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The pathogenicity of the blue stain fungus *Ophiostoma clavatum* in Scots pine seedlings

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Yfirlýsing höfundar

Hér með lýsi ég því yfir að ritgerð þessi er byggð á mínum eigin athugunum, er samin af mér og að hún hefur hvorki að hluta né í heild verið lögð fram áður til hærri prófgráðu.

Pórhildur Ísberg

Abstract

In this thesis the pathogenic potential of the blue stain fungus *Ophiostoma clavatum* in Scots pine (*Pinus sylvestris*) seedlings was explored. Following climate change, in recent decades, an increased severity of the bark beetle *Ips acuminatus* attacks on Scots pine has been observed, both in Finland and in the alpine regions of Europe. *I. acuminatus* vectors *O. clavatum* and these attacks have led to increased tree mortality.

This research was designed to determine if *O. clavatum* alone had pathogenic potential and if it would be a contributor to Scots pine seedling mortality. The method used was to divide 90 Scots pine seedlings into: i) a control group, ii) a mock-inoculated control group, and iii) an inoculated group. The seedlings were kept in an incubator room at stable conditions so nothing bothered them apart from the infection and the mechanical wounding. The seedlings were then observed for 8 weeks before various growth components were measured in addition to being inspected for *O. clavatum* infection, and a visual infection class was given to those infected.

The result was that there was no significant difference in the growth patterns between the groups, except in reduced total dry weight of the inoculated group compared the untreated control group, when small differences in initial seedling size had been accounted for. The main conclusion is, therefore, that *O. clavatum* does not have much significant pathogenic potential on its own on Scots pine seedlings. The harm observed in nature is most likely a combination of climate conditions and the intensity of the bark beetle attacks, with the blue stain infection assisting in the downfall rather than being the causal agent.

Keywords: Bark beetle, *Ips acuminatus*, *Pinus sylvestris*, boreal forest, climate change, forest health.

Ágrip

[Sýkingarmætti grágeitarsveppsins *Ophiostoma clavatum* í fræplöntum skógarfuru]

Í þessari rannsókn var sýkingarmætti grágeitarsveppsins *Ophiostoma clavatum* í fræplöntum skógarfuru kannað. Í kjölfar loftlagsbreytinga undanfarinna áratuga hefur árásarharka barkarbjöllumnar *Ips acuminatus* gagnvart skógarfuru aukist töluvert í Finnlandi sem og í fjallahéruðum Evrópu. Þessi aukna harka hefur orsakað meiri trjádauða en vant er. *I. acuminatus* ber smit af *O. clavatum* á milli trjáa.

Rannsóknin var hönnuð með það fyrir augum að kanna hvert sýkingarmætti *O. clavatum* væri og hvort smitið eitt og sér gæti orsakað trjádauða. Við framkvæmdina voru 90 fræplöntum skipt í viðmiðunarhóp, viðmiðunarhóp með gervismiti, og smithóp. Fræplönturnar voru hafðar í gróðurherbergi þar sem umhverfisaðstæður voru stöðugar og ekkert angraði plönturnar annað en gervismitið og raunsmitið. Fylgst var með plöntunum í átta vikur og í lok þess tímabils voru ýmsar mælingar framkvæmdar sem og smit kannað og smiteinkunn gefin.

Helstu niðurstöður voru þær að engin marktækur munur var á milli hópa nema hvað viðkom lægri þurrvigt smitaða hópsins og viðmiðunarhópsins án gervismits þegar munur milli upphafshæðar var tekinn með í reikninginn. Það má því ganga út frá því að sýkingarmætti *O. clavatum* sé lítið sem ekkert í fræplöntum skógarfuru. Sá skaði sem sýkt tré verða fyrir í náttúrunni er líklegast afleiðing samverkandi þátta veðurfars og annarra umhverfisaðstæðna sem og aukin harka í árásum barkarbjallnanna sem bera smit í tréð sem ásamt öðrum þáttum veikir tréð og getur jafnvel valdið dauða þess.

Lykilorð: Barkarbjöllur, *Ips acuminatus*, *Pinus sylvestris*, barrskógar, loftslagsbreytingar, heilbrigði skóga.

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1. Introduction

1.1 Scots pine

In the boreal forests the Scots pine (*Pinus sylvestris*) is the dominant species. It grows in large swaths of forests across Eurasia (Houston Durrant, de Rigo, and Caudullo 2016; Rehfeldt et al. 2002) (Figure 1), where it has both an economic and an ecological importance (Gauthier et al. 2015; Houston Durrant et al. 2016). In Fennoscandia (Finland, Sweden, Norway, and Kola peninsula and Karelia of Russia), Scots pine is planted and harvested in large industrial scale for timber, pulp, and paper (Houston Durrant et al. 2016), but at the same time Scots pine forests offer ecological services in form of regulating water and nutrients as well as sequestering carbon (Gauthier et al. 2015). Maintaining the health of the forest is, therefore, important so it may continue to provide its ecological services as well as holding its economic value.



Figure 1: Natural range and distribution (green), and introduced and naturalised area (tan) of Scots pine in Eurasia (Caudullo et al., 2018).

Scots pine is both frost and drought tolerant, and is not demanding in regards to soil and can, therefore, grow in even very poor soils. Consequently, Scots pine can be found in ecologically varied habitats (Houston Durrant et al. 2016). Moreover, within the species there are numerous provenances and sub-species adapted to the local climate (Rehfeldt et al. 2002).

In addition to climatic adaptation, pines are physically protected against pests, such as wood boring insects, with thick bark forming a barrier between the living tissues of the tree and

the offending organism. This is the tree's primary defence. In case of penetration, through wounding or lenticels for example, secondary defence mechanisms can be activated. Scots pine, like most plants, have the ability to mobilise chemicals within their systems to counteract infections by various microbes. Also, when attacked by bark beetles, and other wood boring insect, traumatic resin ducts are formed to produce mass defence compounds. Finally, resin flow will be able to seal the wound to allow the tree to repair the damage (Franceschi et al. 2005). These attributes make the Scots pine such a successful species in the boreal forests, and gives them a powerful protection against a wide range of pests and pathogens.

Trees are long living sessile organisms; this can be a disadvantage when it comes to rapid adaptation. A sudden change in the environmental conditions puts the trees under stress. Conifers are, however, resilient and very successful plants. Their defence mechanisms against any sort of environmental aggression has ensured this success (Franceschi et al. 2005). If there is a gradual change in the environment one assumes that the generations can adapt, through natural selection, as evolution slowly mends the gap between the population fitness and new challenges in the environment (Fridley and Sax 2014).

Local adaptations of tree populations, for example, to climate or parasites, occurs, and the local provenances have the highest relative fitness in regards to the environment in their place of origin (Savolainen, Pyhäjärvi, and Knürr 2007). Hence, in Finland, Scots pine provenances have adapted to their environment and the climate conditions of their original habitat. This has been shown with research in a variation of bud set and their frost hardiness according to latitude (Hurme et al. 2011). Indeed, Scots pine grows in all vegetation zones in Finland, in peaty forests, on rocky ground, and in swamps (Ursing and Wanntorp 2005). Research on provenances elsewhere from Europe (Bulgaria, France, Germany, Italy, Poland, and Spain) show provenance-specific differences in drought resistance and water stress sensitivity in relation to summer precipitation rates at their place of origin (Seidel et al. 2016).

From the above it is clear that adaptation to local conditions occurs between Scots pine populations, and a constant gradual shift in the environment will direct the genetic adjustment of the pine populations towards the new climate environment, through the common evolutionary processes, such as natural selection, mutation, and drift. If, however,

the change in their environment is sudden the fitness of the population will be altered due to their sessile existence and inability to simply uproot themselves and migrate to a more favourable environment (Rehfeldt et al. 2002). Hence, even Scots pine populations, which are more drought adapted, like provenances growing in the Mediterranean area (Seidel et al. 2016), are seeing higher mortality rate due to unusually dry and warm summers following climate change (Allen et al. 2010). This indicates that the change is too rapid for the populations to adapt via the normal evolutionary processes.

1.2 Recent climate changes

In recent decades, the changing climatic conditions have been more rapid than previously in the Holocene, with warming of the atmosphere at the forefront. This is widely recognised as being the result of increased greenhouse gasses (GHGs) in the atmosphere, which can mostly be attributed to anthropogenic activity. Calculations show a linear increase of mean annual temperature (MAT) from 1880 to 2012 to have been 0.85°C. The warming has not been regular and the temperature rise between 1983 and 2012 was the most significant 30-year period of warming in the Northern Hemisphere (IPCC 2014).

