviporum* and *H. annosum* were significant ($p < .05$). The greatest differences between *P. gigantea* isolates were in spore production, which varied from 0.7 to 182.8 (mean 20.4 ± 3.30) million per Petri dish.

3.2 | Growth rate of *P. gigantea* and *Heterobasidion*

The four *P. gigantea* isolates grew in all tree species billets except *P. menziesii* and one of the two *P. abies* stems (Figure 2). The highest growth rates were observed in the wood of *Pinus* species. The ranges of growth rates (data pooled for the four *P. gigantea* isolates, zero values excluded) in different tree species were as follows: *P. sylvestris* 5.1–7.2, *P. contorta* 5.0–6.6, *P. abies* 0.7–6.3, *P. strobus* 2.8–6.2, *P. sitchensis* 1.0–3.2 and *L. sibirica* 0.8–1.2 mm/day.

Growth rates of different *P. gigantea* isolates within a tree species did not significantly differ ($p > .05$). All four isolates grew significantly faster in *Pinus* species than in *L. sibirica*, and the growth rate of isolates Gi107P and G1 in pines differed significantly from the growth rate in *P. sitchensis*. There were also significant differences in the growth rate of *P. gigantea* in different *Pinus* species: growth of isolates Gi107P and G1 was significantly faster in *P. sylvestris* and *P. contorta* compared to that in *P. strobus*.

Heterobasidion parviporum and *H. annosum* isolates grew in billets of all tree species. Mean vertical growth of *H. parviporum* varied from 0.8 to 4.8 mm/day and for *H. annosum* from 0.9 to 6.0 mm/day (Figure 2). The growth rate of *H. parviporum* was significantly

TABLE 1 Properties of the *Phlebiopsis gigantea* isolates grown on malt extract agar medium. The isolates are arranged according to their growth rate

Isolate of *Phlebiopsis gigantea*	Host tree species	Growth rate ± *SD*, mm/day[a]	Growth rate over *Heterobasidion* colony, mm/day[b]		Spore production, million per Petri dish[a]
			H. parviporum	*H. annosum*	
Gi107P	*Pinus sylvestris*	8.6 ± 4.3	0.8	0.8	42.5
K4	*Picea abies*	8.6 ± 2.7	1.2	0.9	21.5
E107P	*P. sylvestris*	8.5 ± 4.0	0.8	0.7	44.0
Sk107E	*P. abies*	8.2 ± 3.6	0.6	0.7	8.4
Le307P	*P. sylvestris*	7.9 ± 3.6	1.0	0.7	9.4
O207E	*P. abies*	7.9 ± 4.0	0.7	0.6	35.5
J1707P	*P. sylvestris*	7.8 ± 3.9	0.9	0.6	19.1
K207P	*P. sylvestris*	7.8 ± 3.9	0.7	0.6	3.0
Kd107E	*P. abies*	7.8 ± 4.3	0.9	0.6	2.6
ROTSTOP	*P. abies*	7.8 ± 4.0	0.9	0.8	42.9
Le407P	*P. sylvestris*	7.7 ± 3.9	0.8	0.6	22.8
Le207P	*P. sylvestris*	7.6 ± 3.3	0.8	0.7	24.2
Le707P	*P. sylvestris*	7.5 ± 3.8	0.8	0.6	16.6
O107E	*P. abies*	7.5 ± 3.2	0.8	0.5	19.3
D107P	*P. sylvestris*	7.5 ± 4.1	0.7	0.8	23.3
Gi307P	*P. sylvestris*	7.3 ± 3.4	0.7	0.7	51.6
T107E	*P. abies*	7.3 ± 4.1	0.8	0.6	4.1
J1107P	*P. sylvestris*	7.2 ± 3.6	0.8	0.6	16.6
J607P	*P. sylvestris*	7.1 ± 3.9	0.9	0.7	29.3
J707P	*P. sylvestris*	7.1 ± 3.8	0.8	0.7	35.6
G1	*P. abies/P. sylvestris*	7.1 ± 3.0	1.4	0.9	47.3
J107P	*P. sylvestris*	7.0 ± 3.8	0.7	0.6	21.7
J507P	*P. sylvestris*	7.0 ± 3.8	0.9	0.6	5.4
J1207P	*P. sylvestris*	6.9 ± 4.0	0.8	0.6	6.5
J1307P	*P. sylvestris*	6.9 ± 4.5	0.6	0.6	12.1
J1407P	*P. sylvestris*	6.9 ± 4.4	0.8	0.6	12.6
K407P	*P. sylvestris*	6.9 ± 3.9	0.9	0.7	12.9
Le807P	*P. sylvestris*	6.9 ± 4.2	0.7	0.5	2.9
J1507P	*P. sylvestris*	6.8 ± 4.2	0.6	0.7	0.7
B707E	*P. abies*	6.7 ± 3.2	0.8	0.6	11.1
S207P	*P. sylvestris*	6.7 ± 3.7	0.8	0.7	19.4
K107P	*P. sylvestris*	6.7 ± 3.2	0.7	0.6	22.2
Le507P	*P. sylvestris*	6.7 ± 4.1	0.8	0.7	22.2
J307P	*P. sylvestris*	6.5 ± 3.9	0.9	0.6	9.1
J1007P	*P. sylvestris*	6.5 ± 3.8	0.8	0.7	20.7
Kn107E	*P. abies*	6.5 ± 3.4	0.8	0.7	18.5
T207E	*P. abies*	6.5 ± 4.1	0.7	0.7	182.8
N107P	*P. sylvestris*	6.4 ± 3.6	0.7	0.6	18.4
Kd207P	*P. sylvestris*	6.2 ± 4.0	0.8	0.5	5.2
B307E	*P. abies*	6.1 ± 3.6	0.9	0.6	6.8
J807P	*P. sylvestris*	6.1 ± 3.8	0.7	0.7	20.9
K307P	*P. sylvestris*	6.1 ± 4.0	1.0	0.7	12.5

(Continues)

TABLE 1 (Continued)

Isolate of *Phlebiopsis gigantea*	Host tree species	Growth rate ± SD, mm/day[a]	Growth rate over *Heterobasidion* colony, mm/day[b]		Spore production, million per Petri dish[a]
			H. parviporum	*H. annosum*	
Le107E	*P. abies*	6.1 ± 3.4	0.8	0.6	23.7
N207P	*P. sylvestris*	6.1 ± 4.1	0.6	0.6	13.3
B107E	*P. abies*	6.0 ± 3.5	0.8	0.6	4.5
J407P	*P. sylvestris*	5.9 ± 3.6	0.7	0.5	14.3
B407E	*P. abies*	5.8 ± 4.1	0.8	0.7	14.5
B207E	*P. abies*	5.7 ± 3.5	0.8	0.7	29.3
B607E	*P. abies*	5.7 ± 3.4	0.9	0.7	6.5
Le607P	*P. sylvestris*	5.7 ± 3.0	0.7	0.7	14.2
Gi207P	*P. sylvestris*	5.5 ± 3.2	0.8	0.7	5.3
B507E	*P. abies*	5.5 ± 3.7	0.7	0.6	16.6
J907P	*P. sylvestris*	5.2 ± 3.5	0.9	0.9	5.8
J207P	*P. sylvestris*	5.1 ± 3.9	0.7	0.5	24.6
S107P	*P. sylvestris*	4.7 ± 2.2	0.8	0.8	7.5
J1607P	*P. sylvestris*	4.6 ± 3.7	0.7	0.7	8.5
Kn207P	*P. sylvestris*	4.1 ± 3.4	0.8	0.5	8.0
	Mean (±SE)	6.7 ± 0.13	0.8 ± 0.02	0.7 ± 0.01	20.4 ± 3.30

[a]Three repetitions per isolate.
[b]Two repetitions per isolate.

($p < .05$) higher in *P. sylvestris*, *P. strobus* and *P. abies* compared to *L. sibirica* and *P. menziesii*. Similar results were obtained with *H. annosum*; it grew significantly faster in *P. sylvestris*, *P. strobus* and *P. abies* compared to *P. sitchensis*, *L. sibirica* and *P. menziesii*. The difference in growth rate between *H. parviporum* and *H. annosum* within a tree species was significant only in *P. strobus*.

3.3 | Wood occupation by *P. gigantea*

Phlebiopsis gigantea mainly occupied sapwood. The mean proportions of sapwood (%) on sample discs were as follows: *P. contorta* 87.7 ± 0.8, *P. sitchensis* 66.1 ± 1.0, *P. abies* 47.0 ± 1.8, *L. sibirica* 45.1 ± 2.5, *P. sylvestris* 45.1 ± 2.1, *P. strobus* 40.1 ± 0.7 and *P. menziesii* 39.6 ± 0.8.

As expected, based on the large proportion of sapwood in *P. contorta*, isolates of *P. gigantea* occupied a larger total area on sample discs of this tree species (13%; average of *all P. gigantea* isolates) compared to the other tree species (2%–4%) (Figure 3). The area occupied by *P. gigantea* isolates (all combined) on sample discs varied from 0.2% to 39.4% in *P. contorta*, from 0.04% to 9.7% in *P. sylvestris*, from 0.2% to 8.7% in *P. sitchensis*, from 0.1% to 7.5% in *P. strobus*, from 0.1% to 5.3% in *P. abies* and from 0.4% to 3.0% in *L. sibirica*. Great variation was observed also between tree individuals of *P. contorta* ($p < .001$) and *P. sylvestris* ($p < .001$). There were no significant differences in area occupied by *P. gigantea* isolates between tree individuals of *P. strobus* ($p = .73$), *P. sitchensis* ($p = .87$) and *L. sibirica* ($p = .85$).

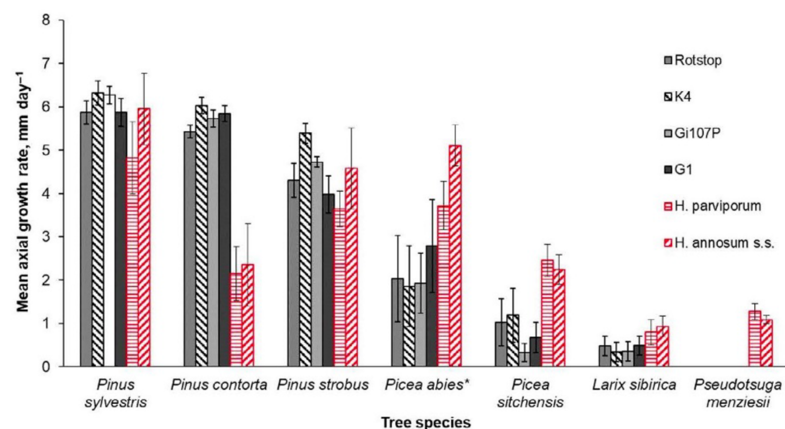

FIGURE 2 Mean growth rate of *Phlebiopsis gigantea* and *Heterobasidion* isolates in billets of seven tree species. Error bars show standard error. **Phlebiopsis gigantea* grew only in one of the two spruce stems

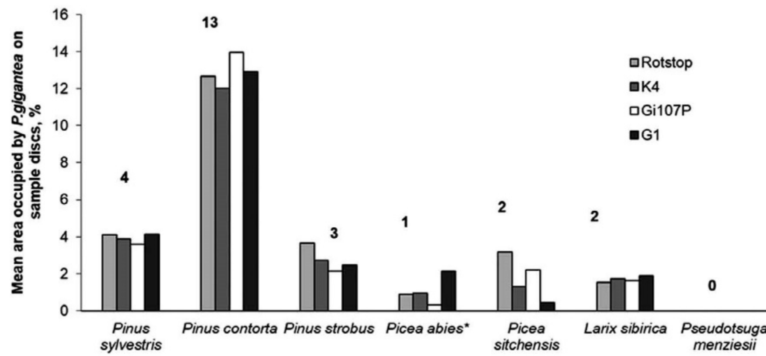

FIGURE 3 Mean relative area occupied by *Phlebiopsis gigantea* on sample discs cut from stem pieces of different tree species (%). *Results from one tree only

The area occupied by isolates of *P. gigantea* on sample discs varied depending on analysed depth. Isolate K4 in *P. contorta* at 3 cm depth had the largest mean area among all *P. gigantea* isolates: 18.3% of the total disc area. The area occupied by isolate Gi107P in *P. contorta* did not decrease significantly with increasing depth from 3 to 14 cm; it even increased at a depth of 16 cm (Figure 4(a)). Mean area occupied by *P. gigantea* isolate G1 at various depths of *P. abies* billets varied from 0.9% to 3.4%, and in *P. sylvestris* billets from 1.2% to 5.3% (Figure 4(b)). Mean area of *P. gigantea* isolates in the billets of *P. abies*, *P. strobus* and *P. sylvestris* reached a maximum of 6% of total disc area.

The observed differences in area occupied by *P. gigantea* isolates on sample discs at various depths were not significant ($p > .05$).

4 | DISCUSSION

4.1 | Growth of *P. gigantea*

Pinus species are primary hosts of *P. gigantea* (Tubby et al., 2008). Therefore, it was not surprising that all four isolates of *P. gigantea* developed successfully in wood of *P. sylvestris* and *P. contorta*. The growth rate was somewhat lower in *P. strobus*. However, our field observations indicated that *P. gigantea* fruited abundantly on large diameter logs of *P. strobus*, indicating well-developed mycelium in wood.

The growth rates of *P. gigantea* isolates in wood of *P. abies* were generally similar with those obtained in other studies (Korhonen et al., 1994; Sun, Korhonen, Hantula, & Kasanen, 2009a). Growth was two times faster (Figure 2) and wood occupation slightly larger in *P. abies* (5%) compared to *P. sitchensis* (2%) (Figure 3). Similarly, Rishbeth (1963) reported that growth of *P. gigantea* was slow in wood of *P. sitchensis*. Webber and Thorpe (2003) noted that the Rotstop isolate occupied a larger area in wood of *P. abies* compared to *P. sitchensis*, while *P. gigantea* isolates of UK origin showed larger occupation in *P. sitchensis*. Differences between our results and those of Webber and Thorpe (2003) might be explained by different techniques used for inoculation of billets, as they applied *P. gigantea* isolates to holes drilled into the bark of the billet.

It was surprising that none of the four *P. gigantea* isolates grew in one spruce individual. These results indicate huge differences between individual trees regarding the ability of *P. gigantea*, and

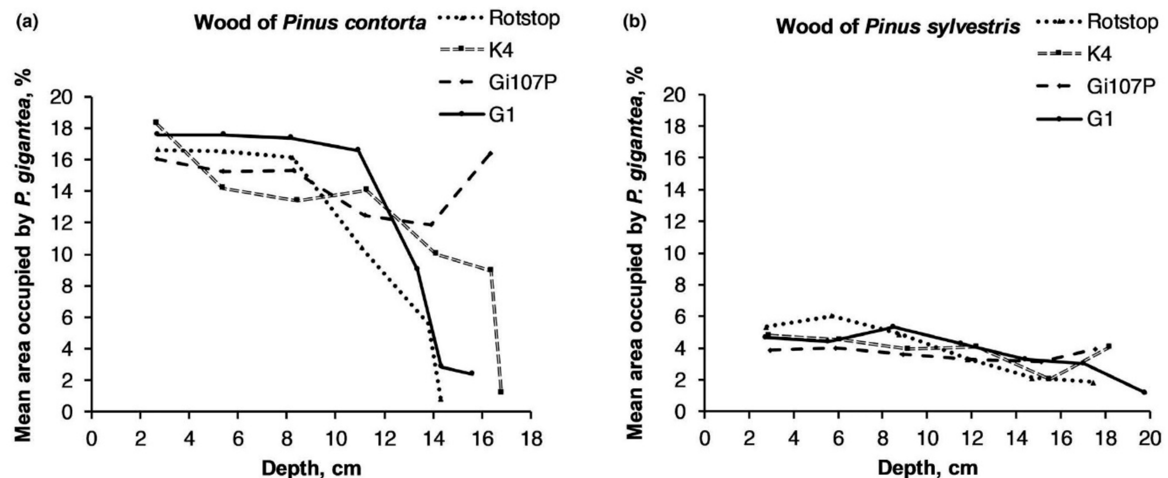

FIGURE 4 Mean area occupied by *Phlebiopsis gigantea* isolates on *P. contorta* (a) and *P. sylvestris* (b) sample discs cut at different distances below the inoculation surface of the billets

probably of other wood-colonizing fungi, including *Heterobasidion*, to grow in them, as shown also by Sun, Korhonen, Hantula, and Kasanen (2009a).

Growth of *P. gigantea* was very poor in *L. sibirica*. A previous study (Rishbeth, 1963) also reported that *P. gigantea* could not protect *L. decidua* from *Heterobasidion* infection. As far as we know, our results describe the development of *P. gigantea* in wood of *L. sibirica* for the first time.

In our study, *P. gigantea* did not grow in the wood of *P. menziesii*. Thomsen and Jacobsen (2003) also concluded that growth of *P. gigantea* was worse on wood discs of *P. menziesii* compared to growth on *Larix* sp. On stumps of *P. menziesii* inoculated with a mixture of *Heterobasidion* and *P. gigantea* spores, *P. gigantea* colonized a smaller area than *Heterobasidion* on stump surfaces (Rishbeth, 1963). However in an earlier study (Meredith, 1960), after inoculation of *Pinus* stumps by a mixture of *Heterobasidion* and *P. gigantea* spores, even with a low proportion of the latter, *P. gigantea* had an advantage in colonization.

Although the data in our study is rather small, it suggests that Latvian *P. gigantea* isolate G1 could be a successful colonizer of pine and spruce stumps compared to the Rotstop isolate. In Sweden, Berglund, Rönnberg, Holmer, and Stenlid (2005) found that local isolates of *P. gigantea* showed higher efficacy than Rotstop against *Heterobasidion*. The results of Żółciak (2007) in Poland also showed faster development of local *P. gigantea* isolates in Scots pine stem and roots, compared to Rotstop.

4.2 | Growth of *Heterobasidion* in wood

Because our study material included only one isolate each for *H. parviporum* and *H. annosum*, and only two individuals of each tree species, we cannot draw conclusions about differences in host specialization of *Heterobasidion* isolates. It has been shown in several studies that *H. parviporum* is better adapted to spruce and *H. annosum* to pine (Korhonen, 2003; Swedjemark & Stenlid, 1995; Vasiliauskas & Stenlid, 1998; Zaluma, Gaitnieks, Arhipova, & Vasaitis, 2015). Our results are in agreement only with the latter observation: the isolate of *H. annosum* used in this study grew faster than the isolate of *H. parviporum* in wood of all three *Pinus* species. However, it also grew faster in *P. abies*, possibly due to the individual properties of the tree, or because the growth rate in dead sapwood does not correlate with growth in living trees.

The growth rate of *Heterobasidion* in *P. sitchensis* wood was slower than in *P. abies*. This is in agreement with previous reports: spread of *Heterobasidion* has previously been shown to be two times faster in stems and roots of Norway spruce compared to Sitka spruce (Stenlid & Redfern, 1998 and literature within). The growth rate in *L. sibirica* and *P. menziesii* wood was significantly slower than in the other tree species. *Larix sibirica* is susceptible to Heterobasidion root rot (Negrutskii, 1986; Piri, 1996), as has also been shown for *Larix decidua* (Greig, 1962; Greig, Gibbs, & Pratt, 2001; Rönnberg, Mårtensson, & Berglund, 2008; Rönnberg & Vollbrecht, 1999). Gonthier, Brun, Lione, and Nicolotti (2012) and Gonthier (2019) reported that *L. decidua* was significantly less often infected than *P. abies*.

4.3 | Significance of *P. gigantea* in protecting different tree species against *Heterobasidion*

In conifer stumps, mycelium of *Heterobasidion* grows as a narrow column downwards and more slowly laterally (Kuhlman & Hendrix, 1964; Redfern, 1993), but in deeper parts of the stump, *Heterobasidion* can occupy larger volumes of wood (Berglund & Rönnberg, 2004; Redfern, 1982, 1993; Varese, Gonthier, & Nicolotti, 2003). Efficacy of *P. gigantea* in controlling *Heterobasidion* is not only related to the ability of *P. gigantea* to rapidly colonize upper parts of stumps, but also to the growth rate of *P. gigantea* in deeper layers of stumps (Berglund & Rönnberg, 2004; Korhonen et al., 1994). Hence, fast-growing *P. gigantea* isolates can limit the spread of *Heterobasidion* into stump roots and its further spread within forest stands (Korhonen et al., 1994; Pettersson et al., 2003). Our results indicated that the total area occupied by *P. gigantea* on sample discs was significantly larger in *P. contorta* compared to the other tree species (Figures 5–7). An obvious reason for this is that the proportion of sapwood in *P. contorta* was larger than in the other tree species, and *P. gigantea*, as well as *Heterobasidion*, grows much better in dead sapwood than in heartwood (Korhonen, 2003; Korhonen et al., 1994). It is also possible that the rapid growth rate of *P. gigantea* in *P. contorta* inhibited growth of *Heterobasidion* near inoculation grooves due to the radial growth of *P. gigantea*. Therefore, the growth rate of *Heterobasidion* in *P. contorta* was slower than in *P. sylvestris* and *P. strobus*.

The development of *Heterobasidion* and *P. gigantea* in the same stem sections of Norway spruce and Scots pine suggested better competitive ability of *P. gigantea* in the latter (Holdenrieder, 1984). It is reasonable to consider that pine stumps are less infected by *Heterobasidion* than spruce stumps (Gaitnieks et al., 2018; Gonthier et al., 2001) because natural *P. gigantea* is more common in pine stumps (Kenigsvalde et al., 2016).

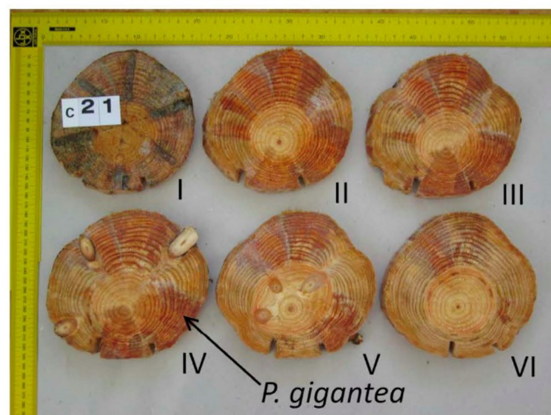

FIGURE 5 Incubated sample discs (I–VI) from a *Pinus contorta* billet. Arrow shows wood discoloration by *Phlebiopsis gigantea*

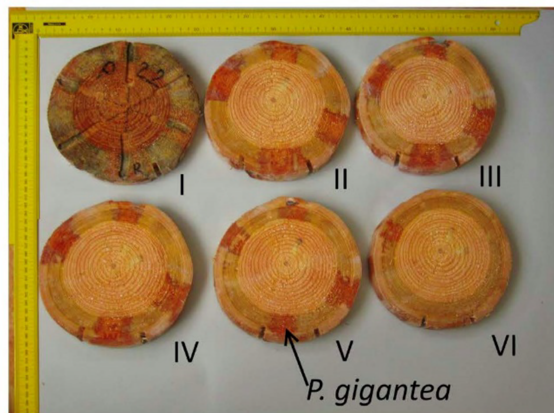

FIGURE 6 Incubated sample discs (I–VI) from *Pinus sylvestris* billet

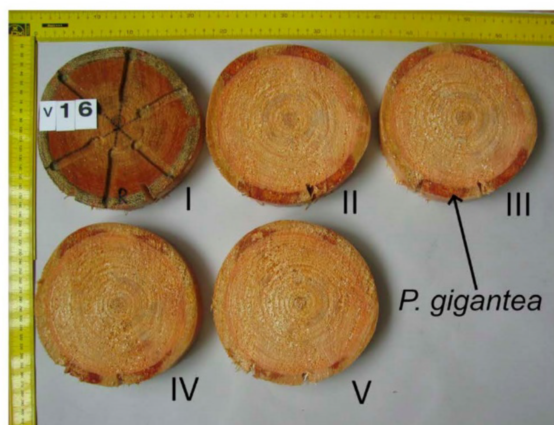

FIGURE 7 Incubated sample discs (I–V) from *Pinus strobus* billet

The case of *L. sibirica* is interesting, since *Heterobasidion* and *P. gigantea* grow slowly in the wood of this species. Several experimental plantations of *Larix* sp. have been established in Latvia (Jansons et al., 2016; Smilga, 1982). Therefore, in further research it would be useful to compare the susceptibility of different *Larix* species to *Heterobasidion* by conducting resistance testing at the tree clone level.

Spruce is economically one of the most important tree species in Latvia. Further research is needed to find spruce reproductive material with increased resistance to root rot and, in addition, to obtain *P. gigantea* isolates that provide highly efficient protection of spruce stumps against *Heterobasidion* infection.

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3.2. Fungi inhabiting stem wounds of *P. abies, P. sylvestris* and *P. contorta* (Paper III, IV, V)

To estimate the importance of mechanical wounds on vitality and spread of stem and root rot causing fungi, including *Herterobasidion* spp., *P. abies* (**Paper III)**, *P. sylvestris* (**Paper IV)**, and *P. contorta* (**Paper V**) stands were investigated.

3.2.1. Wounding patterns, discoloration and decay causing fungi in *P. abies* (Paper III)

ARTICLES

Fungi Inhabiting Bark Stripping Wounds Made by Large Game on Stems of *Picea abies* (L.) Karst. in Latvia

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Burneviča, N., Jansons, Ā., Zaļuma, A., Kļaviņa, D., Jansons, J. and Gaitnieks, T. 2016. Fungi Inhabiting Bark Stripping Wounds Made by Large Game on Stems of *Picea abies* (L.) Karst. in Latvia. *Baltic Forestry* 22(1): 2-7.

Abstract

In the last decade the populations of large game as moose and red deer have increased in Latvia, and the risk of damage to forest stands has increased simultaneously. The aim of this study was to evaluate the extent of bark stripping wounds, decay incidence and associated fungi in 30-year-old *Picea abies* stems damaged by big game. In total, 90 trees were evaluated and 157 bark stripping wounds of different age (1-10 years) were measured. From each wound margin one wood sample was collected for evaluation of presence of decay and subsequent fungal isolation. Decay was found in 13-50% of investigated wounded *P. abies* trees depending on study site (mean 26.7%). All injuries were open wounds. Area of exposed sapwood was 7 – 6142 cm^2. The most commonly isolated fungi were ascomycetes *Neonectria fuckeliana*, *Sarea difformis* and *Phialocephala* sp., and basidiomycetes *Cylindrobasidium evolvens* and *Amylostereum areolatum*.

Key words: *Picea abies*, bark stripping, decay fungi.

Introduction

Norway spruce (*Picea abies* (L.) Karst.) is one of the most economically important conifer species in Latvia. However, root and stem rot caused by various species of fungi can lead to considerable losses in timber production (Arhipova et al. 2011). The most important fungal species causing stem decay in *P. abies* stands are *Heterobasidion annosum* s.l. and *Stereum sanguinolentum* (Korhonen and Piri 2003, Arhipova et al. 2011). Several studies have shown that stem wounds are an important route for tree infection with decay-causing fungi (Vasiliauskas et al. 1996, 2001, Vasiliauskas 2001).

In the last decades, the populations of large game as moose (*Alces alces* L.) and red deer (*Cervus elaphus* L.) have increased in Latvia (Baumanis 2013). The risk of damage to forest stands has increased simultaneously. One type of damages is bark stripping that can reduce value of final wood harvest (Gill 1992, Vospernik 2006, Andersone-Lilley et al. 2010). The most severe damage usually occurs in trees with DBH (diameter at breast height) 5-20 cm and age 4 to 50 years (Gill 1992, Vasiliauskas et al. 1996, Vospernik 2006, Čermák and Strejček 2007, Månsson and Jarnemo 2013). Bark stripping wounds are usually situated at a height of 1-2 m, while injuries caused by timber harvesting machines are mostly on tree roots and butt (Isomäki and Kallio 1974, Vasiliauskas 2001). The area of bark stripping wounds is very variable, from 2 to 4,815 cm^2 (Vasiliauskas et al. 1996, Čermák and Strejček 2007). Vasaitis et al. (2012) showed that wounds on *P. abies* stems greater then 5 cm width are unlikely to be completely occluded and are more prone to infection by fungi causing stem decay. The most common decay-causing fungus is *Stereum sanguinolentum*, which can cause extensive stem rot resulting not only in reduced timber quality, but increased vulnerability to wind or snow damage (Randveer and Heikkilä 1996, Vasiliauskas et al. 1996, Čermák and Strejček 2007, Vasaitis 2013). Only one study on fungi colonizing bark stripping wounds has been conducted in Latvia (McLaughlin and Šica 1996). The aim of this study was to evaluate the wounding pattern caused by bark stripping on stems of *P. abies* and to identify the associated fungi.

Materials and methods

Field work

The study was conducted in Latvia in three 32-34-year-old *P. abies* monocultures (Figure 1), all growing on mineral podzolic soil: two (Šķēde 1 and 2) in an *Oxalidosa* and

one (Kalsnava) in a *Hylocomiosa* forest type, according to the Latvian classification system (Zālītis and Jansons 2013).

Experimental design was similar to that of related work on *Pinus contorta* (Arhipova et al. 2015). In each study site, 30 living *P. abies* trees with bark stripping injuries were selected by choosing the most adjacent wounded tree to the previous measured one. Each tree was numbered and its diameter at breast height (DBH) measured. The number of individual injuries per stem (wounds separated by sound bark) was recorded for each selected tree. For each of 157 recorded injuries, maximum length and width, as well as heights of the lowest and highest wound margins were measured. To estimate wound area, configuration of small wounds (length < 60 cm) was drawn by a waterproof marker on a transparent paper and in the laboratory area was measured using a Tamaya Digital Planimeter "Planix 10-S". For wounds with length more than 0.6 m, the area was calculated as an ellipse using measured maximum length and width.

From each of 157 wounds a single wood sample (10-16 cm long bore core) was taken using an increment borer. Depending on wound height and accessibility, the sample was taken either 1 cm above or below the injury. Wood samples were assessed for the presence/absence of decay or discolouration, individually placed in sterile plastic tubes, and transported to the laboratory for further fungal isolation.

Standard statistics (means, standard deviation) of measurements of wounds and discolourations were calculated (Fowler et al. 1998).

Isolation and identification of fungi

In the laboratory, the bore core was split into two pieces, each piece flame-sterilized and individually placed in 9-cm diameter plastic Petri dishes on malt agar media (15 g malt extract, 12 g agar, 1l H_2O) (300 Petri dishes in total). Petri dishes were incubated at room temperature and inspected twice a week for fungal growth; all emerging mycelia were subcultured on individual Petri dishes and grown as pure cultures. After 3-4 weeks of growth, all pure cultures were examined under a microscope and grouped into mycelial morphotypes according to microscopic features of mycelium.

One to two representatives of each distinct mycelial morphotype were subjected to molecular identification following procedures from Arhipova et al. (2011). In brief, DNA extraction and PCR amplification were made according to established protocols (Kåren et al. 1997). The ready PCR products were purified using FastAP Thermosensitive Alcaline Phosphatase and *Escherichia coli* exonuclease I (Thermo Scientific) and sent to Macrogen Europe (Amsterdam, the Netherlands) for further Sanger sequencing. Sequencing was done in one direction using universal primer Its4 for every specimen. All sequences were manually edited using the Bioedit software (version 7.0.9.0). BLAST searches were performed using the GenBank sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For delimiting fungal taxon (presumed species), the Internal Transcribed Spacer (ITS) sequence homology was set at 98-100 %. For delimiting at genus level, the ITS sequence homology was set at 94-97 %. All obtained ITS sequences were deposited in GenBank (accession numbers KR072493-KR072507).

Results

Mean parameters of surveyed trees and bark stripping wounds are presented in Table 1. Stem DBH of examined trees varied from 9 to 29.5 cm. The maximum number of wounds per stem was four. All 157 injuries represented open wounds. Area of exposed sapwood was in the range from 7 to 6.142 cm^2 of sapwood (589 ± 1.173 cm^2 on average). Wound age varied from 1 to 10 years, and on individual stems wound age varied between 1 and 6 years. The lowest wound height was (0 - 0.4 m) at root collar and the highest one was 199 cm. However, typically the lowest wound margin was recorded at stem height 80 – 1.4 m (102 wounds or 65 %). Decay was found in 13-50 % of the selected *P. abies* trees depending on study site (26.7 % on average).

Table 1. Average parameters (mean ± *SD*) of analyzed *Picea abies* trees (n=90) and bark stripping wounds (*n* = 157)

Parameters / site	Kalsnava	Šķēde1	Šķēde 2	All
Stem diameter at breast height (cm)	17 ± 4	17 ± 5	19 ± 5	18 ± 5
Wounds per stem (no.)	2.4 ± 0.9	1.4 ± 0.6	1.5 ± 0.6	1.7 ± 0.8
Wood discolouration (% of stems)	50.0	13.3	20.0	26.7
Wound width (cm)	9 ± 5	10 ± 5	11 ± 5	10 ± 5
Wound length (cm)	28± 20	38 ± 21	37 ± 19	33 ± 20
Height of lower wound margin (cm)	97 ± 32	100 ± 30	62 ± 23	88 ± 33
Height of upper wound margin (cm)	125 ± 27	139 ± 23	98 ± 24	121 ± 30
Exposed sapwood per wound (cm^2)	591 ± 1206	541 ± 1095	631± 1211	589 ± 1173
Time since damage (years)	5 ± 2	5 ± 1	6 ± 2	5 ± 2

Of 157 wood samples, 92 (59 %) resulted in fungal growth and yielded 160 fungal isolates representing 25 fungal taxa (Table 2). The most common fungi isolated from bark stripping wounds were ascomycetes *Neonectria fuckeliana* (24.8 % of all wounds), *Sarea difformis* (13.4 % of all wounds) and *Phialocephala* sp. (13.4 % of all wounds). Eight species of basidiomycetes were occasionally isolated. The most common species of basidiomycetes were *Cylindrobasidium evolvens* (10.8 % of all wounds) and *Amylostereum areolatum* (3.8 % of all wounds).

