Screening assays for assessing resistance of European larch to larch canker disease

Erik Kügler

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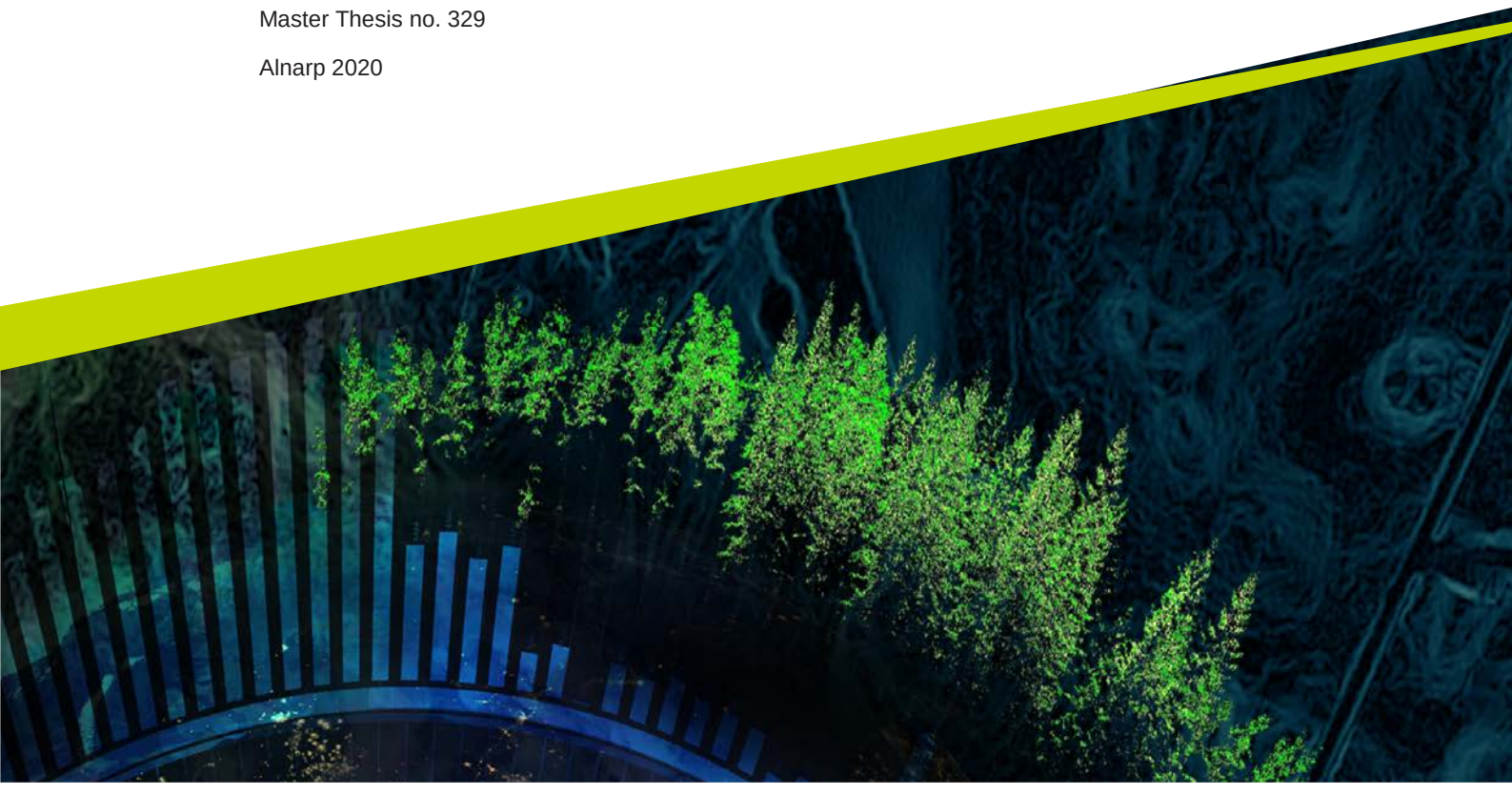

Screening assays for assessing resistance of European larch to larch canker disease

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Abstract

Larch canker disease caused by the ascomycete fungus *Lachnellula willkommii* has made European larch (*Larix decidua*) cultivation unprofitable. The pathogen originates from east-Asia and was introduced to Europe in the 18th century and since then has caused large scale damage in European larch plantations and natural forests. A low level of resistance to *L. willkommii* has been identified in the natural population of European larch, notably from material sourced from Poland which offers potential for resistance breeding in the future. Screening resistance requires reliable methods to accurately phenotype trees. The aim of the study was to determine a proper inoculation method that can be used for resistance testing in the field, considering differences in host response based on the inoculum substrate used and the variation in virulence among isolates of the pathogen. In greenhouse experiments, the differences in lesion size (disease severity) on European larch were determined three months following inoculation with *L. willkommii* using two different inoculum substrates (agar and wood) colonized by the fungus and five different isolates originating from Sweden and the Czech Republic. Analyses of the disease symptoms demonstrated that agar plug colonized by *L. willkommii* caused significantly longer lesion lengths than colonized wood chips. No differences in disease severity were found among isolates. The results indicate the importance of inoculum choice and the negligible effect of isolate choice for future screening of resistance in field trials. Future breeding experiments will potentially result in cultivation of *L. decidua* as a renewed valuable species option for Sweden and northern Europe.

Key words:

Lachnellula willkommii, *Larix decidua***,** inoculation assay, virulence, isolates, resistance, Sweden.

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1 Introduction

1.1 Importance and distribution of European larch

In its native habitat, European larch, *Larix decidua* Miller, is a fast-growing pioneer species, that can reach heights of 50 m during its long lifespan of 600-800 years (Da Ronch et al., 2016; Praciak et al., 2013). European larch is native to central and eastern European mountain ranges, in particular the Alps, the Sudetes and the Carpathian Mountains (Figure 1), where it grows between 180 and 2500 meters above sea level.

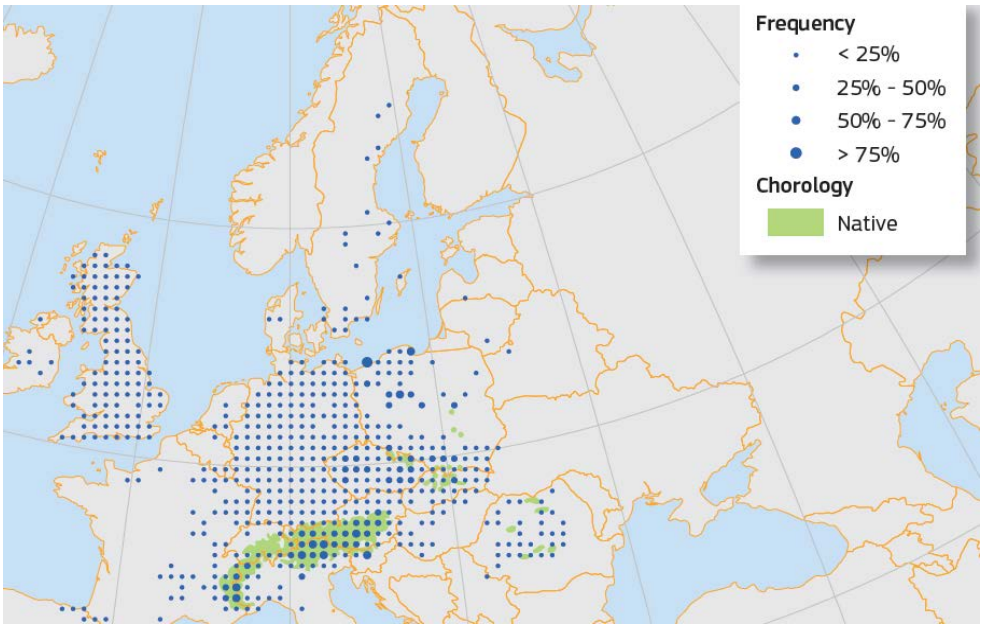

Figure 1. Native distribution range (derived after Wagner et al., 2015) of European larch and its distribution range where it has been introduced throughout Europe. Frequency represents the estimated occurrence among inventory plots from field observations in National Forest Inventories (Source: Da Ronch et al., 2016)

The species has been introduced to large parts of central Europe (Figure 1), Canada, north-eastern United States and New Zealand (Da Ronch et al., 2016).

Compared to other conifer species, European larch has characteristic fast growth, high adaptability and high durability (Praciak et al., 2013) which has made it an important timber tree historically. The species has a high content of tannins and resin (Geburek, 2003) which makes the wood durable and suitable for outdoor use such as wooden houses and furniture. Larch wood can also be utilized for pulp or turpentine production. However, the high susceptibility of European larch to the ascomycete fungus *Lachnellula willkommii* Hartig, the causal agent of larch canker disease, makes it currently unprofitable to cultivate.

1.2 The Larch canker disease

1.2.1 History

Initially, the identity of *L. willkommii* remained unclear, because it was not distinguishable from a native saprophytic fungus, *Lachnellula occidentalis* G.G. Hahn & Ayers, commonly occurring on larch trees. *Lachnellula willkommii* is native to Japan, and probably other Asian countries where larch is native. For example, a 100-year-old canker, caused by *L. willkommii*, was found in 1970 in a natural Japanese larch (*Larix kaempferi* Lambert) stand in Japan (Kobayashi, 1970).