Also in Finland, the global increase in MAT due to increasing GHGs will be reflected in the local climate (Jylhä et al. 2009; Ruosteenoja, Jylhä, and Kämäräinen 2016; Venäläinen et al. 2020), and indeed it has already. In the time period of 1847 to 2013, the MAT in Finland has risen above 2°C. This averages at 0.14°C per decade. The warming is not evenly divided. The winter months (Nov-Jan) show most warming, followed by the spring months (March-May) (Mikkonen et al. 2015). In future projections, warming during the winter period is estimated to be between 3° and 9°C in Finland. In contrast, summer temperatures are estimated to increase by approximately 1° to 5°C. Both these projections are compared to mean temperatures of the period 1971-2000 (Jylhä et al. 2009).

Atmospheric warming will bring about more change to climate than just temperature rise as the change in temperature will also affect evaporation, precipitation, wind, and snowmelt. All these factors will have an impact on the ecosystems and their functions. Higher temperatures in winter will mean that less snow will fall and the precipitation will be in the form of rain instead, as well as the ground will not remain frozen during winters (Jylhä et al. 2009; Venäläinen et al. 2020). It can be assumed that less snow in winter will decrease the snowmelt in spring, further limiting water availability.

1.2.1 Impact on forest ecosystems globally

The forest ecosystems of the world have started to feel the impact of the hazards introduced by the changing conditions. Climate change has altered the frequency, intensity, and timing of many disturbance events in the forest ecosystems (Seidl et al. 2017). Warmer and drier conditions will facilitate fire, drought, and insect disturbances, while the warmer, and sometimes wetter, conditions increase disturbances from wind, and higher humidity will facilitate pathogens (Seidl et al. 2017). Indeed, summer temperatures have been on the rise in Europe in the last 30 years (Jylhä et al. 2009). The higher temperatures have caused longer periods of drought, which in turn have resulted in a decline in the health of forests particularly in southern Europe (Chinellato et al. 2014), and where the southern limits of Scots pine's natural range are, increased tree mortality has been observed since the 1990's due to warmer and dryer conditions (Allen et al. 2010).

It is also worth considering that trees are often key species in terrestrial ecosystems. The impact of climate change resulting in tree mortality can, therefore, have a dramatic impact on the forest ecosystems when the lifecycle of the tree is cut short because they are often long living organisms (Alberto et al. 2013). Furthermore, if the forests are damaged by more frequent disturbances, then the ecosystem services they provide will also be disrupted. This can apply to services such as climate regulation and timber production (Gauthier et al. 2015), and that would have wider consequences than just for the forest itself.

1.2.2 Impact on forest ecosystems in Fennoscandia

Climate change is likely to affect the forests in Fennoscandia in a slightly different manner than that of the more southern forests. The projected higher temperatures and increased rainfall, instead of snow in winter, is predicted to increase the forest growth in the northern part of Fennoscandia. The same will not happen in the southern parts as there the higher temperatures are predicted to bring more drought due to evaporation (Jylhä et al. 2009; Venäläinen et al. 2020). Increased precipitation in the south will, therefore, not benefit forest species more vulnerable to drought such as Norway spruce (*Picea abies*) (Venäläinen et al. 2020). At the same time as the temperature changes other climatic factors will have an impact on the forest. With less frost in wintertime the stability of trees will be jeopardized as windfall is more likely to occur when the roots are not anchored in a frozen soil during the windier season (Ohrn 2012). In general, wind disturbances have been more important in the

boreal forests of Finland and western Russia, in comparison to the boreal forests of North-America, where fire disturbances have had more impact on the forest dynamics (Gauthier et al. 2015). Furthermore, the higher temperatures in winter and decreased snowfall will result in less melting in spring reducing water availability. However, the decreased snowfall, will on the other hand, diminish tree damage from heavy snow (Kinnunen et al. 2013).

In addition to the abiotic disturbances introduced by climate change, the changing conditions will also affect the interaction between the trees and their pests and pathogens. As the climate warms, conditions change and a geographical shift occurs. Natural range of species changes. For example, in Fennoscandia, moths, such as the winter moth, *Operophtera brumata*, and the autumnal moth, *Epirrita autumnata*, both defoliators of mountain birch (*Betula pubescens*), have reached into new territories. The winter moth has established itself at higher elevation than before, even reaching the treeline in northern Norway, while the autumnal moth is expanding its range further north to new territories (Tobin, Parry, and Aukema 2014).

The natural range of trees will also change, as has already been established. The southernmost limits of Scots pine are already suffering from the dryer hotter summers experienced in Europe in recent years (Allen et al. 2010). Hence, it is safe to assume that the natural range will shift northward.

It is so that the type of distribution of tree populations affect their ability to migrate and adapt to different environments (Alberto et al. 2013). In the short term, the response to changing environment will be reflected in physiological plasticity, that is different phenotypes, but in the long term, evolutionary selection processes will help in adjusting the population to the new environment (Rehfeldt et al. 2002), including both abiotic and biotic factors. Continuous populations, such as the Scots pine, are better able to shift than those with fragmented or isolated populations (Alberto et al. 2013).

Of course, the forests of Fennoscandia are largely managed, and the suitability of the timber trees to their environment is monitored, as well as the general health of the forests. The Finnish Natural Resources Institute, for instance, runs a long-term breeding programme designed to improve the genetic stock of the most important tree species. This includes Scots pine. The goal is to improve the timber product, but also to ensure the trees produced are

adapted to the various sites and climates (Rusanen et al. 2021). This will be useful when the climate has changed so much that the vegetation zones and suitability of habitats for various species have shifted northwards.

1.3 Pathogens in the boreal forest

In the boreal forest, the most common pathogens are fungal pathogens (Desprez-Loustau et al. 2016). The root and butt rot caused by *Heterobasidion* spp. is economically the most important pathogen affecting conifers in European forestry. The disease is white rot, which causes butt and root rot, and the eventual death of its host (Woodward 2010), as it functions as both a necrotrophic and a saprotrophic fungus (Garbelotto and Gonthier 2013). The spores develop in the summer or autumn and spread with air to either exposed and wounded roots or other woody tissues or to stumps of felled trees. The infection can also spread in soil from mycelia growing from infected roots to the root system of a healthy tree, and thereby infecting it (Garbelotto and Gonthier 2013; Zúbrik, Kunca, and Csóka 2013). Once infection has been established at a site, it is almost impossible to eradicate it. Control methods are then the only option (Garbelotto and Gonthier 2013). With warmer winters predicted in Finland (Jylhä et al. 2009), and presumably Fennoscandia at large, the risk is that sporulation could increase infection frequency. This could be a threat to forestry if many mild winters with prolonged periods of temperatures above 5°C become a reality (La Porta et al. 2008), and higher temperatures will increase spore formation in general (Venäläinen et al. 2020). *Heterobasidion* spp. might, therefore, become an even greater threat in the future, considering that forest pathogens have a close relationship with the environmental conditions of their habitat (Sturrock et al. 2011).

Another disease prevalent in the boreal forests in Fennoscandia is *Gremmeniella abietina*, Brunchorstia disease (also known as Scleroderris canker), affecting mostly pine (Venäläinen et al. 2020). It is an airborne disease that can enter the softer tissues of the tree through lesions or other openings on the bark. The symptoms are browning of needles, and then death. This will lead to the whole branch dying off, and if the infection reaches the stem it will form cankerous wounds there (Halldórsson and Sverrisson 2014). *G. abietina* has caused major epidemics in the pine forests of Fennoscandia (Halldórsson and Sverrisson 2014; Venäläinen et al. 2020). However, for the disease to be successful the sporulation must coincide with right moisture content of the atmosphere. Cool and wet springs are more likely to encourage *G. abietina* outbreaks. If climate change will herald dryer and warmer springs

for the future, the prevalence of this disease is likely to diminish (Venäläinen et al. 2020). There are after all two sides to each coin and the shifting in the climate can have both negative and positive effects.

Here, in this thesis, the research involves the blue stain fungi *Ophiostoma clavatum*. Blue stain fungi are not considered serious pathogens in forestry (Lundell et al. 2014), but with the change in climate conditions their vectors, the bark beetles, seem to thrive better than before (Chinellato et al. 2014; Siitonen 2014; Wermelinger et al. 2008). The relationship between the vector, the host, and the pathogen, *O. clavatum*, could be changing (see later).

1.4 Bark beetles in the boreal forest

Bark beetles (Scolytinae) are a versatile family of insects that spend most of their life cycle within their host plant. Scolytinae beetles, both adults and larvae, feed in the phloem of their host plant (Hulcr et al. 2015; Sauvard 2014) and they are especially harmful to conifers (Sauvard 2014), which have significant economic value (Houston Durrant et al. 2016; Linnakoski et al. 2012). Scolytids are amongst the most severe pests causing economic damage in the forest industry (Kirisits 2004). Although severe tree mortality caused by Scolytus beetles are usually bound to small localities and are infrequent, larger outbreaks have been reported. These cases arise usually during environmental stress of some sort, related to climate conditions, disease, or accompanying other insect outbreaks (Smith and Hulcr 2015).