Table 2. Fungi isolated from bark stripping wounds of *Picea abies* in Latvia

Fungal taxa	GenBank accession no.*	Frequency of occurrence, %			
		in wounds (*N*=157)	in trees with discolouration (*N*=24)	in trees without discolouration (*N*=67)	in trees, (*N*=90)
Basidiomycetes					
Amylostereum areolatum (Chaillet ex Fr.) Boidin	KR072496	3.8	25.0	-	6.7
Climacocystis borealis (Fr.) Kotl. & Pouzar	KR072506	0.6	4.2	-	1.1
Cylindrobasidium evolvens (Fr.) Jülich	KR072493	10.8	25.0	13.4	16.7
Gymnopilus penetrans (Fr.) Murrill	KR072502	2.5	12.5	1.5	4.4
Heterobasidion parviporum Niemelä & Korhonen	-	0.6	4.2	-	1.1
Peniophorella praetermissa (Karst.) Larss	KR072503	0.6	4.2	-	1.1
Pholiota spumosa (Fr.) Singer	KR072505	0.6	4.2	-	1.1
Stereum sanguinolentum (Alb. & Schwein.) Fr	KR072495	0.6	4.2	-	1.1
All Basidiomycetes	-	4.7	11.8	6.8	7.8
Ascomycetes and anamorphic fungi					
Ascocoryne cylichnium (Tul.) Korf	-	13.4	29.2	11.9	16.7
Aspergillus sp.	-	0.6	-	1.5	1.1
Aureobasidium pullulans (de Bary) G. Arnaud	-	1.3	4.2	-	1.1
Eutypa sp. K7	KR072494	5.1	20.8	4.5	8.9
Neobulgaria sp. K35	KR072504	0.6	4.2	-	1.1
Hypocrea pachybasioides Yoshim	-	1.3	4.2	1.5	2.2
Hormonema dematioides Lagerb. & Melin	-	0.6	-	1.5	1.1
Lachnum sp. K48	KR072507	0.6	-	1.5	1.1
Neonectria fuckeliana (C. Booth) Castl. & Rossman	-	24.8	45.8	17.9	25.6
Ophiostoma piceae (Münch) Syd. & P. Syd	-	0.6	4.2	-	1.1
Grosmannia piceaperda (Rumbold) Goid	KR072497	0.6	4.2	-	1.1
Penicillium sp.	-	1.9	-	4.5	3.3
Pezicula eucrita (Karst.) Karst.	KR072499	1.3	-	3.0	2.2
Phaeomoniella effusa Damm & Crous	KR072500	1.3	4.2	1.5	2.2
Phialocephala sp. K19	KR072498	13.4	33.3	13.4	18.9
Sarea difformis (Fr.) Fr	KR072501	13.4	25.0	19.4	21.1
All Ascomycetes and anamorphic fungi	-	55.6	82.4	56.2	61.1
Zygomycetes					
Umbelopsis isabellina (Oudem.) W. Gams	-	0.6	4.2	-	1.1
All Zygomycetes	-	8.8	29.4	8.2	12.2

Discussion and conclusions

A large proportion (26.7 %) of young spruce (32-34 years) examined in this study had decay and discolouration. Several basidiomycetes commonly isolated from decayed trees usually use open stem wounds as infection courts. *Amylostereum areolatum*, *Stereum sanguinolentum* and *Heterobasidion parviporum* can cause stem decay, which can significantly decrease wood quality. For example, in a ten-year period after injury, average vertical spread of decay due to *A. areolatum* was observed to be 2.8 m, with decay affecting 30-40% of total stem cross area (Vasiliauskas 1999). This fungus is one of few species of basidiomycetes that uses insects for transmission, and usually is introduced to fresh bark wounds by siricid woodwasps during their oviposition (Vasaitis 2013). *Stereum sanguinolentum* is the most important decay-causing fungus colonizing bark stripping wounds of different origin (Roll-Hansen and Roll-Hansen 1980, McLaughlin and Šica 1996, Vasiliauskas et al. 1996, Čermák and Strejček 2007). Rate of vertical spread of decay by *S. sanguinolentum* was observed to be between 13.3 and 19.5 cm per year (Čermák and Strejček 2007). Seven years after wounding, the decay column in spruce stems can reach 1-4 m height and can affect 3 - 84 % of the stem cross-section (Vasiliauskas and Stenlid 1998a). Vasiliauskas et al. (1996) found a positive correlation between wound age and frequency of wounds infected by *S. sanguinolentum*. In Norway, 16% of stem wounds were found to be infected 5-7 years after wounding and 39 % of trees with 15-20-years-old wounding scars were infected by *S. sanguinolentum* (Solheim 2006). The low incidence of this fungus in the current study could be associated with rather young wound age (the mean is five years), and the infection incidence might be expected to increase with time. The method used for detection of decay was not very precise, and approximately 30 % of decayed trees might remain unnoticed, especially in cases of lateral rot (Stenlid and Wästerlund 1986). *Heterobasidion parviporum*, which usually infects trees through root contacts, is able to infect open wounds, especially on roots or close to tree base (Redfern and Stenlid 1998). In the current study *H. parviporum* was isolated from a wound with the lowest margin at 18 cm from root collar. However, stem and root wounds are not as important infection courts for this pathogen as freshly cut stumps (Redfern and Stenlid 1998 and references therein, Rönnberg 2000). Nevertheless, wound infection can play a significant role in unmanaged forest, where stumps are absent (Garbelotto and Gonthier 2013). *Cylindrobasidium evolvens*, the most abundant wound colonizing basidiomycete in the current study, is usually associated with large recent wounds and without considerable stem decay (McLaughlin and Šica 1996, Vasiliauskas et al. 1996). *Climacocystis borealis* typically infects wounds on roots and the lower part of the trunk, causing root and butt rot of spruce in old grown forests (Hallaksela 1984, Solheim 2006), but we isolated it from a wound at height 70 cm. In Scandinavia this fungus is an indicator species of natural forest (Nitare 2000).

The most common fungus isolated from bark stripping wounds was ascomycete *Neonectria fuckeliana*. This species is a common wound invader of several tree species (Vasiliauskas et al. 1996, Vasiliauskas and Stenlid 1998b), but it is not associated with decay and can be isolated also from sound-looking wood (Roll-Hansen and Roll-Hansen 1979, Huse 1981). However, this fungus is a weak pathogen and can cause bark necrosis of spruce (Philips and Burdekin 1982). *Neonectria fuckeliana* has been reported as a significant pathogen causing Nectria flute canker in *Pinus radiata* plantations in New Zealand (Dick and Crane 2009). An experiment established in Latvia in 2011 showed that *N. fuckeliana* can cause cankers on spruce bark three years after artificial inoculation (Brūna, unpublished data).

As spruce trees are vulnerable to bark stripping from the age of 4 years up to 50 years (Gill 1992 and references therein, Vasiliauskas et al. 1996, Gill et al. 2000, Čermák and Strejček 2007), subsequent damage after bark stripping will increase with time. Fresh bark stripping wounds were observed on some of the sampled trees in current study, which means that the number of trees containing stem decay can be expected to increase up to final harvest. The extent of stem decay and frequency of infection is strongly correlated with stand age (Vasiliauskas et al. 1996, Čermák and Strejček 2007, Gaitnieks et al. 2008). In a study conducted in the Czech Republic (Čermák and Strejček 2007), 44% of spruce trees were damaged by red deer and stem decay was observed in 68 % of damaged trees. Bark stripping damage in young conifer stands (including bark stripping and browsing damages) is positively correlated with deer and moose population density (Gill 1992 and references therein, Kiffner et al. 2008, Baumanis 2013).

In comparison with the exotic tree species *Pinus contorta* Douglas ex Loudon, which also is susceptible to bark stripping by deer and moose (Arhipova et al. 2015), our data showed that damage on *P. abies* trees of the same size and age can be much more severe. The maximum area of exposed wood in the current study was considerably higher than in studies made by other authors (Vasiliauskas et al. 1996, Čermák and Strejček 2007), which might be because spruce pure cultures are more severely damaged than mixed stands (Gill 1992 and references therein, Baumanis 2013). The results of this study repeatedly emphasize risks posed to forest stands in areas, where the populations of the big game are disproportionally large.

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3.2.2. Fungi associated with resin tapping wounds of P. sylvestris (Paper IV)

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ORIGINAL ARTICLE

Long-term pathological consequences of resin tapping wounds on stems of Scots pine (*Pinus sylvestris* L.)

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Abstract

Key message **After 5–6 decades since inflicting resin tapping wounds, overmature (> 120 years old) *Pinus sylvestris* stems remain undecayed and vigorous.**

Abstract Overmature trees of *Pinus sylvestris* bearing large wounds made by resin tapping decades ago are still present in woodlands of south-eastern Baltic Sea region. The aim of the present study was to investigate health condition of those trees focusing on fungal infections and to estimate impact of the injury on radial stem growth. The study was conducted in Latvia in three overmature stands of *P. sylvestris*, resin-tapped in 1950–1970 s. On the studied ninety 120–167-year-old trees, exposed sapwood constituted from 1140 to 7755 cm^2 per individual stem. Of the 127 wounds sampled, 52 (41%) showed wood discoloration. The discoloration in its extent was limited, expanding beyond wound margins approx. 1 (max 3) cm in radial, and 6–7 cm in longitudinal directions. Of the 127 wood samples/wounds subjected to fungal isolations, 96% resulted in fungal growth, yielding 236 fungal isolates that represented 47 fungal taxa. The most common among macro-fungi was basidiomycete *Porodaedalea pini*, which was isolated from 9% of stems. The fungus is currently classed not as a tree pathogen, but instead as an indicator species for woodland sites to be considered for nature conservation. Data from tree ring widths have revealed that tree reacted to the resin tapping injury by increasing radial increment of the un-affected part of the circumference of the stem. Current study demonstrated that even on the long term, resin tapping has little influence on health condition and vitality of *P. sylvestris*, even at the very old age. This should be considered as a supporting message in case resin taping practices in the region are to be revived.

Keywords *Pinus sylvestris* · Resin tapping · Stem wounds · Fungal infection · *Porodedalea pini*

Introduction

In Latvia, experimental resin tapping from stems of Scots pine (*Pinus sylvestris* L.) was initiated in year 1903, and already in year 1913, a total of 2290 kg of resin has been obtained from 1280 trees. Since then (based on experience from Germany) resin tapping started to be implemented on an industrial scale. Yet systematic research in this respect was launched in 1923 but only since 1950s, the resin tapping has become a large-scale activity acquiring an important role in forest management (Rasins and Vilsons 1960). Optimal pine age for resin productivity was estimated to be 70–80 years; however, in practice, resin tapping was usually accomplished in 90-year-old and older stands. Stands from all quality classes were selected (site index I–V; Bušs 1997), estimated yield of resin from low productive individual pine being 400–600 g, and from highly productive, 3200–4800 g per season. In all, from 1950 to 1980 total yield of resin in Latvia comprised 2800–3000 tons annually, and in all over 20,000 ha of pine stands were used for resin tapping (Rasins and Vilsons 1960; Baumanis et al. 2014). Since 1980, due to large production of cheaper synthetic resins, resin tapping in pine stands of Latvia has been abandoned.

Resin tapping was done during the vegetation season, normally from May to September. Upon initiating the procedure, surface layer of outer bark had to be scratched off, simultaneously delimiting size of the wound to be inflicted. Then, two opposite approx. 5 mm wide × 5 mm deep

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diagonal (30°–45° angle) sapwood scars were incised in a pairwise manner, encompassing a total of approx. 40 cm of stem circumference. Subsequently, each tree was visited about 3 times per week, during each visit making a pair of new scars, normally at 1–1.5 cm distance either above or below from the two respective previous ones (Rasins and Vilsons 1960; Racinskas 1995). Thus, depending on duration of a tapping season and number of incised scars, size of a wound inflicted on and individual stem per season/year could potentially be very large. For example, in case of 3 visits per week during 5 weeks of harvesting, it would result in a total of 15 pairwise incisions, comprising approx. 40 cm-wide × 25–30 cm-long "fishbone-like" sapwood-exposing injury, thus comprising an open sapwood wound exceeding 1000 cm^2 in size. In case if resin harvesting from a given tree would be accomplished during 5 seasons (years; "usual protocol" being 8 to 10 years), this would result in a wound comprising accumulative length of over 2 m, exposing thousands of cm^2 of sapwood for decades to come (Fig. 1a, b). Moreover, a common practice was to inflict two tapping wounds per individual stem (Fig. 1c). In certain cases, to enhance resin yield, chemical substances as chloride-lime (5 years before felling) and

Fig. 1 Sixty-three-year-old resin tapping wounds on 135-year-old stem of *Pinus sylvestris* (Site 1—Kalsnava); **a** wound on a standing tree; **b** close photo of wound surface; note width of scars of approx. 0.5 cm, and "step" between scars of approx. 1 cm; **c** cross section of two resin tapping wounds showing approx. 3–4 cm lateral closure from each margin in about 4 decades; **d** longitudinal section of a wound showing up to 1 cm-deep reddish sapwood discoloration

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sulfuric acid (2 years) were applied on a cut wood surface (Rasins and Vilsons 1960; Racinskas 1995).

Despite the forestry regulations requiring to cut resin-tapped trees soon after the completion of resin harvesting (1–2 years), considerable numbers of those had remained uncut, thus currently overmature pines bearing large old wounds are still present, and can be found throughout the whole country (Fig. 1a). Moreover, similar situation is being observed also in neighboring countries with the long resin tapping history, as Lithuania, Poland and eastern part of Germany Racinskas 1995; van der Maaten et al. 2017, and references therein). Consequently, such trees currently constitute characteristic (albeit fragmented) feature of overmature pine forests of south-eastern region of the Baltic Sea, nowadays as a rule subjected to nature protection regimes. Surprisingly, to date no information is available regarding impact on tree health of such injuries (e.g., eventual occurrence of associated stem rots, insect attacks, etc.), despite considerable interest by foresters, nature conservationists, but also concerns by a wider society (e.g., while encountering such trees during recreational activities). Moreover, the existing data on the impact of resin tapping on pine growth are fragmented and to a certain extent contradictory Auzins 1995; Genova et al. 2014; van der Maaten et al. 2017, and references therein). The aim of the present study was to investigate fungal infections to old resin tapping wounds on stems of *P. sylvestris*, and to estimate impact of the injury on radial stem growth.

Materials and methods

Field work

The study was conducted in Latvia in three overmature stands of *P. sylvestris*, resin-tapped in 1950–1970 s, all of them growing on mineral soil: Site 1—Kalsnava, approx. 140 years old (56° 7246 N, 25° 9167 E), Site 2—Ventspils, approx. 167 years old (57° 4495 N, 21° 8070 E), and Site 3—Zvirgzde, approx. 140 years old (56° 6863 N, 24° 4462 E). In each stand, 30 living resin-tapped *P. sylvestris* were randomly selected. For each tree, the diameter at 1.3 m height (DBH) was measured (twice in both directions, estimating mean), and the number of individual injuries per stem recorded (Table 1). For each of the 127 recorded injuries, maximal length and width and position in relation to ground level (height of its lowest margin) were measured. The area of exposed sapwood (wound) was estimated based on its length and width. From each of the 127 wounds, a single wood sample (bore core length, 10–26 cm) was taken using an increment borer, targeting 2 cm below the injury. Bark was removed from the core immediately after extracting the sample. After each sampling, the borer was sterilized in 70% ethanol. Immediately after sampling, each wood core was assessed for the presence or absence of discoloration. After assessment, all samples were individually placed in sterile plastic tubes and transported to the laboratory for fungal isolation.

Table 1 Mean parameters (mean ± SD) of analyzed *Pinus sylvestris* trees and resin tapping wounds

Parameters	Site 1 (Kalsnava)	Site 2 (Ventspils)	Site 3 (Zvirgzde)	All
Stems				
No. examined	30	30	30	90
Stem diameter at 1.3 height (cm)	**49 ± 9**[a]	38 ± 6	38 ± 6	42 ± 9
Distance of damage from ground (cm)				
Lowest point	63 ± 8	**129 ± 11**	46 ± 9	74 ± 44
Highest point	180 ± 22	225 ± 10	261 ± 11	219 ± 39
No. of stems with one/two/three wounds	9 /20 /1	29/ 1/ 0	15/ 15/ 0	17.7 / 12/ 0.3
Exposed sapwood per stem (cm^2)	3753 ± 1907	2985 ± 777	**9419 ± 2981**	5361 ± 3538
Wood discoloration (% of stems)	60	43	60	54
Wounds[b]				
No. examined	52	31	42	42
Wound width (cm)	**18 ± 4**	30 ± 4	30 ± 2	25 ± 7
Wound length (cm)	117 ± 23	95 ± 11	**214 ± 11**	146 ± 58
Exposed sapwood per wound (cm^2)	2168 ± 994	2889 ± 590	**6422 ± 583**	3818 ± 2065
Wood discoloration (% of wounds)	42	42	41	41

[a] Values printed bold indicate that the differences between the means are statistically significant (Wilcoxon signed-rank test, $p < 0.05$)

[b] On Site 1 (Kalsnava), resin tapping was accomplished in years from 1955 till approx. 1965. We do not know exact timing for Sites 2 and 3 but, judging accordingly wound width, the tapping should have been taking place during 1970s. This investigation was accomplished in the year 2018

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Isolation of fungi

Isolation and identification of fungi followed our routine procedures (Arhipova et al. 2015; Burnevica et al. 2016). In the laboratory, each tree core was split into approx. 8 cm-long pieces and, after sterilization by flame, each piece was individually placed in a 9 cm plastic Petri dish on malt agar media (15 g malt extract,12 g agar, and 1000 mL H_2O). Petri dishes with samples were incubated at room temperature and inspected in every 3 days for fungal growth; all emerging mycelia were subcultured on individual Petri dishes and grown as pure cultures. After 4–5 weeks of growth, all pure cultures were examined under a microscope and grouped into mycelial morphotypes. Isolates, obtained during the study, are deposited in fungal culture collection of Latvian State Forest Research Institute Silava.

DNA extraction, amplification and sequencing

One to three representatives of each distinct mycelial morphotype were subjected to molecular identification following procedures as in our previous studies (Arhipova et al. 2011, 2015). One to three representatives of each distinct mycelial morphotype were subjected to molecular identification following procedures as in our previous studies (Arhipova et al. 2011, 2015). DNA extraction and PCR amplification were made according to established protocols (Kåren et al. 1997; Padutov et al. 2007).

DNA was extracted using a modified CTAB method (Padutov et al. 2007). For DNA extraction, fungal mycelia were collected from Petri dishes using flame sterilized scalpel and placed into 2 ml microcentrifuge tubes with 150 µl 2% CTAB extraction buffer and three flame sterilized metal bead were added in each of the tubes, and the samples were ground twice in the buffer using a Bead-Beater homogenizer (Mixer Mill MM 440, Germany) for 45 s at 29 r/s. Then the samples were centrifuged at 12,000 rpm for 8 s. An additional volume of 650 µl of 2% CTAB extraction buffer was added to the suspension and the samples vortexed (Bio Vortex V1, Latvia) for 5 s. Then samples were incubated in water bath at 65 °C for 60 min with intermittent shaking every 20 min (MultiSUB Maxi, UK). After heating, the samples were centrifuged at 12 rpm for 20 min and 700 µl of the supernatant was transferred to a new 2 ml microcentrifuge tube with 700 µl of chloroform. The mixture was shaken for 30 s and samples were centrifuged at maximum speed for 20 min. A total of 550 µl of the aqueous phase was moved into a new 2 ml microcentrifuge tube, but the chloroform phase was discarded. One-fifth volume of a 5% CTAB buffer, preheated to 65 °C in the water bath, was added to the aqueous phase and thoroughly mixed. Then samples were incubated in water bath at 65 °C for 15 min with intermittent shaking after 7 min during incubation. The equal volume of chloroform (1:1) was added and the mixture was shaken for 30 s after incubation. Then samples were centrifuged at maximum speed for 20 min. The upper aqueous phase were transferred to a new 2 ml microcentrifuge tube and the DNA was precipitated with 2 volumes of isopropanol. Samples were gently mixed by inversion and incubated for 30 min at +4 °C and then centrifuged 30 min at 13,000 rpm. Isopropanol was removed and the DNA pellet washed with 900 ml of 70% cold ethanol to eliminate salt residues adhered to the DNA. The tubes were centrifuged at 13,000 rpm for 5 min and ethanol was removed from the top of each tube. The samples were put in the fume hood for 30 min with the lid open to dry the ethanol out. 50 µl of TE buffer (pH 8.0) was added and samples were put at +40 °C for 24 h. The DNA concentration was determined using spectrophotometer NanoDrop 8000 (Thermo Fischer Scientific, US).

PCRs were performed in a volume of 10 µl containing approximately 50 ng DNA, 2 µl HOT FIREPol® Blend Master Mix (Solis BioDyne, Estonia) (containing 10 mM MgCl2), 0.3 µM ITS 1 F (CTTG GTCATTTAGAGGAAG TAA) and ITS4 (TCCTCCGCTTATTGATATGC) primers (Kåren et al. 1997). PCR was carried out in a thermocycler (Eppendorf Mastercycler EP gradient) using the following protocol: initial predenaturation step at 95 °C for 15 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 35 s, and 72 °C for 1 min and a final extension step of 72 °C for 10 min.

PCR fragments were visualized UV Transilluminator (FireReader V10, U) after electrophoresis on 2% agarose gel stained with 0.5 µg/ml ethidium bromide.

The PCR products were purified using FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I (EF0651 and EN0581,ThermoFisher Scientific, US) and sent to Macrogen Europe (Amsterdam, the Netherlands) for further Sanger sequencing. Sequencing was conducted in one direction using the universal primer ITS4 for every specimen. BLAST searches were performed using the GenBank sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 10 April 2019). Sequences were manually edited using the BioEdit software (version 7.0.9.0). To determine fungal taxons (presumed species), the internal transcribed spacer (ITS) sequence homology was set at 98–100%. For delimiting at the genus level, the ITS sequence homology was set at 94–97%. All ITS sequences obtained in this study were deposited in GenBank (MK801309–MK801355).

Sample tree

To accomplish detailed examination, on Site 1 (Kalsnava), one pine was felled, 135 years of age, 42.4 cm DBH, showing two resin tapping wounds. The first resin tapping injury on the stem was inflicted in year 1955, thus 63 years previously to the investigation. The larger was 184 cm in length, 15 cm in

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width; area of exposed sapwood was approx. 2760 cm^2. Its lower point was located at 110 cm from root collar, and the higher at 294 cm. The second wound was 115 cm in length and 14 cm in width; area of exposed sapwood was approx. 1610 cm^2. Its lower point was located at 100 cm from root collar, and the higher at 215 cm. The vertical and radial extent of wood discoloration, and extent of mechanical damage inflicted to stem by resin tapping was estimated by cutting the stem into two sections in the middle of the larger wound, after that each section was dissected longitudinally, and radial spread, and the total length of discoloration column was recorded. Extent of wound closure in lateral and longitudinal directions was measured.

Dendrochronological measurements

From 15 resin-tapped pines and from 10 control pines wood cores were taken. The trees were spatially distributed within the same stand (Site 1, Kalsnava) and were of a similar age, being approx. 140 years old (age varied from 120 to 150). A core was extracted from each stem at 1.3 m height using an increment borer (each bore core reached the center of a stem, or more). From resin-tapped stems, the cores were taken from the stem side directly opposite to the wound (in case of a single wound per stem), or, in cases of two wounds per stem, from the middle part in-between of those. Tree-ring width was measured with the precision of 0.01 mm using measurement system LINTAB 5 (RinnTECH, Germany). Measurements were made using WinCELL 2007 software (Regent Instruments, Canada). Sample images, with 24-bit color depth and 1200 dpi resolution, were acquired using EPSON GT 15,000 scanner.

Statistics

Data were inspected for normality using the Shapiro–Wilk test and by manually evaluating Q–Q plots. Using these criteria, obtained data(diameter of trees, size of wounds, exposed sapwood per tree and radial increment of resin-tapped trees) were considered to be not normally distributed. Wilcoxon signed-rank test was performed to compare differences of diameter of trees, size of wounds, exposed sapwood per tree in three stands and to compare tree ring width of resin-tapped trees to non-tapped trees. The statistical analysis was performed in the "R" environment, R studio Version 1.3.1093 (RR Core Team 2020).

Results and discussion

Wounds

The present study examined wounds on overmature *P. sylvestris* stems inflicted during resin tapping 60–50 years prior to the investigation. Characteristics of examined trees and wounds are presented in Table 1. The results demonstrated that, despite the five decades since the injury to the stems has occurred, areas of exposed sapwood had remained extremely large, on average constituting 5361 cm^2 per individual stem. Sizes of individual wounds also varied to great extent, minimal and maximal size of a wound corresponding to 1140 and 7755 cm^2, respectively, indicating that occlusion of resin taping wounds on *P. sylvestris* stems is slow. This was further confirmed by the analysis of the sample tree, in an examined wound of which since about six decades after the injury, only 3–4 cm lateral and 5–20 cm longitudinal closure from its respective margins was observed, corresponding to a total of approx. 8 and 25 cm closure in each direction, respectively (Fig. 1c, d). This can be compared with the occlusion rates of mechanical wounds inflicted to stems of Norway spruce (*Picea abies* (L.) Karst.), which are also notoriously slow. Here, it has been demonstrated that in numerous cases even 3 cm wide artificial mechanical stem wounds failed to occlude over the two decades, and none of initially 15 cm wide has occluded (Vasaitis et al. 2012; Vasaitis 2013). Yet, in case of resin tapping wounds of *P. sylvestris* examined during the present work, we do not know whether the investigated injuries were subjected to chemical treatment for the tapping, although it might have had negative impact on the closure rates.

Fungi, insects and windbreaks

Another restricting factor for the occlusion could be potentially imposed by infections of wood-inhabiting fungi. However, of the 127 wounds sampled in the current study, only 52 (41%) showed (reddish) wood discoloration. Analysis of the sample tree has demonstrated that, in that particular stem, the discoloration in its extent was limited, expanding beyond wound margins about 1 (max 3) cm in radial, and 6–7 cm in longitudinal directions (Fig. 1c, d). Of the 127 wood samples/wounds subjected to fungal isolations, 96% resulted in fungal growth, yielding 236 fungal isolates that represented 47 fungal taxa (Table 2). The most common among macro-fungi, was basidiomycete *Porodaedalea pini* (Brot.) Murrill, which was isolated from 9% of stems. Characteristic for this fungus are the latent infections to trees through natural pathways (e.g., dead twigs), symptomless persisting in wood for many years, and subsequent development of heart-rot in aging overmature stems (Vasaitis 2013, and references therein). Consequently, *P. pini* is currently classed not as a tree pathogen, but instead as an indicator species for woodland sites to be considered for nature conservation (Nitare 2000). Notably, in USA, the fungus has even been used for artificial inoculations of conifers to promote stem decay in trees to be subsequently used as wildlife habitats (Filip

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Table 2 Fungi detected in stem wounds of *Pinus sylvestris*

Fungal taxa	GenBank accession no.	Frequency of occurrence (%)	
		In wounds (n=127)	In stems (n=90)
Basidiomycota			
Bjerkandera adusta	MK801310	1.6	2.2
Coprinellus disseminatus	MK801349	0.8	1.1
Fomitopsis pinicola	MK801342	0.8	1.1
Phlebia acerina	MK801347	0.8	1.1
Porodaedalea pini	MK801329	7.9	8.9
Trichaptum abietinum	MK801344	0.8	1.1
Ascomycota			
Aequabiliella effusa	MK801313	2.3	3.3
Alternaria alternata	MK801345	0.8	1.1
Alternaria infectoria	MK801346	0.8	1.1
Ascocoryne sarcoides	MK801322	14.2	14.4
Botrytis cinerea	MK801334	0.8	1.1
Chaetothyriales sp. P38	MK801333	1.6	2.2
Cladosporium sp. P12	MK801318	0.8	1.1
Cladosporium xylophilum	MK801316	0.8	1.1
Coniochaeta ligniaria	MK801323	0.8	1.1
Coniochaeta velutina	MK801324	0.8	1.1
Crumenulopsis pinicola	MK801332	1.6	2.2
Dothideomycetes sp. P11	MK801317	3.1	4.4
Dothideomycetes sp. P47	MK801337	1.6	2.2
Dothideomycetes sp. P49	MK801338	0.8	1.1
Eurotiales sp.	MK801350	6.3	7.8
Mariannaea elegans	MK801319	0.8	1.1
Paraconiothyrium fuckelii	MK801348	0.8	1.1
Penicillium chrysogenum	MK801311	1.6	2.2.
Penicillium maclennaniae	MK801309	18.9	25.6
Penicillium sp. P76	MK801352	1.6	2.2
Penicillium spinulosum	MK801351	1.6	2.2
Penicillium thomii	MK801325	8.6	12.2
Pestalotiopsis sp. P14	MK801320	2.4	3.3
Pyxidiophorales sp. P77	MK801353	1.6	2.2
Sarea difformis	MK801315	37.8	50.0
Sarea resinae	MK801326	8.7	11.1
Sydowia polyspora	MK801331	0.8	1.1.
Symbiotaphrina microtheca	MK801314	2.4	3.3
Thyronectria pinicola	MK801321	0.8	1.1
Tolypocladium inflatum	MK801343	0.8	1.1
Tolypocladium pustulatum	MK801328	2.4	2.2
Trichoderma harzianum	MK801327	11.8	13.3
Trichoderma viride	MK801354	0.8	1.1
Tymphanis sp. P35	MK801330	2.4	2.2
Tymphanis sp. P46	MK801336	0.8	1.1
Unident. Ascomycota P43	MK801335	0.8	1.1
Unident. Ascomycota P50	MK801339	0.8	1.1
Mucormycota			
Absidia caerulea	MK801340	1.6	2.2
Absidia glauca	MK801341	1.6	2.2
Umbellopsis sp. P5	MK801312	1.6	2.2
Umbelopsis isabellina	MK801355	15.0	21.1

et al. 2011). Moreover, during the present study, fruit bodies (sporocarps) of *P. pini* were observed on stems of five trees, growing either close to the edge of a wound or higher on a stem. Interestingly, in our previous work in Latvia, the fungus was occasionally isolated from much younger (30-year-old) stems of *Pinus contorta* Douglas ex Loudon, exhibiting bark stripping damage caused by game (Arhipova et al. 2015). Yet neither in that nor in the current study, the isolations of *P. pini* were associated with decay.

Among other fungi, characteristic were Ascomycetes from the genera *Ascocoryne*, *Alternaria*, *Cladosporium*, *Penicilium*, *Sarea* known as endophytes and saprotrophs, some of which were reportedly associated with wood discoloration in bark stripping wounds on stems of *P. contorta* (Arhipova et al. 2015). Moreover, several fungi were detected that can be pathogenic to trees, namely *Botrytis cinerea*, *Pestalotiopsis* and some unidentified Dothideomycetes (Table 2). However, representatives of those groups of fungi are known mainly as foliar pathogens and/or decomposers of wood in the last stages of degradation (Capieau et al. 2004; Ferrari et al. 2021; Wang et al. 2019). Yet, a limitation is that the ITS rDNA sequence data are insufficient to delineate species in some ascomycete tree pathogens, and in particular of those from *Ophiostoma* species clusters (Linnakoski et al. 2016). Thus, the possibility cannot be excluded that also during the present work certain ascomycetes have been identified at the species *sensu lato* level. On the other hand, none of ophiostomatoid fungi, and/or other potential tree pathogens apart of discussed above, have been detected by us in wounded stems of *P. sylvestris*. We conclude that the sequences of full ITS region were sufficient for taxon identification for fungi within the focus of the current work.

Exit holes of insects on wood and bark of a tree indicate not only the incidence of the attack as such, but also that insect life cycle in a given substrate has been completed. On living tree stems, this symptom remains exposed for decades. During the present study, no insect exit holes on examined wounds or on stems at wound height have been detected. Windbreaks and symptoms of bacterial tree diseases, e.g., wetwood (Griffiths 2013) were not observed.

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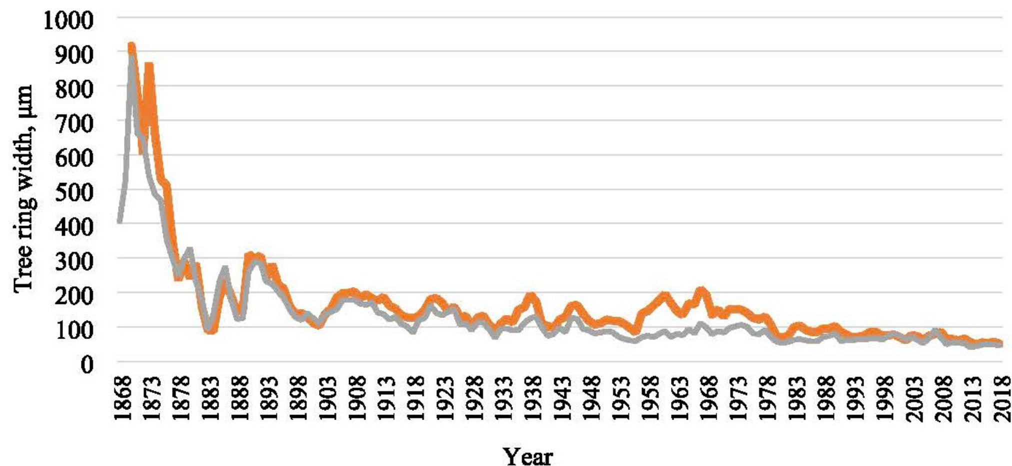

Fig. 2 Mean tree ring width (µm) for resin tapped and control pines. Orange bold line—tree ring width of resin tapped trees, gray line—tree ring width of control trees

Radial increment

Impact of resin tapping on radial increment was investigated on Site 1 (Kalsnava), where the tapping was initiated in year 1955 and lasted for approx. 10 years afterwards. Data from tree ring widths have revealed that radial increment of resin-tapped trees, as compared to non-tapped ones, in years thereafter has notably increased ($p < 0.001$) (Fig. 2). Similarly, increased radial growth at wounded and deformed parts of a stem was observed in resin-tapped *P. sylvestris* stems in German study, and explained by fact that in such instance wood formation is concentrated on the living portion of the bole, as after tapping there is no growth taking place on the exposed sapwood due to removal of cambium (van der Maaten et al. 2017). Yet in this respect, a negative impact of resin tapping on wood quality is asymmetrical stem growth, which notably devalues its otherwise the most commercially valuable part (Auzins 1995). However, resin-tapped stems should be subjected to the final felling soon after completion of the tapping (Rasins and Vilsons 1960). Under current circumstances, resin-tapped *P. sylvestris* trees at least in woodlands of the south-eastern Baltics are the components of sites of overmature forests under nature protection regimes, thus very unlikely to be harvested for wood.