Lachnellula willkommii was probably introduced and became established in Europe during the 18th century (Cech, 2013). In the mid 800s, Willkomm (1866) and Hartig (1880) reported observations of a canker disease on European larch in Germany. Hartig (1880) concluded that the pathogen had spread over the whole distribution of European larch by this time. It was therefore declared a major threat for European larch by Willkomm (1867) in the 19th century. In England, the canker was already fairly common in 1838 (Hiley, 1919) from where it was unknowingly introduced with seedlings to the USA in 1904 and 1907 (Spaulding and Siggers, 1927). In Sweden, the fungus was probably present from the mid-1800s, where it spread from southern Sweden (Skåne) to more northern parts (Schotte, 1917). During the middle of the 19th century, European larch was planted in high amounts

in northern Europe due to its high timber value (Schotte, 1917). The disease was carried via regeneration material to most plantation sites.

1.2.2 Diagnosis

The most prominent sign of infection are the fungal fruiting bodies, called apothecia (Figure 2), which are formed annually on affected stems and branches (Sylvestre-Guinot and Delatour, 1983). The apothecia are typically 1-6 mm in diameter and have a 1 mm high stalk. They are typically cup-shaped or can have a disk-like appearance when completely open in moist conditions. The cup is bright yellow-orange and is surrounded by a white, chalky, and hairy rim. By examining only the fruiting bodies, *L. willkommii* can be distinguished from most *Lachnellula* species apart from the saprophytic *L. occidentalis* (Cech, 2013; Hiley, 1919; Yde-Andersen, 1979b). Individual infected trees can have several perennial cankers on the stem and branches. Cankers are often accompanied with resin outflow, swellings and blackish bark cracks. Small diameter saplings can be girdled and die (Sinclair and Lyon, 2005). Needles on infected branches often turn yellow starting at the canker and continuing to the tip of the branch (Cech, 2013; Hiley, 1919; Yde-Andersen, 1979b).

Figure 2. Apothecia on an infested Larix branch (Photo: Mimmi Blomquist, SLU)

1.2.3 Infection biology

Airborne ascospores of the fungus presumably enter the tree through an existing wound where they germinate, penetrate the cork layer and establish infection. It is not clear if previous wounding is absolutely required (Sylvestre-Guinot and Delatour, 2002, Hiley 1919). Once past the bark, mycelia then grows into the phloem and kills the vascular cambium (Hiley, 1919). Infection on 2-5-year-old shoots appears as an elliptic or circular sunken area (Yde-Andersen, 1979b). Often, resin outflow can be noticed and the bark turns necrotic. Small diameter branches and stems either die from girdling or a perennial canker develops whereby phloem and cambial tissue are killed and subsequently regenerated (via necrophylactic periderm formation and callus formation, respectively) each year. If the tree is able to resist the pathogen invasion, canker growth will cease and the tree usually heals the wound by compartmentalizing the infection and callus formation to overgrow it (Figure 3).

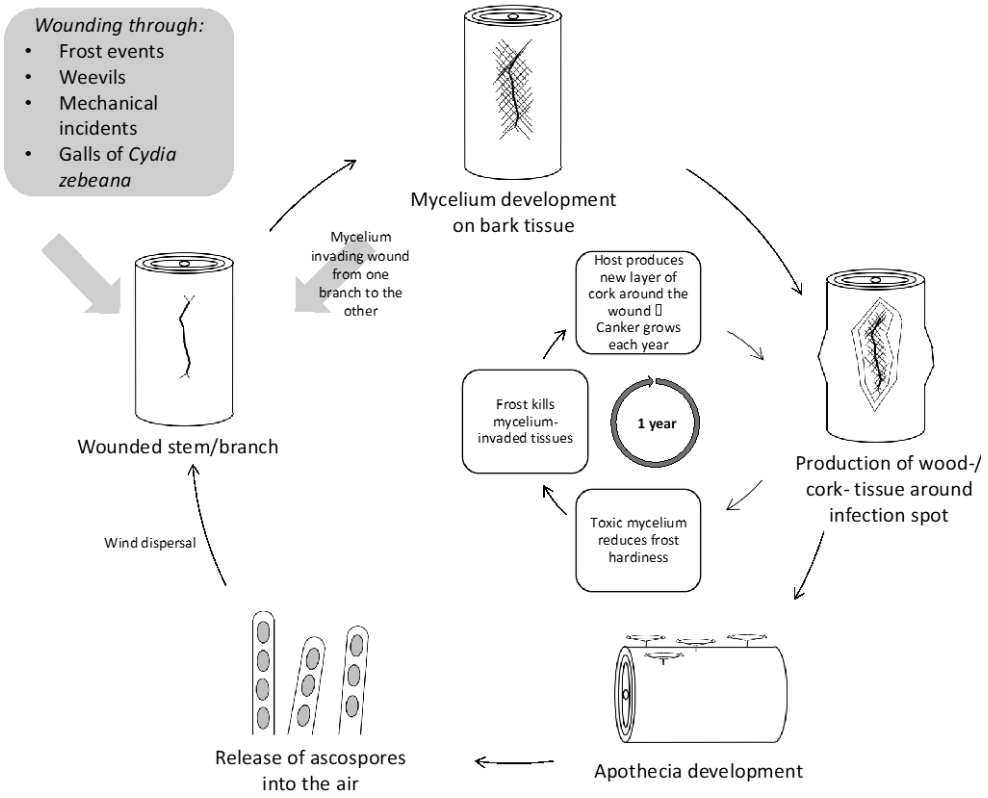

Figure 3. Presumed general life cycle of *L. willkommii* (drawn by Erik Kügler)

1.2.4 Epidemiology

The epidemiology of *L. willkommii* is based on the combination of host susceptibility, climatic factors and site conditions (Cech, 2013). Environmental factors like frost events, *L. willkommii* suiting air temperature, high air humidity and oceanic climate seem to increase the susceptibility of larch individuals and therefore the incidence and severity of disease (Robak, 1964; Cech, 2013; Schober, 1977). Stand structure and species composition may also impact disease development. Schober (1977) found fewer cankers in larch-beech mixtures than in larch monocultures, but (Schotte, 1917) found that mixed larch-Norway spruce stands had more disease than larch monocultures. Overall, host susceptibility seems to be the most important factor determining pathogenicity and virulence of *L. willkommii* (Cech, 2013).

In east Asia, *L. willkommii* presumably does not cause much damage on the native Japanese larch (*Larix kaempferi* Lambert). For this reason, tree breeders have used Japanese larch in tree improvement programmes to produce hybrid larch (*Larix x eurolepis* Henry) which is less susceptible to *L. willkommii* compared to European larch. The hybrids' cultivation is now preferred over the native European larch.

1.3 Hybrid larch and European larch

Hybrid larch was originally bred to create a more valuable larch than European- or Japanese larch, that could be used in forestry. When European larch was moved from its original mountain range to the lowlands, it showed poor adaptation, slow growth and high canker susceptibility (Pâques, 1989). The imported Japanese larch was known for its fast juvenile growth and its canker resistance but it could not be cultivated due to the species high moisture requirements (Pâques, 1989). The expectations of a more valuable plantation species led to the hybridisation breeding of *L. x eurolepis* Henry, the hybrid between Japanese larch and European larch.

Hybrid larch has many advantages in comparison to its parental species (Ekö et al., 2004). Young hybrid larch stands are declared to grow faster than its parental species and other conifers under certain conditions in Sweden (Ekö et al., 2004; Gothe, 1953; Brandt, 1977; Hering, 2002), which makes it a highly economical species. Pâques (1989) explains this outstanding growth with the heterosis effect: the hybrid vigour increases the growth of the hybrid in comparison to its parents (Pâques et al., 2009). Furthermore,

its durable timber for outside construction and its expected high land expectation value (Ekö et al., 2004) makes it a lucrative commercial forest species.

The hybrid's main advantage over European larch is its resistance to *L. willkommii* (Ekö et al., 2004; Gothe, 1953; Sylvestre-Guinot et al., 1999; Pâques 1989) that would not result in any major economic loss. Sylvestre-Guinot et al. (1999) demonstrated resistance in hybrid larch inoculated with *L. willkommii*. In that study, nearly all infected trees had healed and showed no signs of infection after 39 months. However, disease severity varies and some hybrid larch stands can have exceptionally high infection rates, as much as what is observed in European larch stands (Yde-Andersen, 1979a). As hybrid larch has distinct advantage over European larch in terms of its ability to withstand infection by *L. willkommii* without causing large economic losses, the hybrids' cultivation has largely replaced European larch for regeneration material.

Still, European larch, in the absence of the pathogen, has clear advantages over hybrid larch when it comes to growth and timber quality traits. Not only its nativity makes European larch an attractive alternative, but it produces a straighter stem then hybrid larch (Ekö et al., 2004), is more frost resistant (Gothe, 1953) and has similar rate of growth in young (Pâques et al., 1999) and considerably older stands (Gothe, 1953), compared to the hybrid larch.