Bark beetles are often associated with various fungi, most often blue stain, as well as, bacteria, mites, and even nematodes, which can increase the harm to the host tree, in addition to the wood boring activities of the beetle itself (Khadempour et al. 2012). Example of these is the European spruce bark beetle, *Ips typographus*, which can kill trees in large numbers, causing both ecological and economical harm (Davydenko, Vasaitis, and Menkis 2017). In fact, it is the most important tree killing bark beetle in Europe (Ohrn 2012; Seidl and Rammer 2017), and it vectors a necrotrophic fungus, *Endoconidiophora polonica*, which is believed to have an important role to play in overcoming the tree's defence systems, during an attack by *I. typographus* bark beetles (Linnakoski et al. 2017).

In the boreal forests of western North-America, the mountain pine beetle, *Dendroctonus ponderosae*, has the ability to cause tremendous loss in forestry during outbreaks (Ohrn

2012). Like its European counterpart the mountain bark beetle vectors fungal infections and is associated with four different species of blue stain fungi (Khadempour et al. 2012). Aggressive bark beetles, such as these two examples, share common features. These are: i) pheromone communication, which they use to attract others of their kind to a successfully penetrated host tree; ii) they are associated with a virulent fungi infecting the phloem and which may block water transport in the xylem; and iii) highly aggressive bark beetles have good resistance against chemical defence reactions of the host, such as resin flow (Ohrn 2012).

In the changing climate, bark beetle interactions with their hosts will change. Warmer climates will give bark beetles the ability to produce more generations per summer increasing the population density (Ohrn 2012; Wermelinger et al. 2008), and this has been the case with *I. typographus* in Scandinavia (Ohrn 2012). The dryer conditions also reduce tree vigour which, in combination of increased bark beetle density, will increase the likelihood of tree mortality (Ohrn 2012; Wermelinger et al. 2008).

In the case of *D. ponderosae* in North-America, the warming climate has seen its geographical range spread, and new species being targeted. Previously, the mountain pine beetle was mostly associated with lodgepole pine (*Pinus contorta*) and ponderosa pine (*P. ponderosa*), but the beetle has been able to migrate east across the Rocky Mountains, and jack pine (*P. banksiana*) has now become a host to the beetle (de la Giroday, Carroll, and Aukema 2012). Climate change can, therefore, cause both increased population size in the natural habitat of the bark beetle, and by sheer numbers they can cause more damage in each favourable season than normally. Furthermore, they are able to migrate to new habitats, where the local ecosystem might not be used to them and ill equipped to counteract the predatory behaviour, or the fungi associated with the bark beetle.

The above examples were about the effects of the most well-known bark beetles in the boreal forests, *I. typographus* and *D. ponderosa*. In this thesis, though, the focus will, however, be on the much less studied *I. acuminatus* and its associated fungi, *O. clavatum* (see later).

1.4.1 *Ips acuminatus*

The higher temperatures and dryer summers in Finland (Jylhä et al. 2009), and in the alpine regions of Europe, have increased the reproduction rates of the previously considered a minor forest pest, the bark beetle *Ips acuminatus*, which has resulted in more aggressive attacks by the beetle on the Scots pine populations (Chinellato et al. 2014; Siitonen 2014; Wermelinger et al. 2008). Accompanying *I. acuminatus*, is the blue stain fungus *Ophiostoma clavatum* (Kirisits 2004; Linnakoski et al., 2012, 2016).

In the last two decades, a number of cases of dead Scots pines containing galleries bored by the bark beetle *I. acuminatus* have been observed in southern Finland (Siitonen 2014). *I. acuminatus* (Figure 2) is mainly found to live on Scots pine (Siitonen 2014), even though the beetle is also associated with other *Pinus* species (Kirisits 2004; Wood and Bright 1992). It has, however, been observed that *Ips* beetles usually favour a particular genus of trees (Cognato 2015) as in the case of *I. acuminatus* and Scots pine.



Figure 2: *Ips acuminatus*. Average length: 3,5mm. (Photo: Maja Jurc, University of Ljubljana, Bugwood.org).

The geographic range of *I. acuminatus* is wide: the beetle is found all over Eurasia (Siitonen 2014; Wood and Bright 1992) and is native also to Finland. The beetle's numbers dwindled, however, in Finland in the 1950's until the observed increase in the 2000's noted from the number of galleries in dead trees. The observed death of seemingly healthy pines infested by *I. acuminatus*, along with follow-up research, led to the conclusion that their death was linked to the increased aggressiveness of the beetle (Siitonen 2014). This means that the beetles have successfully overcome the defence mechanisms, or the pines were already weakened by other environmental stress to be able to fend off the bark beetle attacks. Bark beetles generally require their host to be dead to be able to infest it. They are also able to penetrate the host defences of dying or severely weakened trees (Kirisits 2004; Krokene et al. 2013). But access to the wood or the secondary phloem underneath the bark is essential for the beetles to complete their lifecycle (Kirisits 2004). The combination of increased bark

beetle population size and availability of susceptible hosts, due to favourable temperatures, but increased aggressiveness has been linked to change in climate conditions, seems to have increased tree mortality (Siitonen 2014; Wermelinger et al. 2008).

1.5 Blue stain fungi

In nature, blue stain fungi are mainly found on fallen trunks (Lundell et al., 2014), in sapwood, under the bark, and in tunnels bored by beetles, and even in roots of diseased trees (Seifert et al., 1993). As discussed previously, it is possible that an intensive bark beetle attack causes the death of the tree. However, bark beetles do prefer dead or dying trees (Kirisits 2004; Krokene et al. 2013). Furthermore, felled tree or otherwise dead trees allow exposure to the sapwood. This allows easier access for the blue stain fungi to spread as it cannot penetrate the bark on its own. Exposed sapwood, on the other hand, makes the spread of the blue stain easier once the spores have settled in the wood. Stacked timber is, for example, vulnerable should infection settle in as the fungi can spread rapidly (Millers et al. 2017).

Blue stain colours the sapwood causing discolouration in the sapwood (Lundell et al., 2014; Millers et al., 2017), and is mainly found in conifers, such as Scots pine (Millers et al., 2017). In reality the blue stain fungi rarely causes structural damage to the wood as they are not decomposers and do not affect the lignocellulose of the xylem (Lundell et al., 2014). The discolouration is, though, a cosmetic flaw and harms the market value of the timber (Millers et al., 2017). Additionally, timber infected by blue stain is of lower quality. It absorbs more moisture and tends to rot sooner than uninfected timber (Nordsteien and Skúlason 2007). Proper sanitation is therefore needed if there is a danger of blue stain infection in timber stacks.

1.5.1 *Ophiostoma clavatum*

When *I. acuminatus* attacks Scots pine it is sometimes accompanied by the blue stain fungus *O. clavatum* (Kirisits 2004; Linnakoski et al. 2012, 2016) (Figure 3). *Ophiostoma* fungi are considered the most common bark beetle related fungi in northern Europe (Linnakoski et al. 2012). The genus contains a variety of species, of which more than 130 have been described (Linnakoski et al. 2016).

Fungi of the genus *Ophiostoma* include both saprotrophs and facultative parasites. Usually, as is the case with *O. clavatum*, they are dependent on a bark beetle vector, even though some do appear independently (Kirisits 2013). *Ophiostoma* species can cause wilt diseases or blue-stain (Kirisits 2013). Largely, though, it has been found that many *Ophiostoma* species are not the causal agent, or if any association, the pathogenicity has been proven weak and only contributing in a limited way to tree mortality (Harrington 1993), and indeed the pathogenicity of most blue stain fungi is strongly linked with the aggressiveness of the insect vector (Kirisits 2013), which is then associated with population size and favourable environmental conditions (Franceschi et al. 2005; Siitonen 2014; Wermelinger et al. 2008).



Figure 3: a) *Ophiostoma clavatum* discoloured Scots pine branch, b) entry holes made by *Ips acuminatus*, and c) galleries under the bark. (Photos: Heikki Nuorteva).

The best known of the pathogenic *Ophiostoma* fungi are the Dutch elm disease pathogens (*O. ulmi* and *O. novo-ulmi*), and is one of few of the genus to cause true vascular wilt disease (Kirisits 2013), but for the most part these fungi are not great destructors of forests.

O. clavatum is a newly defined species complex, including the type species of the complex, *O. clavatum*, which previously might have been identified as *O. brunneo-ciliatum*, as they are nearly identical morphologically apart from the shape of the ascospore and conidiomata (Linnakoski et al. 2016). Both of these fungi are vectored by *I. acuminatus* (Kirisits 2004), even though recent studies show stronger association between *O. brunneo-ciliatum* and the bark beetle *I. sexdentatus* (Linnakoski et al. 2016).