Conclusions

Current study demonstrated that, even on the long term, resin tapping has little influence on health condition and vitality of *P. sylvestris*, even at the very old age. In fact, the present work provided new insights and confirmed that those trees could indeed be of importance for nature conservation. Moreover, in recent years, interest for Non-Wood Forest Products, relevant in supporting sustainable forest management and rural development is being noted in Europe and that, as reported from Spain and Portugal, also includes the option for resin tapping of, e.g., maritime pine (*Pinus pinaster* Ait.) (Genova et al. 2014; Silva et al. 2020). The possibility cannot be excluded that the interest in such renewable natural resource as pine resin will come into agenda also to other areas of Europe, e.g., to more northern parts, where *P. sylvestris* is grown. Should this be the case, current study herewith provides valuable (and supportive) insights regarding the options, outcomes, and consequences of the related eventual activities.

Author contribution statement AZ, RV designed the experiment and made conceptualization; AZ, ZS, RRR collected and analysed the data; AZ, TG, RV developed the first draft; AZ led the writing for the subsequent revisons. All authors conributed critically to the drafts and gave final approval for publication.

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Data availability statement Datasets obtained in this study are openly available in GenBank (MK801309–MK801355).

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Declarations

Conflict of interest Authors disclose financial or non-financial interests that are directly or indirectly related to the work submitted for publication.

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3.2.3. Bark stirpping wounds, wood discoloration of *P. contorta,* and associated fungi (Paper V)

1434

NOTE

Bark stripping of *Pinus contorta* caused by moose and deer: wounding patterns, discoloration of wood, and associated fungi

Natalija Arhipova, Aris Jansons, Astra Zaluma, Talis Gaitnieks, and Rimvydas Vasaitis

Abstract: The aim of this study was to assess the extent of bark stripping wounds, subsequent wood discoloration, and associated fungi in 30-year-old *Pinus contorta* Douglas ex Loudon stems damaged by large game. In total, 90 trees were evaluated, and 170 bark stripping wounds of different ages (1–20 years) were measured. From each wound, wood samples were collected for subsequent fungal isolation. Thirty trees were cut to evaluate the length of the discoloration column. Of 170 injuries, 16 of them represented closed scars and 154 of them represented open wounds that exposed 4–4355 cm^2 of sapwood. The wound length had a strong impact on the length of decay ($r = 0.716$); however, the spread of discoloration beyond the wound margin was limited (0–20 cm). The most commonly isolated fungus was *Sarea difformis* (Fr.) Fr. and, among the Basidiomycetes, *Peniophora pini* (Schleich.) Boidin. The results suggest that when planning to grow *P. contorta* in areas of Europe, the population size of large game animals needs to be considered, in view of potential risk of bark stripping damage.

Key words: lodgepole pine, bark stripping wounds, wood discoloration.

Résumé : Le but de cette étude était d'évaluer l'étendue des blessures d'écorçage, la coloration subséquente et les champignons associés sur des tiges de *Pinus contorta* Douglas ex Loudon âgées de 30 ans et endommagées par le gros gibier. Au total, 90 arbres ont été évalués et 170 blessures d'écorçage de différents âges (1–20 ans) ont été mesurées. Des échantillons de bois ont été prélevés dans chaque blessure pour l'isolation subséquente des champignons. Trente arbres ont été coupés pour évaluer la longueur de la colonne de coloration. Des 170 blessures, 13 représentaient des cicatrices fermées et 154 des blessures ouvertes où 4 à 4355 cm^2 de bois d'aubier était exposé. La longueur de la blessure avait un impact important sur la longueur de la carie ($r = 0,716$), par contre la progression de la coloration au-delà de la marge de la blessure était limitée (0–20 cm). Le champignon qui a été isolé le plus souvent était *Sarea difformis* (Fr.) Fr. et, parmi les Basidiomycètes, *Peniophora pini* (Schleich.) Boidin. Les résultats indiquent qu'on doit tenir compte de la taille des populations de gros gibier, étant donné les risques de blessures d'écorçage, si on envisage de cultiver *P. contorta* dans certaines régions de l'Europe. [Traduit par la Rédaction]

Mots-clés : pin tordu, blessures d'écorçage, coloration du bois.

Introduction

In Latvia, experimental plantations of *Pinus contorta* Douglas ex Loudon were established in years 1979–1981, with native *Pinus sylvestris* L. simultaneously planted as a control (Baumanis and Birgelis 1993). Evaluation of growth in 2008–2010 showed that the standing volume in stands of *P. sylvestris* was 170 $m^3 \cdot ha^{-1}$, compared with a standing volume of 240 $m^3 \cdot ha^{-1}$ (about 25% higher than *P. sylvestris*) in stands of *P. contorta* (Sisenis 2013). The results, therefore, indicate that the exotic *P. contorta* has potential for use in practical silviculture in the Baltic region for wood biomass production. However, it was noted that *P. contorta* commonly suffered bark stripping damage, which was assessed at a level of up to 68% of trees in some stands (Sisenis 2013). The damage occurs by bark removal from stems during feeding by large herbivores, which results in open mechanical wounds. In Latvia, the damage is caused by two species of large game animals, namely red deer (*Cervus elaphus* L.) and moose (*Alces alces* L.) (Andersone-Lilley et al. 2010).

In a review, Gill et al. (2000) reported that the bark stripping wounds of *P. contorta* might lead to the development of wood discoloration and decay. For example, decay was observed in 7.8% of logging injuries of retained *P. contorta* in partially harvested stands of British Columbia (Allen and White 1997). Yet, in the available literature, the extent of fungal damage in wounded stems has not been estimated, and the associated fungi have not been identified. Therefore, more detailed investigations are required to further evaluate the perspectives of growing *P. contorta*, for example, under northern European conditions and particularly in areas that are overpopulated by large game animals. The goals of the present study were to (*i*) describe the wounding pattern caused by bark stripping on stems of *P. contorta*, (*ii*) assess the extent of the subsequent discoloration of wood, and (*iii*) identify the associated fungi.

Material and methods

Field work

This study was conducted in Latvia in three 31- to 32-year-old experimental plantations of *P. contorta* (Fig. 1), with all of them growing on mineral soil: Kuldiga (56°40′29″N, 21°57′31.2″E), Zvirgzde (56°40′45″N, 24°25′60″E), and Kalsnava (56°40′29″N, 25°57′35″E). The stands were chosen for investigation, because they are the three oldest *P. contorta* forest stands in Latvia, they

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Fig. 1. Location of study sites.

have suitable sizes (0.5, 1.2, and 0.2 ha in area, respectively), and they are the only stands in which bark stripping occurred. The proportion of stems showing bark stripping damage exceeded 50% in each of the stands. Selective felling, which potentially could be the only other source of mechanical wounding of stems apart of the damage inflicted by large game, had never been used in the investigated stands.

In each stand, 30 living *P. contorta* trees showing bark stripping injury were selected, mostly by choosing the adjacent wounded tree to the initially measured tree. If the adjacent tree with bark stripping was dead or dying, it was omitted. For each wounded tree, the diameter at breast height (DBH, 1.3 m) was measured, and the number of individual injuries per stem (wounds or scars separated by sound bark, thus representing individual stripping events) was recorded. For each of the 170 recorded injuries, maximal length and width and position in relation to ground level (height of its lowest margin) were measured. To estimate wound area, its configuration was drawn by a marker on transparent paper. In the laboratory, the surface area of each injury was measured using a Tamaya digital planimeter (Planix 10-S, Tamaya Technics Inc., Tokyo, Japan). For wounds with lengths more than 0.7 m, the area was calculated based on length and width, presuming that the wound had an oval form.

From each of the 170 wounds, a single wood sample (bore core length, 10–16 cm) was taken using an increment borer, targeting either 1 cm above or below the injury (depending on the wound height and accessibility). Bark was removed from the core immediately after extracting the sample. After each sampling, the borer was sterilized in 70% ethanol. Immediately after sampling, each wood core was assessed for the presence or absence of discoloration. After assessment, all samples were individually placed in a sterile plastic tube and transported to the laboratory for fungal isolation. In the sample plot in Kalsnava (site at which the largest proportion of stems contained discoloration; see Table 1), all 30 trees, containing a total of 61 wounds, were cut, and the longitudinal extent of wood discoloration from the upper and lower margins of each wound was measured, and other stem damages (asymmetrical stem growth and resin pockets) were recorded. A search for decayed stems was made also after cutting the trees. As a result, one more tree was found to contain discoloration, in addition to those initially detected by borer cores. The number of years since the damage occurred was determined by counting the number of postinjury annual tree rings. On other sample plots, wound age was visually assessed according to the wound closure rate and amount and freshness of resin exudates.

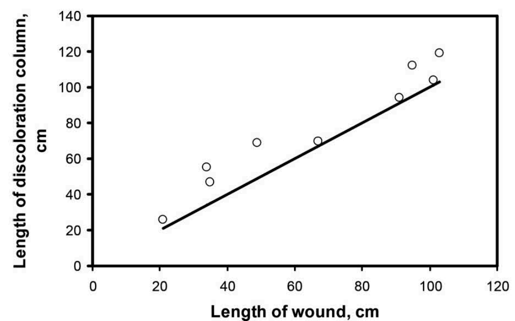
Means, standard deviations, and variation coefficients were calculated (Fowler et al. 1998). Pearson's correlation coefficients were determined between tree and wound parameters.

Table 1. Mean parameters (mean ± standard deviation (SD)) of analyzed *Pinus contorta* trees and bark stripping wounds.

Parameters	Kalsnava	Kuldiga	Zvirgzde	All
Stems				
No. examined	30	30	30	90
Stem DBH (cm)	17±4	19±6	13±4	16±5
Distance of damage from ground (cm)				
Lowest point	83±33	74±20	76±20	78±25
Highest point	138±23	121±19	120±21	126±23
No. of wounds per stem	2.0±0.7	1.8±0.8	1.9±0.9	1.9±0.8
Exposed sapwood per stem (cm^2)	369±668	399±230	470±797	413±619
Wood discoloration (% of stems)	30.0	23.3	6.7	20.0
Wounds				
No. examined	61	54	55	170
Wound width (cm)	5.4±5.9	9.6±6.9	7.8±7.6	7.5±7.0
Wound length (cm)	25.0±26.3	27.6±17.9	28.2±19.3	26.9±21.6
Exposed sapwood per wound (cm^2)	182±492	206±215	252±607	212±469
Wound age (years)	16±5	9±3	9±5	11±5
Wood discoloration (% of wounds)	14.8	13.0	3.6	10.6

Note: DBH, diameter at breast height (1.3 m).

Fig. 2. Longitudinal spread of discoloration column beyond wound margins (open circles) in stems of *P. contorta* in relation to wound length. The solid line represents values for discoloration that did not exceed the wound margins (e.g., 1 m long wound vs. 1 m long discoloration).



Isolation and identification of fungi

In the laboratory, each tree core was split into two pieces (each approximately 5–8 cm long) and, after sterilization by flame, each piece was individually placed in a 9 cm plastic Petri dish on malt agar media (15 g malt extract, 12 g agar, and 1000 mL H_2O; making 342 Petri dishes in total). Petri dishes with samples were incubated at room temperature and inspected twice weekly for fungal growth; all emerging mycelia were subcultured on individual Petri dishes and grown as pure cultures. After 3–4 weeks of growth, all pure cultures were examined under a microscope and grouped into mycelial morphotypes.

One to three representatives of each distinct mycelial morphotype were subjected to molecular identification following procedures from Arhipova et al. (2011). In brief, DNA extraction and PCR amplification were made according to established protocols (Kåren et al. 1997). The PCR products were purified using FastAP Thermosensitive Alkaline Phosphatase and Escherichia coli exonuclease I (Thermo Scientific) and sent to Macrogen Europe (Amsterdam, the Netherlands) for further Sanger sequencing. Sequencing was conducted in one direction using the international primer ITS4 for every specimen. All sequences were manually edited using the BioEdit software (version 7.0.9.0). BLAST

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Table 2. Fungi isolated from stem wounds of *Pinus contorta* in Latvia.

Fungal taxa	GenBank accession no.	Frequency of occurrence (%) in wood samples: Sound looking (n = 152)	Discolored (n = 18)	All (n = 170)	Stems (n = 90)
Basidiomycetes					
Agaricales sp. K44	KP698199	1.3	—	1.2	2.2
Parasola leiocephala (Orton) Redhead, Vilgalys & Hopple	KP698198	0.7	—	0.6	1.1
Peniophora pini (Schleich.) Boidin	KP698183	2.0	5.6	2.4	4.4
Porodaedalea pini (Brot.) Murrill	KP698196	—	5.6	0.6	1.1
All Basidiomycetes		3.9	11.1	4.7	7.8
Ascomycetes and anamorphic fungi					
Alternaria chartarum Preuss	KP698186	—	5.6	0.6	1.1
Ascocoryne cylichnium (Tul.) Korf	KP698202	2.6	11.1	3.5	4.4
Aureobasidium pullulans (de Bary) G. Arnaud	KP698201	2.6	5.6	2.9	5.6
Cadophora fastigiata Lagerb. & Melin	KP698203	1.3	—	1.2	2.2
Chaetomium globosum Kunze ex Fr	—	0.7	—	0.6	1.1
Cladosporium sp.	—	0.7	—	0.6	1.1
Diplodia pinea (Desm.) J.J. Kickx	KP698189	—	5.6	0.6	1.1
Epicoccum nigrum Link	KP698197	1.3	5.6	1.8	2.2
Eutypa lata (Pers.) Tul. & C. Tul	KP698187	0.7	—	0.6	1.1
Helotiales sp. K22	KP698192	0.7	—	0.6	1.1
Hypocrea pachybasioides Yoshim	KP698200	1.3	—	1.2	2.2
Neonectria fuckeliana (C. Booth) Castl. & Rossman	—	1.3	11.1	2.4	4.4
Ophiostoma minus (Hedgc.) Syd. & P. Syd.	KP698185	1.3	—	1.2	2.2
Penicillium sp.	—	14.5	11.1	14.1	23.3
Pezicula eucrita (Karst.) Karst.	KP698190	3.9	11.1	4.7	7.8
Phaeomoniella effusa Damm & Crous	KP698191	2.0	—	1.8	3.3
Phialocephala sp. K13	KP698188	17.1	16.7	17.1	26.7
Physalospora scirpi (Gutner) Arx	KP698184	1.3	11.1	2.4	4.4
Sarea difformis (Fr.) Fr.	KP698193	34.9	50.0	36.5	42.2
Sarea sp. K25	KP698194	3.9	—	3.5	6.7
Tolypocladium pustulatum (Bills, Polishook & J.F. White) Quandt	KP698195	—	5.6	0.6	1.1
Unidentified ascomycetes	—	3.9	11.1	4.7	8.9
All Ascomycetes and anamorphic fungi		51.3	83.3	54.7	61.1
Zygomycetes					
Umbelopsis isabellina (Oudem.) W. Gams	—	6.6	16.7	7.6	11.1
Umbelopsis ramanniana (Möller) W. Gams	—	2.0	—	1.8	3.3
All Zygomycetes		8.6	16.7	9.4	12.2

Note: Identification of fungal taxa for which GenBank accession numbers are not provided was based on morphological characteristics of the mycelia.

searches were performed using the GenBank sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 20 February 2015). To determine fungal taxons (presumed species), the internal transcribed spacer (ITS) sequence homology was set at 98%–100%. For delimiting at the genus level, the ITS sequence homology was set at 94%–97%. All ITS sequences obtained in this study were deposited in GenBank (accession numbers, KP698183–KP698203).

Results

Mean characteristics of examined trees and injuries are presented in Table 1. Stem DBH of examined trees varied from 6 to 32 cm. The maximum number of wounds per stem was five. Of 170 injuries, 16 of them represented closed scars, and 154 of them were open wounds that exposed 4–4355 cm^2 of sapwood. Wound age varied from 1 to 22 years, and wounds with a wide range of age (1–20 years) on some individual stems were also observed. The injuries (lowest edge) occurred as low as the root collar (six trees or 3.5%) and as high as 166 cm at the highest edge of the wound. Yet, typically, the lowest margin (35%) of wounds was recorded at a stem height of 1–1.5 m.

Among the 90 stems examined, discoloration was observed in 18 (20%) of them. Of the 170 wounds on these stems, wood discoloration was present on 11% of the wounds. Among the 61 wounds dissected at the Kalsnava plot, nine (15%) of them had discolored wood. Yet, the longitudinal spread of the discoloration beyond the upper and lower edges of the wound margins was limited (Fig. 2), not exceeding 10 cm above and 20 cm below the margins. Consequently, positive significant correlations were observed between wound length and total length of discoloration column and its longitudinal spread over wound margins (r = 0.72, p = 0.0001; r = 0.45, p = 0.0001, respectively). On the other hand, correlations between area of exposed sapwood per stem and stem DBH (r = −0.04, p = 0.71) and between age of the injury and length of discoloration column (r = 0.10, p = 0.43) were nonsignificant.

Of the 170 wood samples subjected to fungal isolations, 98 (57.3%) of them resulted in fungal growth, yielding 271 fungal isolates that represented 28 fungal taxa (Table 2). The most common fungus isolated was *Sarea difformis* (Fr.) Fr. (36.3% of all wounds, typically growing out from the core at a distance of 5–10 cm from its outer part). Four species of basidiomycetes were occasionally isolated. The most common species of basidiomycetes was *Peniophora pini* (Schleich.) Boidin, which was isolated from 2.3% of wounds.

Discussion

Similar to the present work with *P. contorta*, previous studies on bark stripping damage on spruce, oak, and ash have also shown highly irregular patterns of injury, resulting in wounds that varied in size from a scratch to several thousand square centimetres, located mainly 1–2 m high on a stem (Vasiliauskas et al. 1996; Vasiliauskas 1998; Vasiliauskas and Stenlid 1998). However, among different tree species, pathological consequences of mechanical stem damage differ significantly. Damage in spruce and ash re-

Fig. 3. Bark stripping wounds on stems of *P. contorta*. (*a*) 18-year-old bark stripping wound, (*b*) cross-section of an old bark stripping wound that showed stem deformation and decay; (*c*) cross-section of an occluded wound – resin pocket inside the stem in the place where bark stripping was located.

sults in extensive heart-rot columns expanding metres away from physical wound boundaries, whereas wound decay in oak is more or less confined to the initially damaged part of the stem (Vasiliauskas 2001). Patterns of wound-associated discoloration observed in *P. contorta* most closely resemble those observed in wounded birch stems, for which the discoloration is limited to centimetres in all directions from the damaged area (Vasaitis et al. 2012).

The results, therefore, show that *P. contorta* is relatively resistant to fungal infections in the exposed sapwood of a living tree, which is in agreement with earlier observations (Allen and White 1997; Gill et al. 2000). However, besides decay and discoloration, another negative impact of bark stripping on wood quality was asymmetrical stem growth, especially if the wound was large (Figs. 3*a* and 3*b*). Moreover, even under the surface of completely occluded wounds, resin pockets were commonly observed, further reducing the quality of the wood (Fig. 3*c*). Apart from wood discoloration and the deformation of the damaged stem, yet another pathological consequence of bark stripping was tree death, commonly observed in all three investigated areas. In such cases, bark stripping wounds on individual trees usually had different ages, demonstrating that animals were feeding on the same tree for a number of years until complete debarking of the stem around the whole perimeter occurred.

The most common fungus isolated from *P. contorta* wounds was *S. difformis*, which typically was associated with resin pockets. Previously, *S. difformis* has been isolated from living *P. contorta* stems in Canada, where it was not associated with wood discoloration or decay (Bourchier 1961). Similarly, in Europe, *S. difformis* and the related species *Sarea resinae* (Fr.) Kuntze have been regularly recorded in sound-looking wood of intact stems of living spruce (Vasiliauskas et al. 2001). This demonstrates that the colonization by *Sarea* spp. does not require wounds and that these species can occur in a tree as endophytes. Although *S. difformis* has been reported as a colonizer of branch stubs of spruce following green pruning, the fungus was also observed to be common in branches that have been unpruned, which also suggests an endophytic life cycle (Metzler 1997). *Porodaedalea pini*, which was recorded from one decay column, is known to cause extensive heart rot to *P. contorta* in its native range (Robinson-Jeffrey and Loman 1963) and also to European pines in Europe. This fungus enters intact trees through natural pathways and can remain latent in healthy wood inside a stem for years, which is, in fact, a characteristic feature for numerous wood fungi (see Vasaitis (2013) and references therein). Thus, in this respect also, *P. pini* is not dependent on mechanical injury. *Neonectria fuckeliana* (C. Booth) Castl. & Rossman, which was isolated from several trees, causes pine flute canker in New Zealand and is considered to be a major pathogen in that country (Dick and Crane 2009). However, it is a native and very common fungus in Europe, where it colonizes stems of several tree species without causing the discoloration of wood (see Vasiliauskas and Stenlid (1997) and references therein).

It is known that *P. contorta* is most susceptible to bark stripping at an age of 5–16 (up to 40) years and that trees, if damaged once, are more likely to be damaged repeatedly (Gill 1992; Gill et al. 2000). Cited observations agree with our results, as most of the investigated trees were first damaged 10–22 years ago at an age of 7–20 years old, and some individual stems had injuries ranging in age from 1 year to 20 years. In conclusion, the results of the present study show that bark stripping damage to *P. contorta*, despite initially incurred at young ages, will continue in the 30- to 33-year-old trees. This will have a persisting negative impact on wood quality up until the final harvest, as typically up to 2 m of the most valuable first log will be degraded to low-quality fuel wood. Moreover, besides mortality directly related to debarking, bark-stripped *P. contorta* trees are known to become prone to

breakage by wind (Gill et al. 2000). Therefore, the results suggest that when planning to grow *P. contorta* in areas of Europe, the population size of large game animals needs to be considered, in view of the potential risk of bark stripping damage.

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3.3. Infection, spread and clonality of *Heterobasidion* spp. in *P. contorta* plantations established on forest clear-cuts and agricultural land (Paper VI)

ARTICLE

Infection and spread of root rot caused by *Heterobasidion* spp. in *Pinus contorta* plantations in Northern Europe: three case studies

A. Zaļuma, I. Muižnieks, T. Gaitnieks, N. Burņeviča, Ā. Jansons, J. Jansons, J. Stenlid, and R. Vasaitis

Abstract: This study investigated the origins and spread patterns of *Heterobasidion* root disease in three *Pinus contorta* Dougl. ex Loudon plantations established on forest and agricultural land and subjected to three different management scenarios. Trees with decline symptoms and stumps remaining from the previous rotation were sampled for fungal isolations. Ten isolates of *Heterobasidion parviporum* Niemelä & Korhonen and 425 of *Heterobasidion annosum* (Fr.) Bref. were tested for clonality through somatic compatibility tests. The following conclusions were reached: (*i*) *P. contorta* is highly susceptible to *H. annosum* and *H. parviporum* and both pathogens cause dieback of *P. contorta*; (*ii*) *H. annosum* from previous-rotation *P. sylvestris* stumps can effectively transfer to *P. contorta*; (*iii*) the pathogens may form constantly expanding territorial clones; (*iv*) basidiospores of both pathogens colonise stumps of *P. contorta* (primary infections); (*v*) *H. parviporum* clones expanded more slowly than clones of *H. annosum*; (*vi*) clonal spread proceeded more quickly from stumps with established secondary infections than from stumps with primary infections; (*vii*) *H. annosum* can persist in pine stumps for at least 26 years; and (*viii*) stump treatment should be considered to control *Heterobasidion* primary infections.

Key words: lodgepole pine, *Heterobasidion*, primary infection, secondary infection.

Résumé : Cette étude porte sur l'origine et les patrons de dispersion de la maladie de racines causée par *Heterobasidion* dans trois plantations de *Pinus contorta* Dougl. ex Loudon établies sur des terrains forestiers et agricoles et soumises à trois scénarios d'aménagement différents. Les arbres montrant des symptômes de dépérissement et les souches restantes de la rotation précédente ont été échantillonnés pour la présence de champignons. Dix isolats de *Heterobasidion parviporum* Niemelä & Korhonen et 425 isolats de *Heterobasidion annosum* (Fr.) Bref. ont été soumis à des tests de compatibilité somatique pour déterminer leur clonalité. Les conclusions suivantes ont été tirées : (*i*) *P. contorta* est très sensible à *H. annosum* et *H. parviporum* et les deux pathogènes causent le dépérissement de *P. contorta*; (*ii*) *H. annosum* présent dans les souches de *P. sylvestris* restantes de la rotation précédente peut effectivement se transmettre au *P. contorta*; (*iii*) les pathogènes peuvent former des clones territoriaux en constante expansion; (*iv*) les basidiospores des deux pathogènes colonisent les souches de *P. contorta* (infections primaires); (*v*) les clones de *H. parviporum* se propagent plus lentement que les clones de *H. annosum*; (*vi*) la propagation clonale est plus rapide à partir des souches où sont établies des infections secondaires qu'à partir des souches colonisées à la suite d'une infection primaire; (*vii*) *H. annosum* peut persister dans les souches de pin pendant au moins 26 ans; et (*viii*) on devrait envisager de traiter les souches pour maîtriser les infections primaires causées par *Heterobasidion*. [Traduit par la Rédaction]

Mots-clés : pin tordu, *Heterobasidion*, infection primaire, infection secondaire.

1. Introduction

Since the 1950s, the introduced lodgepole pine, *Pinus contorta* Dougl. ex Loudon, has become increasingly important in forestry in Northern Europe, and to date, hundreds of thousands of hectares have been planted with this species, e.g., in the UK (Redfern 1982) and Scandinavia (Hagner 1983; Karlman 2001). Moreover, in Sweden, tree breeding programmes for *P. contorta* have been developed (Hayatgheibi 2018). *Pinus contorta* is susceptible to root rot caused by fungi from the genus *Heterobasidion* (Redfern 1982; Vollbrecht et al. 1995; Piri 1996; Woodward et al. (1998) and references therein; Greig et al. 2001; Rönnberg and Svensson 2013) — the most economically important pathogens of conifers in the Northern Hemisphere (Woodward et al. 1998). In Northern Europe, there are two species of *Heterobasidion* that cause the damage: *Heterobasidion parviporum* Niemelä & Korhonen and *Heterobasidion annosum* (Fr.) Bref. (Niemelä and Korhonen 1998).

Primary infections of the pathogens are established by airborne basidiospores. After landing on surfaces of freshly cut conifer stumps (in Northern Europe, mainly *Picea abies* (L.) H. Karst and *Pinus sylvestris* L.), the spores germinate and hyphae grow over the stump surface and penetrate down into the inner part of the stump, finally colonising lateral roots (Redfern and Stenlid (1998) and references therein; Swedjemark and Stenlid 2001; Garbelotto and Gonthier 2013). Secondary infections occur when hyphae of the pathogens spread to root systems of adjacent trees via root contacts, which can produce expanding, territorially defined disease centres (Stenlid and Redfern 1998; Garbelotto and Gonthier 2013). Moreover, *Heterobasidion* spp. effectively invades roots of trees replanted on infested forest clearcuts creating intergenera-

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Fig. 1. Location of study sites.

tional secondary infections (Stenlid 1987; Vasiliauskas and Stenlid 1998; Lygis et al. 2004). As a result, established disease centres on forest land potentially have the capacity for indefinite longevity; although the complete arrest of expansion of disease centres was documented in North America (Rizzo et al. 2000).

Surprisingly, *P. contorta* as a *Heterobasidion*-susceptible species has not been mentioned in more recent syntheses on *Heterobasidion* in which the data on tentative susceptibility of numerous tree species (including nine *Pinus* species from Europe and North America) to five biological species of the pathogen were summarised (Garbelotto and Gonthier 2013; Gonthier and Thor 2013). In fact, the first and, to date, the only work demonstrating the susceptibility of *P. contorta* to natural *Heterobasidion* sp. infections was carried out in Finland in the early 1990s. Piri (1996) investigated infections of the disease in two *P. contorta* plantations (8 and 14 years old) established on clearcut *P. abies* sites. It was found that 18.4% and 5.8% (9 and 11 trees) of the *P. contorta* were infected in the respective plantations, and in most of cases (7 and 10 trees), the infections were traced to stumps of the previous generation of *P. abies*. All of the detected infections proved to be due to *H. parviporum*, thus providing evidence that *H. parviporum* on *P. abies* can be transferred from infected stumps to the next generation of *P. contorta* (Piri 1996).

To the best of our knowledge, there are no studies of *Heterobasidion* spp. (*H. annosum*, *H. parviporum*, and *Heterobasidion irregulare* (Underw.) Garbel. & Otrosina) infectiousness, establishment, ability to transfer over forest generation, and rate of spread from tree to tree via root contacts within stands of *P. contorta*. The aim of this work was to investigate the spread patterns of *Heterobasidion* spp. in *P. contorta* plantations established on forest and former agricultural land under three different management scenarios.

2. Materials and methods

2.1. Fieldwork

This study was performed in three *P. contorta* plantations in Latvia (Fig. 1). Characteristics of the investigated plantations are presented in Table 1. The management scenarios were as follows: (*i*) stand 1 (56°40′1.9″N, 25°49′59″E), a 26-year-old unmanaged plantation (established on a *Heterobasidion*-infested clearcut site of approximately 100-year-old *P. sylvestris*); (*ii*) stand 2 (56°41′06″N, 24°27′42″E), a 27-year-old unmanaged plantation (established on a *Heterobasidion*-infected clearcut site of 100-year-old *P. sylvestris*), which was thinned directly after the first sampling, and (*iii*) stand 3 (57°03′47″N, 21°57′11″E), a 28-year-old plantation established on former agricultural land (assumed to be free of *Heterobasidion* infections), thinned 16 years earlier. Stands 1 and 3 were sampled once, and stand 2 was sampled twice: before thinning and 4 years after thinning. Thinnings in stands 2 and 3 were conducted during September–October and stumps were not treated with control agents (neither urea nor biological control agents) to prevent new (primary) infections by *Heterobasidion* spp. to freshly cut stumps.

In each stand, a visual evaluation of tree health condition allocated the trees to three categories: (1) crowns visually healthy; (2) crowns with dieback characteristic of *Heterobasidion* symptoms, i.e., chlorotic foliage, resin flow, reduced growth (Piri, 1996); and (3) dead or windthrown. All trees and previous-generation stumps were examined for fruit bodies of *Heterobasidion*. To detect the pathogen, category 2 and 3 trees and all trees with fruit bodies were cut, and then 3 cm thick wood discs were taken from stump surfaces. Previous-generation stumps could not be sampled by taking discs, as they were heavily decomposed. Consequently, decomposed parts of stumps were removed with an axe, and wood samples were cut from the solid parts of the stumps and (or) roots. All of the wood discs taken from the felled trees and wood samples taken from old stumps were individually placed in plastic bags and brought to the laboratory. The numbers of surveyed, sampled trees and previous-generation stumps are shown in Table 2. In each plot, the sampled trees and stumps were numbered and mapped.

2.2. Isolation and identification of *Heterobasidion* genotypes

In the laboratory, the discs were debarked and washed with a stiff brush under tap water (the brush was washed in the tap water immediately after each sample was brushed). Discs were incubated for 5–8 days at room temperature in open plastic bags. Using a dissection microscope (at 20–30× magnification), the presence of *Heterobasidion* spp. was confirmed by observing its characteristic asexual sporulation (conidiophores) on disc surfaces. The *Heterobasidion* colonies were sampled with sterilised surgical forceps and individually placed on malt extract agar (MEA) in Petri dishes. Outgrowing *Heterobasidion* mycelia were subcultured. Wood samples taken from stumps were cut with a knife to an approximate size of 1 × 0.5 cm, flame surface sterilized, and incubated for 7 days at room temperature on MEA in Petri dishes. Outgrowing *Heterobasidion* mycelia were isolated. All of the isolates obtained were individually stored on MEA in test tubes at 4 °C until used in subsequent pairing tests for somatic compatibility.

Isolates originating from the same sample plot were subjected to somatic compatibility tests by pairwise confronting their mycelia on MEA in 9 cm Petri dishes in all combinations. The genets were identified either by recording the line of demarcation in the

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Table 1. Description of investigated 26- to 31-year-old plantations of *Pinus contorta* (+ <5% admixture of *Pinus sylvestris*).

	Stand 1	Stand 2[a]	Stand 3
Location	57°06′N, 21°95′E	56°68′N, 24°46′E	56°60′N, 25°49′E
Area, ha	0.2	0.8	0.2
Age, years	26	27 (31)	28
Tree DBH, cm	12	12 (15)	14
Planting density, no.·ha^{-1}	5000	5000	5000
Density at sampling, no.·ha^{-1}	2290	2986 (1471)	2350

Note: Previous forest generation in stands 1 and 2 (established on clearcuts) consisted of ~100-year-old *P. sylvestris*: stand 1, established on clearcut, unthinned; stand 2, the first sampling was done in the unthinned plantation and second sampling was done 4 years after thinning. Stand 3 was established on agricultural land, thinned 16 years previously. DBH, diameter at breast height.

[a]In this column, numbers in parentheses are from the second sampling in stand 2.

Table 2. Tree dieback and infections by *Heterobasidion* spp. in 26- to 31-year-old plantations of *Pinus contorta* (+ <5% admixture of *Pinus sylvestris*).

	Stand 1	Stand 2[a]	Stand 3
***Pinus contort* trees**			
Visually surveyed, total no.	439	2270 (1124)	438
Dieback and windthrown, sampled, no.; %	128; 29	257; 11 (120; 11)	220; 50
1st and 2nd sampling pooled	—	(377; 17)	—
Heterobasidion-infected,[b] no.; %	84; 19	192; 9 (96; 9)	72; 16
1st and 2nd sampling pooled	—	(288; 13)	—
Dieback symptoms and infected, %	66	75 (80)	33
1st and 2nd sampling pooled	—	(76)	—
***Pinus sylvestris* trees**			
Visually surveyed, total no.	19	119 (53)	32
Dieback symptoms, sampled, no.; %	3; 16	0; 0 (1; 2)	16; 50
Heterobasidion-infected,[b] no.; %	3; 16	0; 0 (1; 2)	2; 6
Heterobasidion fruit bodies, tree no.; %	36; 8	96; 4	18; 4
***Pinus sylvestris* stumps from previous generation**			
Sampled, no.	67	244	—
Heterobasidion isolated, no.; %	2; 3	1; 0.4	—

Note: Stand 1, established on clearcut, unthinned; stand 2, the first sampling was done in the unthinned plantation and second sampling was done 4 years after thinning. Stand 3 was established on agricultural land, thinned 16 years previously.