Münch (1933) observed that in European larch stands in eastern Germany, trees less than 80 years showed considerably more signs and symptoms of larch canker disease than older individuals. Münch (1933) suggests that the observed differences in ontogenic susceptibly are actually due to seed origin, as the younger individuals were from alpine regions in Tyrol, whereas the older trees were from the Sudetes. A possible option to find more resistant European larch may lie in finding the right provenance. Later, other authors agreed that European larch from different provenances show different susceptibility to larch canker (Zimmerle, 1941; Yde-Anderson, 1979a). Some even suggest similar rankings in which the Tyrol-larch is the most susceptible and the Sudetes-larch is considered the most resistant in Europe (Göhrn, 1957; Troeger, 1962; Schober, 1977). Pâques et al. (1999) presented however in an artificial inoculation experiment, that even individuals from the expectably most resistant provenance from Poland, showed infection rates between 10-30 %. Nevertheless, individuals from the Sudetes provenance tend to show high resistance against larch canker and possess a broad ecological adaptability and high vigour (Stern, 2003).

Commercial forestry in Sweden is heavily reliant on two species: Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*). Sweden's forests are vulnerable to several threats such as climate change, invasive and endemic pests and pathogens, because of its low resilience caused by the lack of biodiversity (Skogsdata SLU, 2019). New options are therefore needed to fit future needs. Hybrid larch is currently advertised as a valuable alternative by leading forestry advisors and companies like Skogsforsk, Skogsplantor, and Skogsunskap (Arvidsson, 2006; Skogskunskap.se, 2018; Stern et al., 2005). Hybrid larch was the second most planted foreign tree species in Sweden during the last decades (Skogstyrelsen, 2018) and is promoted by Skogstyrelsen (2018) as a commercial species that contributes to increased variation of spruce dominated forests. The more open canopy in hybrid larch stands can result in richer field layers and increases the recreational value of the landscape (Skogstyrelsen, 2018).

1.4 Improved European larch: Reintroducing a new option for Swedish forestry

European larch possesses the same ecological traits and has comparable or even faster growth and higher quality timber compared to other common conifers (Ekö et al., 2004). It is therefore of great interest for Swedish forestry to reconsider European larch as an option should suitable material become available that shows better resistance to *L. willkommii*.

In 2017, seeds were picked from approximately 85 half-sib families of European larch from the area of Sudetes in southern Poland in order to establish cultivation tests to study growth, stem quality and resistance to *L. willkommii* in southern Sweden (M. Liziniewicz, Skogforsk, personal communication). This material was propagated and recently established in field trials to test and select the best families to carry forward for potential propagation and establishment of seed orchards. However, to properly screen these trees for resistance, a suitable screening test needs to be established that would allow for some discrimination among individuals.

An appropriate inoculation method should be able to test for resistance against *L. willkommii,* both in the short-term and long-term. The degree of damage to the host may depend on several external factors, but also on the

used substrate and infecting isolate (Kim et al., 2005). The choice of substrate may influence the “inoculum potential” of the fungus. The inoculum potential is the energy a certain inoculum provides the fungus with, at the infection site (Garrett, 1960). It depends essentially on the size and the nutritional content of the inoculum. For virulence studies of pathogens various inoculum substrates are used. These are either derived from natural occurring material, like any plant material, or prepared media (commonly malt extract agar or potato dextrose agar). In other virulence studies involving *L. willkommii*, sorghum straws (Pâques et al., 1999; Sylvestre-Guinot et al., 1994, 1999), malt extract agar and oat flakes **(**Sylvestre-Guinot and Delatour et al., 1983) have been used as inoculum sources, however the reasons for the choice of inoculum substrate in these tests is not discussed, but would nonetheless be useful for standardizing screening methods.

In addition, it is commonly known that different isolates of a fungal species often possess different degrees of pathogenicity and virulence. This is the case for plant pathogens of both herbaceous (Keeling, 1985) and woody plants (French, 1978; Griffin, 1983; Steenkamp et al., 2012). Currently, there is no information regarding differences in virulence among several *L. willkommii* isolates. Often the isolate that has proven pathogenicity once, is used in follow-up studies without being questioned (e.g. Pâques et al., 1999; Sylvestre-Guinot et al., 1983, 1999), but this warrants further investigation.

1.5 Project Aim

The overall aim of the project is to determine a suitable screening method for testing the Sudetes European larch families against the fungal pathogen *L. willkommii*, by determining the variation in disease severity based on i) different inoculation methods, and ii) different isolates of the fungus.

2 Materials and Methods

2.1 Sampling locations and tissue collection for isolation of *L. willkommii*

In order to obtain isolates from which further experiments could be conducted, we needed to locate and collect samples from diseased larch for isolation in the lab. The first site was Sävar in Västerbotten, where Skogforsk has a research station and where the *Larix* species, clone and precise location of infected trees were known previously. Branch cankers exhibiting characteristic symptoms (branch swellings, necrotic lesions, resin flow) and signs (i.e. fruiting bodies) of larch canker (Figure 4) were collected from seven trees on the 11th of June 2019 with the assistance of an elevated hydraulic lift. For each infected tree, three symptomatic branches were collected by cutting a section of the branch using saws (Figure 4). Apothecia from each lesion were picked by hand and placed separately into small paper bags and labelled according to the clone- and branch number.

Figure 4. Left: Canker with resin outflow, Right: Removal of apothecia from infested branch (Photos: Erik Kügler)

The whole cut section of branch was then labelled and packaged for shipment to the Forest Pathology Lab in Alnarp. In-between sampling of individual trees, tools were disinfected with 70% ethanol.

The second site was Kättnas in Södermanland and was visited on the 5th of September 2019. Here, infected *Larix spp.* individuals were observed previously. Apart from these, the nearby forest stands and tree individuals along the road were checked for both infected European larch and hybrid larch individuals. The presence of cankers was noted based on visual observations from ground level. Branch samples were collected from four trees following the same procedure as described at the first site. (Figure 11)

2.2 Isolation and identification of *L. willkommii*

In order to confirm the identity of *L. willkommii* on samples, and to exclude the possibility of having collected and isolated the similar species *L. occidentalis*, further diagnostics were needed. Morphology of single spore isolations has historically been used to identify fungi (Choi et al., 1999), but the advent of genetic sequencing permits more precise identification and classification (Cech, 2013). This is the only way to distinguish *L. willkommii* from the morphologically similar *L. occidentalis* (Cech, 2013), and therefore DNA analysis was of crucial importance to carry forward the work.

In the lab, apothecia from the branches were removed with a sterile scalpel and stored at 4°C in petri dishes sealed with Parafilm until further processing. Apothecia were surface sterilized with alternating 30 second treatments of 70% ethanol, 0.5% sodium hypochlorite and again with 70% ethanol. The fruiting bodies were dissected into approximately 3 mm sized pieces (Figure 5) and placed into petri dishes containing 2% malt extract agar (MEA) (Appendix 1). The plates were sealed with Parafilm and stored at room temperature in the dark. Reported optimal *in vitro* growing temperatures for *L. willkommii* vary from 12°C to 26°C (Hiley, 1919; Ito et al., 1963; Dharne, 1965). After 10-14 days, outgrowth from the apothecia forming chalky white colonies with velvety aerial mycelia characteristic of *L. willkommii* (Cech, 2013) were selected for further subculturing to fresh 2% MEA (Figure 5).

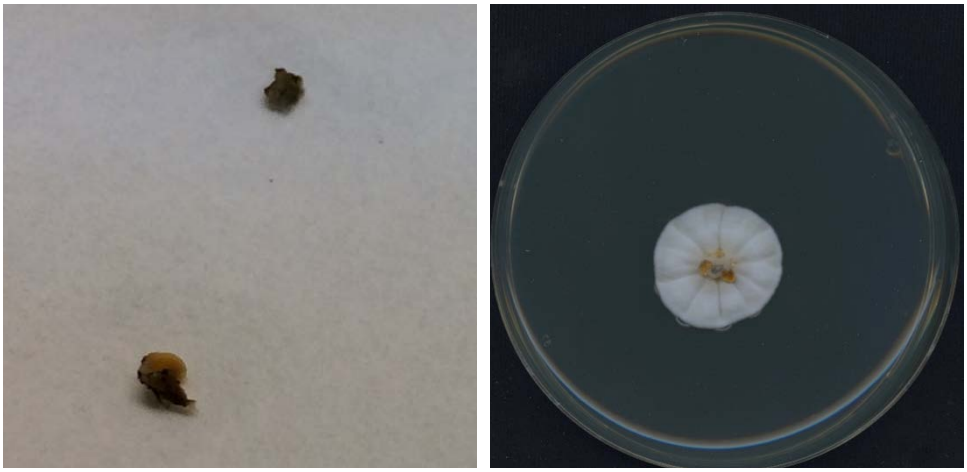

Figure 5. Left: Dissected apothecia, Right: Pure culture (Photos: Erik Kügler)

After 30-40 days of growing in pure culture, a 1 mm diameter plug containing mycelia was transferred to a 50 ml Falcon tube containing malt extract broth (MEB) (Appendix 1) and placed onto a shaker for 10 days at 320 rpm at room temperature.