Regarding the similarity in morphology, the *Ophiostoma* fungi can be recognised by their sexual spores (ascospores), which are slimy and sit on top of dark stalks (Seifert, Webber, and Windfield 1993). These stalks are the ascomata bearing the disposed asci (Malloch and Blackwell 1993). The difference between the *O. brunneo-ciliatum* and *O. clavatum* comes down to the size of the ascomata and conidiophores, where *O. clavatum* is smaller (Linnakoski et al. 2016). Figure 4 shows *O. clavatum* culture on an agar medium and its distinct conidiophore aggregations, brown submerged hyphae and whitish-grey aerial hyphae.



Figure 4: Typical morphology of *Ophiostoma clavatum* (Photo: ÞÍ).

1.5.2 Interactions between *I. acuminatus*, *O. clavatum*, and Scots pine

The main host of *I. acuminatus* and *O. clavatum* is the Scots pine (Kirisits 2004; Linnakoski et al. 2016). Scots pine, like other conifers, has developed a defence system against bark beetles, which are estimated to have co-evolved for approximately 3 million years (Krokene et al. 2013). The outer most defence is perhaps the most important one, when thinking of bark beetle attack, especially when considering the pathogens that could be introduced through the breached bark and infect the sapwood within (Franceschi et al. 2005), like is the case with *I. acuminatus* and *O. clavatum* (Kirisits 2004; Linnakoski et al. 2012, 2016). The fungal infestation initially brought on by the insect vector is thought to assist in weakening the trees' defences (Lieutier, Yart, and Salle 2009), as it disrupts the hydraulic function of the xylem (Guérard, Dreyer, and Lieutier 2000; Villari et al. 2012). This disruption is evident by the discolouration of needles and eventual needle loss (Wermelinger et al. 2008).

At a certain attack density threshold the trees are overwhelmed and can no longer withstand the attack (Guérard et al. 2000). The weakened state of the host trees enables the beetles to continue their attack with the end result of the tree's death (Guérard et al. 2000; Lieutier et al. 2009). It is the high number of bark beetles, during the epidemic phase, caused by the

environmental conditions discussed earlier, that enable aggressive bark beetle attack to be successful. The reality is, though, that only a small number of bark beetles in northern Europe are aggressive. Most, or about 90%, are non-aggressive (Krokene et al. 2013). However, when they are able to breach the defences of conifers, associated with their mass attacks, instigated by their pheromone coordination (Ohrn 2012), their numbers will overwhelm the tree. The association with pathogenic *Ophiostomatoid* fungi could increase the impact, as well as tree monoculture (Krokene et al. 2013). Homogeneity of the forests such as the single-species plantations of Scots pine, common in Finland and elsewhere where industrial forestry is practiced, will give the bark beetles more habitat options.

In addition to the increased number of beetles attacking the pines, the prolonged heat and drought periods cause stress within the pines and weaken them (Wermelinger et al. 2008). One of the physiological responses to drought is the closing of stomata, which in Scots pine happens early in comparison to many other species in a similar forest ecosystem (Zweifel, Rigling, and Dobbertin 2009), resulting in low transpiration rates and reduced photosynthesis as CO₂ is not getting into the needles and hence less energy available to the tree to drive its physiological functions. Loss of needles due to drought weakens the pines even further (Dobbertin 2005). Studies have also shown significant increase in deaths of live standing Scots pine in the summer following a summer of drought (Dobbertin et al. 2007), showing the long lasting effect of drought on the trees. The combination of restricted water availability and more intense bark beetle attacks will exhaust the defence mechanisms of the pines. Add on the fungal infection and you have a sure recipe resulting in tree mortality.

1.6 Relevance to Iceland

Scots pine is not native to Iceland. The only native coniferous species is the common juniper (*Juniperus communis*), which does not grow into a tree but is a relatively low growing shrub (Kristinsson 2007). Conifers have, however, been planted in Iceland, and of these, the most common species is the Siberian larch (*Larix sibirica*), which has been successfully grown in Iceland, notably in Northeast-Iceland, for a good part of a century. It has particularly thrived inland in Northeast- and East-Iceland where mainland climate is more prevailing than oceanic climate (Blöndal and Gunnarsson 1999; Snorrason and Sigurgeirsson 2006). Other common forestry species in Iceland are the Sitka spruce (*Picea sitchensis*), and of pines, the lodgepole pine (*Pinus contorta*). Both are introduced species from the Pacific coast of North-

America, and have thrived well in the Icelandic climate in recent decades (Snorrason and Sigurgeirsson 2006).

Scots pine has also been planted in Iceland. First in 1903, and was extensively planted in the 1940's and into the 1960's. The Scots pine population was though almost completely destroyed by the pine woolly aphid (*Pineus pini*) soon after, and by the early 1960's, Scots pine planting had ceased. However, new research looking into the generations of Scots pine, grown of seeds of the few that survived the aphid attacks, show a measure of resistance against the pine woolly aphid (Heiðarsson et al. 2020). Clearly, some evolutionary selection has occurred and the Scots pine might, therefore, have a comeback of sorts into Icelandic forestry in the future.

Climate change is likely to have an effect on Icelandic flora and fauna, and therefore, the forest ecosystem, species selection, and the survival of various pests and pathogens. The changes already observed are in line with climate change observed globally (Halldórsson et al. 2013).

The average warming since mid-19th century in Iceland, when systematic weather monitoring began, has been 0.8°C. In the period 1980-2015, a warming of 0.5°C per decade has been observed, with more warming detected in West-, and Northwest-Iceland. Along with the warming, precipitation has increased during the same period (1980-2015) from annual measurement of 1500 ml to 1600-1700 ml (Björnsson et al. 2018).

Since the effects of climate change in Iceland are that of warming and increased precipitation, the likelihood of the emergence of new fungal pathogens to establish increases, but fungal and fungal-like pathogens form the majority of forest diseases (Desprez-Loustau et al. 2016). The ability of fungal disease to spread is a major indicator of how well they can establish themselves (Philibert et al. 2011). For that to happen the environment has to accommodate their requirements of survival, but most plant pathogens have a close relationship with the environmental conditions of their habitat. Abiotic factors such as temperature and precipitation affect host and pathogen interaction in both how susceptible the host is to the pathogen and how successful the pathogen will be in relation to reproduction and infection ability (Sturrock et al. 2011).

Of forest pathogens in Iceland the most common ones are rust fungi (Uredinales) in *Betula*, *Salix*, and other deciduous trees and shrubs. In conifers, the rust fungi *Chrysomyxa abietis* has caused some damage in Norway spruce. In pine, *Gremmeniella abietina*, has become more prevalent, but so far it has not yet become a worrying trend in lodgepole pines (Halldórsson and Sverrisson 2014), which are the most common pine species planted (Snorrason and Sigurgeirsson 2006). Larches have also been extensively planted, but are relatively healthy. They have though been sensitive to diebacks caused by fluctuating winter temperatures and weather conditions that can both cause an untimely flushing and increase their disease susceptibility during summer (Halldórsson and Sverrisson 2014).

Blue stain fungi are not a regular occurrence in Iceland. When *Ophiostoma* fungi are detected, they are usually in timber structures of roofs. The fungal spores have then most likely been imported with the timber as *Ophiostoma* are not found in nature in Iceland. When the roof has started to leak, or carelessness at the building site has allowed moisture in, an *Ophiostoma* growth is triggered (Guðríður Gyða Eyjólfsdóttir, personal communication). Usually, moisture above 20% and temperatures at 5°C or above will spur the growth of the fungi (Nordsteien and Skúlason 2007).

Insect pests in Icelandic forests are few at present (Halldórsson et al. 2013). There are approximately 50 native species, which live partially or entirely on woody vegetation in the country. In the last century around 30 additional species have been introduced to the forest ecosystem in Iceland. Of these, most are moths and Hemiptera, such as aphids. The forest insects are mostly benign, causing limited damage to trees and shrubs, though occasional outbreaks have been documented throughout history. The most impactful aggressors of the forest insects in Iceland are aphids and moth larvae (Halldórsson and Sverrisson 2014).

Bark beetles and other wood boring insects are also sometimes found in imported timber material but traces of them have, on occasion, been found in nature. These are for example *Urocerus gigas* that is a regular find in Iceland (Ólafsson 2013). In 2011, larvae of the giant woodwasp were found in larch trunk that had been felled. The larvae were raised in a cage and all the wasps turned out male. Most likely a lonely female had laid her eggs there (Ólafsson, 2013; Brynja Hrafnkelsdóttir, personal communication). This, however, shows that eggs and larvae of some wood boring insects will survive the winter in Iceland. If, or

rather when, they establish themselves, Iceland might have to prepare for the insect associated diseases as well.

Another example are entry holes in wood (Figure 5), that those with a trained eye can spot now and then in the woods. Perhaps there are somewhere populations of wood boring insects still lurking in isolated vegetation oases that survived the land degradation and the almost complete annihilation of the native forests in the first few centuries of human settlement in Iceland (Blöndal and Gunnarsson 1999). Whatever the reason, there are traces found in nature, and there are likely to be some microscopic stowaways in timber, soil, and plant material imported to the country. As the climate condition continues to change in Iceland, as elsewhere, more insect pests as well as pathogens are likely to find their way to the country and some of them will be able to establish themselves (Björnsson et al. 2018).