[a]In this column, numbers in parentheses are from the second sampling in stand 2.

[b]Conidia of *Heterobasidion* observed on stem cross-section surface.

contact zone of confrontation (demonstrating that the genotypes are different) or by observing free fusion of the mycelia (implying that the genotypes were identical) (Stenlid 1985). Based on these data, delineation of genets (or territorial clones when comprised of at least two isolates) was accomplished by transferring their boundaries onto the constructed field maps. The species of each genet was determined by mating tests with *H. annosum* and *H. parviporum* single spore tester strains (Korhonen 1978).

2.3. Data analysis

Figures 2, 3, and 4 were created using the geospatial tool QGIS 2.18.3 (https://qgis.org/en/site/), and the area of genets was measured using the "Measure Area" tool. The Wilcoxon test (unpaired or paired samples) was used to compare width and area of genets in each plantation, and their rates of expansion between stands 2 and 3. For calculations, R version 3.4.3 was used (R Core Team 2017).

3. Results

3.1. Incidence of dieback

In plantations established on former forest land (clearcut sites of 100-year-old *P. sylvestris*), the incidence of trees showing crown dieback symptoms was lower (29% in stand 1 and 11% in stand 2) (Table 2) than in the plantation established on *Heterobasidion*-free former agricultural land (50% in stand 3), which had been thinned 16 years previously (Table 2; Figs. 3 and 4). In stand 3, 115 trees (52% of declining or dead trees) were windthrown (storm in January of 2005) and 70 trees were wounded by large game animals (red deer and moose).

Despite the removal of all trees with dieback in stand 2, symptoms developed in 120 (11%) of the retained trees during the subsequent 4 years after thinning, and the proportion of trees with dieback symptoms after 4 years was similar to that initially observed (Table 2). The additional decline observed in residual trees of stand 2 4 years after first sampling increased the proportion of total observed diseased trees in stand 2 from 11% (first survey) to 17% (pooled first and second surveys) (Table 2).

3.2. Observed infections of *Heterobasidion*

Although *Heterobasidion* root disease was suspected as the primary cause of the disease symptoms observed in the sampled trees, the presence of the pathogen was not confirmed in all cases. After incubation of stem discs, conidiophores were observed on 66% of the samples from stand 1, 75%–80% from stand 2, and 33% from stand 3 (Table 2). As with dieback, the incidence of trees in which *Heterobasidion* infections were observed on cut discs from stump surfaces also differed between the two plantations established on former forest land (19% and 9% in stand 1 and stand 2 — first sampling, unthinned, respectively). Thinning and removal of symptomatic trees did not prevent the spread of disease to retained trees. The baseline (i.e., prethinning) incidence of disease was 9% (192 infected trees) and a subsequent survey 4 years later

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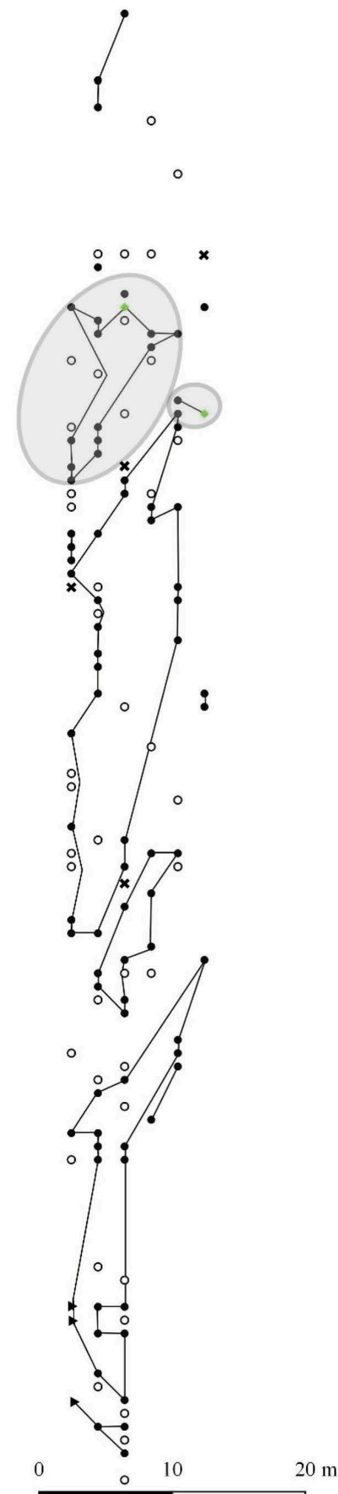


Fig. 2. Distribution of *Heterobasidion annosum* genotypes in stand 1. *Heterobasidion annosum* isolated from *P. contorta* (●), *P. sylvestris* (▲), and previous-generation *P. sylvestris* stumps (◆). *Heterobasidion* not isolated from *P. contorta* with dieback symptoms and *Heterobasidion* conidiophores at stump (×) and *P. contorta* with dieback symptoms (○). The lines connect trees and (or) stumps from which somatically compatible *H. annosum* strains were isolated. The two ellipsoid areas, shaded grey, cover approximate areas of two *H. annosum* clones, the origins of which have been traced to previous-generation stumps. Note that for technical reasons in drawing ellipses, the larger clone also encircles six trees with dieback symptoms from which the pathogen has not been isolated. For a similar reason, the smaller clone encircles one tree with *H. annosum* belonging to an adjacent clone. [Colour online.]

found once again that 9% (an additional 96 trees) of the retained trees were diseased. Thus, the cumulative incidence of disease in the stand increased to 13% (288 trees). In stand 3, the plantation established on former agricultural land, the incidence of infected trees (conidia observed in 16%) was similar to that in stands 1 and 2. A total of 150 trees with *Heterobasidion* fruit bodies were observed: 36, 96, and 18 in stands 1, 2, and 3, respectively (Table 2). *Heterobasidion* fruit bodies were observed on one (0.3%) stump out of 311 inspected previous-generation stumps.

3.3. Isolates and genets of *Heterobasidion*

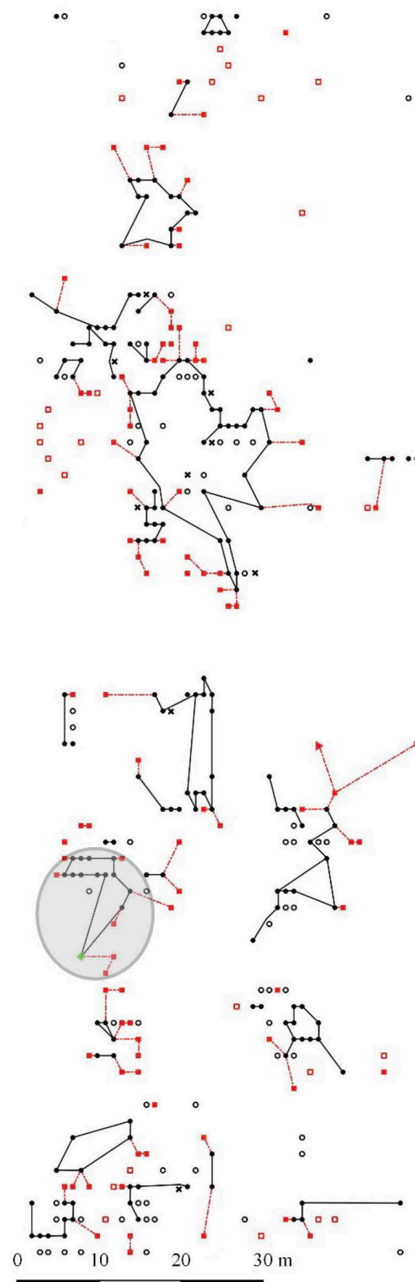
Culturing success from stem discs bearing *Heterobasidion* conidiophores was high. In total, 432 isolates from *Heterobasidion* spp. infected trees were obtained: 83, 280, and 69 (95.4%, 96.8%, and 93.2%) from stands 1, 2, and 3, respectively (Table 3); 422 of those were identified as *H. annosum* and 10 were identified as *H. parviporum* (the latter only in stand 3; Fig. 4). Spatial distributions of *Heterobasidion* genets in the plantations are shown in the Figs. 2, 3, and 4. In total, 98 *Heterobasidion* genets were detected. In the unthinned stand 1 (former forest land), four genets (31%) occurred only in single trees, while nine (69%) were detected in two or more standing trees. In stand 2, single-tree genets were detected in seven (20%) *P. contorta* prior to thinning and in 15 (33%) *P. contorta* 4 years after the thinning, while the respective numbers of territorial genets increased from 28 to 30. Somatic compatibility tests revealed that the proportions of single-tree vs. territorial genets were at least two times lower on former forest land sites (stands 1 and 2) than on the former agricultural land site (stand 3). In stand 3, 28 of 40 genets (70%) were single-tree occurrences. The mean number of trees per genet was three to four times greater on former forest land than on former agricultural land, respectively: 6.4 trees per genet in stand 1, 5.2 in stand 2 (increased to 6.2 in the 4-year period after thinning), and 1.7 in stand 3 (Table 3).

3.4. Territorial clones (including > 1 tree): forest vs. agricultural land (stands 1 and 2 vs. 3)

The maximum number of trees comprising a territorial genet was 24 (mean 8.8) in stand 1, 29 (mean 6.3 trees) in stand 2, which increased to 49 (mean 8.8 trees) after thinning, and only 8 (mean 3.4 trees) in stand 3. Thus, the extent of clonality on the former agricultural land was two to three times less than on forest land.

The mean width of territorial genets was about 8–14 m (maximum width, 30–40 m) on former forest land (stands 1 and 2) compared with mean width of 5 m (maximum width, 12 m) on former agricultural land (stand 3) (Table 3); thus, in the latter stand, the territorial genets were 1.5 to 3 times smaller in width (not significant). The number of *H. parviporum* territorial clones was limited (two clones) and the maximum width reached was 2.8 m (Fig. 4). Comparisons of mean clone width prior to and after thinning in stand 2 demonstrated that the width increased significantly ($p < 0.001$) during the 4 years after thinning from 7.8 to 10.3 m (Table 3).

Fig. 3. Distribution of *Heterobasidion annosum* genotypes in stand 2. *Heterobasidion annosum* isolated from *P. contorta* at first sampling (●), *P. contorta* at second sampling (4 years after thinning) (■), current-generation *P. sylvestris* at second sampling (▲), and previous-generation *P. sylvestris* stump (◆). *Heterobasidion* not isolated from *P. contorta* with dieback symptoms and *Heterobasidion* conidiophores at stump (×), *P. contorta* with dieback symptoms at first sampling (○) and at second sampling (□). Continuous lines connect trees and (or) stumps from which somatically compatible *H. annosum* strains were isolated in the first sampling, and the dashed–dotted lines connect trees and (or) stumps from which somatically compatible *H. annosum* strains were isolated in the second sampling (in online version, all symbols and lines from the second sampling are in red). Ellipsoid area, shaded grey, covers the approximate area of the *H. annosum* clone, the origin of which has been traced to the previous-generation stump. Note that for technical reasons in drawing the ellipse, the clone also encircles two trees with dieback symptoms from which the pathogen has not been isolated and excludes one tree with *H. annosum* belonging to the encircled clone. [Colour online.]



The maximum rate of territorial clone active expansion per year in width, calculated from the expansion of clones between the first and the second sampling in stand 2 (the maximum distance of red dotted lines in Fig. 3), was almost nine times greater than that on agricultural land in stand 3 (calculated by dividing the distance between the two furthest trees of a clone by 16 years and by 2 (infection centre assumed to be in the middle): 3.5 vs. 0.4 m, respectively. Also, the mean rate of active expansion (0.9 vs. 0.15 m·year^{-1}, respectively, in stand 2 vs. stand 3) calculated from expansion of territorial clones on former forest land (mean of all red-dotted lines in Fig. 3) was significantly (p = 0.0015) higher (approximately sixfold) than on former agricultural land (mean of all distances between trees comprising territorial clones; Fig. 4).

The maximum area of the territorial clones on the former forest land sites was ~7–8 times greater (190 m^2 in stand 1; 210 m^2 in stand 2) than on the former agricultural land site, stand 3 (27 m^2) (Table 3). The mean area of territorial clones was also larger (not significant; p > 0.05) on former forest land (~21 m^2 and 43 m^2) than on former agricultural land (~11 m^2), thus differing by ~two- to four-fold (Table 3). Significant differences were not found between the mean widths and areas of territorial clones stand 1 and stand 3, which may have been due to the low number of observations (9 and 12, respectively) and high variation in the datasets (Table 3).

In stand 2, the mean area occupied by genets increased significantly, from 30 to 41 m^2 (p = 0.0016), during the 4 years between the first and the second surveys (Table 3). The maximum expansion rate in area from already established clones on forest land in stand 2 was ~16 times greater than that on the former agricultural land of stand 3: 13.5 vs. 0.8 m^2·year^{-1}, respectively. The average area expansion rate in stand 2 (forest land, 2.3 m^2·year^{-1}) was significantly (p = 0.0001) greater (by ~eight times) than that in the stand 3 plantation on former agricultural land (0.3 m^2·year^{-1}).

3.5. *Pinus sylvestris*

A total of 170 *P. sylvestris* trees were surveyed in the study. The incidence of those showing dieback symptoms differed among the plots: 16%, 0% (2%), and 50% for stands 1, 2, and 3, respectively (Table 2). *Heterobasidion* conidiophores were observed and the pathogen was isolated from five symptomatic *P. sylvestris* (Table 2; Figs. 2, 3, and 4). Of the 311 wood samples taken from *P. sylvestris* stumps of the previous generation and subjected to fungal isolations, only three (1%) of them yielded pure cultures of *Heterobasidion* (Table 2), while *Trichoderma* spp. and *Ascocoryne* spp. were frequently isolated (168 and 86 isolates, respectively). Each genet from previous-generation stumps also has been isolated from next-generation *P. contorta* (Figs. 2, 3).

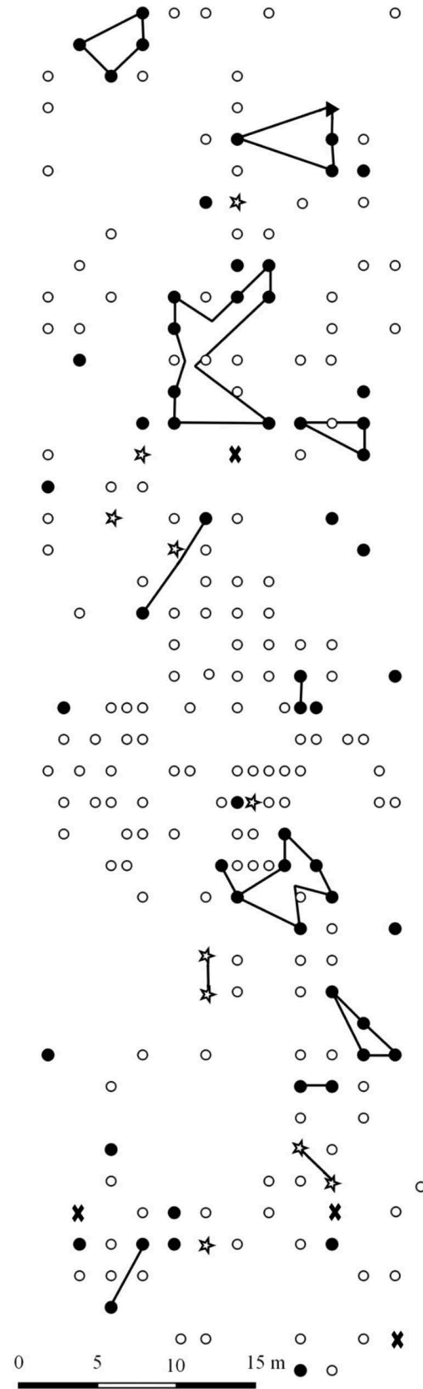
4. Discussion

This study presents the most extensive data on *H. annosum* and *H. parviporum* root rot disease in plantations of *P. contorta* to date. For the first time, comparative investigations on population structures and expansion of *Heterobasidion* spp. were conducted in *P. contorta* stands.

4.1. Susceptibility of *P. contorta* to *Heterobasidion* and sources of new infections

This study provides new evidence that airborne basidiospores of both *H. annosum* and *H. parviporum* are able to colonise cut

Fig. 4. Distribution of *Heterobasidion* spp. genotypes in stand 3. *Heterobasidion annosum* isolated from *P. contorta* (●) and from *P. sylvestris* (▲); *H. parviporum* isolated from *P. contorta* (☆). *Heterobasidion* not isolated from *P. contorta* with dieback symptoms and *Heterobasidion* conidiophores at stump (×) and from *P. contorta* with dieback symptoms (○). The lines connect trees from which somatically compatible *H. annosum* or *H. parviporum* strains were isolated.



stumps of *P. contorta* (primary infections), and that following primary infection, the pathogens spread to adjacent trees via root contacts (secondary infections) (Figs. 2, 3, and 4). Numerous studies had reported that only freshly cut stumps unoccupied by other decay fungi are susceptible to colonisation by *Heterobasidion* spp., and that after 4–8 weeks, they become inaccessible to the pathogen (Redfern and Stenlid (1998) and references therein). Consequently, our work provides new evidence that *H. annosum* from diseased stumps of previous-generation *P. sylvestris* can transfer to planted trees of the next generation of *P. contorta* (Figs. 2 and 3). As the previous-generation *P. sylvestris* stumps were cut at least 26 years previous to investigations (according to the age of replanted next generation; Table 1), our work demonstrates that *H. annosum* can persist and remain viable in stumps of cut, mature *P. sylvestris* for at least 26 years. This finding is comparable with earlier studies, which reported the longevity of *H. annosum* in conifer stumps to be 35–68 years (Greig and Pratt 1976; Piri (1996) and references therein).

4.2. Implications for silviculture

The findings of this study confirm the susceptibility of *P. contorta* to both *H. annosum* and *H. parviporum* via both primary and secondary infections and have silvicultural implications, for example, when planning reforestation on an infected site, scheduling a thinning (during high or low risk times of the year), or considering an option for stump treatment to prevent primary infections. For example, our results (Table 2) demonstrate that the disease can occur in plantations established on infection-free areas. On former agricultural land (stand 3), we observed that 50% of trees had dieback symptoms, and 16 years after thinning, 16% of all trees had *Heterobasidion* infection on the root collar. This was a higher incidence rate than on already-infested forest land (stands 1 and 2 had 29% and 17% trees with symptoms and 19% and 13% of all trees were confirmed to be infected, respectively). Stand 3 had been thinned during the autumn, which is a high-risk season for *Heterobasidion* infection in Latvia (Brauners et al. 2014), and stumps were not treated with preventive substances such as urea or a spore suspension of the biological control agent *P. gigantea*.

4.3. Probable infections by *Heterobasidion*

In all stands, there was a large proportion of declining and dead pines in which the pathogen was not detected (Table 2); therefore, the possibility cannot be excluded that causes other than *Heterobasidion* were responsible for a certain proportion of declining or dead trees. Other possible causes of their decline have not been investigated; however, in stand 3, a large proportion of declining or dead trees were windthrown (115 out of 220 or 52%), which indirectly indicates that their root systems might have been damaged by *Heterobasidion* during the 16 years since thinning but that pathogen has not yet reached the stump surfaces.

Fruit bodies of *Heterobasidion* have been observed only on one out of 311 inspected (0.3%) previous-generation stumps. In fact, this provides the evidence for low vitality of the pathogen in those stumps and explains low frequency of pure culture isolations. By contrast, fruit bodies of *Heterobasidion* were observed on 150 dead or declining trees (5%; Table 2), constituting comparatively fresh woody substrates. This indicates that environmental conditions on investigated sites were otherwise suitable for fruiting of *Heterobasidion*.

4.4. Territorial clones, size, and rates of expansion

As *Heterobasidion* fungi possess a sexual reproductive system, each individual is genetically unique. Thus, each territorial clone must originate from a single fungal individual established within a single stump. Therefore, the current study provides evidence

Table 3. Isolates, genets, and territorial clones of *Heterobasidion* spp. in 26- to 31-year-old plantations of *Pinus contorta* (+ <5% admixture of *Pinus sylvestris*).

	Stand 1	Stand 2[a]	Stand 3
Isolates obtained			
Total no. (including previous generation stumps)	85	184 (97)	69
Isolations from infected trees, no.; %	83; 95.4	183; 95.3 (97; 100)	69; 93.2
Genets detected			
In a single tree, no.; no.·ha^{-1}	4; 20	7; 9 (15; 19)	28; 140
In two or more trees (territorial clones), no.; no.·ha^{-1}	9; 45	28; 35 (30; 38)	12; 60
Total no.; no.·ha^{-1}	13; 65	35; 44 (45; 57)	40; 200
Trees per genet, no.	6.4	5.2 (6.2)	1.7
Characteristics of territorial clones[b]			
Maximum no. of trees and (or) stumps per clone	24	29 (49)	8
Average no. of trees per clone	8.8	6.3 (8.8)	3.4
Maximum width, m	39.9	29.5 (35.1)	11.7
Mean width ± SD, m	13.5±13.9AB	7.8±6.4A (10.3±8.0)B	4.9±2.5A
Maximum rate of expansion,[c] m·year^{-1}; mean ± SD, m·year^{-1}	—	3.5; 0.9±0.6A	0.4; 0.2±0.1B
Maximum area, m^2	191	209 (263)	27
Mean area ± SD, m^2	43±63ABC	21±40B (29±50)C	11±8ABC
Maximum rate of expansion, m^2·year^{-1}; mean ± SD, m^2·year^{-1}		13.5; 2.3±3.0A	0.8; 0.3±0.2B

Note: Stand 1, established on clearcut, unthinned; stand 2, the first sampling was done in the unthinned plantation and second sampling was done 4 years after thinning. Stand 3 was established on agricultural land, thinned 16 years previously. Mean values followed by different letters indicate that the differences between the means are statistically significant (Wilcoxon signed-rank test, $p < 0.05$).

[a]In this column, numbers in parentheses are from the second sampling in stand 2.

[b]Approximate estimates on the extent of their occupied area and rates of spread.

[c]For stand 2, calculations are based on lengths of red dotted lines from Fig. 2 divided by 4 years (since thinning), and in stand 3, calculations are based on the distances between the trees of territorial clones in Fig. 3 divided by 2 and by 16 years (since thinning).

that the territorial clones are each from a single stump. The sizes of the clones (tens of metres in width) were comparable with those observed in previous studies on pre-infected forest sites in plantations established on clearcuts of both *P. abies* and *P. sylvestris*. It is also interesting to note that in a number of these studies, transfer of the pathogen to different tree species was observed (Stenlid 1985; Lygis et al. 2004; Piri 1996; Piri and Korhonen 2001; Vasiliauskas and Stenlid 1998). Moreover, the data from stand 2 provide evidence of territorial expansion of *H. annosum* over the period of 4 years after a thinning (Fig. 3). The observation that newly expanded areas of infection were occupied by the same genotypes as those detected during the first sampling provides proof that these infections originated from old stumps of the previous generation of *P. sylvestris*, either directly or by transfer from infected root systems of thinned *P. contorta*. The present work did not estimate the exact borders and size of *Heterobasidion* genets (for this, a thorough analyses of excavated root systems would be required) but approximated the extent of their occupied area and rates of spread. As only the stems have been sampled, our work generates minimum estimates of size and expansion rates of the observed genets, as shown in the Table 3 and Figs. 2, 3, and 4.

Eight of 10 new genets detected in stand 2 during the second sampling (Table 3) were confined to a single tree (Fig. 3) and thus might have originated from new primary infections from nearby cut stumps of thinned *P. contorta*, which were transferred later by root contacts. However, it is most likely that the genets originated from the previous *P. sylvestris* rotation and were present in the host tree but were not detected during the first sampling. Such a scenario was previously described by Rönnberg et al. (2006) and Piri and Korhonen (2008). In this regard, it has been reported that development of territorial clonality following primary infections of *Heterobasidion* takes at least 7–8 years (Vasiliauskas 1989; Swedjemark and Stenlid 1993; Stenlid and Redfern 1998). In our study, the yearly territorial expansion on former forest land (stand 2) was in agreement with estimations by Hodges (1969) and Rizzo et al. (2000), who reported spread of about 2 m·year^{-1} in forests of southern pines and cedar in the USA. According to Bendz-Hellgren et al. (1999), the average growth rate is 25 cm·year^{-1} in spruce stump roots and 9 cm·year^{-1} in living tree roots, which is comparable with *Heterobasidion* spp. expansion rate in stand 3. Consequently, the 4-year time period monitored in this study was insufficient for development of territorial clonality of a new pathogen > 2 m; however, high planting densities in stands 1, 2, and 3 could have enhanced expansion of *Heterobasidion* territorial clones.

The situation observed in stand 3 (established on infection-free former agricultural land) was markedly different, as the spread originated exclusively from primary infections (Fig. 4). In this stand, the area occupied by the clones was much smaller, trees encompassed by the clones were fewer in number, and the rate of expansion was significantly slower than was observed on the pre-infected forest sites of stands 1 and 2 (Fig. 4 and Table 3). Although earlier studies indicate that the spread of *Heterobasidion* spp. should be faster on previous agricultural land (Woodward et al. (1998) and references therein), our study found that the pathogen spread more quickly from stumps with established secondary infections than from freshly cut stumps with primary airborne infection. The importance of infected previous-generation stumps on infection transfer has been noted in earlier studies (Stenlid 1987; Woodward et al. 1998; Bendz-Hellgren et al. 1999; Greig et al. 2001; Piri and Korhonen (2008) and references therein). In stand 2, the proportion of new trees with dieback and infection 4 years after thinning was once again equal to what was observed before thinning; therefore, our results support earlier observations that spruce stumps left after thinning transmit root rot to residual trees (Pettersson et al. 2003; Piri and Korhonen 2008). In general, lower numbers of trees in clones occupying a smaller stand area indicate that these clones are relatively younger than those comprised of more trees and covering a larger area, which corresponds well with the history of the plantations included in this study (Swedjemark and Stenlid 1993).

4.5. Transfer to the next forest generation and to different tree species

This study demonstrates effective transfer of *H. annosum* from *P. sylvestris* to *P. contorta* (stands 1 and 2). Previous studies on local

population structure and territorial clones of biological species of *Heterobasidion* focused mainly on stands of *P. abies* previously infected by *H. parviporum*. In a pioneering investigation conducted in Sweden, Stenlid (1985) studied a 120-year-old *P. abies* stand thinned approximately 30 and 15 years previously and detected a number of territorial clones reaching up to 30 m in diameter, all of which belonged to *H. parviporum*. Similarly, up to ~40 m long territorial clones were observed in an intensively thinned *P. abies* stands in Serbia, and all of them also consisted of *H. parviporum* (Keča and Keča 2013). In Finland, related studies investigated the spread of *Heterobasidion* from infested *P. abies* stands to trees of the next forest generation (Piri 1996; Piri and Korhonen 2001) in which efficient transfer of the disease from the previous forest generation to subsequently established 10- to 53-year-old trees was observed. In that study, maximal length of territorial clones in each study varied between approximately 10 and 30 m and *H. parviporum* was isolated from over 98% of infected trees; the rest were *H. annosum*.

Two studies on *H. annosum* investigated its interspecific transfer from previously grown tree species to another species of the subsequent forest generation. Vasiliauskas and Stenlid (1998) reported that 43.7% of all *Heterobasidion* isolates (38) obtained in a *P. abies* stand self-established on a clearcut of *P. sylvestris* belonged to *H. annosum*, while the others belonged to *H. parviporum*. Lygis et al. (2004) provided evidence for the transfer of *H. annosum* from an infested and clearcut *P. sylvestris* to the next generation of planted *Betula pendula* Roth. In the first study, maximal diameter of a territorial clone of *H. annosum* was up to 20 m, while in the second, its maximal diameter was 48 m.

5. Conclusions

In this study, we examined 444 *Heterobasidion*-infected *P. contorta* trees in three Latvian *P. contorta* plantations subjected to different management regimes. For the first time, we detected 51 territorial clones of the pathogen, demonstrated secondary tree-to-tree infections by *H. annosum*, traced the infections to a previous generation of *P. sylvestris*, and reported observations of 40 disease centres initiated by primary airborne infections of both *H. annosum* and *H. parviporum*. This study generated new data on the ecology and patterns of spread of *Heterobasidion* spp. in *P. contorta* plantations. From this body of novel data, we concluded the following: (*i*) *Pinus contorta* is susceptible to root rot and dieback caused by *H. annosum* and *H. parviporum*; (*ii*) airborne basidiospores of both pathogens colonise cut stumps of *P. contorta*, establishing primary infections; (*iii*) following establishment in stumps and root systems through primary infections, secondary spread to adjacent trees results via root contacts; (*iv*) *H. annosum* from diseased stumps of a previous generation of *P. sylvestris* can transfer to planted next-generation *P. contorta*; (*v*) *H. annosum* can persist and remain viable in stumps of cut mature *P. sylvestris* for at least 26 years; (*vi*) infections by both *H. annosum* and *H. parviporum* can produce large (up to 263 m^2) territorial clones causing extensive tree dieback and mortality; (*vii*) development of territorial clones of *H. parviporum* proceeds at a slower rate than that of *H. annosum*; and (*viii*) when planning thinning of *P. contorta* plantations established on uninfested areas (e.g., agricultural land), stump treatment with agents that prevent primary infection by *Heterobasidion* (particularly urea) should be considered, as was also more recently pointed out by Gonthier (2019).

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3.4. Protection of stumps against *Heterobasidion* basidiospore infection (Paper VII, VIII)

The potential of local *P. gigantea* populations to restrict *Heterobasidion* basidiospore infection in conifer stands in Latvia was evaluated (**Paper VII**). The efficacy of native Latvian *P. gigantea* isolates, the biological control Rotstop® agent and urea treatments against *Heterobasidion* spp. basidiospore infection in *P. abies* stands was compared (**Paper VIII**).

3.4.1. The potential of natural colonization by *P. gigantea* to restrict the *Heterobasidion* primary infection (Paper VII)

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Natural infection and colonization of pre-commercially cut stumps of *Picea abies* and *Pinus sylvestris* by *Heterobasidion* rot and its biocontrol fungus *Phlebiopsis gigantea*

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Scots pine

A B S T R A C T

Fungi from the genus *Heterobasidion* are among the most important pathogens of forest trees in Northern Hemisphere causing root rot and wood decay, while *Phlebiopsis gigantea* is a very common saprotrophic wood decay fungus. Both fungi are primary colonizers of freshly cut conifer stumps (through which *Heterobasidion* spp. accomplishes primary infections of tree root systems), thus both fungi are competing for the substrate. To date, *P. gigantea* is widely used as *Heterobasidion* spp. biocontrol agent. Hypothesis has been proposed that natural colonization of stumps by *P. gigantea* might also to some extent restrict infections by the pathogen. The main aim of the study was to assess the potential of natural infections of *P. gigantea* to restrict infection and spread of *Heterobasidion* spp. in *Picea abies* and *Pinus sylvestris* stumps. In total, 793 *P. abies* stumps and 1158 *P. sylvestris* stumps were examined in 24 sample plots located in the eastern part of Latvia. Of these, 325 (41.0%) *P. abies* stumps were infected by *Heterobasidion* spp., and 59 (7.4%) by *P. gigantea*, and 168 (14.5%) *P. sylvestris* stumps were infected by *Heterobasidion* spp., and 846 (73.1%) by *P. gigantea*. In *P. abies*, the observed *Heterobasidion* spp. infection frequencies were significantly ($p < 0.05$) higher than those of *P. gigantea*, while the respective situation in *P. sylvestris* was reverse and *P. gigantea* infections were more frequent ($p < 0.05$). The mean surface area colonized by *Heterobasidion* spp. in *P. abies* and *P. sylvestris* stumps was 5.7 and 5.3 cm^2 and did not differ significantly (mean coverage of stump surface area respectively 18% and 13%; $p = 0.41$). In contrast, the mean surface area colonized by *P. gigantea* was significantly different in the two tree species, respectively, 3.9 and 21.3 cm^2 (16% and 59%; $p < 0.05$). The mean surface area colonized by *Heterobasidion* spp. in *P. abies* stumps was significantly larger ($p < 0.05$) than the area colonized by *P. gigantea*, while conversely, the mean area colonized by *P. gigantea* in *P. sylvestris* stumps was significantly larger ($p < 0.001$) than that colonized by *Heterobasidion* spp. Both fungi were co-occurring in *P. abies* stumps in 33 cases (4.2% of all investigated stumps), and in *P. sylvestris* stumps in 138 cases (11.9%). There were no correlations between the sizes of colonized areas of *Heterobasidion* spp. and *P. gigantea* in *P. abies* stumps ($r = 0.06$; $p = 0.76$), or *P. sylvestris* stumps ($r = 0.009$; $p = 0.27$). In conclusion, the results of this study strongly suggest that even in stumps of *P. sylvestris*, that otherwise are much preferred for natural colonization by airborne spores of the biocontrol agent *P. gigantea*, natural colonization by *P. gigantea* is not able to restrict infections by *Heterobasidion* spp. This clearly indicates that for effective biocontrol of *Heterobasidion* spp. infections, the necessity for thorough treatment coverage of cut *P. abies* and *P. sylvestris* stumps at early stages of plantation management, during pre-commercial thinning.

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1. Introduction

Fungi from the genus *Heterobasidion* represent the most important pathogens of forest trees in the Northern Hemisphere where, by the end of the 20th century, annual losses caused by disease to forest industry constituted up to 800,000 million euro per year, and are bound to increase in the next decades and centuries (Woodward et al., 1998). In Northern Europe, two species from this genus cause the most damage: *Heterobasidion parviporum* Niemelä & Korhonen and *H. annosum* (Fr.) Bref. *Heterobasidion parviporum* preferably infects *Picea abies* (L.) H. Karst. (Norway spruce), while *H. annosum*, infects *P. sylvestris* L. (Scots pine), but also *P. abies* (Niemelä and Korhonen, 1998). In *P. abies*, the disease results in extensive heart-rot of stems (reaching up to 10 m in length) while the sapwood remains mainly intact, thus the trees generally remain non-symptomatic before harvesting (Vasiliauskas and Stenlid, 1998). By contrast, in roots and stems of *P. sylvestris*, sapwood is attacked, leading to tree mortality already a few years after infection, resulting in disease centers containing dead trees (Lygis et al., 2004).