Samples were prepared for DNA extraction by lyophilizing harvested mycelia for three days, placing them into 1.5 ml microcentrifuge tubes and pulverizing to fine powder using a Retsch MM44 ball mill (Retsch GmbH, Haan, Germany) for 30 seconds. DNA was extracted using the Omega "E.Z.N.A SP Plant DNA kit" (Omega Bio-Tek, Inc., Norcross, USA) which uses silica column to capture DNA while removing contaminants such as proteins, cellular debris and polysaccharides. DNA concentrations were determined using a NanoDrop® ND-1000 (Wilmington, USA) which makes a spectrophotometric measurement of UV absorbance at 260nm. This enables to measure the nucleic acid concentration of the sample.

To identify the isolates using genetic sequencing, a specific, diagnostic region of the genome is targeted. For fungi, the most commonly used region is the internal transcribed spacer (ITS) region. The ITS region is a non-coding section of DNA between the small subunit ribosomal RNA and the large subunit ribosomal RNA, and is generally well conserved within a species, but highly variable between species, making it an ideal target area for species identification. The target DNA fragment was copied using polymerase chain reaction (PCR). A reaction mix, usually containing a buffer, a thermostable DNA polymerase, four deoxynucleotides, two oligonucleotide primers and the template DNA (Weising, 1995) was used to conduct PCR. Three temperature controlled steps are repeated in a certain amount of cycles. In

the first step, the denaturation step, the strains of the template DNA are melted at around 94 °C to make the template DNA single stranded. The second step, the annealing step enables the primers to anneal to their target sequence on the DNA. During this procedure, the reaction mix is cooled to a temperature between 25 °C and 65 °C. Primers are short nucleic acid sequences that provide a starting point for DNA synthesis. In the elongation step, the temperature is increased to 72 °C. Here the polymerase extends the 3' ends of the DNA-primer hybrids, that were formed in the second step, towards the other primer site. This final step creates a complete replicate of the target fragment of the DNA.

In the second, and subsequent cycles, both the original template DNA and the fragment copy are desaturated in the first PCR step and the repetition of the cycles results in an exponential amplification of the target fragment of the DNA.

For the present study, the reaction mix consisted of "DreamTaq PCRMaster Mix (2X)" (Thermo Fisher Scientific™, Waltham, USA) primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC), 1 µl of temple DNA and water were added resulting in a 20 µl mixture. PCR was run in an automated thermal cycler with the following program: Denaturation at 95 °C for 120 sec and 60 sec at 95 °C, annealing at 55 °C for 45 sec, extension at 72°C for 90 sec. This cycle was repeated 35 times. The PCR product was cleaned using "Exo-SAP-IT High-Zhroughout PCR Product Cleanup" (Affymetrix, Santa Clara, USA) according to manufacturer's instructions to prepare it for subsequent sequencing.

Agarose gel electrophoresis was used to confirm the presence of PCR products. This technique is routinely used for separating mixtures of macromolecules such as nucleic acids. Agarose is a polysaccharide that forms a solid gel when mixed with water and heated. The resultant gel has a certain porosity depending on the agarose concentration used. When DNA is loaded into the gel and an electric current is applied, the DNA travels through the pores of the gel at a speed determined by its length and the gel's pore size. The DNA is visualized by intercalating staining. A 1.2% agarose gel was prepared and the DNA was visualized with RedGel fluorescent dye. (Figure 6).

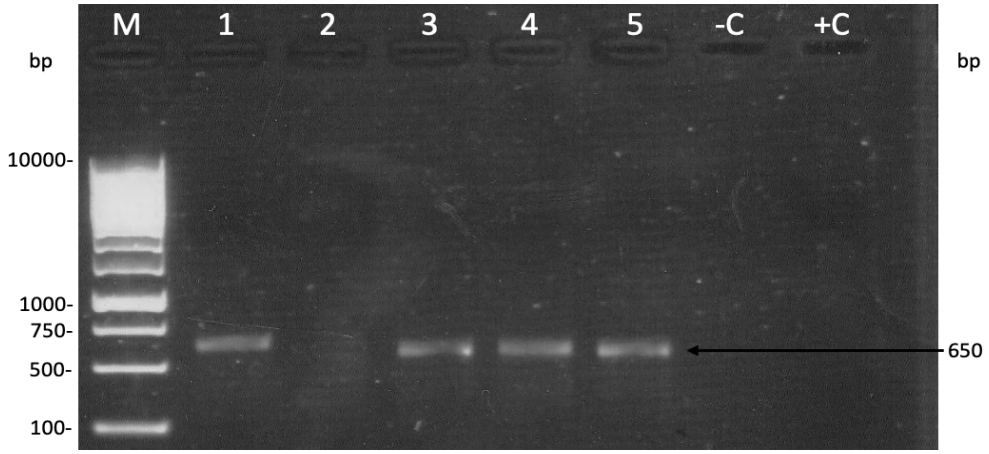

Figure 6. Gel electrophoresis showing PCR products under UV-light. 1kb DNA ladder (M), samples (1-5), negative control (-C), positive control (+C) (Scan: Erik Kügler)

Before sending the DNA material for automated Sanger sequencing to KIGene at CMM (Karolinska Institutet, Stockholm, Sweden) the DNA concentrations were measured using the "Qubit 3.0 Fluorometer" (Thermo FIsher Scientific™, Waltham, USA). The sequencing, reveals combination of the four nucleobases adenine (A), guanine (G), cytosine (C) and thymine (T) in the nucleic acid of the DNA (Weising, 1995). The obtained raw sequences were processed using "BioEdit Sequence Alignment Editor" (Ibis biosciences, Carlsbad, USA) and queried against fungal ITS sequences using BLASTn on the NCBI website. Of the 50 isolates obtained from the tests, two isolates were identified as *L. willkommii* with a sequence homology percent of 99,79 % (Isolate IS 61 R1) and 98,47% (Isolate IS 329 R1) and these were used for further experimentation. (Figure 11)

2.3 Greenhouse inoculation experiment

The plant material consisted of 185 1-year-old European larch seedlings from a single half-sib family from Sudetes in Poland which was provided by Skogsforsk. Seedlings were planted in February 2019 under controlled greenhouse conditions at Ekebo nursery, near Svalöv, Sweden, and then later transported to growing beds outside. The plants were transported to SLU, Alnarp in mid-September 2019 where they were immediately put in controlled climate chambers in the Biotron. The conditions of the chamber were: 12 h light and 12 h dark per day, light intensity 250 µ mol/m^2/s, temperature during light hours 18°C and during dark hours 14°C. The plants were allowed to acclimate to the growing conditions for about 4 weeks prior

to inoculation. The plants were watered by hand twice a week throughout the duration of the experiment.

For all inoculations, the seedling's bark was peeled back to expose the cambium in a window shape (Figure 7) using a sterile scalpel, size 1,15 cm, at between 20 cm and 21,15 cm above the soil line. All inoculations took place between the 15th and 18th of October 2019.

Two separated experiments were conducted:

2.3.1 Experiment 1: Test of substrates

In this experiment, two inoculation methods were tested on seedlings: agar plug of mycelia and a colonized woody segment (Figure 7). For the latter, inocula was prepared by first cutting in half and sterilizing 1 cm long matchsticks, and then autoclaving at 120 °C for 20 min before being placed into a petri dish containing a pure culture of either isolate (#IS 61 R1 or #IS 329 R1) of *L. willkommii* and allowed to grow for 21 days. After that period of time, woody segments appeared to be fully colonized by the fungus. (Figure 8)

One of 6 treatments was applied to individual seedlings:

1. Wounding + sterile woody segment (control to 3&5)
2. Wounding + sterile agar (control to 4&6)
3. Wounding + woody inoculum (Isolate IS 329 R1)
4. Wounding + agar inoculum (Isolate IS 329 R1)
5. Wounding + woody inoculum (Isolate IS 61 R1)
6. Wounding + agar inoculum (Isolate IS 61 R1)

All artificial stem wounds were covered using Parafilm and another ribbon to prevent desiccation of the inoculum.

Figure 7. Left: Window cut with agar plug, Right: Window cut with woody inoculum (Photos: Erik Kügler)

In total, 100 European larch individuals were inoculated; 40 with woody inoculum (containing 20 of each of the two isolates), 40 with agar inoculum (containing 20 of each of the two isolates), 10 with sterile woody segments, and 10 with sterile agar.