Figure 5: Entry holes by unknown beetles in a) spruce at Mógilsá in SW-Iceland, by the coast, and b) in birch in Grímsnes, S-Iceland, inland (Photos: Brynja Hrafnkelsdóttir).

In the future, considering the climate change that has already happened, and is likely to occur, the introduction of new pests and pathogens is a serious risk in Iceland (Björnsson et al. 2018). Pathogens, such as the root rot fungus *Heterobasidion* spp., might find its way to Iceland, and cause serious risk, considering that this is a soilborne disease, which mycelia can live on plant debris in the soil until it finds a more permanent host to infect (Garbelotto and Gonthier 2013).

In Iceland, two types of soil material are imported. Sphagnum or peat moss, used in nurseries, and soil in pots with imported potted plants. Both of these are required to have a phytosanitary certificate (Regulation on importation of plants and plant material, nr.189/1990). There is though always a risk involved, which cannot be overlooked, when importing soil, that microorganisms hitch a ride and get inadvertently introduced into the forest ecosystems in Iceland. Insect pests are also able to, and perhaps more easily, travel to Iceland with imported goods. Awareness of the risk is necessary, and to build a knowledgebase on how to control or eradicate them is important.

1.7 The aim of the study

The aim of the research presented in this thesis was to determine the pathogenic potential of the *I. acuminatus* associated fungus *O. clavatum*, and to ask the question if *O. clavatum* is pathogenic on its own when Scots pine seedlings are inoculated with the fungus. As explained earlier in this chapter, the increased infestation of *I. acuminatus* has been detected in Finland and elsewhere in Europe. The environmental conditions have favoured the growth of the beetle's populations, while weakening the vitality of their host tree, Scots pine (Chinellato et al. 2014; Siitonen 2014; Wermelinger et al. 2008). The fungal infection that is associated with the beetle is likely to play a part in the demise of the tree and possibly it is a combined effort of the fungus, the beetle, and climatic factors that causes the eventual tree death (Lieutier et al. 2009). The aim of this research is though not to assess the harm caused by the beetle, but it is important to remember that the fungus is vectored by it. The actual pathogenicity of the fungus by single infection should shine some light on the severity of the fungus as a pathogen.

1.7.1 The chosen method

The method used in this research was to inoculate Scots pine seedlings with *O. clavatum*, and compare the growth with two control groups: one which was mock-inoculated and another that was left unharmed. The seedlings were kept in a controlled environment to ensure there were no other environmental disturbances or stressors that could affect the outcome of the inoculation. By maintaining stable conditions, the hope was that any signs of stress, discolouration, and resin leakage could be attributed to the fungal infection.

Following the observation period, several measurements were taken, as described in chapter 2 Method and materials, as well as completing the Koch's postulate. Koch's postulate is a method, first introduced by Robert Koch in the late 19th century, where the pathogen is isolated from the host and introduced to a new healthy host to prove that the pathogen is causing the symptoms of the original host. That is to say, Koch's postulate proves causation (Byrd and Segre 2016). In this research Koch's postulate was applied by taking samples of the infected area of the seedlings and placing them in 2% malt extract agar in Petri dishes, for cultivation and to confirm if the fungal infection detected was indeed *O. clavatum*.

Finally, statistical methods were applied to confirm if inoculated seedlings of Scots pine were significantly affected by the *O. clavatum* infection.

2. Method and materials

2.1 Fungal strain

In this study a strain of *Ophiostoma clavatum* originating from an adult beetle infesting Scots pine (collected by Heikki Nuorteva) and isolated in pure culture at the University of Helsinki was used. Agar slants were cut from the fresh 1-2 weeks old culture, using a sterilised scalpel (blade size 11), and placed on 10 separate 70mm petri dishes containing 2% malt extract agar (MEA). The scalpel was sterilised in 70% ethanol and in gas flame, and then cooled in MEA on a plate between each cut. The cut pieces were placed in the centre of the fresh medium. Each dish was then closed with a Parafilm® and grown upside down in an incubator in stable conditions at 25°C. After a 12-day incubation period the fungal hyphae had grown to cover the whole agar plate (Figure 6). Out of the 10 dishes, three were selected for the seedling inoculation. The chosen plates all had the most even growth radiating from the centre to the edge of the dish. This method secures the likelihood of even distribution of fungal hyphae on the sample seedlings. The cultures were stored in a fridge at 4°C while waiting for the seedlings to be ready.

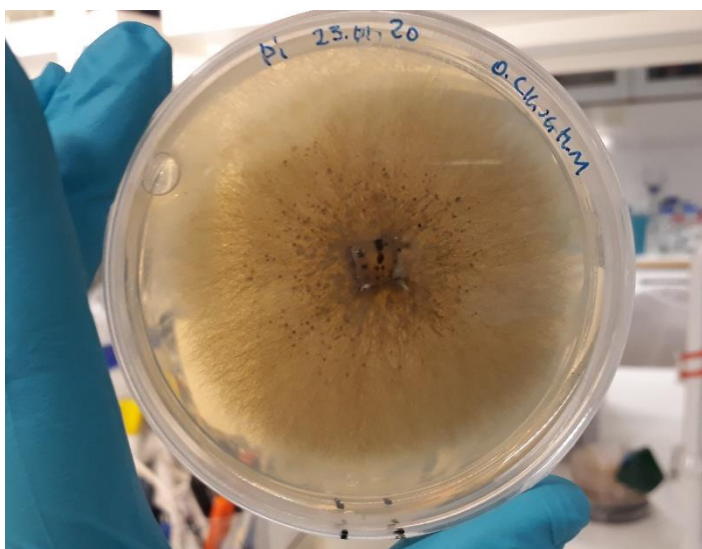


Figure 6: Inspection of fungal growth (Photo: PÍ).

2.2 Seedlings

Frozen one-year old Scots pine seedlings of the provenance PL81 were procured from Fin Forelia Oy nursery, situated in Nurmijärvi, Finland. The seedlings were placed in a fridge with a diurnal light cycle and at 3-4°C to allow them to thaw in conditions mimicking nature. After an approximately 2-week period the seedlings were planted individually in pots sized 10x10x10cm, and placed in an incubator room to adjust to the potted life (Figure 7). In total, 91 seedlings were potted. The pots were placed on a tray with 15 pots in each tray, six trays in total. The trays were then placed on shelves within the temperature and light controlled

incubator room. The incubator room had diurnal light cycles and the temperature was a steady 20°C. Additionally, a fan kept the air moving. Furthermore, the trays and the pots within the trays, were rotated on weekly basis.

2.3 Selection and measurements

After another two-week adjustment period the inoculation of the seedlings was performed. One seedling was of low quality and was rejected at the start, leaving the



Figure 7: Newly potted seedlings (Photo: ÞÍ).

seedling total at 90. The seedlings were randomly divided into three groups according to the treatment they would receive. One group was inoculated with *O. clavatum*, and the other two groups were control groups. The random selection was made by labelling plastic strips with numbers 1 to 70, CM1 to CM 10, and C1 to C10, and then the strips were randomly selected from a box, and the seedling labelled with the plastic strip selected. The seedlings which received numbers 1 to 70 were inoculated with *O. clavatum*; numbers CM1 to CM10 were mock-inoculated with 2% MEA, and C1 to C10 were left untouched with no inoculation or wounding into their bark. Before starting the inoculation, the height of the seedlings was measured, and each seedling was examined to determine its quality. One additional plant (C9) was rejected because the top had been broken off in the handling of it, and it was therefore unusable. Additionally, seedling 11 had a stunted growth with a side branch competing to become the new leader, seedling 54 had no woody part on its stem, and seedling 58 had an overly narrow stem for a successful inoculation. Seedlings 11, 54, and 58 were consequently moved to the control group as C11, C54, and C58. The final number of seedlings in the experiment was 89: 67 inoculated, 10 CM mock-inoculated, and 12C control seedlings.

2.4 Inoculation method

The inoculation was performed by stripping the woody part of the stems of needles on an area approximately 2cm in length. A 3x4mm ‘window’ was cut into the bark to expose the

sapwood. To prevent contamination, the CM control group was inoculated first with 2% MEA, and then the test group was inoculated with the fungus. A similar sized piece of the malt agar or fungal cultures was cut and placed on the wound. Parafilm® was used to seal in the MEA and fungus (Figure 8). The inoculation was conducted in a clean environment, but not completely sterile. All tools used, scalpel and tweezers, were sterilised between use in 70% ethanol, and gloves were used in handling of the seedlings and fungal samples and the MEA.



Figure 8: Seedling CM6. From left: small window opened on the bark; MEA used for mock inoculation; and the wound closed with Parafilm® (Photo: PI).