Primary infections of the pathogens are accomplished by basidiospores released from perennial sporocarps formed on previously colonized woody substrates: logs, trunks, snags, stumps, infected trees (Redfern and Stenlid, 1998). The basidiospores, although mostly dispersed in the vicinity of a sporocarp, can be spread by air over hundreds of kilometers (Rishbeth, 1959). After landing on surfaces of freshly cut conifer stumps (in north Europe predominantly *P. abies* and *P. sylvestris*), spores germinate, mycelia grow over the stump surface, penetrate down into the inner part of stumps, and finally colonize lateral roots (Rishbeth 1951, 1957). If trees are cut at temperatures below + 5 °C, then the risk for primary (airborne spore) infections are minimal, but the risk increases significantly if cutting is done during the vegetation period, from April to October (Brandtberg et al., 1996; Lindén and Vollbrecht, 2002).

Secondary infections occur when mycelia of the pathogens grow into root systems of adjacent trees via root contacts. Consequently, since root contacts are a characteristic feature of forest stands, the pathogens can spread indefinitely, which leads to the formation of long lasting disease centers (Stenlid and Redfern, 1998). For example, it has been demonstrated that the pathogen is able to persist and remain active in a *P. sylvestris* stump for over 60 years (Greig and Pratt, 1976). Moreover, *Heterobasidion* spp. are effective invaders of roots of trees replanted on infected forest clear-cuts, leading to transfer of the disease to new forest generations (Stenlid and Redfern, 1998; Vasiliauskas and Stenlid, 1998; Lygis et al., 2004; Zaluma et al., 2019). As a result, established disease centers in forest areas with an increasing number of infected root systems possess the capacity for indefinite longevity.

Phlebiopsis gigantea (Fr.) Jülich is also a white-rot basidiomycete fungus, and also a primary colonizer of freshly cut stumps of *P. abies* and *P. sylvestris* (Vasiliauskas et al. 2005b, and references therein). However, it is a saprotroph unable to spread into and colonize root systems, and cause active decay in living trees. However, it has been demonstrated that following colonization of a stump surface, *P. gigantea* effectively competes with *Heterobasidion* spp. for space and nutrients (Rishbeth, 1959, 1963; Sun et al., 2009). Consequently, the hypothesis has been proposed that application of *P. gigantea* spores to cut surfaces could have the potential to restrict (or exclude) the ability for the *Heterobasidion* spp. pathogens to enter root systems of stumps, consequently reducing (or eliminating) their ability to accomplish secondary infections to adjacent trees. In a pioneering study, artificial application of a mycelial suspension of *P. gigantea* to freshly cut *P. sylvestris* stumps was proven to have a restrictive effect on their colonization by *Heterobasidion* spp. (Rishbeth, 1963). Consequently, a protective preparation of *P. gigantea* spores has been developed on a commercial scale (Korhonen et al., 1994). Stump treatment with *P. gigantea* spore suspension is regularly used and believed to be economically beneficial on a variety of tree species (but mainly on *P. abies* and *P. sylvestris*) in many European countries (Holdenrieder and Greig, 1998; Oliva et al., 2017, and references therein).

Several authors have hypothesized that natural deposition of airborne *P. gigantea* spores might also have potential to restrict airborne infections by *Heterobasidion* spp. (Rishbeth, 1952, 1963; Meredith, 1960; Greig, 1976; Negrutsky, 1986; Holdenrieder and Greig, 1998). However, detailed systematic studies in this respect are scarce. One example is a previous study in Latvia, where both *P. gigantea* and *Heterobasidion* spp. co-occurred in 37% of *P. abies* stumps (out of 174 analyzed) and 12% of *P. sylvestris* stumps (out of 134). Detailed analysis of these stumps showed that relative wood area colonized by *Heterobasidion* spp. both in *P. abies* and *P. sylvestris* was significantly smaller in the presence of *P. gigantea* ($p = 0.001$) (Kenigsvalde et al., 2016). Those observations indicate that simultaneous co-occurrence of the pathogen and its biocontrol agent within a single conifer stump is not uncommon, and that the possibility of their interactions in this niche cannot be excluded. Therefore, it would be of interest to assess in more detail simultaneous infection frequencies and extent of stump surface colonization by both the pathogen and its biocontrol agent resulting from natural "spore rain".

The aims of the present study were: i) on a large scale, to investigate the frequency of natural colonization of rot fungus *Heterobasidion* spp. and its biocontrol agent *P. gigantea* on pre-commercially cut stumps of *P. abies* and *P. sylvestris*; ii) to determine the extent of stump surface colonization by their mycelia; iii) to compare whether, and to what extent these processes differ between the pathogen and its biocontrol fungus, and between the two tree species; iv) to assess the potential of natural infections of *P. gigantea* to restrict infection and spread of *Heterobasidion* spp. in *P. abies* and *P. sylvestris* stumps.

2. Materials and methods

2.1. Field work

Twenty-four sample plots were established in stands of *P. abies* (9 plots) and *P. sylvestris* (15), planted on former forest land, and not showing dieback symptoms. The previous forest generation consisted of *P. sylvestris* and/or *Betula pendula* Roth. Age of the plantations was 11–39 years (Table 1), and were located in the eastern part of Latvia (52°42′N/25°50′E) within a radius of approx. 5 km, and at approx. 120 m altitude. In the sample plots, a total of 793 *P. abies* (diameter 2–12 cm) and 1158 *P. sylvestris* (diameter 2–12 cm) trees were cut using a chainsaw during June–August in years 2012 and 2013, making ca. 50 cm high stumps. Each stump was numbered. None of them showed symptoms of discoloration or decay. Numbers and frequencies of stumps colonized by each of the fungi were recorded in each plot. Characteristics of the sample plots, trees and stumps are presented in Table 1, and respective means for each tree species in Table 2.

In each plot, stumps were sampled once after 15–56 weeks (Table 1). Two 2–3 cm thick disks were cut from each stump. The first (top) disc was discarded and the second disc was taken to the laboratory for further analyses.

2.2. *Heterobasidion* spp. infection assessment and identification, isolation of *P. gigantea*

According to methods described by Gaitnieks et al. (2018), the discs were incubated for 5–7 days at room temperature. Afterwards, a transparent grid (consisting of 0.5 cm^2 squares) was attached to the lower surface of the disc (i.e. 4–6 cm below the original stump surface). Using a dissection microscope, the presence of *Heterobasidion* spp. was estimated by observing its characteristic asexual sporulation (conidiophores) (Gaitnieks et al., 2018), and the presence of *P. gigantea* by orange brown coloring in the wood and morphological characteristics of mycelium (Oliva et al., 2015, 2017; Gaitnieks et al., 2018). Specifically, for *P. gigantea*, mycelial features included crystal-crusted lamprocystidia (Eriksson et al., 1981; Breitenbach and Kränzlin, 1986).

Table 1
Description of the sample plots, stumps and infection frequencies by *Heterobasidion* spp. and *Phlebiopsis gigantea*.

Sample plot*	Tree species	Age of cut trees (years)	Age of stumps (weeks)	Number of analyzed stumps	Number of infected stumps/infection frequency (%)	
					Heterobasidion spp.	*P. gigantea*
1	*Picea abies*	24	44	40	19/47.5	14/35.0
2A	*P. abies*	30	20	56	20/35.7	0
2A	*P. abies*	31	36	121	63/52.07	6/5.0
3	*P. abies*	39	20	95	14/14.74	0
4	*P. abies*	33	20	90	65/72.2	3/3.3
5	*P. abies*	26	27	103	60/58.3	16/15.5
6	*P. abies*	17	48	96	10/10.4	19/19.8
7	*P. abies*	34	33	45	33/73.3	1/2.2
8A	*P. abies*	24	35	147	41/27.9	0
8A	*Pinus sylvestris*	24	35	49	4/8.2	39/79.6
9	*P. sylvestris*	15	18	84	4/4.8	80/95.2
10	*P. sylvestris*	11	44	90	0	73/81.1
11A	*P. sylvestris*	15	56	71	0	71/100
11A	*P. sylvestris*	20	39	88	14/15.9	72/81.8
12	*P. sylvestris*	19	18	68	1/1.5	64/94.1
13	*P. sylvestris*	27	46	96	0	66/68.8
14	*P. sylvestris*	19	44	79	0	58/73.4
15A	*P. sylvestris*	14	15	78	20/25.6	13/16.7
15A	*P. sylvestris*	15	39	104	21/20.2	65/62.5
16	*P. sylvestris*	14	15	55	4/7.3	4/7.3
17	*P. sylvestris*	28	39	45	0	38/84.4
18	*P. sylvestris*	16	38	75	36/48.0	64/85.3
19	*P. sylvestris*	16	35	95	60/63.2	69/72.6
20	*P. sylvestris*	15	40	81	4/4.9	70/86.4

* The same number and letter represent two sample plots in the same forest stand, sampled twice.

From 38 stumps symptomatic for *P. gigantea*, pure culture isolations were attempted by cutting pieces of wood from the colonized surfaces, flame sterilizing, and placing samples on Petri dishes containing agar medium (Vasiliauskas et al., 2004, 2005a, b). Of these 38 stumps, *P. gigantea* was isolated from 31 stumps (81.6%), while the rest of samples were contaminated by fast-growing moulds (mainly *Mucor* spp., *Mortadiella* spp., *Penicilium* spp.).

In each *Heterobasidion* and/or *P. gigantea* infected stump the total number of marked grid squares containing *Heterobasidion* spp. were counted and the area of *Heterobasidion* spp. infection calculated. The area colonized by *P. gigantea* was redrawn on a transparent sheet and measured using a planimeter (PLANIX 10S "Marble", Tamaya, Japan). For each colonized stump, the relative area occupied by each investigated fungus was calculated by dividing their occupied areas by the total area of the disc (Kenigsvalde et al., 2016).

2.3. Statistical analyses

Differences of means between tree species in stand and stump characteristics were compared using *t*-tests. The difference between mean age of the investigated *P. abies* and *P. sylvestris* stands was significant, the differences in mean diameter of analyzed stumps and mean age of the stumps were non-significant (Table 2). This, taking into account that sample plots of this study were located within a radius of ca. 5 km, provides an opportunity for comparative analysis of both frequencies of infection and the extent of surface colonization observed in spruce and pine stumps. The mean stump surface areas colonized by *Heterobasidion* spp. and *P. gigantea* in both tree species was compared by non-parametric Mann–Whitney *U* test. Analyses of data were performed in RStudio (R Core Team, 2015). *P. gigantea* and *Heterobasidion* spp. infection frequencies (number of colonized stumps) between tree species were compared using a chi-square test. Strength of the association between *P. gigantea* and *Heterobasidion* occurrence in *P. abies* and *P. sylvestris* stumps was calculated using odds ratio (OR). A generalized linear model (GLM) was used to analyze the impact of tree age, stump age on the presence of *Heterobasidion* spp. and *P. gigantea* in stumps, and on stump surface area colonized by fungi. Spearman's rank correlation between infection frequency, area colonized by *Heterobasidion* spp. and *P. gigantea* and stump diameter were calculated.

3. Results

Infection frequencies by *Heterobasidion* spp. and *P. gigantea* varied within a broad range among the sample plots of both tree species

Table 2
Mean characteristics of investigated stands, stumps, infection frequencies and stump surface areas colonized by *Heterobasidion* spp. and *Phlebiopsis gigantea* in *Picea abies* and *Pinus sylvestris*.

	P. abies	*P. sylvestris*	p-value at $\alpha = 0.05$
Stand age, years (mean ± SD)	28.7 ± 6.6	17.9 ± 5.0	$p = 0.001$
Stump age at sampling, weeks (mean ± SD)	31.4 ± 10.5	34.7 ± 12.5	$p = 0.55$
No. of investigated stumps	793	1158	–
Stump diameter, cm (mean ± SD)	5.7 ± 2.5	6.0 ± 2.0	$p > 0.05$
Infected stumps by *Heterobasidion* spp./*P. gigantea* (no.)	325/59	168/846	$p < 0.05$
Infection frequency of *Heterobasidion* spp./*P. gigantea* (%)	41.0/7.4	14.5/73.1	$p < 0.05$
Stump surface colonized by *Heterobasidion* spp., cm^2 (mean ± SD)	5.7 ± 6.2*	5.3 ± 5.5**	$p = 0.41$
Stump surface colonized by *P. gigantea*, cm^2 (mean ± SD)*	3.9 ± 7.3*	21.3 ± 17.6**	$p < 0.05$

* Difference between means in row significant at $p < 0.05$.
** Difference between means in row significant at $p < 0.001$.

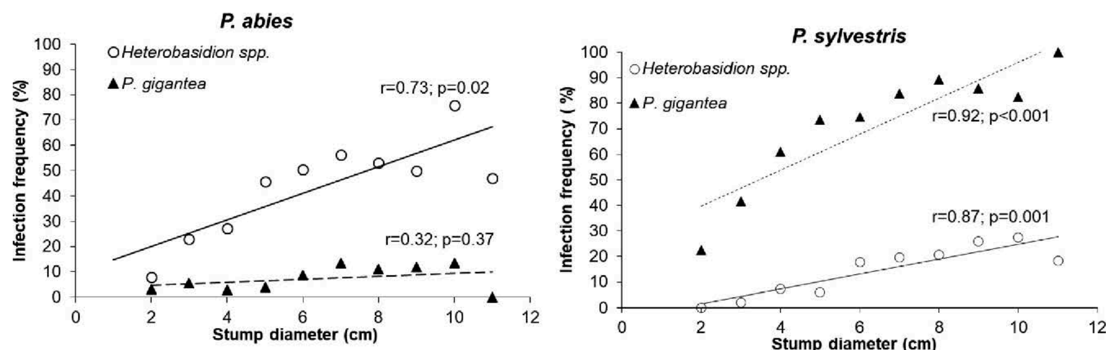

Fig. 1. Infection frequency of *Heterobasidion* spp. and *P. gigantea* in relation to stump diameter. Solid line – mean infection frequency by *Heterobasidion* spp., dashed line – mean infection frequency by *P. gigantea*. Each symbol (data-point) represents mean value derived from 17 to 127 *P. abies*, and 11 to 225 *P. sylvestris* stumps.

(Table 1). In *P. abies* plots, 10–73% stumps were infected by *Heterobasidion* spp. (mean 41%), and 0–35% by *P. gigantea* (mean 7%), while in *P. sylvestris* plots corresponding figures were 0–63% (mean 15%) for *Heterobasidion* spp., and 7–100% (mean 73%) for *P. gigantea* (Table 1). In summary, of all 793 investigated *P. abies* stumps, 325 (41.0%) were infected by *Heterobasidion* spp., and 59 (7.4%) by *P. gigantea*. Of all 1158 investigated *P. sylvestris* stumps, 168 (14.5%) were infected by *Heterobasidion* spp. and 846 (73.1%) by *P. gigantea* (Table 2).

In *P. abies* stumps, infection frequencies by *Heterobasidion* spp. were significantly higher ($p < 0.05$) than those of *P. gigantea*, while in *P. sylvestris* infection frequencies of *P. gigantea* were significantly higher ($p < 0.05$) (Fig. 1 and Table 2). Grouping the infected stumps according to diameter classes demonstrated that infection frequency of *Heterobasidion* spp. increased significantly with increasing stump diameter both in *P. abies* stumps ($r = 0.73$; $p = 0.02$) and in *P. sylvestris* stumps ($r = 0.87$; $p = 0.001$). In this respect, infection frequency of *P. gigantea* also showed a similar trend, but the increase was significant only in *P. sylvestris* stumps ($r = 0.92$; $p < 0.001$), while it was insignificant in *P. abies* ($r = 0.32$; $p = 0.37$) (Fig. 1).

The extent of surface colonization was subsequently investigated for each infected stump. Among individual stumps of both tree species, the proportion of colonized surface area varied within a broad range. In *P. abies*, it varied between 1 and 77% (mean 16%) for *Heterobasidion* spp., and 1–44% (mean 6%) for *P. gigantea*. Respective values for *P. sylvestris* were 1–76% (mean 9%) and 1–100% (mean 52%) (Table 2). Mean surface area colonized by *Heterobasidion* spp. in *P. abies* and *P. sylvestris* stumps was 5.7 and 5.3 cm^2 and did not differ significantly (Table 2). By contrast, for *P. gigantea* those values differed significantly between the two tree species, 3.9 and 21.3 cm^2, respectively (Table 2). When the extent of colonization was compared between the two fungi, in *P. abies* stumps *Heterobasidion* spp. colonized a larger area than *P. gigantea* (5.7 vs. 3.9 cm^2; $p < 0.05$), while, the mean area colonized by *P. gigantea* in *P. sylvestris* stumps was significantly larger than that colonized by *Heterobasidion* spp. (21.3 vs 5.3. cm^2; $p < 0.001$) (Table 2).

The surface area colonized by *Heterobasidion* spp. increased with increasing stump diameter both in *P. abies* stumps ($r = 0.82$; $p = 0.004$) and in *P. sylvestris* stumps ($r = 0.69$; $p = 0.03$). In this respect, the area colonized by *P. gigantea* also showed similar trends, both in *P. abies* ($r = 0.47$; $p = 0.20$) and in *P. sylvestris* ($r = 0.96$; $p < 0.001$), although in case of *P. abies* the correlation was non-significant (Fig. 2).

In *P. abies* stumps, the surface area colonized by *Heterobasidion* spp. correlated positively with tree age ($p = 0.02$) and stump age ($p < 0.001$), but there were no significant correlations for the area colonized by *P. gigantea* either with tree age ($p = 0.41$) or with stump age ($p = 0.15$). In *P. sylvestris* stumps, there were no significant correlations for *Heterobasidion* spp. either with tree age ($p = 0.48$) or with stump age ($p = 0.43$), while the area colonized by *P. gigantea* correlated positively with tree age ($p < 0.001$), but there was no correlation with stump age ($p = 0.27$).

Both fungi were found in the same *P. abies* stump in 33 cases (4.2% of all investigated stumps), and in the same *P. sylvestris* stump in 138 cases (11.9% of all stumps). In *P. sylvestris* stumps, *Heterobasidion* spp. and *P. gigantea* co-occurred ($\chi 2 = 7.71$; $p < 0.01$). A similar association was observed in *P. abies* stumps ($\chi 2 = 5.24$; $p < 0.05$). An odds ratio (OR) shows, that the strength of the association of *P. gigantea* and *Heterobasidion* spp. in *P. abies* stumps is higher than in *P. sylvestris* stumps: 1.921 and 0.327, respectively.

There were no correlations between the sizes of colonized areas of *Heterobasidion* spp. and *P. gigantea* either in *P. abies* ($r = 0.06$; $p = 0.76$), or *P. sylvestris* ($r = 0.009$; $p = 0.27$) stumps. In five *P. sylvestris* stumps, the extent of surface colonization by *P. gigantea* was high, comprising approx. 78–97% of the total stump surface area (Fig. 3 and Table 3). Yet, in none of these cases, natural colonization (accomplished exclusively by airborne spores of *P. gigantea*) had prevented infections by *Heterobasidion* spp., and the presence of the pathogen was detected in each of these stumps.

4. Discussion

The results of this study explicitly demonstrate the strong preference of the pathogen to colonize of *P. abies* stumps, while, in contrast, the strong preference by the saprotroph to colonize *P. sylvestris* stumps. A similar trend has also been reported by Kenigsvalde et al. (2016). Frequencies of infection of both fungi in the present study correlated positively with the diameter of investigated stumps of both tree species. Regarding *Heterobasidion* spp., this is in a good agreement with the results obtained in our previous study, carried out in stands with similar characteristics, where both the infection frequency and stump area colonized by the pathogen were significantly larger in *P. abies* stumps than *P. sylvestris* stumps: 25 vs. 10%, and 7.5 vs. 4.1 cm^2, respectively (Gaitnieks et al., 2018). In addition, results of other studies in have shown, that under natural conditions, *P. abies* stumps are more often infected by *Heterobasidion* spp. than by *P. gigantea* (Vasiliauskas et al., 2004, 2005a; Gaitnieks et al., 2019).

Both *Heterobasidion* spp. and *P. gigantea* are primary colonizers of coniferous stumps and occupy similar ecological niches (Rishbeth, 1959, Meredith, 1960). This is confirmed by our results, indicating that *P. abies* stumps were co-colonized by *P. gigantea* and *Heterobasidion* spp. This was also the case with *P. sylvestris* stumps although to a lesser degree. Regarding *P. gigantea*, some previous studies have noted its predominant natural infections of cut stumps of pine (Meredith, 1960; Jokinen, 1984; Lipponen, 1991). For example, Jokinen (1984) examined 1375 stumps of *P. sylvestris* and found that 68.4% of them were

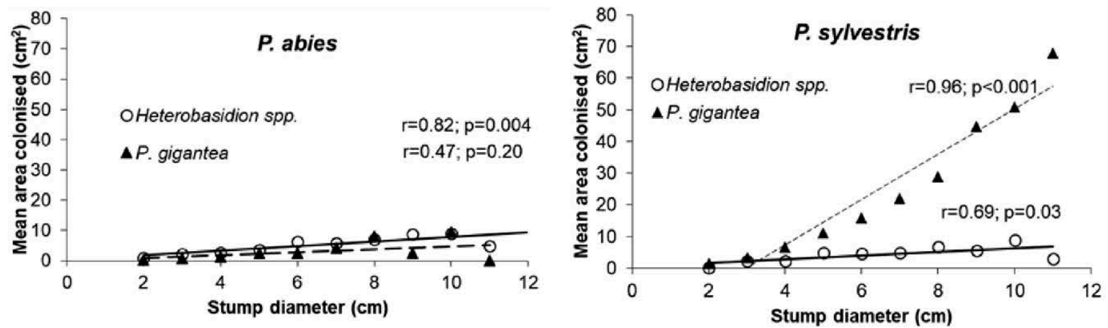

Fig. 2. Surface areas of stumps colonized by *Heterobasidion* spp. and *P. gigantea* in relation to stump diameter. Solid line – mean infection frequency by *Heterobasidion* spp., dashed line – mean infection frequency by *P. gigantea*. Each symbol (data-point) represents mean value derived from 2 to 54 *P. abies*, and 2 to 170 *P. sylvestris* stumps.

colonized by *P. gigantea*, while only 3.4% by *Heterobasidion* spp. Lipponen (1991) reported that in two *P. sylvestris* stands *Heterobasidion* spp. infection was related to occurrence of *P. gigantea.* In the present study, stump surface area colonized by *P. gigantea*, but also *Heterobasidion* spp., increased with increasing stump diameter, both in *P. abies* and *P. sylvestris.* This correlation was particularly pronounced for *P. gigantea* in *P. sylvestris* (Fig. 2), demonstrating the pronounced adaptiveness of this fungus to colonize freshly cut wood of *P. sylvestris* Effective spread of *P. gigantea* on freshly cut wood of pine has been noted also in the study by Korhonen et al. (1994).

In the present study, the surface areas colonized by *Heterobasidion* spp. and by *P. gigantea* in stumps where both fungi were observed, were not correlated, in either *P. abies*, or *P. sylvestris* (Fig. 3). This to some extent contradicts the results of our previous work where a significant negative correlation was established between the extent of stump surface colonization by *Heterobasidion* spp. and *P. gigantea* (Kenigsvalde et al., 2016). However, in the previous study, larger diameter stumps were examined (14–26 cm), which might have influenced the result. Predominant natural colonization of several *P. sylvestris* stumps by *P. gigantea* did not hinder infections by *Heterobasidion* spp. Yet notably, these five stumps with the largest surface colonization area by *P. gigantea*, originated exclusively from a single sample plot, which might indicate the importance of a local spore source of this biocontrol agent for its predominant colonization of suitable woody substrate. Production of *Heterobasidion* and *P. gigantea* spores depends not only on abundance of sporocarps, but also on weather conditions (Rishbeth, 1959, 1963). Spore production of *P. gigantea* decreasing significantly during dry periods (Rishbeth, 1959).

Results of the present study strongly suggest that even in stumps of *P. sylvestris* that are much preferred for natural colonization by airborne spores of *P. gigantea*, natural colonization by *P. gigantea* is not able to restrict infections by *Heterobasidion* spp.

A similar conclusion was made in a previous extensive study (Jokinen 1984). Moreover, our recent studies in *Pinus contorta* Dougl. ex Loudon plantations has demonstrated high susceptibility of its stumps to primary infections both by *H. annosum* and *H. parviporum* (Zaluma et al., 2019). Tubby et al. (2008) reported that complete stump treatment coverage using the biological control product *Phlebiopsis gigantea* PG Suspension is required to protect stumps of Corsican pine (*Pinus nigra* Arn. ssp. *laricio*) from infections by *H. annosum*, and that decreasing levels of coverage allow increasing areas of the stump surface to be colonized by the pathogen. Similar results have been obtained in Sweden using mechanically treated *P. abies* stumps (Rönnberg et al., 2006). Furthermore, in controlled inoculations of *P. abies* stumps by *P. gigantea*, quantitative interactions between the pathogen and biocontrol agent largely depended on the extent of surface coverage by *P. gigantea* (Rotstop S – commercial *P. gigantea* strain from Sweden). In well covered stumps, Rotstop S application decreased *Heterobasidion* spp. biomass by almost 200 times, while the situation when poor coverage was applied, resulted in a 2-fold biomass increase of surviving *Heterobasidion* spp. colonies (Oliva et al., 2017).

Study in Finland has shown that *Heterobasidion parviporum* can

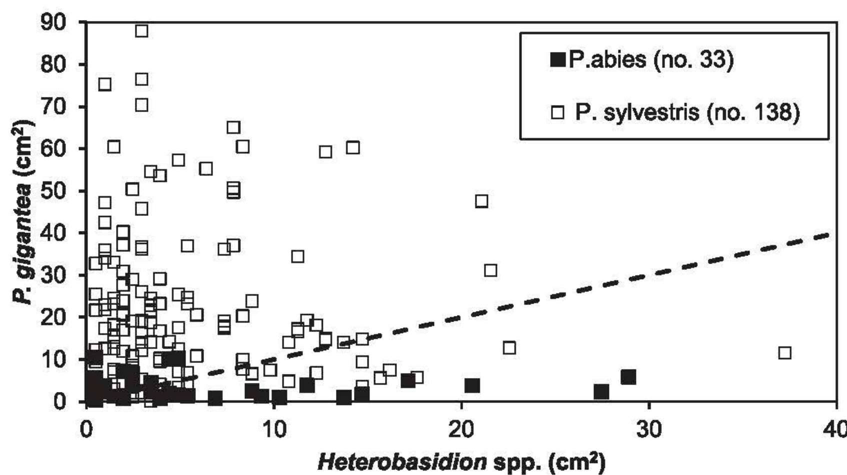

Fig. 3. Surface areas colonized by *Heterobasidion* spp. vs. *Phlebiopsis gigantea* on *Picea abies* and *Pinus sylvestris* stumps where both fungi were detected. Each symbol (data-point) represents a single stump. Occurrence on the diagonal line would demonstrate that colony sizes of *Heterobasidion* and *P. gigantea* in a particular stump are of (almost) equal size. Location of a symbol above diagonal line indicates predominant colonisation of a given stump by *P. gigantea*, while below, by *Heterobasidion*.

Table 3
Extent of colonization of five stumps of *Pinus sylvestris* by *Heterobasidion* spp. and *Phlebiopsis gigantea* on which maximal colonization rates by *P. gigantea* were observed (in Fig. 3, five symbols at the top left corner). All five were located within the same sample plot No. 18 (Table 1).

Stump parameters		Surface area colonized (cm^2)		Coverage of surface area (%)	
Diameter (cm)	Surface area (cm^2)	*Heterobasidion* spp.	*P. gigantea*	*Heterobasidion* spp.	*P. gigantea*
10.5	88.2	2.9	85.3	3.3	96.7
10.0	76.9	2.9	70.4	3.8	91.5
10.5	84.9	1.0	75.3	1.2	88.6
10.5	87.4	2.9	76.5	3.4	87.5
10.5	83.3	7.8	65.1	9.4	78.1

remain viable in infected relatively thin *P. abies* roots (1.5 cm in diameter) for up to 74 months (Piri and Hamberg, 2015). This indicates that even small stumps can accumulate and harbor infection potential of the pathogen during prolonged periods. Our study demonstrated common occurrence of *Heterobasidion* spp. in relatively small (2–12 cm diameter) stumps during 20–48 (spruce) and 15–56 (pine) weeks since cutting (Table 1), thus long-term studies on persistence of the pathogens in the root systems of such small-sized stumps would be of interest. In conclusion, this study demonstrated that pre-commercially cut stumps of *P. abies* and *P. sylvestris* are prone to colonization by the pathogen, and that natural "spore rain" of its biocontrol species cannot efficiently restrict *Heterobasidion* basidiospore infections. This clearly indicates the necessity for thorough treatment coverage of cut *P. abies* and *P. sylvestris* stumps at early stages of plantations management, during their pre-commercial thinning.

CRediT authorship contribution statement

Tālis Gaitnieks: Conceptualization, Funding acquisition, Project administration, Methodology, Writing - original draft, Supervision. **Astra Zaļuma:** Investigation, Data curation, Project administration, Writing - original draft, Writing - review & editing. **Kristīne Kenigsvalde:** Investigation, Data curation, Visualization, Writing - original draft. **Lauma Brūna:** Investigation. **Dārta Kļaviņa:** Investigation. **Natālija Burņeviča:** Investigation. **Jan Stenlid:** Writing - original draft. **Libor Jankovský:** Writing - original draft. **Rimvydas Vasaitis:** Conceptualization, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2020.104208.

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3.4.2. Efficacy of Rotstop® *versus* treatments with the native *P. gigantea* strains and urea against *Heterobasidion* basidiospore infection (Paper VIII)

Article

Control of Heterobasidion in Norway Spruce Stands: The Impact of Stump Cover on Efficacy of Urea and *Phlebiopsis gigantea* and Implications for Forest Management

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Abstract: This study investigated the efficacy of Rotstop®, a native Latvian *Phlebiopsis gigantea* strain and 35% urea solution in combination with a stump cover treatment to control against natural spore infection by *Heterobasidion* spp. upon precommercial thinning of Norway spruce in three stands growing on former agricultural lands. The major findings were that (i) infection rates of *Heterobasidion* spp. on stumps treated with the native *P. gigantea* strain, Rotstop® or urea are similar when stumps are uncovered, and (ii) stump cover promotes stump colonization by the Latvian *P. gigantea* strain and Rotstop®, leading to a significantly smaller relative area colonized by *Heterobasidion* spp., as well greater efficiency against *Heterobasidion* in comparison with urea. Covering of stumps appears beneficial for controlling Heterobasidion stump colonization and may be valuable to forest owners if used in small-scale operations, but it is impractical in automatized thinnings, where managers should consider using regular Rotstop® without covering the stumps.

Keywords: biological control; Rotstop®; urea treatment; root rot; basidiospores; agricultural land

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1. Introduction

Heterobasidion annosum sensu lato (Fr.) Bref. is a species complex of necrotrophic, root and white rot pathogens of conifers, comprising five species distributed in the Northern Hemisphere [1]. Three of the species are native to Europe: (i) *Heterobasidion annosum* sensu strictum (Fr.) Bref., primarily a pathogen of Scots pine (*Pinus sylvestris* L.) but also other pines and conifers; (ii) *Heterobasidion parviporum* Niemelä & Korhonen, a pathogen of Norway spruce (*Picea abies* (L.) Karst.); and (iii) *Heterobasidion abietinum* Niemelä & Korhonen, largely a pathogen of silver fir (*Abies alba* Mill.) and other *Abies* species. *Heterobasidion irregulare* (Underw.) Garbel. & Otorsina is native to North America; however, it was introduced into Europe in the 1940s and became invasive by spreading in *Pinus pinea* L. and *Querqus* spp. stands [2]. In intensively managed forests and plantations, *Heterobasidion* spp. is a major threat to timber production, owing to growth reduction and increased tree mortality, with financial losses estimated as more than 790 million euros per year in Europe alone. Moreover, these calculations do not include wind and storm damage in decay-affected stands, damage that may be or may become (due to climate change) extremely significant [1 and references therein]. Disease development is largely dependent on forest management practices [3–5]. Primary infection of the fungus occurs by airborne spores infecting newly exposed wood surfaces [3,6]. Secondary infection from *Heterobasidion* spp.

infected stumps and trees to healthy trees may occur belowground through interconnected root systems [3,6–9]. Stumps also serve as the main structure for developing fruiting bodies [10]. In spruce stands of Latvia, approximately 23% of cut trees are colonized by rot-causing fungi, most often *H. parviporum* [11].

While practically impossible to eradicate once established in a stand, the disease can be managed in healthy stands by certain preventative measures that limit primary infections. Stump and root removal of infected and neighboring tree material is an effective method, but it is expensive, requires specialized machinery and is hence rarely used in practice [12]. Harvesting and thinning during nonsporulation times greatly reduces the risk of new infections and should be done when possible. When logging during periods of sporulation, stump surfaces should be treated with a chemical or biological control agent (BCA) [13]. There are a few chemicals shown to be effective at reducing *Heterobasidion* colonization, the most important being urea. In efforts to reduce the use of chemicals in forestry, many countries in Europe have opted for the BCA Rotstop®. Rotstop® is a commercial formulation containing spores of the fungus *Phlebiopsis gigantea* (Fr.) Jülich, which is a naturally occurring saprotrophic fungus that effectively outcompetes *Heterobasidion* spp. for nutrients in the woody stumps. Different versions of Rotstop have been formulated based on local strains of *P. gigantea*, such as the PG suspension in the UK and PG IBL in Poland. BCA has been further developed to be compatible with the increased mechanization and use of harvesting machines and can now be applied directly to cut stumps at the time of felling through specialized sawblades [14–20]. BCA containing various strains of *P. gigantea* show higher efficacy in pine stumps in comparison to spruce stumps [21–23]. Urea is a chemical alternative to BCA [16,20,24,25] and registered for use in Finland, United Kingdom, Denmark, France, Ireland [2,16] and Latvia [19].