2.3.2 Experiment 2: Test of isolates

In experiment 2, five different isolates were used to inoculate trees: The same two from samples obtained in Sweden and used in experiment 1, and three additional isolates obtained from samples collected on diseased larch in Teplice and Most, Czech Republic (kindly provided by Karel Černý). Inocula in this experiment was comprised of only colonized agar plugs. For each of the five isolates, 15 seedlings were inoculated similar to that described in experiment 1. Ten plants were used as a control by wounding and applying a sterile agar plug (i.e. no fungus). The five used isolates were composed out of the 2 isolates from Swedish material, (IS 61 R1 and IS 329 R1) and the 3 isolates from Czech Republic (S.053.15, S.054.15, and S.055.15). (Figure 9)

For both experiment 1 and experiment 2, seedlings were randomly placed on the trays to ensure equal growing conditions. (Figure 11)

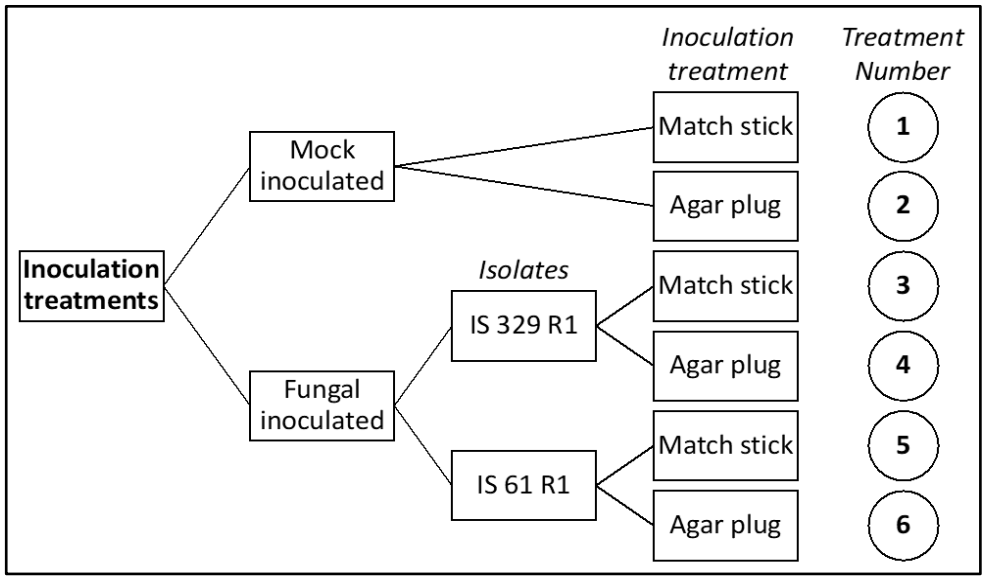

Figure 8. Illustration of inoculation and mock-inoculated (i.e. control) treatments for Experiment 1

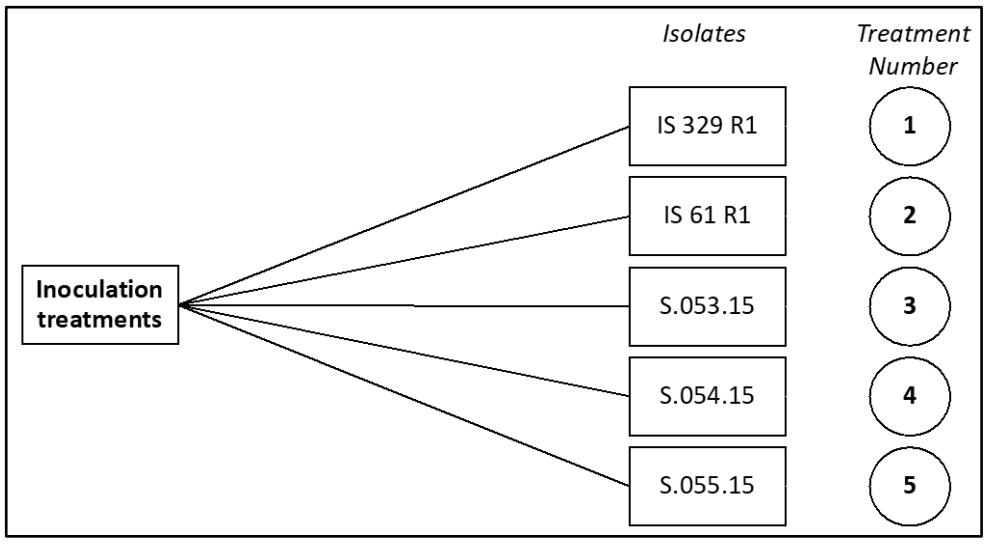

Figure 9. Illustration of inoculation and control treatments for experiment 2

2.3.3 Harvesting plants and scoring disease severity

All plants were harvested and measured from January 20th to January 24th. The seedling's stems were cut 10 cm above and below the inoculation point. The 20 cm cut section was taken to the lab. Two measurements were taken using a digital ruler:

1. Outer lesion length: the length (in mm) of necrosis along the bark of the treated stem (Figure 10)
2. Inner lesion length: the stem was cut in half along the stem axis (longitudinally) to be able to see necrosis in all the cambium and xylem tissue, and examined under a stereoscope to obtain the length of necrosis. (Figure 10)

Any particularities such as callus formation or resin exudation were written down, when observed. (Figure 11)

Figure 10. Left: Measurement of outer lesion length, Right: Measurement of inner lesion length (Photos: Erik Kügler)

2.3.4 Re-isolation of the pathogen

Robert Koch developed a four-step procedure called Koch's postulates in the late 19th century, to prove that a specific organism may be the cause of a certain disease (Walker et al., 2006). The postulates are the following:

1. The disease must be consistently present in the host, and absent in a healthy host.
2. The disease organism must be isolated from the lesions and grown in pure culture.
3. The cultured upon inoculation to a healthy host, should develop symptoms of the disease.

4. The pathogen must be re-isolated and proven identical to the disease organism.

The first three steps were followed throughout the experiment. To ensure recovery of *L. willkommii* from inoculated trees, a smaller subset of samples (12 in total; 1 from each treatment), were selected and re-isolation attempted by taking a small section of the necrotised tissue, disinfecting it for 15 seconds in 70 % Ethanol and then placing it on 2% MEA, and left to incubate in the dark for 1 month. Thereafter, species identification was conducted using based on morphological characterization in comparison with the reference isolates. (Figure 11)

2.4 Statistical analysis methods

For both experiments 1 and 2, the inner and outer lesion lengths were analysed separately. Normal distribution of data was assessed using Shapiro Wilk test. QQ-plots of the data were used to graphically analyse the distribution. Equality of variance was tested using Levene's test. When normal distribution and equality of variance was assured, one-factor analysis of variance (ANOVA) or two factor ANOVA was applied to assess any differences among treatments. When significant effects were detected using ANOVA (as determined at the $\alpha < 0,05$ level), multiple comparisons were made using Tukey's honestly significant difference (HSD) test. Pearson's correlation and subsequent regression analysis were used to study correlations between inner and outer lesion lengths. All tests were conducted using R and R-Studio (RStudio, Inc., Boston, USA).