2.5 Observation period

Observations were made on a weekly basis and the seedlings were watered twice per week. On observation days, notes on the health of each seedling were taken. Discolouration of needles, resin production around the lesions, and any other signs of stress were observed and documented. Two weeks after the inoculation the Parafilm® was removed to prevent it from growing into the new tissue forming in the wound. Observations continued on a weekly basis for six more weeks.

2.6 Examination

When the observation period was over, after 8 weeks, the seedlings' height was measured and documented. Seedling 1 was rejected at this stage as it had four stems competing for dominance, and seedling 44 turned out to be two seedlings instead of being a two-stemmed seedling. The uninoculated seedling in pot 44 was discarded. Each seedling was removed from their individual pots, the soil cleaned off, and the wounds examined. Both the mock-inoculated, and the inoculated seedlings' bark was first polished off with a fine-grained sandpaper (P400), and then peeled with scalpel around the lesions to examine if any infection was manifesting around the inoculation site. Any infection detected was measured using a digital Vernier calliper. Depending on the depth and size of the infected area, the seedlings were assigned to one of four classes: 0 no infection detected; 1 minimal infection (barely visible $< 0,5\text{mm}$); 2 some infection (infection surrounding the wound $0,5\text{mm}-2\text{mm}$); and, 3 considerable infection (infection growing $> 2\text{mm}$ along the stem from the wound). How the infection was classified was also based on how the infection had grown in the seedling. To be classed as class 3 infection the infection needed to be of some depth as well or $>1\text{mm}$.

Before the seedlings were peeled, random inoculated seedlings were chosen using a randomising command in Excel. From these seedlings, samples were taken from the infected area to regrow on agar plates. Later new samples were taken from these samples to complete the Koch's postulate.

2.7 Biomass measurements

Additionally to analysing the infection rate, and categorise the seedling into classes according to the size and depth of the infection, the biomass of the seedlings was measured. The seedlings were weighed at a wet weight and then set to dry in an incubator for approximately 48 hours after which they were weighed again to get the dry weight. Furthermore, roots, stem, and needles were picked off the dried samples and weighed separately to determine if there was any difference in the biomass of composition of the infected and non-infected seedlings.

2.8 Statistical analysis

The measurements and observations made were fed into SAS Basic 9.4® software for statistical analysis. The variables measured were: height at inoculation (H1), height at the end of the observation period (H2), the difference between the two height measurements in

cm (Hdiff) and in percentage (Hdiff_rel), infection class (Infection), dry weight (DW), weight of the dry needles (Needle), the dry roots (Root), and the dry stem and branches (Stem). Finally, the ratio of the needles or needle-mass ratio (NMR), root-mass ratio (RMR), and stem and branches ratio (SMR) was compared against each other and between treatments, as well as the root-shoot ratio (RSR).

First, descriptive statistics were run on the whole assembly to see treatment means, medians, and standard error, for all treatments: 1 control, 2 mock-inoculated, and 3 inoculated. Then a Shapiro-Wilk test was used to see if the results within each treatment were significantly different from normal distribution. The Shapiro-Wilk test showed that the data in each treatment group had normal distribution, except for the infection class (categorical variable), as well as RMR and RSR, where treatment 1 (control) and 3 (inoculated) were not normally distributed, and in SMR, where treatment 3 (inoculated) did not have a normal distribution. Next, a Spearman's rank correlation was used to assess if the relationship between the infection class, which is not a continuous, normally distributed variable, and any of the other normally distributed variables measured. Finally, a One-Way ANCOVA analysis was run, where initial height was also included in the model as a covariate, thereby correcting for the individual plant size differences between treatments in the beginning.

3. Results

3.1 Observation period

The seedlings were allowed to adjust to the containers before being measured for height and being inoculated. After the two-week adjustment period the seedlings looked to have grown somewhat from the initial 10 cm average height (as guaranteed by the nursery but not confirmed by the researcher) and were just about ready for the inoculation. The average height of the potted seedlings after the approximately two weeks of adjustment period was 18.58cm with the standard deviation of 3.05cm. During the 8-week observation period the seedlings showed no signs of stress. There was no discolouration of needles at the top of the seedlings. There were yellowing on needles above and below the mechanical wounding on the stem, but new, green, needles grew instead. The height of the seedlings increased. On average the control seedlings and the inoculated seedlings grew by 11% and mock-inoculated seedlings by 10%. There was no visible discolouration in the mechanical wounding, though dark and dried-up hyphae leftovers created a film in top of many of the wounds. Resin was visible in and around the wounds (Figure 9).



Figure 9: Sample 50 shows dried up resin droplets in the wound and dark residual hyphae from initial inoculation. Sample 50 was found to be of infection class 1 (Photo: PÍ).

3.2 Infection examination

When examining each seedling more carefully, there was evidence of infection in 45%, or 30 of the total 66 seedlings, which were inoculated by the fungus. This left 55% with no infection at all. The unaffected seedlings were given the infection class 0, 23% had very little infection (class 1), 19% some infection (class 2), and only 2 seedlings, or 3%, had visibly, and measured, deeper infection (class 3) (Figure 10). Most of the infection were superficial

with little or no depth. The infection detected, which covered more areas than just around the wounding site grew lengthwise and upwards. None of the mock-inoculated seedlings had other injuries or showed evidence of infection.

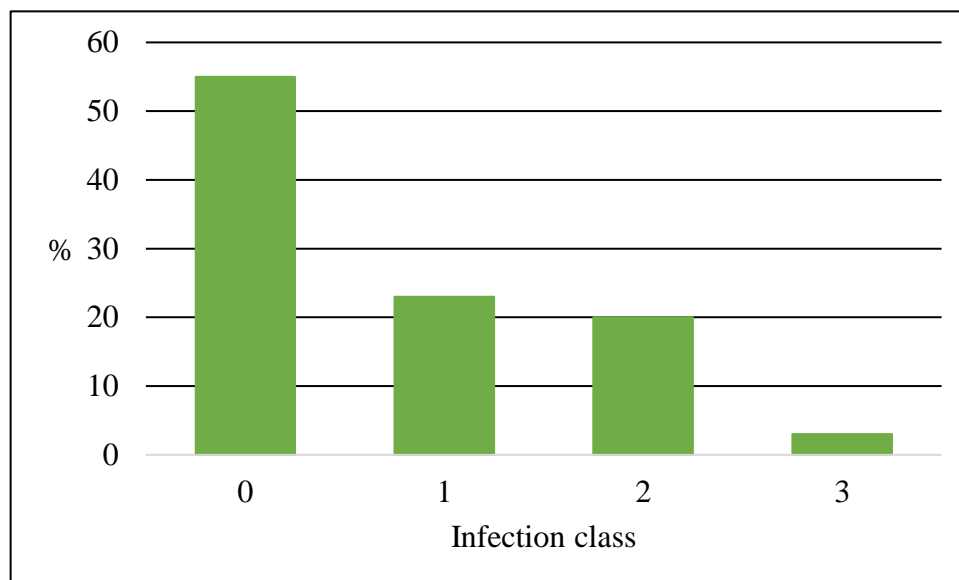


Figure 10: Percentage of inoculated seedlings in each infection class.

Samples (10 in total) for re-isolations of the fungus were taken of randomly chosen seedlings: 4, 22, 27, 29, 45, 47, 48, 56, 67, and 70. All but number 4 (infection class 0) were growing fungal hyphae in the 2% MEA the samples had been placed in. To complete Koch's postulate the samples were reisolated. This time the samples 29, 45, and 67 did not show typical morphology of *O. clavatum*, but grew green and white mould. Each seedling had been placed in different infection class; 29 in class 2, 45 in class 1, and 67 in class 3. New samples were taken from the Petri dishes containing samples 29, 45, and 67, but with no result. The initial samples had though shown morphological structures and culture characteristics of *O. clavatum*. That is, brown to black submerged hyphae with white aerial hyphae and brush-like conidiophores as can be seen in Figure 11.