Urea and Rotstop® generally have various efficacy rates in spruce stumps [26–28]; however, BCA are considered to be more susceptible to biotic and abiotic factors, while urea is more stable [29]. The efficacy of Rotstop® and urea is dependent on stump coverage [28,30]. Oliva et al. [31] showed that urea is a reliable, long-term (at least 15 years) protection method against root and butt rot of Norway spruce. Only a few studies have directly compared the efficacy of BCA to urea in spruce stumps in the same experiment [20,28,30,32,33], and these yielded inconsistent results. The efficacy of stump treatment with urea solution and spore suspension of *P. gigantea* against infection by *Heterobasidion* spp. has been compared in field conditions in *Abies cilicica* wood, and urea showed higher efficacy than BCA [34]. Data obtained in Denmark showed that urea more effectively prevented the spread of *Heterobasidion* root rot to adjacent *P. abies* than Rotstop® or local strains of *P. gigantea* [33]. In Italy, the efficacy of urea at different concentration levels (10–30%) and Rotstop® has been compared [5], the data showing similar efficacy for a 30% urea (w/v) concentration and BCA. Contrastingly, Anselmi and Nicolotti [27] reported that the efficacy of *P. gigantea* was higher than that of 30% urea. In addition, the type of treated wood surface can have an impact; urea showed higher efficacy in logs, whereas Rotstop® and local strains of *P. gigantea* were more efficient in spruce stumps [26].

Heterobasidion spp. infection risk is particularly high in stands on former agricultural land [35,36]. Therefore, it is very important to analyze treatment agents against primary infection in spruce stands planted on former agricultural soil. In the literature available, there are only limited data where the efficacy of both BCA and urea against basidiospore infection in spruce stands on former agricultural lands has been compared.

Stumps are sometimes covered with wood discs, moss and soil to increase BCA efficacy [37–39]. However, stump cover could promote the development of other fungi, including *Heterobasidion* spp. [40,41]. Yet, the influence of stump cover with discs on the efficacy of urea against *Heterobasidion* spp. basidiospore infection is unknown. The aims of this study were to test the control efficiency of Rotstop®, a native Latvian *Phlebiopsis gigantea* strain and urea as control agents against natural spore infection of *Heterobasidion* spp. on pre-commercial thinning stumps of Norway spruce on former agricultural lands, and to analyze the effect of stump coverage on urea and BCA efficacy.

2. Materials and Methods

2.1. Plant and Fungal Material

The experiment was established in 2018 on three, first-rotation Norway spruce stands in Rezekne (Eastern Latvia). Site characteristics are detailed in Table 1. In Stands 1 and 3, precommercial thinning was conducted in 2016, prior to our experiment. To reduce the risk of secondary infections via root contacts from these thinned trees, a 3 m buffer zone between trees used in this experiment and old stumps was implemented. Commercial Rotstop® (*Phlebiopsis gigantea* strain VRA 1835) and Latvian *P. gigantea* strain 422 (in text *P. gigantea* 422), initially isolated from Norway spruce and previously characterized in vitro on malt agar for growth, asexual spore production and antagonism against *H. annosum* and *H. parviporum* [42,43], were used as BCA for stump treatments.

2.2. Experimental Description

At each of the three sites, 160 trees were cut using a chainsaw in July 2018 (in total 480 trees) to a stump height of 70 cm. None of the stumps showed signs of discoloration or decay and were presumed to be free of *Heterobasidion* infection at the time of cutting. Stumps were left at a 70 cm height for one week until they could be further treated. Outer bark was disinfected by treating them with 70% ethanol to reduce the unintended introduction of microbes to cut surfaces before treatment application [44]. For all sites, half of the stumps were cut to a height of 40 cm, while the other half were cut to 45 cm. The 45 cm high stumps then had a 5 cm thick disk cut from the top of the stump, which was kept and used for the subsequent stump cover treatment. After cutting, each stump was treated with one of four stump treatments: Rotstop® spore suspension, *P. gigantea* 422 spore suspension, 35% urea solution or distilled water. Rotstop® and *P. gigantea* 422 spore suspensions were prepared as described by Kenigsvalde et al. [45]. The amount applied varied according to the diameter of the stump surface so that the solution covered the surface with a thickness of about 1 mm [46].

After stump treatment, the 5 cm thick wood discs were replaced on top of their respective stumps, while the other stumps were left uncovered to create 8 unique treatment combinations per site (Rotstop® covered (n = 20), Rotstop® uncovered (n = 20), *P. gigantea* 422 covered (n = 20), *P. gigantea* 422 uncovered (n = 20), 35% urea covered (n = 20), 35% urea uncovered (n = 20), water covered (n = 20) and water uncovered (n = 20)). All stumps were subjected to natural *Heterobasidion* spp. infection. To avoid clustering of a certain treatment to one area of the site, treatments were assigned to stumps according to a randomized complete block design that was identical for all experimental sites. During the establishment of the experiments and the three subsequent weeks, the air temperature fluctuated between 8.9 and 30.5 °C, with a mean of 20.3 °C. Total precipitation in the three-week period following establishment was 51 mm.

2.3. Sampling, Heterobasidion spp. Infection Assessment and Identification of P. gigantea

The stumps were disinfected by treating them with 70% ethanol and sampled 14 weeks after cutting (Table 1). Identification tags from four stumps disappeared prior to sampling, so these trees were excluded, and samples were taken from the remaining 476 stumps. Two 3 cm thick discs were cut from each stump with a chainsaw. The top disc was discarded, and the second disc was taken to the laboratory and assessed for *Heterobasidion* spp. infection. Discs were examined for the presence of *Heterobasidion* spp. conidiophores [47], and the presence of *P. gigantea* was estimated by morphological inspection of the mycelia and presence of oidia (e.g. [17,18,30,48]). The area colonized by *P. gigantea* (either Rotstop®, *P. gigantea* 422 or naturally infected by airborne *P. gigantea* spores (in the text referred to as wild *P. gigantea*)) and *Heterobasidion* spp. was redrawn on a transparent sheet and measured using a planimeter (PLANIX 10S "Marble", Tamaya, Japan). Re-isolations from 20 of the Rotstop® and *P. gigantea* 422 treated stumps were done to confirm successful colonization of the stumps. Somatic incompatibility assays for all isolates were

performed. Isolates were paired on malt agar with the original strain used for inoculation to test for compatibility to confirm their identity [49].

Table 1. Description of experimental sites and stump characteristics.

Site	Latitude, Longitude	Stand Age (Years)	Area (ha)	Forest Type	Number of Stumps	Mean Stump Diameter ±1 SD (cm) [5]	Stump Diameter, min–max, (cm)
1	56.24088, 27.88769	15 [1]	5.83	*Oxalidosa* [2]	160	11.5 ± 5.9 A	8.4–16.0
2	56.22804, 27.97499	15 [1]	2.38	*Oxalidosa turf. Met.* [3]	160	11.8 ± 5.9 A	8.4–14.2
3	56.22430, 27.83745	15 [1]	8.44	*Hylocomisa* [4]	160	7.8 ± 5.7 B	4.1–14.5

[1] No visual signs of heartwood; [2] Mesotrophic *P. abies* stands on mineral soil at the age of 100 years, tree height is 28–33 m [50]; [3] Highly productive mixed spruce and broad-leaved stands on eutrophic-rich drained peat soils [50]; [4] Mesotrophic *P. abies* on mineral soils at the age of 100 years, tree height is 30–33 m [50]; [5] Different letters represent significant differences in stump diameters as determined by the Kruskal–Wallis test at an $\alpha < 0.05$ level.

2.4. Calculations and Statistical Analyses

The relative area colonized by *P. gigantea* (Rotstop® or Latvian strain) and *H. annosum* was calculated by dividing their occupied areas by the total area of the disc (Kenigsvalde et al., 2016). Control efficacy, expressed as the reduced proportion of stumps colonized by *Heterobasidion* spp. and the reduced proportion of wood colonized by this pathogen, for each treatment, was calculated according to the formula: $E(\%) = 100 - \left(100 * \frac{n_t}{n_u}\right)$, where n_t represents the proportion of colonized stumps or proportion of colonized wood for treated stumps, and n_u represents the proportion of colonized stumps or proportion of colonized wood for control stumps [45]. Control efficacy was calculated within site, method and treatment.

Data were inspected for normality using the Shapiro–Wilk test and by manually evaluating Q–Q plots. Using these criteria, total area of discs, area of disc surface covered by *P. gigantea* and area of disc surface covered by *Heterobasidion* were considered to be not normally distributed (p = 0.00021, <2.2e−16 and <2.2e−16, respectively). The differences in diameter were determined using the Kruskal–Wallis test. The relationship between method (i.e., covered and uncovered stumps) and treatment effect (i.e., BCA, urea and untreated control) on the presence of *Heterobasidion* infection was determined using a generalized linear model (GLM) with a binomial distribution and logit as the link function. The relationship between method and treatment on relative infected areas for both *Heterobasidion* and *P. gigantea* was investigated with a GLM with a Poisson distribution and log as the link function. In order to determine differences between coverage methods and stump treatments on the frequency of *Heterobasidion* infection, and the relative areas occupied by *Heterobasidion* spp. and *P. gigantea*, pairwise comparisons of the model's estimated marginal means (EMM) were carried out with a 95% confidence level, with p-value adjustment according to Tukey's method. All statistical analyses were performed in the "R" environment [51].

3. Results

3.1. Effects of Treatments on Heterobasidion Incidence and Stump Colonization

Site did not have a significant influence on colonized area (p = 0.907) or infection frequency by *Heterobasidion* (p = 0.56). In the uncovered stumps, *Heterobasidion* infection frequency was significantly decreased compared to the untreated controls for the urea, Rotstop® and *P. gigantea* 422 treated stumps, but no statistical differences were found between the three treatments (Table 2). *Heterobasidion* infection frequency was significantly higher in the covered control stumps compared to the uncovered control stumps (p = 0.004). A similar trend was observed for urea-treated stumps, where coverage significantly increased *Heterobasidion* incidence (p = 0.026). Significantly fewer stumps were infected by *Heterobasidion* spp. when stumps were covered and treated with either Rotstop® or *P. gigantea* 422 compared to covered and uncovered urea and untreated control stumps. Stump

coverage also decreased *Heterobasidion* infection in both Rotstop® and *P. gigantea* 422 treated stumps compared to the uncovered stumps.

Table 2. Mean infection frequencies (%) of *Heterobasidion* spp. in Norway spruce stumps and percent of stump surface colonized by *Heterobasidion* spp. and *P. gigantea* treated with Rotstop®, native Latvian *Phlebiopsis gigantea* strain or urea (% ± standard deviation).

Treatment	Uncovered	Covered	*p*-Value [2]
***Heterobasidion* spp. Infection Frequency, %**			
Rotstop®	14 a [1]	3 a	$p = 0.561$
P. gigantea 422	13 a	5 a	$p = 0.795$
Urea	17 a	38 b	$p = 0.026$
Control stumps	35 b	53 c	$p = 0.004$
Relative Stump Surface Colonized by *Heterobasidion* spp.			
Rotstop® (min–max)	0.89 ± 5.6 a [1] (0.5–39.3)	0.01 ± 0.3 a (1.6–2.09)	$p < 0.001$
P. gigantea 422 (min–max)	1.08 ± 3.7 a (2.9–20.5)	0.43 ± 2.1 b (3.5–13.5)	$p = 0.002$
Urea (min–max)	0.92 ± 3.0 a (0.9–13.0)	2.72 ± 5.3 c (0.4–24.2)	$p < 0.001$
Control stumps (min–max)	3.39 ± 5.9 b (1.8–22.1)	10.18 ± 10.9 d (2.2–48.6)	$p < 0.001$
Relative Stump Surface Colonized by *P. gigantea*			
Rotstop® (min–max)	60.47 ± 34.3 e (0–100)	85.17± 20.8 e (5–100)	$p < 0.001$
P. gigantea 422 (min–max)	56.51 ± 36.3 e (0–100)	89.77 ± 46.8 e (0–100)	$p < 0.001$
Urea (min–max)	4.43 ± 8.6 f (0–79.6)	10.03 ± 23.4 d (0–98.2)	$p < 0.001$
Control stumps (min–max)	10.68 ± 22.1 d (0–100)	11.32 ± 20.9 d (0–100)	$p = 0.9661$

[1] Values with different letters in columns. "Uncovered" and "Covered" are significantly different at $\alpha < 0.05$ (Appendix A and B). [2] The *p*-values indicate the significance of differences between values in the same row.

Significant differences in relative area occupied by *Heterobasidion* spp. between covered control stumps and other treatments were observed ($p < 0.001$; Table 2; Appendix A). Relative stump surface area occupied by *Heterobasidion* spp. was significantly less when Rotstop® or *P. gigantea* 422 (irrespective of coverage) were applied in comparison to covered and uncovered control stumps and covered urea-treated stumps (Table 2; Appendix A).

Mean relative area of *P. gigantea* was significantly greater ($p < 0.001$) than the area colonized by *Heterobasidion* spp. both in stumps treated with BCA and in control stumps (Table 2). Moreover, the surface area colonized by *P. gigantea* was significantly larger in uncovered control stumps than the area occupied by *Heterobasidion* spp. ($p < 0.001$). All re-isolations were vegetatively compatible with each respective inoculated strain.

A total of 47% of covered control stumps and 38% of uncovered control stumps were colonized by wild *P. gigantea*. Additionally, 24% of stumps (33% of covered and 15% of uncovered) had both *Heterobasidion* spp. and *P. gigantea* present. For these stumps, relative surface area colonized by *Heterobasidion* spp. varied from 3 to 49% (average 14%) and by *P. gigantea* from 1 to 69% (average 21%). The presence of naturally occurring *P. gigantea* had no influence on the natural infection rate of *Heterobasidion* spp. ($p = 0.739$). Eighteen percent of the uncovered urea-treated stumps were colonized by wild *P. gigantea* and 35%

of the covered urea-treated stumps were infected by naturally occurring *P. gigantea*. However, the area occupied by wild *P. gigantea* was considerably smaller than that occupied by Rotstop® or *P. gigantea* 422 (for both $p < 0.001$; Table 2; Appendix B).

3.2. Control Efficacy

Control efficacy was calculated based on the proportion of infected stumps and area occupied by the pathogen. Based on infection frequency, Rotstop® and *P. gigantea* 422 showed the highest efficacy both in uncovered stumps (60.58% and 62.0%, respectively) and covered stumps (95.29% and 92.93%, respectively). For both covered and uncovered urea-treated stumps, the efficacy did not exceed 50% (47.71% and 45.78%, respectively).

The highest control efficacy was also found in BCA-treated and covered stumps in comparison to urea based on the relative surface area occupied by *Heterobasidion* spp., (99.39% for Rotstop®, 95.69% for *P. gigantea* 422 and 72.71% for urea). Also compared to uncovered stumps, the efficacy of both covered and Rotstop® and *P. gigantea* 422 treated stumps were higher (Figure 1).

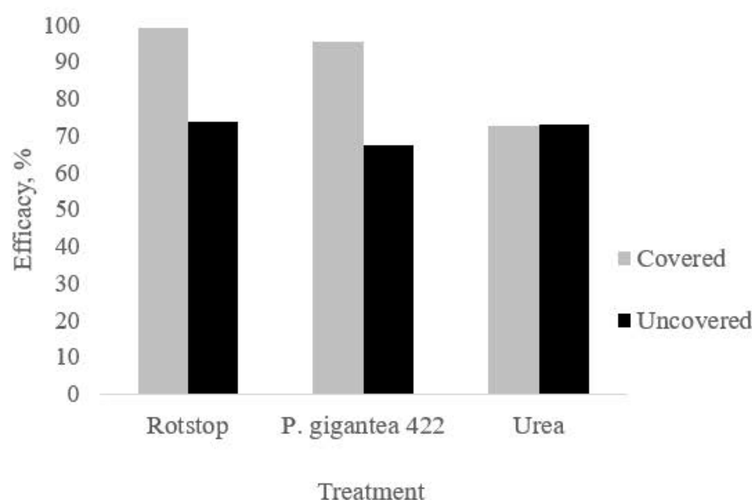

Figure 1. Control efficacy (%) against *Heterobasidion* spp. based on the relative area of colonized wood.

4. Discussion

4.1. Effects of Treatments on Heterobasidion Incidence and Stump Colonization

The incidence of *Heterobasidion* infection did not differ between stumps treated by urea or *P. gigantea* suspensions. Treatment with either BCA significantly decreased the frequency of *Heterobasidion* spp. in comparison to control stumps. However, infection by *Heterobasidion* spp. was not completely prevented, as more than 13% of uncovered BCA-treated Norway spruce stumps were still infected. Such failure in preventing *Heterobasidion* infections is not uncommon for BCA such as Rotstop®. For example, Berglund and Rönnberg [30] regularly observed *Heterobasidion* infections (as high as 70% disease incidence at some sites) on Norway spruce stumps even when fully covered with Rotstop®. The efficacy of Rotstop® could be at least partially associated with the high natural infection rate of *Heterobasidion* spp. [30,45,52]. In Latvia, Rotstop® has proven to be an effective control agent against *Heterobasidion* spore infection [45], and *P. gigantea* 422 was equally effective. Several studies have reported that native isolates of *P. gigantea* are capable of achieving similar, if

not higher, efficiency as Rotstop® [21,45,52]. Therefore, it seems possible to complement the conventionally used Rotstop® with a native strain also in Latvia (*P. gigantea* 422).

In this study, efficacy based on the proportion of infected stumps treated with urea did not exceed 50%; however, if efficacy was based on *Heterobasidion* infected area, then the efficacy of urea was almost the same as BCA. These results are in agreement with those obtained in other studies, where the efficacy of urea and *P. gigantea* were similar [5,20,29]; however, urea has been documented to have higher efficacy in comparison to *P. gigantea* in some studies [26,33,34]. Moreover, development of *P. gigantea* depends on (i) stump treatment coverage quality [28,30,53]; (ii) stump and root wood moisture content, which in turn depend on the humidity during the treatment period [53], weather conditions and seasonality [54–56]; (iii) growth characteristics of different *P. gigantea* isolates [43]; (iv) enzymatic activity of the fungi; (v) the characteristics of the wood; and (vi) the richness of the fungal biota [57]. Furthermore, Wang et al. [29] found that treatment of *Larix x eurolepis* stumps with urea resulted in more stable effects in control of *Heterobasidion* than using BCA. The average air temperature during experiment establishment was close to the optimal for *P. gigantea* development [57], and our data indicate that, although the total precipitation in the three-week period following establishment of the experiments was low, it was sufficient to ensure favorable conditions for fungal growth.

4.2. The Effect of Stump Cover on Heterobasidion spp. and P. gigantea Development

Although not used in practical forestry, stump cover treatments have been examined under experimental conditions, typically using plastic sheets or bags to protect stumps from environmental conditions and to the improve efficacy of *P. gigantea* [52,58–60] and other BCA, consisting of *Hypholoma fasciculare* (Huds.) P. Kumm., *Phanerochaete velutina* Karst., *Vuilleminia comedens* (Nees) Maire and *Trichoderma harzianum* [37].

As we analyzed covered and uncovered stumps, we had a possibility to compare results between these two groups. If the stump surface was uncovered, Rotstop® and *P. gigantea* 422 reached more than 60% efficacy based on the proportion of infected stumps and at least 65% efficacy based on the relative infected area. The results obtained about BCA efficacy based on incidence and colonized area are in agreement with previous research with Norway spruce in Finland, Sweden and Latvia [16,45,61–63]. Our results showed that the covered stumps had a greater relative surface colonized by *P. gigantea*. In two of the sites, treatment with BCA combined with stump cover completely excluded *Heterobasidion* infection (data not shown). Our data confirm that the development of both *P. gigantea* and *Heterobasidion* spp. increases with stump cover. This is in agreement with Redfern [41], who reported that covering stumps with freshly cut branches decreases variation in microclimate, thereby stimulating the development of various fungi, including *Heterobasidion*. Increased formation of *Heterobasidion* spp. fruiting bodies on covered Norway spruce stumps has also been reported by Paludan [40]. Redfern [55] found that Sitka spruce (*Picea sitchensis* (Bong.) Carr.) stumps covered with a polyethylene sheet 60 cm above their surface tended to be more infected by *Heterobasidion* spp. spores compared to uncovered stumps. Both *Heterobasidion* spp. and *P. gigantea* are primary colonizers of conifer stumps [64,65], so factors that positively affect *P. gigantea* likely favor *Heterobasidion* spp. as well. Despite this, our results indicate that covering of stumps with wooden discs significantly promotes *Phlebiopsis gigantea* growth over that of *Heterobasidion* spp. in treated stumps only. This was not the case in the control stumps. Our research demonstrates that stump cover can increase the efficacy of BCA by up to 90%. This may be of value for small-scale forestry, where cuttings are not mechanized, and manual placement of discs is feasible. Moreover, this study provides additional information about processes typically happening during commercial thinning and final felling, when stumps often become covered (with branches, leaves, logging residues, sawdust, moss and soil). However, in both large- and small-scale forestry, stump coverage increases efficacy of BCA only if stumps are treated correctly; otherwise, it may increase the risk of *Heterobasidion* colonization (clearly shown by high *Heterobasidion* infection frequency in covered control stumps; Table 2).

4.3. Treatment effects on wild *P. gigantea*

Wild *P. gigantea* was observed in 43% of the control stumps, which is higher than previous studies in Latvia, where wild *P. gigantea* inefficiently colonized spruce stumps at final felling [45,66]. Trees in this experiment were young and did not contain any heartwood yet. This likely benefited *P. gigantea*, as it prefers to colonize sapwood [17,59], unlike *Heterobasidion* spp., which is better adapted to heartwood in spruce stumps [67]. Moreover, it has been reported that *Picea sitchensis* (Bong.) Carr. heartwood remains susceptible to *Heterobasidion* basidiospores for longer than sapwood [68].

In uncovered control stumps, the mean relative surface area colonized by wild *P. gigantea* was three-fold larger than the area infected by *Heterobasidion*. Kenigsvalde et al. [45] showed that *Heterobasidion* spp. infection in untreated spruce stumps was low when wild *P. gigantea* covered more than 10% of the stump cross-section. However, our data indicate that stumps should be treated either with Rotstop® or *P. gigantea* 422 (equally effective) to protect stumps, as the area occupied by wild *P. gigantea* was at least sox-fold smaller than the area colonized by Rotstop® and *P. gigantea* 422. Moreover, colonization by wild *P. gigantea* did not show any significant effect on the occurrence of *Heterobasidion* infection.

Besides the treatment efficiency against *Heterobasidion* spore infection, the impact of different control agents on other stump-colonizing fungi and surrounding vegetation should be taken into account [64]. Previous studies have asserted that urea has a more negative effect on fungal biodiversity in treated stumps. In comparison to Rotstop®, short-term treatment with urea causes both radical changes in the fungal community structure and damage to bryothytes and vascular plants, while Rotstop®-treated stumps were mainly colonized by the same fungal species as untreated stumps, and no effect on ground-vegetation species was reported [69,70]. However, Varese et al. [37] concluded that the negative effects of urea treatment on fungal diversity are largely short term. We observed no difference in the colonization of wild *P. gigantea* in urea-treated stumps compared to the untreated controls, and hence the long-term effect from use of urea may be questioned and regarded as less important for fungal or biodiversity in general. When deciding between urea or Rotstop/*P. gigantea* as management options, managers should consider relevant factors that can affect treatment efficacy, fungal biodiversity and cost, including season, weather conditions, soil type and equipment availability. However, these issues were outside the scope of this study.

5. Conclusions

Overall, this study clearly shows that the efficacy of *P. gigantea* against *Heterobasidion spp.* in Norway spruce stumps is significantly increased by covering the stump surface with an autochthonous disk. Such a treatment is laborious and not practical for large-scale forestry. However, during manual cutting in private or urban forests stump cover should be considered. Commercial foresters should continue to protect against *Heterobasidion* infection by using urea or Rotstop® when appropriate. There is also a possibility to utilize native *P. gigantea* strains from Latvia rather than Rotstop® without compromising efficacy, which may lead to a higher acceptance by the public and contractors for using BCA.

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Appendix A

Table A1. Output of statistical tests reflecting relative *Heterobasidion* infected area, colonies per cm^2. Relative infected area, Family = Poisson, Factors = Method + Treatment.

Treatment vs. Treatment	Estimate	SE	z	*p*-Value
		Method: Covered		
Rotstop vs. Control	5.114	0.5225	9.786	$p < 0.001$
Rotstop® vs. *P. gigantea* 422	1.969	0.5561	3.540	$p = 0.0095$
Rotstop® vs. Urea	−3.796	0.5267	−7.207	$p < 0.001$
P. gigantea vs. Control	3.145	0.1989	15.809	$p < 0.001$
P. gigantea vs. Urea	−1.828	0.2097	−8.715	$p < 0.001$
Urea vs. Control	1.317	0.0882	14.934	$p < 0.001$
		Method: Not Covered		
Rotstop® vs. Control	1.333	0.1556	8.570	$p < 0.001$
Rotstop® vs. *P. gigantea* 422	0.198	0.1860	1.604	$p = 0.9641$
Rotstop® vs. Urea	−0.035	0.1932	−0.181	$p = 1.000$
P. gigantea 422 vs. Control	1.136	0.1422	7.985	$p < 0.001$
P. gigantea vs. Urea	0.163	0.1826	0.892	$p = 0.9868$
Urea vs. Control	1.298	0.1514	8.574	$p < 0.001$

Appendix B

Table A2. Output of statistical tests reflecting relative *P. gigantea* colonized area, colonies per cm^2. Relative colonized area, Family = Poisson, Factors = Method + Treatment.

Treatment vs. Treatment	Estimate	SE	z	*p*-Value
		Method: Covered		
Rotstop® vs. Control	−2.0176	0.0414	48.679	$p < 0.001$
Rotstop® vs. *P. gigantea* 422	0.0525	0.0195	2.687	$p = 0.126$
Rotstop® vs. Urea	2.1386	0.0431	49.636	$p < 0.001$
P. gigantea vs. Control	−2.0700	0.0413	50.091	$p < 0.001$
P. gigantea vs. Urea	2.1911	0.0430	−50.991	$p < 0.001$
Urea vs. Control	0.1211	0.0564	2.146	$p = 0.385$
		Method: Not Covered		
Rotstop® vs. Control	−1.7333	0.0430	40.353	$p < 0.001$
Rotstop® vs. *P. gigantea* 422	−0.0676	0.0241	2.806	$p = 0.0934$
Rotstop® vs. Urea	−2.6114	0.0636	41.091	$p < 0.001$
P.gigantea 422 vs. Control	−1.6658	0.0431	38.677	$p < 0.001$
P.gigantea vs. Urea	2.5438	0.0636	39.979	$p < 0.001$
Urea vs. Control	0.8781	0.0729	12.046	$p < 0.001$

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3.5. Persistence of *Heterobasidion* species and other wood-inhabiting fungi in root remnants on forest clear-cuts six years after stump removal (Paper IX)

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Initial and long-term fungal diversity and occurrence of *Heterobasidion* spp. in Norway spruce root fragments remaining in soil after stump extraction

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ABSTRACT
Stump removal is considered as the most effective method to reduce losses caused by root rot fungi, including Heterobasidion root rot. To evaluate the persistence of *Heterobasidion* spp. in root fragments left on site after stump removal, and to analyse the ecological impact of stump removal, five permanent sample plots were established in Latvia and samples were taken immediately after stump removal and 6 years later. In total, 1008 roots of 200 *Picea abies* stumps were measured and sampled immediately after stump removal. In total, *Heterobasidion* was detected in 0.7% of healthy looking roots, 2.0% of discoloured roots and 21.4% of decayed roots. Six years later, a total of 203 root fragments were collected in stump removal areas, and *Heterobasidion* was isolated from 1.5% of the collected root pieces. Fungal diversity was higher in root fragments collected in sample plots 6 years after stump removal compared with fungal diversity in roots immediately after stump extraction, and the species were significantly different. In conclusion, stump removal could be used in areas heavily infected by root rot fungi, and this method will likely not have a negative effect on the total amount of saproxylic fungi.

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Introduction

Fungi from the genus *Heterobasidion* cause root and butt rot affecting various conifer and some deciduous tree species worldwide (Korhonen and Stenlid 1998; Gonthier and Thor 2013). Several authors have emphasized that annual economic losses caused by root rots in the northern hemisphere forest sector could comprise more than 500 (Pratt 1998)–800 million euro (Asiegbu et al. 2005). In Latvian Norway spruce (*Picea abies* (L.) H. Karst.) stands, Heterobasidion root rot, caused by two Heterobasidion species: *Heterobasidion annosum* (Fr.) Bref. and *H. parviporum* Niemelä & Korhonen is one of the most devastating fungal diseases, estimated annual losses are ~4000 EUR ha^{-1} (Arhipova et al. 2011; Gaitnieks et al. 2019). Primary infection routes are freshly cut stumps as well as basal wounds on standing trees (Stenlid and Redfern 1998). For the prevention of primary infection during the vegetation period, treatment with biological/chemical preparations of freshly cut stumps is performed in several countries (Gonthier and Thor 2013). In Latvia, Finnish preparation Rotstop® is used for stump protection from *Heterobasidion* spore infection (Kenigsvalde et al. 2016), however, in heavily infected areas stump treatment against primary infection will have low efficacy because of the ability of this fungus to infect neighbouring trees through root contacts (Stenlid and Redfern 1998). Thus stump removal should be considered to control several root rot diseases, including Heterobasidion root rot (Korhonen et al. 1998; Vasaitis et al. 2008; Cleary et al. 2013; Gonthier and Thor 2013). The other alternative is planting deciduous tree species on heavily infected areas (Korhonen et al. 2008; Arhipova 2012; Gonthier and Thor 2013), however, some species, such as *Betula* spp. could be quite susceptible to *Heterobasidion annosum* infection (Lygis et al. 2011).

The first experiments about the impact of stump removal on stand regeneration and tree growth were performed in Latvia in the nineteenth century by Bode (1840). A country scale inventory performed in 2005–2006 revealed that on average, 21.8% of spruce stumps in Latvia contained rot at stump level, and the main causal agent was *H. parviporum* (Arhipova et al. 2011), therefore stump removal could be one option to reduce timber losses, especially in heavily infected areas. However, information about the efficacy of stump removal on reduction of Heterobasidion root rot in Latvian conditions is needed. In addition, the possible impact on forest biodiversity should be evaluated. Data about the influence of stump removal on fungal diversity is very scarce, and was mostly focused on mycorrhizal fungi (Menkis et al. 2010; Kataja-Aho et al. 2012; Ranius et al. 2018 and references wherein) and soil microflora (Modi et al. 2020). Only two studies on the impact of stump harvesting on abundance of saproxylic fungi are available. Toivanen et al. (2012) evaluated occurrence of fruit bodies in 20 forest sites (10 stump harvest and 10 clear cut) during September 2006. Whereas Hiron et al. (2017) evaluated fungal, beetle and lichen diversity in stands (24,449 ha in total) where different block treatments were performed, including stump removal, slash removal and stump and slash removal for three of the most common commercial tree species – pine, spruce and birch.

The aim of this study was (1) to determine the diameter of root fragments left in the soil after stump removal; (2) to analyse persistence of *Heterobasidion* spp. in root pieces

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remaining 6 years after stump removal; (3) to compare fungal diversity in root fragments on stump removal sample plots immediately after stump extraction and 6 years later.

Material and methods

Field work

Stump removal trials were established during 2011–2012 in five sample plots in different regions of Latvia (Figure 1) characterized by high root rot incidence (Arhipova et al. 2011). The criteria for selecting forest sites were – domination of *P. abies*, proportion of decayed spruce stumps at least 50% and area of stand more than one hectare. Each established sample plot was at least 0.5 ha. Clear-cuts on all sample plots were performed 2 years previously. Three sample plots were established in *Hylocomiosa* forest type, and two in *Oxalidosa* forest type (Bušs 1997). Each sample plot was divided into two areas – control area and area where stumps were removed. Areas were separated by a 20-m-wide buffer zone. Decayed Norway spruce stumps within sample plots were sampled in 2011 and again in 2012 using a Presler increment borer; abundance of *Heterobasidion* spp. was surveyed. Each stump was sampled twice (approximately 10–15 cm above ground from opposite sides (180°)), all sampled stumps were numbered and their height and diameter measured. Number of sampled stumps in sample plot varied from 78 to 189 (depending of incidence of decayed stumps). In total, 1208 stumps were sampled.

In November–December 2012 stumps on all sample plots (excluding control areas) were removed using a caterpillar excavator Komatsu PC210LC with CBI stump extractor (two sample plots) and New Holland E215B with MCR-500 stump extractor prototype (three sample plots) (Zimelis et al. 2013). All *P. abies*, *Populus tremula* L., *Betula pendula* Roth. and *Pinus sylvestris* L. stumps smaller than 50 cm in diameter were removed. *Betula pendula* and *P. sylvestris* stumps greater than 50 cm, as well as all deciduous tree stumps excluding aspen and birch stumps were left in the stump removal area, as well as all stumps 4 m around ecological trees (living pines left for biodiversity conservation and natural regeneration). In total, 1796 stumps of different tree species were removed on sample plots; spruce stumps comprised 83% of all removed stumps. Mean diameter of spruce stumps was 34 cm, height – 29 cm (Table 1).

From excavated spruce stumps in each sample plot, 20 sound looking and 20 decayed spruce stumps infected by *Heterobasidion* spp. (detected during 2011–2012 survey) and *Armillaria* spp. (if stumps infected by *Heterobasidion* were not available) were selected for further sampling (200 stumps in total). The remaining extracted stumps were stacked at the outer edge of sample plots. From each of 20 chosen stumps, five to six of the largest roots close to the breakage point (20–40 cm) were cut using the chainsaw or axe (depending on root size). In total, 200 root fragments from each sample plot were collected. Each root fragment was numbered, placed in separate plastic bag and transported to the laboratory. Before further processing, root samples were stored at 4°C.

In August 2018 (6 years after stump removal), approximately 40 root residuals were collected in each sample plot where stump removal was conducted. Root fragments were collected at random throughout the sample plot, numbered and placed in plastic bags. Most of root fragments collected were half – buried in soil. If it was not possible to extract the whole entire root fragment from soil, it was cut using an axe. All root fragments were placed in plastic bags and transported to laboratory. Before processing, root fragments were stored at 4°C for not more than 48 h.