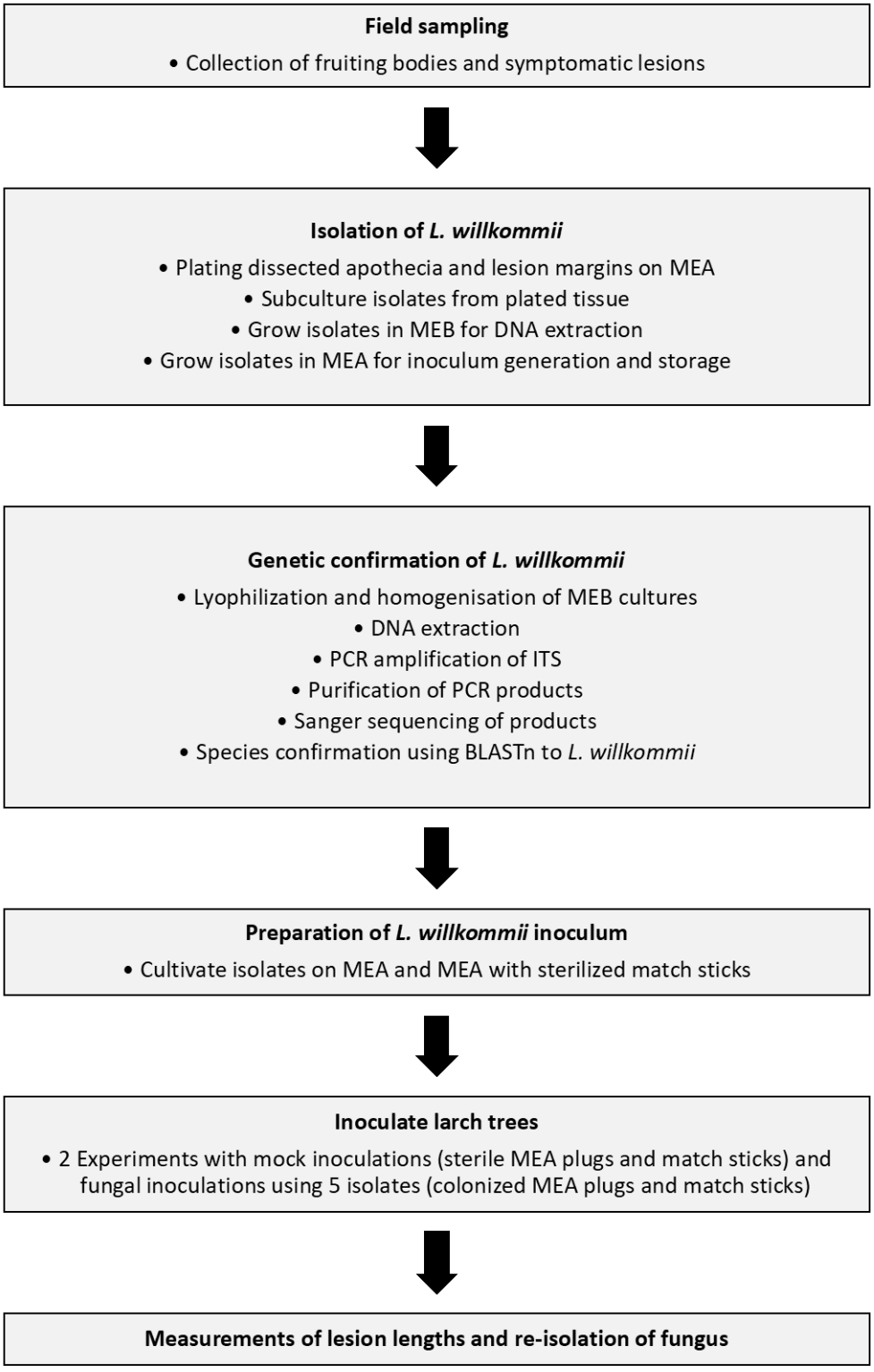

Figure 11. Overview of workflow involved in the thesis work

3 Results

Out of 185 plants, 182 were assessed, as three individuals were pre-emptively studied. None of the trees died. All seedlings showed necrosis around the artificial wound three months after inoculation. Moreover, callus and resin outflow was observed on some individuals (Figure 12). When the branch was dissected longitudinally, necrosis was observed along the cambium tissue as well as necrosis in xylem tissue (Figure 12).

In experiment 1, the average observed outer lesion length was 13,24 mm (range between 11,50 and 16,49 mm), the average observed inner lesion length was 15,61 mm (range between 11,52 and 27,51 mm). In experiment 2, the average observed outer lesion length was 12,64 mm (range between 11,50 and 15,66 mm), and the average observed inner lesion length was 14,27 mm (range between 11,55 and 24,78 mm) (Table 1).

Table 1. Summary of number of individuals, means, ranges and standard deviations (SD) of outer and inner lesion lengths of Experiment 1 and Experiment 2

		Nr.	**Mean in mm**	**Range in mm**	**SD in mm**
Exp. 1	Outer lesion length	97	13,24	11,50 - 16,49	1,27
	Inner lesion length	97	15,61	11,52 - 27,51	2,52
Exp. 2	Outer lesion length	85	12,64	11,50 - 15,66	1,06
	Inner lesion length	85	14,27	11,55 - 24,78	2,22

Inner lesion length was used to compare the differences among treatments (Figure 12). Necrosis spreads in tissue internally that cannot be seen from the outside. The usage of outer lesion length would therefor lead to inaccurate estimates.

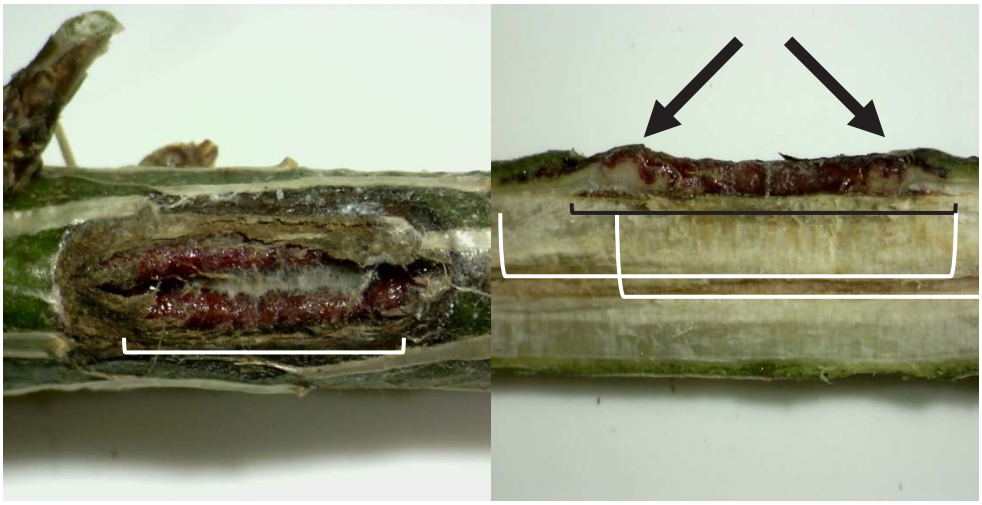

Figure 12. Left: Wound with calluses (white bracket), outer necrosis and resin outflow, Right: Longitudinal cut showing necrophylactic periderm and callus formation at the margin of the lesion (arrows), extent of xylem necrosis (black bracket) and cambium necrosis (white bracket) (Photos: Erik Kügler)

3.1 Experiment 1: Test of substrates

Data was assessed using Shapiro Wilk Test ($p < 0{,}001$) which proved significant deviation from a normal distribution. As ANOVA is robust against certain violations of normality (Schmider et al., 2010), data was still considered normally distributed taking the manual inspection of the QQ-plot of the data into account. Levene's test for homogeneity of variance showed equality of variances ($p = 0{,}133$). A two-factor ANOVA was used to test for differences in lesion length between substrates, between isolates + control and between the combination of substrate and isolate (Table 2). Significant differences were found between substrates ($p < 0{,}001$) and between isolates + control ($p < 0{,}001$). There were no significant differences found between their interaction ($p = 0{,}417$).

Table 2. *ANOVA table results for Inoculum, Isolate and Inoculum:Isolate; including source, degrees of freedom (df), mean square (MS), F-value and p-value*

Source	df	MS	F-value	p-value
Inoculum	1	134,98	31,34	< 0,001
Isolate	2	38,22	8,87	< 0,001
Inoculum:Isolate	2	3,81	0,88	0,417

Tukey's HSD found that trees inoculated with IS 329 R1 on agar and IS 61R1 on agar had equivalent and significantly longer lesions than trees of all other treatment combinations (Figure 13). Lesion lengths for the other treatments did not differ significantly from each other, meaning that there was no statistically significant effect of the fungal inoculations compared to the control in the woody inoculum type.

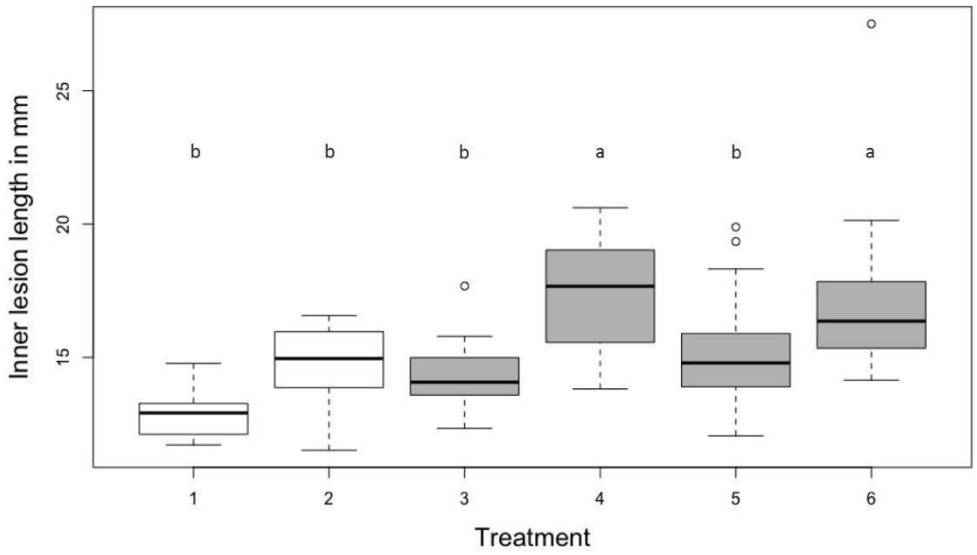

Figure 13. Boxplots of inner lesion lengths from Experiment 1 comparing six treatments. Letters denote significant differences in lesion lengths as determined by Tukey HSD at the α = 0,05 level; Treatments: 1 = Wood control, 2 = Agar control, 3 = Wood IS 329 R1, 4 = Agar IS 329 R1, 5 = Wood IS 61 R1, 6 = Agar IS 61 R1

3.2 Experiment 2: Test of isolates

Data was not normally distributed according to Shapiro Wilk test ($p < 0{,}001$), but inspection of residuals found these to be minor and acceptable violations. Homoscedasticity was not violated as determined by Levenes Test ($p = 0{,}050$). A one-way ANOVA testing for differences in lesion length among the five isolates and control showed a minor statistically significant difference between isolates ($p = 0{,}0485$) (Table 3). Tukey HSD showed that lesion lengths of different isolates however do not differ significantly (Figure 14). Lesion lengths of fungal treatments were not significantly different than those of mock treatments, apart from IS 329 R1. (Figure 14).