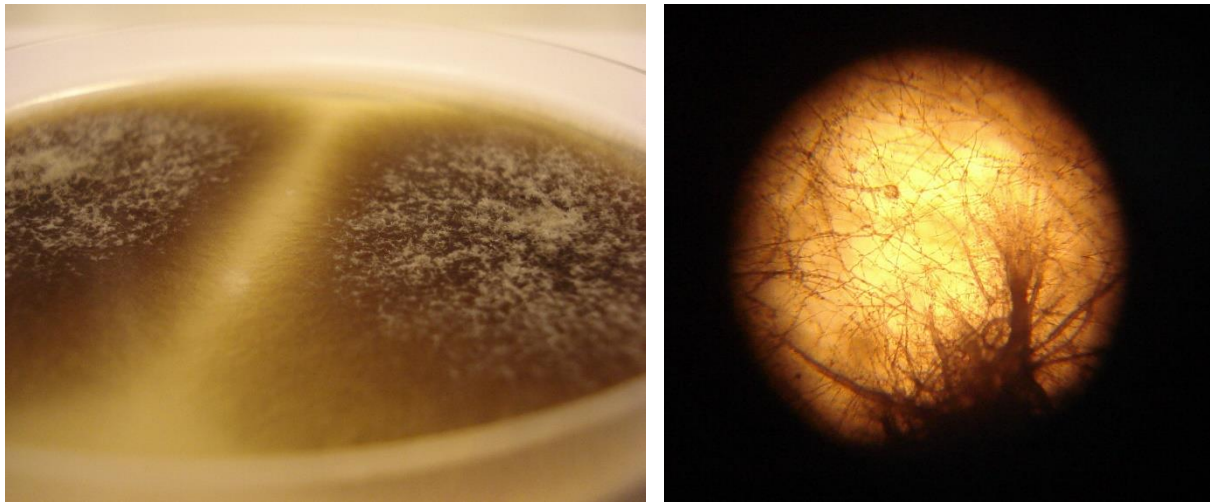


Figure 11: On the left: morphological characteristics of culture of *Ophiostoma clavatum*. On the right: typical brush-like conidiophore of *O. clavatum*. Image taken through the lens of an Olympus SC 30 microscope, 4x/0.10 enlargement (Photos: PÍ).

To determine if there was any relation to be found between the visually estimated infection class and other variables, the Spearman's rank correlation was used across all treatments. The Spearman's test showed no significant correlation to be found between the infection class and measured components (Table 1).

Table 1: Spearman's rank correlation coefficients, N = 88, showing no relationship between the infection class and other categories. Table adapted from SAS 9.4 ® Basic software.

	r	p
H1	-0.0508	0.6386
H2	-0.0753	0.4857
Hdiff	-0.0717	0.5069
Hdiff_rel	-0.0842	0.4353
DW	-0.1353	0.209
Needle	-0.0701	0.5163
Root	-0.117	0.2777
Stem	-0.0753	0.4854
NMR	0.04239	0.695
RMR	-0.0138	0.8983
SMR	-0.026	0.8101
RSR	-0.022	0.8386

3.3 Treatment effects

Even though no significant relationship was found between the infection class and the different variables, additional statistical analysis was applied, to determine if different treatments would have significant effect on any of the measured components. In the further analysis, where the small initial size differences between the treatment plants was included in the ANCOVA model as a covariate, there was a significant difference at the 0.05 level in DW (dry weight) between the control group and the inoculated group (Figure 12 and Table 2). That is to say, the dry weight of the whole seedling was significantly reduced in seedlings that were inoculated, suffered both a mechanical wounding and fungal infection, compared to the untreated control group, after the height difference between the seedlings in the beginning had been corrected for. There was, however, no significant difference between the mock-inoculated seedlings and the control seedlings or the mock-inoculated seedlings and the inoculated seedlings for DW. No other measured variables were significantly different between treatments in the ANCOVA analysis (data not shown). No further analysis was applied, because as is shown in Table 2, the means for different treatments are close to being identical.

Table 2: Results of Analysis of covariance (ANCOVA) where initial height (H1) is the covariate value. Other variables are: height at the end of the observation period (H2), the difference between the two height measurements in cm (Hdiff) and in percentage (Hdiff_rel), weight of the dry needles (Needle), dry roots (Root), and dry stem and branches (Stem), needle-mass ratio (NMR), root-mass ratio (RMR), stem and branches ratio (SMR), and root-shoot ratio (RSR). RMR, SMR, and RSR, were not determined because not all the treatment results had a normal distribution.

Variable	Control		Mock-inoculated		Inoculated		Significance
	Mean	SE	Mean	SE	Mean	SE	Pr > F
H1	18.35	1.23	18.42	0.80	18.65	0.36	n/a
H2	20.33	1.37	20.26	0.87	20.71	0.41	0.7589
Hdiff	1.98	0.26	1.84	0.21	2.07	0.11	0.7589
Hdiff_rel	0.11	0.01	0.10	0.01	0.11	0.01	0.8227
Needle	2.20	0.21	2.01	0.08	2.04	0.06	0.3870
Root	0.81	0.05	0.79	0.04	0.78	0.02	0.7722
Stem	1.00	0.11	0.95	0.08	0.99	0.03	0.7561
NMR	0.55	0.02	0.54	0.02	0.53	0.00	0.6663
RMR	0.21	0.01	0.21	0.01	0.21	0.00	nd.
SMR	0.25	0.01	0.25	0.01	0.26	0.00	nd.
RSR	0.27	0.02	0.27	0.01	0.26	0.01	nd.

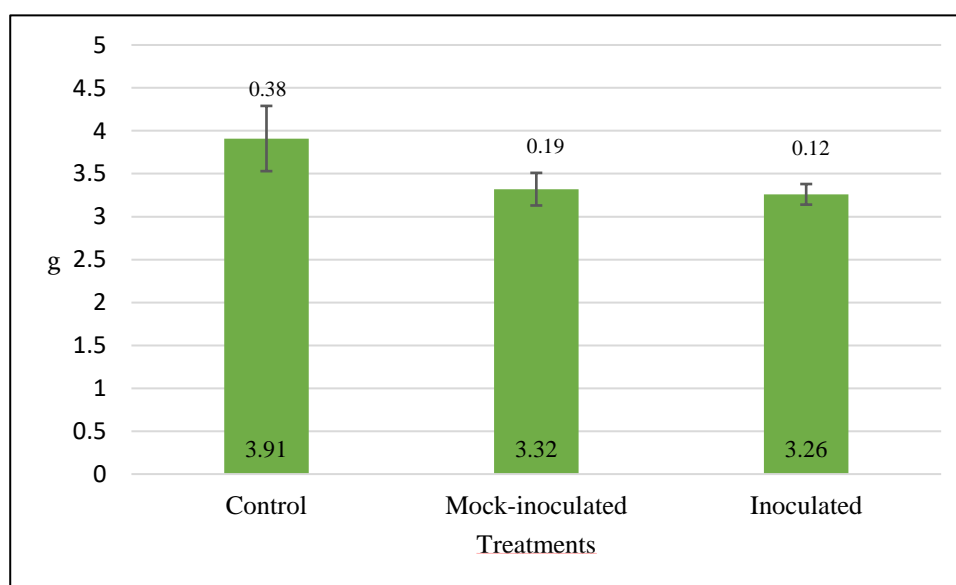


Figure 12: Results of ANCOVA test showing means and standard error of dry weight (g) of Scots pine seedlings. Significant difference is between the control group and inoculated seedlings, $P < 0.05$.

4 Discussion

The results of this study show that the level of *O. clavatum* infection in Scots pine seedlings was not very high. Neither were the effects of the infection on various components significant apart from one. The only significant measurement was the difference between the dry weight of the untreated control group seedlings and the inoculated group. Visually it seems as the mock-inoculated group is also affected, but it is not statistically significant from either the untreated control seedlings or inoculated seedlings (Figure 12). This finding seems to indicate that the mechanical wounding was also contributing to the apparent growth reduction in the inoculated seedlings. How this became the result can be discussed from several perspectives, but in the end, the pathogenic potential of *O. clavatum* is relatively small. Let us now discuss how this result was reached.

4.1 Infection and classification

Both the inoculated and mock-inoculated treatment groups showed some resin in and around the mechanical wounding (Figure 9). Resin droplets were observed where needles had been plucked off the stem to make room for the mechanical wounding and the Parafilm®. Resin production is a natural way for the pine to protect itself against bark beetle attack (Franceschi et al. 2005), and indeed any wounding will cause resin flow. Resin flow, however, dries up quite quickly or within three days from the wounding occurring, whatever the nature of the wound is (Lieutier 2004). This explains the resin formation in the two treatment groups, and why there was no increase in the resin formation during the observation period.

There was some difficulty on deciding the classes of infection when inspecting the seedlings at the end of the observation period. Mainly due to the different nature of several cases, where the infection grew lengthwise in a narrow strip up from the lesion but had no depth to it. Often the infections would grow upwards, rather than downwards. As blue stain fungi survives in the sapwood and feeds on starch and sugars found there (Millers, Magaznieks, and Gzibovska 2017) the upwards growth could be explained by the infection growing upwards in the phloem, towards the source of sugars. The mechanical wounding was applied in the lower part of the stem with few needles to photosynthesise below the infection site.

Usually, though, blue stain fungi grow inwards towards the core (Ballard and Walsh 1984), but here we had only two infected seedlings that showed any considerable depth of infection.

At first the visual infection classes were only three: 0, 1, and 2. The fourth class, 3, was added as two seedlings (47 and 67) had clearly deeper infection than the rest. The type of infection in each class was described in chapter 2.6. Most of the infected seedlings, or 23%, were in class 1, with barely visible infection but still categorised to have infection. This was sometimes difficult to determine due to the very faint and narrow strip of infection. The discoloration of the sapwood did though differ from the taint in the stem originating from the core, which was more reddish in contrast to the greyer and duller colouration of the fungal infection (not shown).