Isolation and identification of fungi

In the laboratory, each root fragment was washed in tap water and dried at room temperature overnight. The diameter of each root fragment was measured using callipers close to the breakage point (in 2012) or in the middle part of root fragment (in 2018). Diameter measurements were performed in two directions and the average diameter for each root fragment was calculated. Presence of fungal fruit

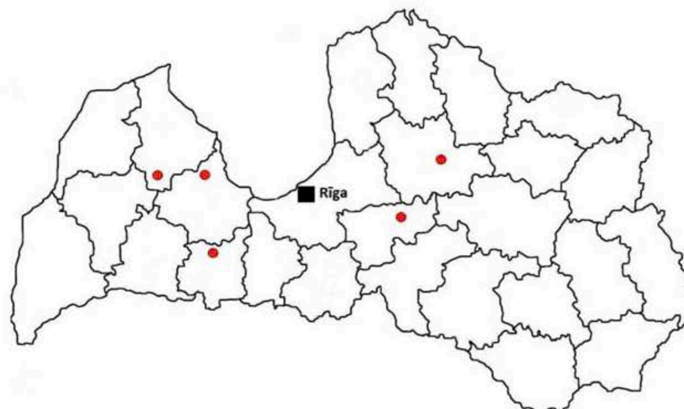

Figure 1. Distribution of stump removal sample plots.

Table 1. Description of sample plots.

Sample plot ID	Forest type	Soil type	Soil pH	Mean diameter of decayed stumps, cm	Mean diameter of healthy stumps, cm	Total number of spruce stumps	Proportion of decayed stumps, %	Proportion of decayed stumps, infected by Heterobasidion, %
82-05-07-712-437-8	*Hylocomiosa*	Sand	3.9	36	37	160	71	3
83-05-07-603-326-7	*Oxalidosa*	Sandy loam	4.7	30	29	357	37	15
65-03-07-410-58-34	*Hylocomiosa*	Loamy sand	4.3	38	36	215	38	24
80-29-07-501-360-9	*Hylocomiosa*	Loamy sand	3.3	33	35	799	23	26
82-04-07-714-188-9	*Oxalidosa*	Loamy sand	4.3	29	27	265	40	6

bodies was evaluated and species identified using morphological features (Breitenbach and Kränzlin 1986; Lesoe 1998). From each root fragment, small wood pieces (2–3 × 0.5–1 cm) from the inner part of the root close to the breakage point were cut using an axe, and root condition (sound looking, decayed or discoloured) was recorded. Each wood sample was briefly surface sterilized by flame, put on a Petri dish (2–5 wood pieces per plate with two replicates) containing Hagem agar medium (5 g glucose, 0.5 g NH_4NO_3, 0.5 g $MGSO_4 \cdot 7H_2O$, 5 g malt extract, 20 g agar, 1000 mL distilled H_2O at pH 5.5) and incubated at room temperature for 5 weeks. Each third–fourth day samples were examined and all emerging fungal mycelia transferred to a separate Petri dish. All obtained fungal isolates were examined under a Leica DM4000B microscope and grouped in morphotypes according to mycelial features. From these, eight species/genera were identified using microscopic features: *Aspergillus niger*, *Aureobasidium pullulans*, *Botrytis cinerea*, *Chaetomium globosum*, *Cladosporium* sp., *Ophiostoma piceae*, *Mucor* sp. S18, *Umbellopsis* sp. (Watanabe 2002). Intersterility tests were performed for identification of *Heterobasidion* (Korhonen 1978) and *Armillaria* species (Guillaumin et al. 1991) using homokaryotic test cultures (courtesy Dr. Kari Korhonen, Finnish Forest Research Institute "Metla" (since 2015 – Natural Resources Institute Finland LUKE)). In addition, the identification of some *Armillaria* isolates was confirmed using molecular methods (PCR). One to five representatives from the remaining unidentified fungal morphotypes were subjected to molecular identification using the universal fungal primers ITS1F and ITS4 using a similar protocol as in Arhipova (2012). Molecular work (DNA extraction, PCR amplification and PVR product purification) was performed in the Finnish Forest Research Institute Metla (in 2013) and in the Latvian State Forest Research Institute Silava Genetic Resources Centre (in 2018–2019). Sanger sequencing (in one direction) was performed by Macrogen Europe using the ITS4 primer. All sequences were manually edited using the Lasergene software package SeqMan (DNASTAR, Madison, Wisconsin). BLAST searches were performed using GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The Internal Transcribed Spacer (ITS) sequence homology was set at 98–100% for delimiting fungal taxon and at 95–98% for delimiting at the genus level. ITS of each sequenced mycelial morphotype was added to the GenBank database (Table 3).

Statistical analyses

For data analysis (analysis of proportions (chi-squared tests), *t*-test, ANOVA), Excel and R software (ver. 3.2.4) (R core team 2015) were used. Significance was evaluated according to Fowler et al. (2001). For analysis of similarity between fungal communities, the Sorrensen similarity index and Shannon diversity index were calculated (Magurran 1988) using the software EstimateS (Chao et al. 2005).

Results

Incidence of Heterobasidion *spp. in roots of* P. abies *immediately after stump extraction*

In total, 1008 samples from roots of extracted spruce stumps were collected (507 root samples from healthy looking and 501 from decayed stumps). The diameter of collected roots varied between 0.9 and 19 cm with mean diameter 4.2 ± 2.6 cm (Figure 2). In most cases (75.3%), the diameter of collected root fragments was less than 5 cm. There were no significant differences between stump extractors used regarding the diameter of roots left in the stump extraction area (on average 4.4 ± 2.5 vs. 4.0 ± 2.7) ($p > 0.01$). Overall, 20% roots of healthy looking stumps and 28% roots of decayed stumps contained rot, but the proportion of roots with wood discoloration was even larger – 52% of roots from sound looking stumps and 46% of roots from decayed stumps. The rest of the collected root fragments were sound looking. The diameter of decayed roots from decayed stumps was significantly larger ($p < 0.01$) than other categories of roots. In addition, those were the most infected by *Heterobasidion* spp. – 32%, but this tendency was not found in sound looking stumps, where differences in diameter of healthy looking and decayed roots was not significant (Table 2). In total, *H. parviporum* was isolated from

Table 2. Occurrence of *Heterobasidion* spp. in roots of healthy looking and decayed stumps.

	Healthy looking stumps			Decayed stumps			
	Sound looking roots	Roots with wood discoloration	Decayed roots	Sound looking roots	Roots with wood discoloration	Decayed roots	In total
Number of roots	141	261	105	130	230	141	1008
Mean diameter (cm ± SD)	4.5 ± 2.5[a*]	3.8 ± 2.3[b]	4.1 ± 2.4[a]	4.3 ± 2.5[a]	3.6 ± 2.1[b]	5.3 ± 3.6[c]	4.2 ± 2.6
Proportion of *Heterobasidion* infected roots (%)	0	0.8[a]	7.6[b]	1.6[a]	3.0[a]	32.0[c]	6.4
Mean diameter of *Heterobasidion* infected roots (cm ± SD)	–	5.8 ± 0.1	5.9 ± 1.5	4.8 ± 0.6	7.1 ± 3.6	5.9 ± 3.5	5.8 ± 3.1

*Values within a row followed by a different letter differ significantly (diameter means compared using a *t*-test, $p < 0.01$; proportion of roots infected by *Heterobasidion* spp. compared using χ^2 tests, $p < 0.01$).

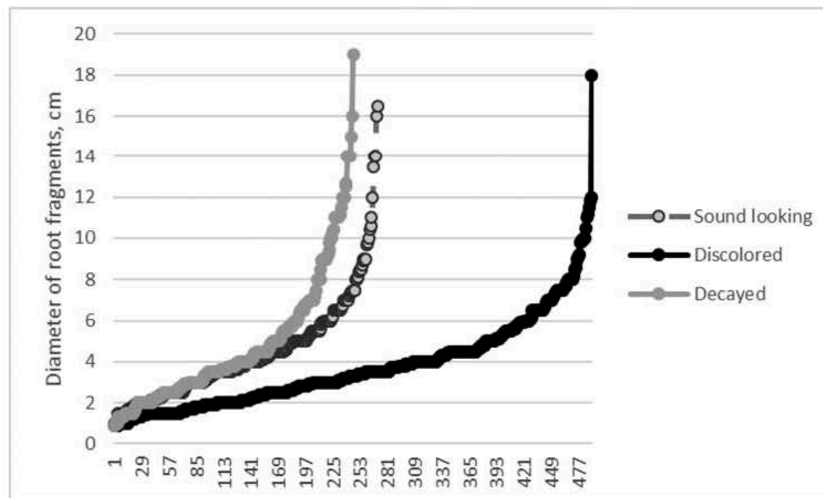

Figure 2. Diameters of root fragments collected from extracted stumps immediately after stump removal.

11% of roots from decayed stumps and 2% of roots from healthy looking stumps. *Heterobasidion* fruit bodies were detected on two roots. The diameter of Heterobasidion-infected roots (5.8 ± 3.1) was significantly larger than of non-infected roots (4.0 ± 2.5) ($p < 0.001$), however, the smallest root from which *H. parviporum* was isolated was 1.5 cm in diameter. In sound-looking stumps (7%), *H. parviporum* was isolated only from roots with discoloration and from decayed roots. The number of infected roots per stump varied from 0 to 3, but in most cases (71%), only one root per stump was infected. In total, from 30 decayed stumps (30%) *H. parviporum* was isolated from discoloured (3.0%) and decayed (32.6%) roots, but also from two (1.6%) sound looking roots. The number of infected roots per stump varied from 0 to 5. In most cases, only one or two roots per stump were infected (73%), however, in 10% of stumps all five collected roots were infected. In two western sites, the proportion of *Heterobasidion* initial infection was quite low (Table 1) and decay was caused mostly by *Armillaria borealis*, which was isolated from 7.8% of sound looking, 14.8% of decayed and 12.4% of discoloured spruce roots. A second *Armillaria* species was found – *A. cepistipes* was subsequently isolated, from 14.5% of sound looking, 20.4% of discoloured and 12.8% of decayed roots.

Survival of Heterobasidion *spp. in partially buried root fragments 6 years after stump removal*

In 2018, 37–48 root pieces were collected from each stump removal sample plot (203 root pieces in total). The diameter of collected root pieces varied from 1.4 to 8.7 cm (mean diameter 3.8 ± 1.7 cm). *Heterobasidion parviporum* was isolated from two (1%) root pieces (with diameter 5.9–8.2 cm) and *H. annosum* from one (0.5%) root piece (with diameter 6.6 cm).

Fungal diversity in root fragments immediately after stump extraction and 6 years later

From 1008 root fragments collected in 2012, 971 (96%) resulted in fungal growth and 49 fungal taxa were isolated: 11 basidiomycetes, 35 ascomycetes and anamorphic fungi and 3 zygomycetes (Table 3). The rest of the samples (37) remained sterile or gave only bacterial growth. Among 203 root fragments collected in 2018, all 203 (100%) resulted in fungal growth and 73 fungal taxa were isolated: 18 basidiomycetes, 48 ascomycetes and anamorphic fungi and 7 zygomycetes.

The most abundant basidiomycetes in 2012 were *A. cepistipes* (17.3%), *A. borealis* (11.7%) and *H. parviporum*

Table 3. Percentages of *Picea abies* roots from which fungal taxa were isolated immediately after stump removal and 6 years later.

Fungal taxa	GenBank access number	Fungi inhabiting stump roots			Fungi inhabiting root pieces 6 years after stump removal (*n* = 203)
		Sound looking roots (*n* = 271)	Discolored roots (*n* = 492)	Decayed roots (*n* = 246)	
Basidiomycetes					
Agaricales sp. S77	MK911640	–	–	0.4	–
Armillaria borealis Marxm. & Korhonen	MK911654	7.8	12.4	14.8	3.4
Armillaria cepistipes Velen.	MK911613	14.5	20.4	12.8	7.4
Bjerkandera adusta (Willd.) Karst	–	–	–	–	0.5
Calocera cornea (Batsch) Fr.	–	–	–	–	0.5
Chondrostereum purpureum (Pers.) Pouzar	MK911642	–	–	0.8	–
Fomitopsis pinicola (SW.) Karst.	MK911616	–	–	–	0.5
Gloeophyllum sepiarium (Wulfen) Karst.	MK911695	–	–	–	2.0
Gymnopilus sapineus (Fr.) Maire	MK911681	–	–	–	3.0
Gymnopus androsaceus (L.) Mata & Petersen	–	–	–	–	2.4
Heterobasidion annosum (Fr.) Bref.	MK911666	–	–	–	0.5
Heterobasidion parviporum Niemelä & Korhonen	–	0.7	2.0	21.4	1.0
Hypholoma capnoides (Fr.) Kumm	MK911639	–	0.2	–	–
Hypholoma fasciculare (Huds.) Kumm	MK911689	–	–	–	2.0
Hypochnicium subrigescens Boidin	MK911615	–	–	–	0.5
Lachnocladiaceae sp. S67	MK911633	–	–	0.4	–
Leucogyrophana mollusca (Fr.) Pouzar	MK911674	–	–	–	1.5
Mycena alnetorum Favre	MK911685	–	–	–	1.5
Mycena galopus (Pers.) Kumm.	MK911630	0.7	0.6	0.4	–
Phanerochaete sanguinea (Fr.) Pouzar	MK911704	–	–	–	0.5
Phanerochaete sordida (Karst.) Erikss. & Ryvarden	MK911663	–	–	–	1.5
Phlebiopsis gigantea (Fr.) Jülich	MK911641	0.4	0.2	–	–
Postia ptychogaster (Ludw.) Vesterh	MK911696	–	–	–	0.5
Postia stiptica (Pers.) Julich	MK911673	–	–	–	2.0
Resinicium bicolor (Alb. & Schwein.) Parmasto	MK911661	0.7	1.8	2.9	16.7
Rhodonia placenta (Fr.) Niemelä, Larss. & Schigel	MK911677	–	–	–	0.5
Sistotrema brinkmannii (Bres.) Erikss	MK911637	0.4	0.2	0.8	–
Tomentella terrestris (Berk. & Broome) Larsen	–				1.5
Tritirachium oryzae (Vincens) de Hoog	MK911694	–	–	–	0.5
All basydiomycetes		25.5	36.3	52.4	36.9
Ascomycetes/anamorphyc fungi					
Alternaria infectoria Simmons	MK911688	–	–	–	1.5
Arthrinium arundinis (Corda) Dyko & Sutton	MK911662	–	–	–	1.5
Ascocoryne cylichnium (Tul.) Korf	MK911653	23.8	22.4	20.2	20.2
Ascomycete sp. SR94	MK911700	–	–	–	1.0
Aspergillus niger Tiegh	–	2.6	1.2	2.1	0.5
Aspergillus versicolor (Vuill.) Tirab	MK911620	5.2	1.2	2.5	–
Aureobasidium pullulans (De Bary) Arnaud	–	0.4	–	–	–
Beauveria bassiana (Bals.-Criv.) Vuill.	MK911629	–	0.2	–	–
Botrytis cinerea Pers.	–	–	0.2	0.4	–
Cadophora fastigiata Lagerb. & Melin	MK911614	–	–	–	0.5
Cadophora malorum (Kidd & Beaumont) Gams	MK911672	–	–	–	17.2
Cenococcum sp. SR98	MK911706	–	–	–	3.4
Chaetomium globosum Kunze ex Fr	–	–	–	–	15.8
Cladosporium spp.	–	0.4	–	–	1.0
Ciliolarina ligniseda (Velen.) Svrcek	MK911628	–	0.2	–	–
Colpoma sp. SR48	MK911680	–	–	–	3.0
Coniochaeta fasciculata (Beyma) Khan, Gené & Guarro	MK911664	–	–	–	4.4
Coniochaeta hoffmannii (Beyma) Khan, Gené & Guarro	MK911657	–	–	–	23.2
Coniochaeta ligniaria (Grev.) Massee	MK911691	–	–	–	1.0
Coniochaeta sp. S69	MK911634	–	0.2	–	–
Cordana pauciseptata Preuss	MK911671	–	–	–	3.9
Corinectria fuckeliana (Booth) González & Chaverri	MK911707	3.0	5.7	6.6	2.0
Cosmospora viridescens (Booth) Gräfenhan & Seifert	MK911617	9.7	5.1	4.5	–

(*Continued*)

Table 3. Continued.

Fungal taxa	GenBank access number	Fungi inhabiting stump roots: Sound looking roots ($n = 271$)	Discolored roots ($n = 492$)	Decayed roots ($n = 246$)	Fungi inhabiting root pieces 6 years after stump removal ($n = 203$)
Drechslera sp. SR45	MK911678	–	–	–	3.0
Epicoccum nigrum Link	MK911660	–	0.2	–	2.5
Fusarium sporotrichioides Sherb.	MK911670	1.1	1.4	0.4	8.9
Jackrogersella multiformis (Fr.) Wendt, Kuhnert & Stadler	MK911668	–	–	–	2.0
Leotiomycetes sp. SR95	MK911701	–	–	–	0.5
Mariannaea elegans (Corda) Samson	MK911686	5.2	6.9	5.3	8.9
Neobulgaria sp. S4	MK911619	–	–	0.4	–
Ophiostoma pallidulum Linnakoski, de Beer & Wingf	MK911643	4.8	8.8	8.2	–
Ophiostoma piceae (Münch) Syd. & Syd	–	9.7	11.0	6.6	1.5
Paraconiothyrium fuckelii (Sacc.) Verkley & Gruyter	MK911699	0.7	0.2	–	0.5
Paraphaeosphaeria michotii (Westend.) Erikss	MK911665	–	–	–	1.5
Paraphaeosphaeria neglecta Verkley, Riccioni & Stielow	MK911627	–	0.2	–	–
Penicillium citreonigrum Dierckx	MK911683	0.7	1.2	0.8	4.9
Penicillium glabrum (Wehmer) Westling	MK911622	21.9	11.2	11.1	–
Penicillium lanosum Westling	MK911625	3.3	3.7	4.5	–
Penicillium maclennaniae Yip	MK911687	–	–	–	47.8
Penicillium melinii Thom	MK911623	–	–	0.4	–
Penicillium sp. S66	MK911632	5.9	6.5	3.7	–
Penicillium thomii Maire	MK911650	–	–	–	6.4
Periconia sp. SR109	MK911705	–	–	–	0.5
Phacidium lacerum Fr.	MK911631	–	0.2	–	–
Phialocephala fortinii Wang & Wilcox	MK911636	1.9	3.5	2.5	–
Phialocephala sp. Sr16	MK911655	–	–	–	27.6
Phialocephala sp. SR34	MK911669	–	–	–	1.0
Phoma herbarum Westend.	MK911682	0.4	0.2	–	0.5
Pseudeurotium bakeri Booth	MK911621	12.6	10.0	8.2	–
Pseudogymnoascus pannorum (Link) Minnis & Lindner	MK911626	0.4	–	–	–
Rhinocladiella atrovirens Nannf	MK911679	–	–	–	6.4
Scytalidium album Beyer & Klingström	MK911698	–	–	–	1.0
Scytalidium lignicola Pesante	MK911656	1.1	0.2	3.7	11.8
Sordariomycetes sp. SR62	MK911684	–	–	–	1.0
Sordariomycetes sp. SR99	MK911702	–	–	–	0.5
Sporothrix inflata de Hoog	MK911635	–	0.4	–	–
Stagonospora sp. SR43	MK911676	–	–	–	1.0
Symbiotaphrina sp. SR88	MK911697	–	–	–	4.4
Talaromyces purpureogenus Samson et al.	MK911693	–	–	–	3.0
Talaromyces wortmannii (Klöcker) Benj	MK911618	4.1	3.1	3.3	–
Tolypocladium inflatum Gams	MK911638	–	–	0.4	–
Trichoderma crassum Bissett	MK911703	–	–	–	1.5
Trichoderma deliquescens (Sopp) Jaklitsch	MK911658	3.7	1.2	2.5	15.3
Trichoderma longipile Bissett	MK911644	–	–	–	37.9
Trichoderma koningii Oudem.	MK911651	–	–	–	35.0
Trichoderma minutisporum Bissett	MK911647	–	–	–	27.6
Trichoderma polysporum (Link) Rifai	MK911675	44.2	48.5	35.0	9.4
Trichoderma viride Pers	MK911659	–	–	–	3.0
Truncatella angustata (Pers.) Hughes	MK911692	–	–	0.4	1.4
Unidentified ascomycetes	–	9.3	6.7	7.4	1.5
All Ascomycetes and anamorphic fungi		88.2	88.0	78.5	98.0
Zygomycetes					
Absidia sp. SR6	MK911648	–	–	–	16.3
Mucor fragilis (Tode) Traverso	MK911646	–	–	–	5.9
Mucor hiemalis Wehmer	MK911690	–	–	–	2.0
Mucor sp. S18	–	19.0	15.5	12.3	–
Mucor sp. SR2	MK911645	–	–	–	4.9
Umbelopsis isabellina (Oudem.) Gams	MK911652	4.5	2.6	5.3	33.0
Umbelopsis dimorpha Mahoney & Gams	MK911624	–	–	0.4	–
Umbelopsis sp. S15	–	2.6	2.9	2.5	24.1
Umbelopsis vinacea (Dixon-Stew.) Arx	MK911667	–	–	–	6.4
All Zygomycetes		23.6	21.2	16.7	58.1
All fungi		93.7	96.5	96.3	100.0

(6.3%). From those, *A. cepistipes* was more frequently isolated from discoloured roots (20.4%), but *H. parviporum* and *A. borealis* – from decayed roots (21.4% and 14.8%, respectively). The most common ascomycetes isolated from roots were *Trichoderma polysporum* (43.8%), *Ascocoryne cylichnium* (22.1%), *Penicillium glabrum* (14.0%), *Pseudeurotium bakeri* (10.2%) and *Ophiostoma piceae* (9.5%). The fungal communities in sound looking, discoloured and decayed roots were highly similar (Sorensen similarity index varied between 0.8 and 0.9).

However, fungal communities in root fragments left on stump removal area 6 years later were significantly different from the fungal communities found in 2012 (Sorensen similarity index – 0.3), and the diversity of fungal taxa was also higher (Shannon diversity index 3.6 in 2018 vs. 3.3 in 2012). Six years after stump removal (in 2018), the most abundant basidiomycetes were *Resinicium bicolor* (16.7%) and *A. cepistipes* (7.4%), the most abundant ascomycetes were *P. maclennaniae* (47.8%), *Phialocephala* sp. SR16 (27.6%), *Coniochaeta hoffmannii* (23.2%), *A. cylichnium* (20.2%), *Cadophora malorum* (17.2%), *Chaetomium globosum* (15.8%) as well as several *Trichoderma* spp. (*T. longipile* – 37.9%, *T. koningii* – 35.0% and *T. minutisporum* – 27.6%).

Immediately after stump extraction, fungal fruit bodies were detected on only 0.2% of root fragments, and all were *Heterobasidion* spp. Six years later fungal fruit bodies of various species were detected on 5.4% of root fragments: *Heterobasidion* spp., *Gloeophyllum sepiarium*, *Jackrogersella multiformis*, *Bjerkandera adusta*, *Postia stiptica*, *Tomentella terrestris*, *Calocera cornea*, *Ascocoryne cylichnium*, *Hypholoma fasciculare* and *Gymnopus androsaceus*. From those, four species – *B. adusta*, *T. terrestris*, *C. cornea* and *G. androsaceus* were observed only as fruit bodies but were not isolated from roots.

Discussion

This study shows that in areas heavily infected by root rot, 74% of stumps with visible decay symptoms and 65% of sound looking stumps were infected by root rot fungi (*Armillaria* spp. and *H. parviporum*), and this may play a major role in disease transfer to the next tree generation (Redfern and Filip 1991; Piri 2011; Gonthier and Thor 2013). Secondary infection via root contacts is the main reason why stump removal could be one of the best measures for reduction of such diseases, as root rots can be considered to be site diseases rather than diseases of individual trees (Gonthier and Thor 2013; Guillaumin and Legrand 2013). Numerous studies have proven a long-term positive impact on limitation of spread of diseases by stump removal, including root rot caused by *Heterobasidion* species (Stenlid 1987; Vasaitis et al. 2008; Shaw III et al. 2012; Cleary et al. 2013).

During this study, several fungal species considered as root rot fungi were isolated: *A. borealis*, *A. cepistipes*, *H. parviporum* and *H. annosum*. The incidence of the most devastating fungal pathogens: *A. borealis* and *H. parviporum* was significantly higher in root fragments immediately after stump removal (11.7 and 6.3%, respectively). In comparison, 6 years later, the frequency of *A. borealis* and *Heterobasidion* spp. in root fragments left in stump removal areas decreased (3.4 and 1.5%, respectively). A study by Piri and Hamberg (2015) showed that 6 years after stump removal, 18% of buried root pieces were still infected by *Heterobasidion* spp., and 8% of replanted *P. abies* seedlings were infected. In this study only 1% of root fragments were still infected by *Heterobasidion*, however, seedling survival was not evaluated during this study. Immediately after stump removal, *Heterobasidion* was isolated from small diameter roots (1.5 cm), however 6 years later it was found only in larger diameter roots (5.9–8.2 cm). This generally agrees with Omdal et al. (2001), who assumed that broken root fragments less than 5 cm in diameter will decompose in a few years and pose no infection threat for future tree generations. Whereas, in the study of Piri and Hamberg (2015), viable *H. parviporum* mycelium was isolated also from root fragments with diameter 1.5 cm, which had been buried in soil for 74 months. Our results could be explained by the fact that most of collected root fragments were severely decayed, most of them completely soft, which could indicate faster decomposition of root fragments in Hylocomyosa and Oxalidosa site conditions. In addition, soil characteristics, such as pH and soil texture may also have impact on survival of *Heterobasidion* spp. in root fragments (Brūna et al. 2019 and references wherein). Piri and Hamberg (2015) in their study found out, that soil pH had no effect on Heterobasidion survival in buried root fragments, however probability of finding Heterobasidion was greater in sandy and sandy moraine soils. Also, microbial activity was high in collected root pieces – at least seven *Trichoderma* species were isolated from half-buried root fragments, and several authors reported that soil-borne *Trichoderma* sp. readily invade wood fragments infected by root rot fungi (Nelson 1964; Munnecke et al. 1976). Moreover, in our study, roots were half-buried, which may reduce wood moisture, especially of smaller diameter roots, which have a negative effect on survival of *Heterobasidion* spp. mycelia.

Some studies showed that careful removal of all root fragments thicker than 5 mm could not completely eradicate root rot disease from infected stands (Stenlid 1987), but even if it is not possible to completely eradicate Heterobasidion root rot disease from infected forest stands, it will still considerably reduce infection pressure on the next tree generation (Vasaitis et al. 2008). Therefore, this makes preventive measures, such as stump treatment, of major importance, especially if used in combination with other root rot limitation activities. The fungus *P. gigantea* (used in many countries for stump treatment) was found in several roots of spruce stumps immediately after stump removal, which suggests the ability of some isolates to colonise not only stumps, but also spread stump root system. Risbeth (1951) also reported the ability of some isolates of *P. gigantea* to replace *Heterobasidion* in stump roots. In this study we found that in most cases, *Heterobasidion* infects 1–2 roots per stump. Antagonistic fungi, which could colonise the remaining sound-looking roots faster than *Heterobasidion*, could play an important role in limitation of the spread of infection, even in cases when the stump is already infected by root rot (Korhonen et al. 1994; Pettersson et al. 2003). The use of such fungal

isolates could increase the effectiveness of stump treatment, however, more research is required. In addition, several other fungal species, such as *F. pinicola*, *S. brinkmannii*, *Hypholoma* sp., *R. bicolor* and several *Trichoderma* spp. could be further tested for their antagonistic qualities and ability to replace *Heterobasidion* in already infected wood (Holdenrieder and Greig 1998).

One of the main considerations when considering the use of stump removal for limiting the extent of root rots is the possible ecological impact. However, stumps are not a naturally occurring substrate, and are initially colonised by common fungal species, which could use various other woody substrates for sporocarp production (Vasaitis et al. 2016). Most threatened fungal species inhabit large dimension logs, but are very rarely observed on stumps (Penttilä et al. 2004; Arhipova et al. 2011; Hiron et al. 2017). In this study, it was found that fungal diversity in root fragments left in soil increased over time and was significantly different 6 years after stump removal. In addition, some basidiomycetes found in root fragments left in stump removal areas usually inhabit spruce logs, not stumps – such as *Postia* spp. or *Rhodonia placenta*, which is rare in Latvia (Dāniele and Meiere 2020). In a study about long-term impact of stump removal on soil microflora Modi et al. (2020) reported that the relative abundance of ectomycorrhizal fungi increased in areas where stumps were removed, but abundance of saprotrophic fungi decreased, which suggests that stump removal in heavily infected areas will have a positive impact on forest regeneration and productivity. Toivanen et al. (2012) found that the occurrence of polypore fruit bodies was lower on fuel harvesting sites, however, that effect becomes less prominent with increasing of area of sample plots. In addition, most near threatened and red listed fungal species were found on substrates other than stumps, which suggests that stump removal will not have a significant impact on those species (Toivanen et al. 2012). That generally agrees with our data, as only one rare species was isolated from root fragments 6 years after stump removal. Regarding common wood decomposing polypore species, we could not agree with conclusions made by Toivanen et al. 2012. Stump removal, performed in limited extent (only for root rot eradication) could not threaten common saproxylic fungal species, as the amount of stumps and coarse woody debris in thinned stands as well as on clear-cuts not affected by root rot will remain the same, which mainly agrees with the results of a review by Ranius et al. (2018). Hiron et al. (2017) showed that if the extraction level was high (70% of stumps and slash), many insect, lichens and fungal species were estimated to lose more than 50% of their population size, however, if extraction level is moderate (30% of stumps or slash), the effect on populations was predicted to be small (<10%) for most species. It is clear that more research needed to evaluate the ecological impact of stump removal, however, regarding saproxylic fungal species, our results suggest that stump removal done for reducing Heterobasidion root rot will not significantly affect fungal biodiversity.

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4. DISCUSSION

This thesis evaluated a) the susceptibility of native and introduced conifer species to *Heterobasidion* spp.; b) possible control measures against *Heterobasidion* root rot infection in Latvia.

4.1. Relative susceptibility to *Heterobasidion* spp. infection of different conifer species (Paper I, II)

The growth rate of both *P. gigantea* and *Heterobasidion* mycelium is strongly dependent on the host tree species. The efficacy of biological control agent is based and evaluated on competition between the growth rate of the pathogen and *P. gigantea* in wood (Pettersson *et al.* 2003; Sun *et al.* 2009). In the context of climate change and increasing interest in planting introduced tree species to reduce the risk of *Heterobasidion* infection, additional information about the development of *Heterobasidion* spp. and *P. gigantea* in different conifer species is needed.

4.1.1. *Heterobasidion* development in living sapwood of *P. sylvestris* vs. *P. abies* (Paper I)

Paper I provides evidence that *H. annosum* and *H. parviporum* differ in their pathogenicity in *P. sylvestris* ($p < 0.05$), however in spruce the difference between two pathogen growth rate was not significant, which is in agreement with earlier artificial inoculation studies (Swedjemark *et al.* 1999). This is contradictory to results obtained from field experiments, where development of both pathogens was analysed in mature *P. abies* trees, and *H. parviporum* grew faster (Vasiliauskas and Stenlid 1998). In stumps of *P. abies*, *H. parviporum* even outcompeted *H. annosum* (Oliva *et al.* 2011). Meanwhile on *P. sylvestris* stumps after artificial inoculation, heartwood colonization by both fungi was unsuccesful and in sapwood only development of *H. annosum* was observed (Oliva *et al*. 2013). Results presented in **Paper I** and Swedjemark *et al*. (1999) provide additional evidence that the spread of both pathogens, but especially *H. parviporum*, might be related to the presence of resin of wood and the amount of heartwood. The same pattern (importance of presence of heartwood) has been observed in *P. abies* stumps (Oliva *et al.* 2011; 2013). More recent greenhouse experiments provide evidence that reduced water availability (stressed trees) can increase necrosis length in *P. abies* saplings after inoculation with *H. parviporum* or *H. annosum* and

water deficit, stress especially, enhanced the growth rate of *H. parviporum* (Terhonen *et al.* 2019).

The higher pathogenicity (infection rate and mean longitudinal fungal growth rate) of *H. annosum* (**Paper I**) in *P. sylvestris* might be related to enzymatic activity. *H. annosum* produces five to six times more laccase than *H. parviporum*, increasing its wood degrading ability (Daniel *et al*. 1998; Asiegbu *et al*. 2005). The higher virulence of *H. annousm* might result in wider distribution even in *P. abies* stands, for example, the proportion of *H. annosum* in spruce stands (up to 10% admixture with *P. sylvestris*) is approximately 30% in Lithuania and Belorussia, while no *H. parviporum* was found in *P. sylvestris* stands (Korhonen *et al.* 1992).

Only one isolate per pathogen species was used in **Paper I**. In Sweden, 11 isolates of *H. parviporum* were used for inoculation experiments in *P. abies*, and the obtained results proved that fungal growth of different *H. parviporum* isolates were similar (Swedjemark and Stenlid 1997). Therefore, it is possible to use one or few fungal isolates for inoculation experiments (Swedjemark and Stenlid 1997). The results of **Paper I** confirm that sapwood of *P. abies* is highly susceptible to both pathogens and wounds may be an important role of infection also in saplings as has been reported in this thesis for mature trees (**Paper III**).

The variation of pathogen growth within sapwood was analyzed in **Paper I** and results revealed that there are differences within-host species (among individual plants). This result provides confidence that *Heterobasidion* resistant *P. sylvestris* clones can be identified, as has been previously worked with *P. abies* in Sweden (Swedjemark and Karlsson 2006, and references therein). Significant differences in pathogen growth rate in different *P. abies* clones has been observed after atrifical inoculation of branches of 15-year-old Norway spruce (Swedjemark and Karlsson 2006). Moreover, in more recent research significant variation was observed in *Heterobasidion parviporum* lesion lengths in the inner bark both between *P. abies* families and between clones within families – 21% of this variation can be assigned to genotypic variation (Skrøppa *et al*. 2014). Other factors, for example plant condition and abiotic stress (Terhonen *et al.* 2019) also can influence fungal development. Identification of *Heterobasidion*-resistant genotypes of *P. sylvestris* in field conditions might take a long time. Recent works on *P. sylvestris* proved genotypic variation of susceptibility against *H. annosum* and in future use of selected *P. sylvestris* families may increase resistance against *Heterobasidion* root rot (Marčiulynas *et al.* 2019).