Table 3. *ANOVA table results for Isolate (5 isolates + 1 mock); including source, degrees of freedom (df), mean square (MS), F-value and p-value*

Source	df	MS	F-value	p-value
Isolate	5	10,70	2,35	0,0485

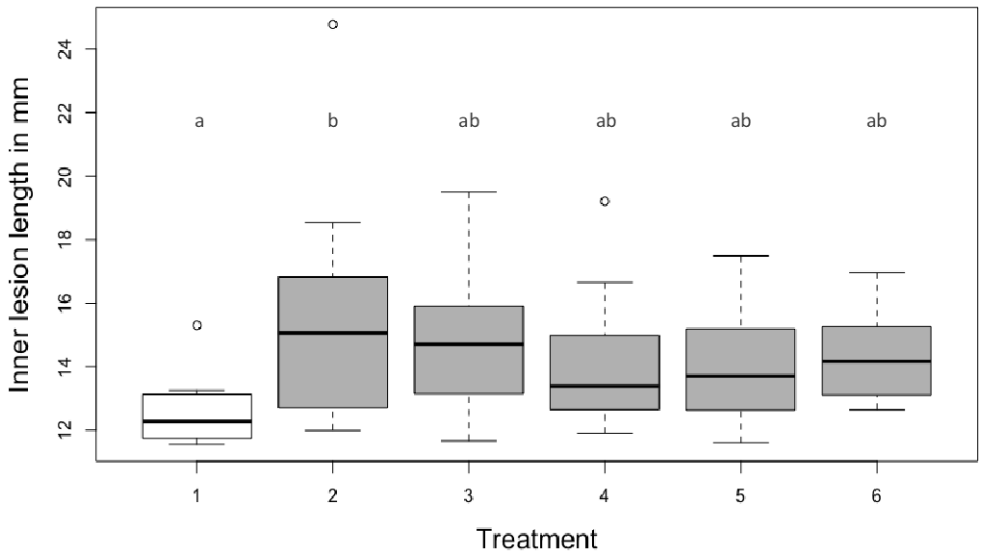

Figure 14. Boxplots of inner lesion lengths from Experiment 2. Letters denote significant differences in lesions lengths as determined by Tukey HSD at the α = 0,05 level: 1 = Control, 2 = IS 329 R1, 3 = IS 61 R1, 4 = S.053.15, 5 = S.054.15, 6 = S.055.15

3.3 Correlation analysis outer lesion length / inner lesion length

To find out if the measurement of inner lesion length is needed and cannot be calculated by the outer lesion length, a correlation analysis between inner- and outer lesion lengths was carried out.

Pearson's correlation test showed that the outer lesion length gives a reliable estimate for inner lesion length ($p < 0{,}001$). Values of outer lesion length can explain 37 % of the variation in inner lesion length ($R^2 = 0{,}372$) (Figure 15). This indicates a fairly inaccurate correlation.

Inner lesion length shows more variation within the dataset than outer lesion length (Figure 16).

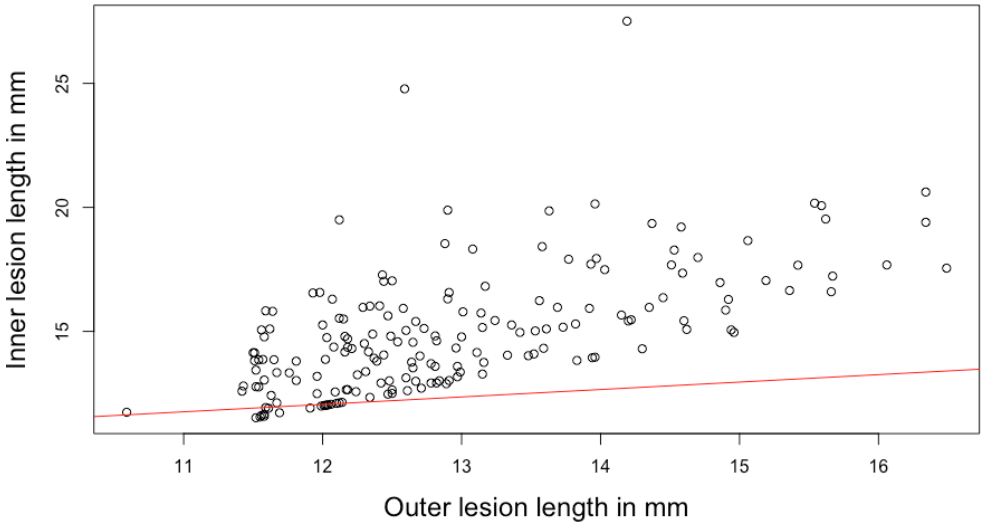

Figure 15. Correlation plot inner lesion length – outer lesion length, Red = Regression line

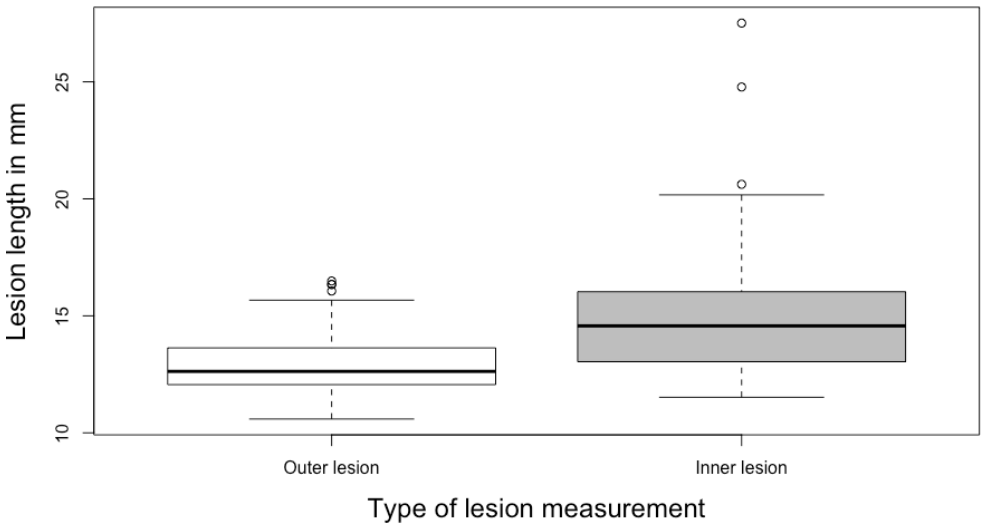

Figure 16. Boxplot comparing variation of inner lesion length and outer lesion length

3.4 Re-isolation

Out of 12 attempted re-isolations of the fungus, in 4 samples, *L. willkommii* was successfully isolated based on morphological characteristic and comparison to reference isolates. All of those *L. willkommii* identified samples were taken from inoculated seedlings. *Lachnellula willkommii* was not isolated from any of the samples taken from control trees.

4 Discussion

The overall goal was to find an appropriate screening method for testing Sudetes European larch families against *L. willkommii* by discovering a suitable inoculation substrate and pathogen isolate. The suitability of methods was assessed using disease severity as a measurement.

4.1 Inoculation substrate

The use of MEA as an inoculum substrate results in longer lesion lengths. The inoculum potential of the agar plug seems thus higher than of the woody inoculum. This can have several reasons: MEA contains a higher water content than the sterilized woody substrate. Fungi like *L. willkommii* prefer a moist growing environment. The high sugar content of MEA could also be preferred fungus. Mycelia was already growing faster on MEA than on wood chips when culturing which confirms the good supply of nutrients. Wood as an inoculum substrate was harder to cultivate but was suspected to be more virulent than MEA as it resembles the natural host tissue. At the same time, the woody substrate, already preconditioned by the fungus colonizing it during several weeks prior to inoculation, may found a preferred niche, which is not worth leaving. This could explain the greater virulence observed in the mycelia infested agar plug. The jelly nature of the agar plug made the placement of the inoculum fit closer to the artificial wound than the stiff woody inoculum. This could have facilitated the fungus to have more direct contact with the exposed cambium and spread more easily than from the woody inoculum. Both MEA and woody inoculum were chosen because of their accessibility, the simplicity of culturing and because they are used as standards in other studies (e.g. Sylvestre-Guinot and Delatour, 1983). Based on this experiment, MEA stands out as the easier inoculum substrate to use in

screening tests, not only for giving reliable necrosis measurements on seedlings but also the ease of producing mass inoculum in the lab for later field tests. *Lachnellula willkommii* studies in France used sorghum straw inocula combined with different wounding methods (Pâques et al., 1999; Sylvestre-Guinot et al., 1994, 1999). These studies were however conducted on older, bigger trees, where other wounding methods and hence different inocula seem appropriate. For the planned future field trials, agar plugs should be suitable as inoculation substrate.