The pathogenicity of blue-stain fungi is usually dependant on how aggressive the insect vector is (Kirisits 2013). From this it can be surmised that the single mechanical wounding administrating the infection was not enough to severely affect the seedlings in this experiment. One wound mimicked the entry point of one single *I. acuminatus* beetle. It can scarcely be considered a major attack. Additionally, when bark beetle attack takes place the beetles and their larvae dig tunnels inside the wood, and if the wood is a living tree, the tunnels will cut through the phloem and xylem disrupting the flow of nutrients and water (Guérard et al. 2000; Villari et al. 2012) and cause much more damage than a simple blue stain fungal infection would. Obviously, it is difficult to simulate bark beetle galleries in the laboratory environment using seedlings, and the extent of the relationship between the bark beetle and the *Ophiostoma* in contributing to tree mortality is tricky to determine. Here, the pathogenicity of the fungus alone seems to be limited and indeed weak.

Furthermore, the Scots pine seedlings were kept at optimal conditions in the present study: stable temperature and light, and consistent watering. The regular rotation of the seedlings ensured that whatever environmental irritant could be disturbing them, location wise in the incubator room, was minimized. All in all, the seedlings were not disturbed or bothered by any other environmental factors except the fungal infection and the single mechanical wounding (where applicable). This can also be a reason enough for why the seedlings were able to combat the infection and why less than half, or 45%, of the seedlings showed any sign of infection, and half of those showed next to no sign of infection.

As Guérard et al. (2000) observed, there is a critical threshold to how many wounds are needed to have mortal consequences to the trees. That research did deal with older plants but

not seedlings, however, some lessons can be drawn from those results, which were that the intensity of the attack on a given area of pines needs to be considerably high for it to result in their death. Here, in this research, the mortality rate was perhaps not the end goal but to see what, if any, affects the infection would have on the growth of the seedlings. For the student researcher, more significant results would have been more exciting, but for the forest industry and from the perspective of forest health, little uptake of infection in pine seedlings is a positive outcome. If, however, multiple wounds would have been cut into the seedlings and/or they had been exposed to environmental stress the conclusion might have been different. Those co-factors warrant further studies.

4.2 Biomass calculations and statistical analysis

The results showed no significant correlation between the biomass of the different components of the seedlings: the roots, stems, and needle mass (Table 1), even though the total dry weight of the seedlings was significantly lower in the inoculated group than the unharmed control group (Figure 12). To answer why that was the case a few aspects need to be investigated.

Dry vegetative matter absorbs moisture from the environment. This results in the measurements made after the specimens were removed from the incubator show the absolute dry weight. However, in this experiment the different mass components: roots, stems, and needle mass, were weighed the day after the initial dry weight of the whole seedling was measured. This should result in higher total weight (not shown) of the different components but in this experiment some numbers were lower. There are two possible explanations for that. Firstly, a different scale was used in a different room and on different floor of the faculty's building when measuring the total dry weight directly from the incubator, than the separate components. Secondly, some material was lost during the work of taking the seedlings apart. The samples were fragile, dry, and very light. Needles got lost, fine roots dissolved, and stem debris could easily have gotten lost as the stems had been peeled when they were being examined for signs of infection. Most likely the human hand of the researcher, and the error it brings, was the main cause of the weight loss of the samples, and caused the results to be inaccurate and useless for the purpose of this research. Discovering this was a good reminder for the student in how strict protocols and training in laboratory work is essential to ensure that minor changes can be detected.

Why only the total dry weight category showed significant difference, but not the others, can perhaps be related to the bulking of the seedlings. Also, as discussed here above there was a possible loss of material during the process, and minor inaccuracies in measurements of weight due to moisture absorption of the different components of the seedlings and the use of different scales. Due to small size of the seedlings the stem circumference was not measured. Perhaps there lies the difference. The inoculated seedlings were possibly putting on less bulk, which was then best reflected in the total biomass numbers.

It was visually observed, but not measured, that the seedlings height grew considerably from the time they were potted up and placed in the incubator room until they were measured and divided into groups and the actual observation period began. The average growth in height across the treatment groups was very similar, or 11% for the untreated control group and the inoculated group, and 10% for the mock-inoculated group. The difference is not huge and given the small number of samples perhaps not a massive indicator of hampered growth in any of the treatment groups. Therefore, the assumption is that the difference in dry weight is a result of less bulking of the inoculated seedlings in contrast to the control groups. In that way the fungal infection might have caused enough stress on the seedlings to reduce their growth.

Considering that there was no statistically significant difference between the groups in terms of growth, except between the dry weight of the control group and inoculated group. It can, therefore, be concluded that it is not primarily the injury itself that hindered the growth, but the combination of the injury and the infection. The injury alone was not severe enough to trouble the seedlings or significantly reduce their total dry weight, but introduce an infection to the wound, and a statistical difference will be observed. This can perhaps also be an indicator that without the fungal infection the activities of *I. acuminatus* would not alone unduly stress the pines in the woods.

The seedlings used for the experiment varied somewhat in initial height. Correcting for the difference was vital to understand if there was any effect to be detected from the infection. As it turned out, this was the only way to show if there was any significant difference between the treatment groups. There was no further analysis applied as the means for different treatments were close to being identical.

Bias in the research is possible. The seedlings used for the control group were not only randomly selected, but also contained rejected seedlings from the inoculation group due to physiological reasons. That is, the seedlings that had stunted growth and stems that had not turned woody at the time of inoculation were rejected and ended up in the control group. The inoculation needed to be applied to a wooden stem, and a stunted growth of the main stem would most likely lead to a new stem selection by the plant, that is to say a new branch would take over. This is probably the main reason why the statistical correction to initial size differences was needed to see any significant treatment effects.

4.3 Fungal reisolations

Samples from ten seedlings were collected of the inoculation area on the stem for reisolation of the fungus in 2% MEA. All samples grew fungal hyphae except the sample which was categorised in infection class 0. The samples were then all reisolated. This time three samples did not show clear morphology of *O. clavatum*, but grew mould instead (not shown). No genetic test was done on the samples to determine that the strain was indeed the correct one. However, there is a reason to believe the samples were contaminated when they were being observed for the first time rather than that the identification was incorrect. Figure 11 shows clearly the morphology of *O. clavatum* growing the first time around from a sample from seedling 29. Quite possibly contamination from the environment was a contributor to the failed regrowth. Possibly the samples did not tolerate the long storage. They were stored in a fridge at 4°C for just under five months before being examined again. This is, though, unlikely as the other samples were fine. Environmental contamination is, therefore, a more plausible explanation.

To complete the Koch's postulate the samples should have been fully isolated and indeed healthy plants reinoculated with the strain. This is the classic approach but currently molecular techniques can be applied as well considering how advanced genome sequencing has become (Byrd and Segre 2016). It can, however, be argued that the classic approach has been applied to a certain extent in this research as the original strain used had been isolated, at the Forest pathology lab Helsinki University, from infected Scots pines. By doing a genetic test and extract the DNA from the samples taken from the random selected seedlings, a definite confirmation would have been guaranteed. This was not done due to lack of facilities and time. This research was done at the height of the covid-19 pandemic and restricted access to laboratories, people, and equipment hampered a little in the execution

and completion of the research. This, though, does not minimize the results that the *O. clavatum* blue stain fungus causes little stress in Scots pine seedlings grown at optimal conditions over a short period of time.

5 Conclusion

The aim of the study was to estimate the pathogenic potential of *O. clavatum* in Scots pine seedlings. The results show that there is no significant effect on the seedlings by a single inoculation apart from its total dry weight. This can be interpreted that the mechanical wounding and the fungus have a small physiological effect on the growth of Scots pine seedlings when they grow in optimal conditions with no disturbances other than the wounding and the fungal infection. However, this only applies if the seedling is unable to thwart the infection after it has been introduced. It must be remembered that less than half (45%) of the seedlings showed some sign of infection and 23% only very little.

In this research, the infected seedlings were kept at optimal conditions with no external stressors apart from the mechanical wounding, and the infection. Optimal conditions, rarely appear in nature. It would, therefore, be interesting to see how seedlings would react in more natural environment and if the infection percentage would then remain as low. Another interesting area to explore is how older trees would react to inoculation. Yet another experiment that would be interesting, is to inoculate plants with different severity, to follow the study by Guérard et al. (2000) to test the intensity threshold required.

In conclusion, the blue stain fungus *O. clavatum* does not seem to seriously stress Scots pine seedlings when a single infection is introduced through mechanical wounding on the bark. *O. clavatum*, does not appear to have any significant pathogenic potential in seedlings when those seedlings are kept at optimal conditions. Therefore, the null hypothesis, on *O. clavatum* not having pathogenic potential, holds.

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