4.1.2. Growth of *Heterobasidion* and *P. gigantea* in cut wood of seven conifer species from the genera *Picea*, *Pinus*, *Pseudotsuga*, and *Larix* (Paper II)

All analysed tree species were susceptible to *H. parviporum* and *H. annosum* (**Paper II**), whereas *P. gigantea* isolates did not colonize *P. menziesii.* The obtained data is in agreement with Thomsen and Jacobsen (2003); in they work weak growth of *P. gigantea* on wood discs of *P. menziesii* was reported. Our research reveals that biological control agents based on *P. gigantea* might be ineffective for *P. menziesii* and other stump treatment methods should be considered. The growth rates of *H. parviporum* and *H. annosum* in wood of *L. sibirica* and *P. menziesii* were comparable to each other, but significantly slower than in other trees. Information about the development of *P. gigantea* in *L. sibirica* wood is limited (Woodward *et al.* 1998; Gonthier and Thor 2013). Previously Rishbeth (1963) reported that *P. gigantea* could not protect *Larix decidua* Miller. from *Heterobasidion* infection. The growth rate of *P. gigantea* described in **Paper II** was very low in *L. sibirica*. The efficacy of biological control agents is based on their growth rate and ability to colonize stump surfaces before *Heterobasidion* spp. (Pettersson *et al.* 2003; Sun 2009), therefore only fast-growing *P. gigantea* isolates could be used. In **Paper II** the growth rate of local Latvian strains of *P. gigantea* and Rotstop® were comparable and both local strains of *P. gigantea* and biocontrol Rotstop® can be equally effective as biocontrol agents against *Heterobasidion*. The comparable efficacy of local Latvian *P. gigantea* isolates and Rotstop® in *P. abies* has been published previously (Kenigsvalde *et al.* 2016).

Paper II revealed that the growth rate of *P. gigantea* isolates was dependent on tree species. Surprisingly, in one *P. abies* stem we did not observe *P. gigantea* development. Our data prooved that the individual tree properties may significantly affect the results of infection experiments, and differences between pathogen development in different *P. abies* individuals has been observed previously (Sun *et al.* 2009). In **Paper II** observed growth rates of *P. gigantea* mycelium in *P. abies* wood were comparable with growth rates observed in previous studies (Korhonen *et al.* 1994; Sun *et al.* 2009). As spruce is one of the economically more important tree species, more research is needed to obtain the best stump protection method or to identify spruce reproductive material with low susceptibility against *Heterobasidion* infection. We observed a higher growth rate of *P. gigantea* in *P. sylvestris* wood in comparison to *P. abies* and this gives advantages for the use of *P. gigantea* as a biocontrol agent in *P. sylvestris* compared to *P. abies* (**Paper II**). These results confirm that the primary hosts for *P. gigantea* are pine species (Tubby *et al.* 2008).

In six out of seven analyzed tree species, the relative area occupied by *P. gigantea* isolates did not exceed 5%, whereas in *P. contorta* stems, the fungus occupied a larger area (13%, on average over all *P. gigantea* isolates) (**Paper II**), indicating that stump treatment with biological control agents containing spores of *P. gigantea* might be more effective in *P. contorta*. In **Paper II** *P. gigantea* mainly colonized the sapwood of *P. contorta*. The proportion of sapwood differed between species, as described in **Paper II**. Previous research indicates the *H. annosum* also prefer sapwood in *P. sylvestris* stumps (Oliva *et al.* 2013). An obvious reason for this is the high concentration of resin acids in *P. sylvestris* heartwood, which might limit the degradative ability of various fungal species (Martínez-Inigo *et al.* 1999).

Paper I revealed that growth rate in functional sapwood of *P. abies* and *P. sylvestris* saplings was significantly dependent on tree species, whereas difference of growth rate of both pathogens in *P. abies* and *P. sylvestris* billets was similar (**Paper II**). The obtained results presented in **Paper I** and **Paper II** demonstrate that there were significant differences (more than sixfold) between average growth rate of both *H. annosum* and *H. parviporum* in *P. abies* and *P. sylvestris* functional sapwood (**Paper I**) in comparison to wood billets (dead sapwood/heartwood) (**Paper II**). Our data confirms Bendz-Hellgren *et al.* (1999) observations – where the growth rate of *Heterobasidion parviporum* in *P. abies* stump roots was more than two times higher than in living tree roots. Differences of *H. annosum* and *H. parviporum* growth rate in functional sapwood and dead wood in both tree species could be explained by active resin flow, and enzymatic activity in different tree species (Daniel *et al.* 1998; Asiegbu *et al.* 2005), especially in *P. sylvestris*, which is characterized by a resinous heartwood (Gonthier and Thor 2013). The data obtained in **Paper I** and **II** indicate that inoculation experiments measuring growth rate of pathogens in seedlings and billets might be contradictory. Temperature during the incubation period in both experiments might significantly affect mycelium growth rate as optimum range from 22 to 28°C (Korhonen and Stenlid 1998). Terhonen *et al.* (2019) also emphasized the importance of experiments on mature trees. Our data indicated that experiments conducted in dead wood cannot draw direct conclusions about the growth rate or host specialization of *Heterobasidion* in living trees but might give valuable information about infection patterns, and this information is crucial when analyzing fungal distribution patterns within a tree or stand, as well developing infection prediction models.

4.2. Fungal infections of stem wounds from the perspective of *Heterobasidion*: *P. abies* (Paper III), *P. sylvestris* (Paper IV) and *P. contorta* (Paper V)

Stem wounding reduces timber quality due to stem deformations and structural defects, and our research also proved that wounds are pathways for fungal infections. Weakened trees may break after windstorms or because of snow pressure (Woodward *et al.* 1998) and trees affected by biotic or abiotic stress are more vulnerable to bark beetle attack (Netherer 2022, and references therein).

The most common stem damages were assessed in *P. abies* (**Paper III**), *P. sylvestris* (**Paper IV**) and *P. contorta* (**Paper V**) stands. In previous years, moose (*Alces alces* L.) and red-deer (*Cervus elaphus* L.) populations have increased (Baumanis 2013). Some tree species such as *P. abies* are highly attractive to deer and are frequently subjected to bark stripping (McLaughlin and Šica 1996). Previous research indicates that *P. abies* stems are vulnerable to fungal attack due wounds made by moose and deer, and that trees are subjected to wounding up to 50 years old (Gill 1992, and references therein; Vasiliauskas *et al.* 1996; Gill *et al.* 2000; Čermák and Strejček 2007). While *P. sylvestris* and *P. contorta* usually are attacked at ages of up to 16 years (Gill 1992). However, in Poland, *P. sylvestris* was subjected to deer attack at the age of 19 years on average (Cukor *et al.* 2022). Bark stripping damages in pine forests in later stages has been rarely observed, and for that reason bark stripping of *Pinus* species has been studied less, resulting in a lack of knowledge about *Pinus* spp. stem wound colonizing fungi. **Paper V** reports that in Latvia, bark stripping damages in *P. contorta* stands occur on average in 20-year old stands and did not cause considerable dieback of trees. Nevertheless, wounded *P. sylvestris* individuals can be found in overmature pine forests. Resin tapping in the Baltic and central Europe region (Poland and eastern part of Germany) played important role for several decades since the 1950's (Rasiņš and Vilsons 1960; Racinskas 1995; Baumanis u.c. 2014; van der Maaten *et al.* 2017, and references therein), and may become a new trend also in the future as currently, harvesting of non-timber products such as bark, leaves and resin represents an important source of income to many people (Ticktin 2004).

Our results from **Paper IV** revealed that resin tapping wounds are approximately nine times larger than bark stripping damages in *P. contorta* and *P. abies* stands and represent the largest possible stem damages during forest management. Resin synthesis is a key defense mechanism against pathogen infestations (Cheng *et al.* 2007). The obtained results in **Paper IV** and **V** showed that both *P. sylvestris* and *P. contorta* are relatively resistant to stem rot causing fungal infections in the exposed sapwood of a living tree, and the obtained results are in agreement with previous publications (Allen and White 1997; Gill *et al.* 2000; Cukor *et al.*

2022). Meanwhile in **Paper III** 26.7% of *P. abies* stems were discoloured and decayed, several basidiomycetes, like, (*Amylostereum areolatum* (Chaillet ex Fr.) Boidin, *Stereum sanguinolentum* (Alb. & Schwein.) Fr. and *H. parviporum*) were isolated from analysed *P. abies* stems (**Paper III**), which can colonize open stem wounds and later cause stem decay, and as a result significantly decrease wood quality (Vasaitis 2013).

According to the prediction model – based on 40 sampled *P. sylvestris* individuals, the spread of stem rot reaches 0.9 cm yr^{-1}, but rot (fungal species was not detected) did not spread more than 50 cm in stems (Cukor *et al*. 2022). This is in agreement with our results in **Paper V**, where in wounded *P. contorta* stems, the longitudinal spread of the discoloration in approx. 13 years did not exceed 10 cm above and 20 cm below the wound margins (growth rate of fungi from inoculation point was on average 0.8 cm and 1.5 cm per year, accordingly and 2.3 cm in total). Long-term fungal growth in *P. sylvestris* may be restricted, as shown by our results in **Paper IV**, where discoloration was restricted (max. 3 cm in radial and 7 cm in vertical directions) even in 50-year old open wounds. Fungal species composition may affect length of discoloration, for example, *A. areolatum* in *P. abies* stems may spread on average 28 cm (Vasiliauskas 1999), while the rate of vertical spread of decay by *S. sanguinolentum* in *P. abies* reaches 19.5 cm per year (Čermák and Strejček 2007).

In **Paper IV** and **V**, *Sarea difformis* Fr. was commonly isolated from *P. contorta* and *P. sylvestris* wounds; this fungus is not typically associated with stem decay, but with resin pockets. In both **Paper IV** and **V**, *Porodaedalea pini* (Brot.) Murrill was observed in *P. contorta* and *P. sylvestris*. *P. pini* has been characterized as indicator species for old woodland sites (Nitare 2000). This fungal species is not dependent on mechanical injury and characterized as the cause of latent infections through natural entries such as dead twigs (Vasaitis 2013, and references therein).

In **Paper III** the most common fungus isolated from bark stripping wounds of *P. abies* was the ascomycete *Neonectria fuckeliana* (C. Booth) Castl. & Rossman, which causes canker and has become an increasing problem in *P. abies* stands in Nordic countries (Pettersson *et al.* 2018), and also results in disease syptoms in *P. abies* after artificial inoculation in Latvia (Kļaviņa, unpublished data). *Heterobasidion* species have been detected in *P. abies* stem wounds (**Paper III**), which gives additional evidence that *P. abies* stem wounds serve as a route for the spread of *Heterobasidion* infections in stands.

The effect on resin tapping on radial increment was analyzed in **Paper IV**. Zevgolis *et al.* (2022) reported that resin tapping reduces the average annual growth of *Pinus brutia* Ten., however the results indicated that the number of tapping scars on the stems affected the results. Our data indicated that resin tapping altered the radial growth of *P. sylvestris* stems at the

beginning, as reported in van der Maaten *et al.* (2017). An additional study revealed that stem damage on the circular perimeter had no significant effect on wood production, and the effect on tree height was negligible (Cukor *et al.* 2022).

4.3. Spread patterns and clonality of *Heterobasidion* spp. in *P. contorta* plantations (Paper VI)

The main goal of growing *P. contorta* in Latvia is to achieve high timber production, developing an alternative to *P. sylvestris* (Sisenis 2013). However, data about *Heterobasidion* infection of this tree species in Nordic Europe is limited (Piri 1996; Rönnberg and Svensson 2013). Results of the previous publications and other literature resources suggested that pines could be better alternative than *P. abies* in *Heterobasidion* infected stand regeneration (**Paper I**, **II**, **V**, **IV**; Piri and Korhonen 2008). In **Paper V** one of the most detailed data on the spread of *H. annosum* and *H. parviporum* in *P. contorta* stands has been obtained.

In **Paper VI**, *Heterobasidion* infection frequency in *P. contorta* stands varied from 9 to 19% although numbers of symptomatic trees were considerably higher and varied from 11 to 50%. Moreover, symptoms developed within a short time; four years after removal of all symptomatic trees – 120 (11%) of the remaining *P. contorta* individuals became symptomatic. High infection frequency may be explained by both the fast growth and development of *P. contorta* and the large number of infected previous generation stumps. In **Paper VI**, only trees with symptoms were sampled, therefore we cannot exclude that some trees may be infected but were asymptomatic. In previous studies in Sweden it was proved that 36-year-old *P. sylvestris* stand crown vitality might not a reliable *Heterobasidion* infection indicator as symptoms were not observed but infection frequency in root systems reached more than 85% (Wang *et al*. 2014). To estimate the real infection frequency in **Paper VI**, more detailed sampling is required, for example, root sampling. Moreover, in **Paper VI** *Heterobasidion* sporocarps were absent in a significant proportion of symptomatic trees, and we cannot exclude the possibility that there may be more than one factor causing tree dieback. For example, in **Paper VI** in stands established on former agricultural lands, more than half of the sampled trees were windthrown. Therefore, weak root systems indicate that trees could be damaged due to infection by *Heterobasidion* spp. (Wang *et al*. 2014).

Our data in plantation established on agricultural land proved that *Heterobasidion* infection can establish and develop in infection free *P. contorta* stands. *P. contorta* stumps are susceptible to infection by basidiospores of both *Heterobasidion* species, followed by development of the pathogens in root systems and establishment of pathogen territorial clones. Additionally, in **Paper VI** stands on former forest lands were analyzed; occurrence of

Heterobasidion annosum in 26-year-old previous-generation *P. sylvestris* stumps was observed. Obtained results in **Paper VI** confirmed that in the Baltic region, viable *Heterobasidion* mycelium can persist in stumps for more than 25 years. Persistence of *H. annosum* mycelium in *P. sylvestris* stumps reach up to 62 years (Greig and Pratt 1976).

Our data in **Paper VI** demonstrated the transfer of *H. annosum* from the previous generation of infected *P. sylvestris* to replanted *P. contorta*. Only a few previous reports describe the intraspecific transfer of *Heterobasidion* species between tree species. In previous publications where intraspecific transfer was analysed the maximal width of territorial clones reached up to 48 m (Vasiliauskas and Stenlid 1998; Lygis *et al.* 2004a). Previously territorial clones and relevant population structures of *Heterobasidion* were mainly analysed in *P. abies* stands (Stenlid 1985; Gonthier and Thor 2013). In **Paper VI**, in stands established on forest land, large territorial clones of *H. annosum* were detected, with a diameter of up to 30–40 m. This demonstrated the high frequency of secondary infections (via root contacts) of the pathogen, that occurred over the 30 years since the previous generation of *P. sylvestris* was felled. As mentioned before, data analysis in **Paper VI** revealed that stumps and living *P. contorta* are highly susceptible to both *Heterobasidion* species, however *H. parviporum* territorial clones expanded more slowly than *H. annosum* territorial clones. Higher growth rate of *H. annosum* in pines has been observed previously (Swedjemark *et al.* 1999; **Paper I)**. In general, *H. annosum* is better adapted to *P. sylvestris*, but is capable of infecting other species as well (Asiegbu *et al.* 2005).

The expansion rate of *H. annosum* and *H. parviporum* observed (on average 0.2 m), (**Paper VI**) in plantation established on agricultural land is comparable to growth in roots of cut *P. abies* stumps and in roots of living trees (Bendz-Hellgren *et al.* 1999). The average expansion rate of the *H. annosum* in forest sites was 0.9 ± 0.6 m, and maximal yearly expansion rate was 3.5 m, therefore high planting densities in stands and fast development of *P. contorta* could have enhanced spread of *Heterobasidion*.

4.4. The potential of *P. gigantea* and urea to prevent primary infections by *Heterobasidion* (Paper VII, VIII)

In many European countries solutions containing *P. gigantea* mycelium and spores are widely used as *Heterobasidion* spp. biocontrol agents (Holdenrieder and Greig 1998). The best known in Northen Europe and also widely used in Latvia is the Verdera produced Rotstop®. However, there might be several factors may serve as excuses to avoid stump treatment:

a) concerns about widely distributing a single isolate distribution in natural ecosystems and the possibility that this isolate could outcompete local fungal societies

(Vasiliauskas *et al.* 2005);

b) the hypothesis that natural colonization of stumps by *P. gigantea* might restrict infections by the pathogen (Holdenrieder and Greig 1998);
c) variable efficacy of *P. gigantea* (Piri *et al.* 2023);
d) increased price for forest management.

Two publications, **Paper VII** and **VIII**, evaluated various possibilities for limitation of *Heterobasidion* basidiospore infection in conifer stands.

Primary infections through surfaces of freshly cut stumps are the main route for *Heterobasidion* infection in current and the next rotations (Piri 1996; Gonthier and Thor 2013). The basidiospores may infect stumps for only a limited period (2–4 weeks), and after that the possibility of infection is negligible (Redfern and Stenlid 1998). In Latvia stump treatment is not obligatory after tree cutting. However, since 2007 in state-owned forests stump protection procedures using Rotstop® (after thinning) has been implemented (Kenigsvalde *et al.* 2011).

Rotstop® preparation consists of *P. gigantea* strain (Pratt *et al.* 2000). Fungus *P. gigantea* is natural also to Latvian conditions. Hypothetically, natural colonization of stumps by *P. gigantea* might affect distribution of primary infections by *Heterobasidion* spp. (Holdenrieder and Greig 1998, and references therein; Kenigsvalde *et al*. 2016). However, detailed systematic studies in this respect are scarce. The main aim of the **Paper VII** was to prove that natural infections of *P. gigantea* do not restrict infection and spread of *Heterobasidion* spp. in *P. abies* and *P. sylvestris* stumps.

4.4.1. Natural stump colonization by "wild" *P. gigantea* strains (Paper VII)

Colonization by native *P. gigantea* was observed in **Paper VII** and **VIII**. However, *P. gigantea* colonization success of *P. abies* stumps in both studies was negligible, but more than 70% of *P. sylvestris* stumps were colonized (**Paper VII**). Results of our study (**Paper VII**) proved that the mean diameter of *P. sylvestris* stumps could affect the occurrence of *P. gigantea* and *Heterobasidion* spp., meanwhile in *P. abies* stumps significant correlation was observed only for *Heterobasidion* spp. Similar pattern was observed in related work in Latvia in understory *P. abies* stand (Gaitnieks *et al.* 2019). Our data confirmed that co-occurrence and interactions of both *P. gigantea* and *Heterobasidion* spp. in the same niches is not uncommon. In **Paper VII**, 4% and 12% of untreated *P. abies* and *P. sylvestris* (respectively) were colonized by both *P. gigantea* and *Heterobasidion* spp., however in **Paper VIII**, 37% of untreated *P. abies* were colonized by both fungi. In previous work in Latvia (Kenigsvalde *et al.* 2016) similar frequencies were observed – 37% of *P. abies* stumps and 12% of *P. sylvestris* stumps were inhabited by both fungi.

Kenigsvalde *et al.* (2016) proved, that *Heterobasidion* spp. infection in untreated spruce stumps might be affected by local *P. gigantea*, and that if *P. gigantea* occupied more than 10% of the stump cross section, then infection of *Heterobasidion* spp. decreased. However, Blomquist *et al.* (2023) observed, that in practice *P. abies* stump treatment coverage with Rotstop® should reach at least 85% to successful reduce *Heterobasidion* infection. Yet, our data in **Paper VII**, **VIII** demonstrated that colonization by natural antagonistic fungi did not show significant effect or strong correlation with the colonized area of *Heterobasidion* in *P. sylvestris* and *P. abies* stumps, which could be explained by the relatively low area of stump coverage by *P. gigantea*. In addition, Tubby *et al.* (2008) reported that full stump surface coverage of the biological control agent containing *P. gigantea* (mycelium and spores) is mandatory to ensure protection of *Pinus nigra* Arn. ssp. Laricio stumps. Incomplete biological control agent coverage allows infection by *Heterobasidion* spp. **Paper II** proved that *P. sylvestris*, and especially *P. contorta* wood ensures relatively fast development of *P. gigantea*. However, **Paper VI** demonstrated high natural infection by both *Heterobasidion* species in untreated *P. contorta* stumps. If trees are cut at temperatures below +5°C, then the risk for primary (airborne spore) infections is minimal, but the risk increases significantly if cutting is done during the vegetation period, from April to October (Donis *et al.* 2014).

However, the results of **Paper VII** demonstrated differences in the ability of the *Heterobasidion* spp. to colonize stumps of *P. abies* and *P. sylvestris*. More than 40% of *P. abies* stumps were infected in comparison with *P. sylvestris* stumps, where only 14.5% of were infected. Studies carried out in Sweden had also show that under natural conditions spruce stumps are more often colonized by *Heterobasidion* than by *P. gigantea* (Vasiliauskas *et al.* 2004). Annesi *et al.* (2005) observations in *P. pinea* are agreement with our results (**Paper VII**) in *P. sylvestris* and *P. abies*, that natural presence of *P. gigantea* was not able to prevent *Heterobasidion* infection.

4.4.2. Comparative efficacy of treatments with Rotstop®, urea, and native *P. gigantea* strains (Paper VIII)

Urea is a chemical alternative to biological control agents (Thor 2003; Blomquist *et al.* 2020) and is registered for use in Finland, the United Kingdom, Denmark, France and Ireland (Korhonen and Holdenrieder 2005).

In **Paper VIII** the efficacy of treatment agents (urea, Rostop® and native Latvian *P. gigantea*) vs. natural *P. gigantea* colonization was compared in *P. abies* stumps on former agriculture land. Assessments of the efficacy of biological control agents in *P. abies* stumps are crucial (Kenigsvalde *et al.* 2016). Additionally, in **Paper VIII** the effect on the stump cover

with wood discs was analyzed. Our data proved that all treatments in uncovered stumps provide similar efficacy against *Heterobasidion* infection, while more than one third of untreated uncovered stumps (left for natural *P. gigantea* colonization) were infected. Subsequently, the efficacy against *Heterobasidion* basidiospore infection was evaluated by proportion of relative infected stump surface area and proportion of infected stumps using all treatment methods (Rotstop®, urea and native Latvian *P. gigantea* isolates). This is the first work comparing chemical and biological control agent efficacy in Latvian conditions. *Heterobasidion* spp. was not completely excluded from treated stumps, as more than 13% of uncovered *P. abies* stumps treated with biological control agents were infected by *Heterobasidion* spp. Relatively low effectiveness of biological control agents in preventing *Heterobasidion* infections has been previously observed in fully covered stumps (Berglund and Rönnberg 2004). **Paper VIII** proved that stump cover promoted efficacy of treatment by biological control agents containing *P. gigantea* by up to 90% of total efficacy (based on relative infected area), unfortunatelly in large-scale forestry this option is seemingly impractical. However, stump cover with wood discs had deleterious effect on the efficacy of urea treatment, where covering promoted development of *Heterobasidion* infection. If the stump surface was left uncovered (as normally done in forest management), biological control agents efficacy reached 60%, while the efficacy of urea was only 50% based on the proportion of infected stumps. Efficacy of both biological control agents and urea were 65% efficacy of urea and biological control agents based on the relative *Heterobasidion* infected area, indicating that infection area was not significantly affected by stump treatment (urea, biological control agents), which is in agreement with earlier studies (Nicolotti and Gonthier 2005). Results of **Paper VIII** on the efficacy of a *P. gigantea* native isolate and Rotstop® (based on both infection incidence and colonized stump area) were comparable with previously obtained data in Finland, Sweden and Latvia (Korhonen *et al.* 1994; Thor 2005; Rönnberg *et al.* 2006; Kenigsvalde *et al.* 2016) and both native strains from Latvia and Rotstop® could be used in stump treatment. However, recent studies in spruce stands in Sweden and Finland after precommercial thinning showed that *P. gigantea* performs significantly worse than urea (Blomquist *et al.* 2020; Piri *et al.* 2023). These results may be affected by abiotic factors such as humidity during the treatment period (Tubby *et al.* 2008), growth characteristics of different *P. gigantea* isolates (Kļavina *et al.* 2023), enzymatic activity of the fungi, wood characteristics and richness of the fungal biota (Żółciak *et al.* 2020). Yet, the decisive factor for prevention of *Heterobasidion* infection is stump coverage quality (Blomquist *et al.* 2023). In many other studies, the relationship between stump surface coverage of *P. gigantea* and control efficacy against *Heterobasidion* infection was observed (Korhonen 2003; Berglund and Rönnberg 2004; Tubby *et al.* 2008; Kenigsvalde *et al.* 2016; Blomquist *et*

al. 2023). Kenigsvalde *et al.* (2016) showed that treatment with *P. gigantea* significantly decreased area occupied by *Heterobasidion* and, *vice versa*, high *Heterobasidion* occurrence in stand might affect the efficacy of *P. gigantea* (Berglund and Rönnberg 2004). In **Paper VIII**, in uncovered spruce stumps treated with urea, the distribution of *P. gigantea* mycelium on wood discs was significantly smaller than in untreated controls, that indicates that native *P. gigantea* populations could be affected by urea treatment in the short term. Results obtained earlier showed that the negative effect of urea treatment on fungal diversity decreases in the long term, which is important for sustainable forest management (Varese *et al.* 2003).

To sum up the results, both **Paper VII**, **VIII** indicate the necessity for full coverage of cut *P. abies* stumps either with urea or *P. gigantea* to prevent infection. Moreover, in the subsequent experiments, it was shown that *P. gigantea* has the potential to limit *Heterobasidion* infection deeper in the wood, in that way reducing spread of *Heterobasidion* via root contacts (Bruna *et al.* 2020) and pathogen longevity.

4.5. The potential of stump removal from infested sites to restrict probability of secondary infections by *Heterobasidion* to the next forest generation (Paper IX)

Wood biomass is one of the most widely used, reliable and renewable fuels and its importance is increasing in the context of emission goals set by the European Union (Jansons *et al.* 2013). As previous studies have shown, *P. abies* stumps, due to their shallow root system (Štofko and Kodrík 2008), are a potential bioenergy resource in Baltics (Uri *et al.* 2015). Moreover, stump extraction has been suggested as an effective method to control root rot diseases (Vasaitis and Stenlid 2008; Cleary *et al.* 2013; Gonthier and Thor 2013). **Paper IX** demonstrated that the frequency of root rot causing fungi *Armellaria borealis* Marxm. & Korhonen and *H. parviporum* decreased (from 11.7% to 3.4%, and from 6.3% to 1%, respectively) six years after stump removal in plots where stump removal was performed. In a related study in Finland, six years after stump removal, 18% of buried root pieces were colonized by *Heterobasidion* (even small roots with a diameter of 1.5 cm), and 8% of *P. abies* saplings were infected (Piri and Hamberg 2015). **Paper IX** and a previous study (Piri and Hamberg 2015) indicated that in heavily infected sites, even after stump removal, viable mycelium can be found in root fragments 6 years after stump removal. In **Paper IX** we observed viable *Heterobasidion* infection in larger roots < 5 cm, implying that smaller roots decompose faster and a proportion of them were completely decomposed. The root pieces analysedin **Paper IX** were half buried which might affect moisture content in wood samples and survival of *Heterobasidion*. Development of *Heterobasidion* spp. is also affected by soil properties and activity of soil fungi and bacteria (Bruna *et al.* 2019).

Although in **Paper IX** we did not obtain evidence that stump removal significantly decreases fungal species diversity, more research is needed to evaluate the impact of stump removal on fungal biodiversity. Also, previous works show that although it is not possible to eliminate root rot causing fungi from stands, stump removal may considerably reduce infection pressure on the subsequent tree generation (Stenlid 1987; Vasaitis *et al.* 2008; Shaw III *et al.* 2012; Cleary *et al.* 2013).

5. CONCLUSIONS

1. *H. annosum* is more pathogenic to *P. sylvestris* than *H. parviporum*, while *P. abies* is equally susceptible to both pathogens, implying higher pathogenicity of *H. annosum.*
2. Although growth rates of both *Heterobasidion* species and *P. gigantea* was highly dependent on tree species, stem-wood colonization of *Picea*, *Pinus* and *Larix* by local (Latvian) strains of *P. gigantea* and biocontrol Rotstop® isolate proceeds at a similar rate; thus, local strains of *P. gigantea* can be effectively used for biocontrol against *Heterobasidion*.
3. *P. abies* stem wounds are susceptible to *H. parviporum*, also to numerous other decay fungi, but conversely, *P. contorta* and *P. sylvestris* stem wounds are relatively resistant to decay causing fungi.
4. Stumps of *P. contorta* are susceptible both to primary (airborne spore) and secondary (mycelial via root contacts to adjacent trees) infections of *Heterobasidion* spp., thus stump treatment is required to control the disease.
5. In *P. sylvestris* and in *P. abies*, natural colonization of stumps by "wild" *P. gigantea* populations does not prevent infections by *Heterobasidion* spp.
6. Stump treatment using urea, biocontrol Rotstop® or a local *P. gigantea* isolate ensures equal *Heterobasidion* control efficacy in *P. abies* stumps on former agricultural land.
7. In *P. abies* stands, stump removal does not eliminate infection potential of *Heterobasidion* from infested sites but may decrease infection frequency.

6. PUBLICATION LIST

1. **Zaluma A.**, Arhipova N., Gaitnieks T., Vasaitis R. 2015. Growth rates of *H. annosum* s.s. and *H. parviporum* in functional sapwood of *P. sylvestris* and *P. abies*. *Forest Pathology*, 45(5): 437–439. DOI: 10.1111/ef*P.*12220.
2. **Zaluma A.**, Bruna L., Klavina D., Burnevica N. Kenigsvalde N., Lazdins A., Gaitnieks T. 2019. Growth of *P. gigantea* in wood of seven conifer species. *Forest Pathology*, 49(6): e12555. DOI: 10.1111/efp.12555.
3. Burņeviča N., Jansons Ā., **Zaļuma A.**, Kļaviņa D., Jansons J., Gaitnieks T. 2016. Fungi inhabiting bark stripping wounds made by large game on stems of *P. abies* (L.) Karst. in Latvia. *Baltic Forestry*, 22(1): 2–7.
4. **Zaluma A.**, Strike Z., Rieksts-Riekstiņš R., Gaitnieks T., Vasaitis R. 2022. Long-term pathological consequences of resin tapping wounds on stems of Scots pine (*P. sylvestris* L.). *Trees*, 36: 1507–1514. DOI: 10.1007/s00468-022-02307-y.
5. Arhipova N., Jansons A., **Zaluma A.**, Gaitnieks T., Vasaitis R. 2015. Bark stripping of *Pinus contorta* caused by moose and deer: wounding patterns, discoloration of wood, and associated fungi. *Canadian Journal of Forest Research*, 45(10): 1434–1438. DOI: 10.1139/cjfr-2015-0119.
6. **Zaļuma A.**, Muižnieks I., Gaitnieks T., Burņeviča N., Jansons Ā., Jansons J., Stenlid J., Vasaitis R. 2019. Infection and spread of root rot caused by *Heterobasidion* spp. in *Pinus contorta* plantations in Northern Europe: three case studies. *Canadian Journal of Forest Research*, 49(8): 969–977. DOI: 10.1139/cjfr-2018-0507.
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8. **Zaluma A.**, Sherwood P., Bruna L., Skola U., Gaitnieks T., Rönnberg J. 2021. Control of *Heterobasidion* in Norway spruce stands: The impact of stump cover on efficacy of urea and *P. gigantea* and implications for forest management. *Forests*, 12, 679. DOI: 10.3390/f12060679.
9. Burņeviča N., **Zaļuma A.**, Kļaviņa D., Brūna L., Legzdiņa L., Gaitnieks T. 2021. Initial and long-term fungal diversity and occurrence of *Heterobasidion* spp. in Norway spruce root fragments remaining in soil after stump extraction. *Scandinavian Journal of Forest Research,* 36(2-3): 117–125. DOI: 10.1080/02827581.2021.1890814.

7. APROBATION

7.1. Publications related to dissertation

1. Bruna L., Klavina D., **Zaluma A.**, Kenigsvalde K., Burņeviča N., Nikolajeva V., Gaitnieks T., Piri T. 2020. Efficacy of *P. gigantea* against *Heterobasidion* conidiospore and basidiospore infection in spruce wood. *iForest*, 13: 369–375. DOI: 10.3832/ifor3279-013.
2. Bruna L., Lione G., Kenigsvalde K., Burnevica N., **Zaluma A.**, Klavina D., Gaitnieks T., Gonthier P. 2021. Inferences on the susceptibility of wood of different tree species to *H. annosum* sensu lato primary infections and on the range of pathogen spores dispersal. *Forests*, 12(7), 854.
3. Brūna L., Kļaviņa D., Korhonen K., **Zaļuma A.**, Burņeviča N., Gaitnieks T. 2020. Effect of soil properties on the spread of *Heterobasidion* root rot. *Proceedings of the Latvian Academy of Sciences, Section B: Natural, Exact, and Applied Sciences*, 73(6): 466–471. DOI: 10.2478/prolas-2019-0072.
4. Burņeviča N., Kļaviņa D., Lione G., Pellicciaro M., Silbauma L., **Zaļuma A.**, Nikolajeva V., Gonthier P. 2022. In vitro screening of Latvian isolates of *Bjerkandera adusta* and *Sistotrema brinkmannii* as potential biocontrol agents against *Heterobasidion* root and butt rots. *Baltic Forestry*, 28(1), 637. DOI: 10.46490/BF637.
5. Gaitnieks T., Brauners I., Kenigsvalde K., **Zaļuma A.**, Brūna L., Jansons J., Burņeviča N., Lazdiņš A., Vasaitis R. 2018. Infection of pre-commercially cut stumps of *P. abies* and *P. sylvestris* by *Heterobasidion* spp. – a comparative study. *Silva Fennica*, 52(1): 1–7. DOI: 10.14214/sf.9911.
6. Gaitnieks T., Bruna L., **Zaluma A.**, Burnevica N., Klavina D., Legzdina L., Jansons J., Piri T. Development of *Heterobasidion* spp. fruit bodies on decayed *P. abies*. 2021. *Forest Ecology and Management*, 482, 118835.
7. Gaitnieks T., Silbauma L., Muižnieks I., **Zaļuma A.**, Kļaviņa D., Burņeviča N., Grosberga M., Lazdiņš A., Piri T. 2022. Spread of *Heterobasidion* genotypes in Norway spruce stands on drained peat soil in Latvia. *Canadian Journal of Forest Research*, 52(4): 499–510. DOI: 10.1139/cjfr-2021-0309.
8. Gaitnieks T., **Zaļuma A.**, Kenigsvalde K., Klavina D., Brauners I., Piri T. 2019. Susceptibility of small-diameter Norway spruce understory stumps to *Heterobasidion* spore infection. *Forests*, 10(6), 521. DOI: 10.3390/f10060521.
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7.2. Book chapter

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