4.2 Virulence of isolates

Virulence studies suggest, that different isolates of one pathogen often cause different pathogenicity. The use of different isolates causing *Hypoxylon* canker on *Populus tremuloides* even caused differences in canker morphology besides differences in pathogenicity (French, 1978). Also, isolates of different geographic locations might cause different disease severity as demonstrated in studies of pitch canker on *Pinus spp.* (Steenkamp et al., 2012) and *Pseudotsuga menziesii* (Gordon et al., 2006). The choice of isolates is hence crucial for pathogenicity and any screening assays that may be used in tree improvement programs. Nevertheless, no *L. willkommii* study comparing the pathogenicity of different isolates, to my knowledge, can be found. Based on the results of this work, neither geographic location nor individual differences of isolates from the same geographic location seem to influence disease severity. For later tests the choice among the five available isolates is hence of minor importance. Only IS 329 R1 showed significantly longer lesions than the control treatment and should therefore be used in future trials.

4.3 Re-isolation

Despite resulting in relatively small lesions, the pathogen was successfully re-isolated in some of the inoculated trees, which proves that the pathogen is alive and may cause further disease given enough time. Therefore the chosen time of ending the experiment may have prevented us from seeing the full course of disease. Future resistance screenings should therefore consider this and include longer term lesion measurements.

4.4 Lesion length as a proxy for disease screening

The analyses of the study are based entirely on the measurement of lesion lengths. Literature suggest that shorter lesions indicate stronger defences of the host (Bonello and Blodgett, 2003; Bonello et al., 2006; Krokene et al., 2008). In previous studies, dissecting the stem longitudinally has given a good visual for assessing necrosis in the xylem, cambium and phloem tissue (Nevill et al., 1995; Jung et al., 1996). The same procedure applies to this study as mycelia of *L. willkommii* colonises phloem and cambium (Hiley, 1919) and should be studied from the inside of the stem. Besides this method, the lesion length on the bark visible from the outside of the stem was assessed to see if it correlates well to the inner lesion length, which could save time in the future by only measuring the external lesion. However, the correlation between the two measurements was not sufficient to deliver accurate estimates. Therefore, inner lesion length should be used as a precise measurement in future investigations.

In other larch canker studies (Pâques et al., 1999; Sylvestre-Guinot and Delatour, 1983) presence of callus, resin outflow and apothecia and length of necrosis were measures for infection. The present study assessed infection using only necrosis measurements from bark- and inner-stem- tissues. The reason for not assessing other symptoms was the short time between inoculation and harvest compared to the longer growing period in other studies. In the present study, calluses and resin outflow could only be observed on view individuals. Apothecia cannot be formed in this short time. Nevertheless, necrosis served as a reliable indicator for measuring infection by *L. willkommii* over that induced by artificial wounding.

Lachnellula willkommii exhibited slow growth *in vitro* so a rapid formation of necrosis and large lesions lengths *in planta* were not anticipated. The slow growth in the host is also plausibly affected by the high resistance of the host. The tree family used in this study came from the supposedly resistant regions in Poland. The slow growth after inoculation, seemingly influenced by both fungus growth and host resistance, led to small variances in lesion length.

The reactions of the seedling to the inoculation depend predominantly on the host. Not only genetic variations influence the hosts susceptibility. Inoculated plant organs can vary among individual seedlings which can cause differences in disease severity (Sylvestre-Guinot and Delatour, 1983) that cannot be influenced by host genetics. A high number of repetitions per treatment however should overcome that drawback.

Certain times of the year provide favourable climate conditions for fungi to infest and grow on hosts in a natural environment (Buczacki, 1975). Also, the physiological state of the host during and post-inoculation can influence fungal development in the host (Sylvestre-Guinot and Delatour, 1983). The timing of the artificial inoculation can therefore effect the natural growing patterns of the fungus and the host's susceptibility. Sylvestre-Guinot and Delatour (1983) and Buczacki (1973) have noted the seasonal variation in lesion lengths in European larch. Furthermore, Ito et al. (1963) and Sylvestre-Guinot and Delatour (1990) clarify that inoculations during growing season of European larch leads to less successful inoculations than inoculations before or after the growing season. In the present study, inoculations were conducted in mid-October, usually when dormancy has set in, and it cannot be excluded the possibility that this factor may have affected symptom development.

The use of artificial inoculations facilitates infection by a pathogen. The method of mounting mycelia infested inocula on an artificial wound does not mimic the natural course of infection by *L. willkommii* which happens through wind dispersed spores. Whether spores need some sort of wounding to invade the plant is still unclear (Sylvestre-Guinot and Delatour, 2002)**.** The applied inoculation method presumes that fungus enters through wounding and ignores possible preformed bark defences, that may play a role in the host response to infection. A more natural inoculation method would be via spore inoculation. An earlier study investigated the effects of spore dispersion with those of mycelium inocula, however only mycelium inocula induced high infection rates (Sylvestre-Guinot et al., 1994). Even though the use of mycelium inocula does not mimic natural inoculation, it is still the most effective method to test for virulence of *L. willkommii* (i.e.: Pâques et al., 1999; Sylvestre-Guinot et al., 1994, 1999) and provides comparable measurements for screening in studies.

4.5 Recommendations for future investigations

The previously discussed factors lead the way to the future of *L. willkommii* inoculation experiments. Several experiments could help to fill current knowledge gaps.

The choice of a suitable inoculation method and inoculum substrate is poorly discussed in literature. The present study provides some new information that can be adjusted for future screening of European larch genotypes for

future breeding programmes. However, it would still be interesting to conduct further research comparing other inoculum substrates such as different woody substrates, different media and the sorghum straw (Pâques et al., 1999; Sylvestre-Guinot et al., 1994, 1999). Especially the use of non-impregnated European larch wood instead of the chemically treated matchsticks would be interesting to test. The same applies for differing wounding strategies and inoculation methods. Different wounding techniques that vary in size and the number of wounds may be factors that influence disease severity. Even though the use of ascospores induced lower infection rates than mycelium infested inocula in a previous study (Sylvestre-Guinot et al., 1994), the use of ascospores should be tested again. Combined with different wounding strategies, this method could possibly be closer to mimic the natural infection process. A longer growing period of the fungus on the seedling would lead to more clear disease symptoms (Pâques et al., 1999; Sylvestre Guinot and Delatour, 1983) and should therefore be considered in the design of future studies.

The isolates used in this study originated from only two regions. As Gordon et al. (2006) and Steenkamp et al. (2012) suggest, isolate location can lead to differences in pathogenicity. Testing the virulence of different *L. willkommii* isolates from more locations could add more information. In addition, testing differences between combinations of isolates from different locations and different host provenances could result in new knowledge about location dependent host-pathogen interactions.

The present experiment is strongly affected by the host's resistance to *L. willkommii*, especially considering that the material tested was already selected for its presumed tolerance to the fungus. It would have been ideal to test inoculation methods on less resistant European larch individuals and it may have given more variation in the symptom expression. Another host characteristic worth investigating is how age and size of tree is influencing the host response.

European larch is native to central and eastern European mountain ranges, where it is growing in dry continental climates on well drained soils (Da Ronch et al., 2016). On the contrary, *L. willkommii* is favoured by high air humidity and oceanic climates (Robak, 1964; Cech, 2013). As infection with *L. willkommii* is mostly observed out of European larch's native distribution range, namely in more oceanic climates, it might be worth investigating why European larch is confined to the dry alpine regions. This could provide information about suitable cultivation locations in the future.

5 Conclusion

Both tested inoculum substrates, wood and agar, produced disease symptoms on the host. Disease symptoms were quantifiable and comparable using measurements of varying lesion lengths. Mycelium infested agar plugs produce significantly longer lesion lengths than infested woody inocula. Considering future screening trials, inoculations using agar inocula can give reliable measurements of disease severity for comparing among individual genotypes. Another advantage of using agar inocula is that it is rather easy to mass produce in the lab and quick to adhere on the seedling.

As all tested isolates showed the same virulence, the choice of isolates is negligible. In the goal of standardisation, IS 329 R1 would be suffice for further inoculation experiments. This isolate showed significantly longer lesion lengths than the control treatment. It could possibly be better adapted to local growing conditions as it was found in Sweden.

Climatic factors and site conditions play an important role in influencing host-pathogen interactions (Cech, 2013). The future breeding experiments should therefore take the intensified symptoms in cold, moist sites with little air drainage such as valley bottoms or hollows into account (Cech, 2013; Schober, 1977). To improve future inoculation experiments for screening disease resistance, more research should be invested in understanding the mechanisms affecting disease tolerance. Standardising methods are critical for this purpose and therefore more efforts into optimizing inoculum substrates and testing methods, may be warranted.

The present study helps to perform upcoming field trials more accurately and carefully. The described method is a crucial step to proceed with screening efforts that will lead to tree-improvement programmes for resistant European larch in Sweden. In the long-term, if a breeding program finds suitable

resistant genotypes of European larch, that would give new or renewed possibilities for reintroducing a valuable tree species alternative for Sweden and northern Europe.

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Appendix

Agar recipes

Malt Extract Agar (MEA)

Ingredients

- 20 g Malt extract
- 18 g Agar
- 1000 ml deionised water

Method

- Mix all ingredients
- Autoclave at 121°C for 15 minutes
- Pour into Petri dishes
- Cool the plates in room temperature

Malt Extract Broth

Ingredients

- 20 g malt extract
- 1000 ml deionised water

Method

- Mix the ingredients and stir thoroughly
- Autoclave at 121°C for 15 minutes
- Pour 35 ml into falcon tubes