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Master's Thesis

PaLAR3 locus in Norway spruce genome and its link to resistance against Heterobasidion parviporum in drought stress

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Tiivistelmä

Heterobasidion –sukuun kuuluvien patogeenisten sienten aiheuttama juurikääpäinfektio on merkittävin hävikkipuuta aiheuttava patogeeni kuusella (*Picea abies*) Pohjoismaissa. Kuusen genomissa leucoantosyanidini reduktaasi -entsyymiä koodaavan PaLAR3 –geenin tiedetään vaikuttavan puuyksilön resistenssiin *H. parviporum* -juurikääpäinfektota vastaan. Geenin lokuksessa kuusella voi olla AA, AB tai BB alleelit. B-alleelin läsnäolon kuusen PaLAR3 lokuksessa on todettu vaikuttavan kuusen entsyymituotantoon siten, että (+)-catechiini pitoisuudet ovat korkeammat juurikääpäinokulaation jälkeen niillä yksilöillä, joiden PaLAR3 lokuksesta löytyy B-alleeli. Ilmastonmuutoksen seurauksena häiriöt ja muutokset hydrologisissa kierroissa lisääntyvät, ja sään ääri-ilmiöt yleistyvät. Tämän seurauksena kuivuuden uskotaan yleistyvän etenkin pohjoisilla alueilla, lisäten patogeenien ja tuholaisten leviämisen riskiä. Metsänjalostusohjelmilla pyritään jalostamaan metsiä siten, että ne olisivat taloudellisesti tuottavia ja kannattavia tulevaisuudessakin. Tutkimalla kuusen genetiikkaa jalostusohjelmaan voidaan lisätä sellaisia yksilöitä, jotka todennäköisimmin kestävät tulevaisuuden ilmastohaasteet paremmin.

Tässä tutkielmassa tutkittiin kuivuuden vaikutusta juurikääpäinfektion leviämiseen kuusessa kuivuusstressin alaisena. Kuusten PaLAR3 geenin alleelit määritettiin ja tuloksissa tarkasteltiin inokuloidun juurikäävän aiheuttaman nekroosin pinta-alaa suhteessa alleeleihin, puiden kasteluolosuhteisiin, sekä inokuloinnissa käytettyyn juurikääpäkantaan. Tulosten valossa PaLAR3 geenin alleeleilla ei ole vaikutusta nekroosin pinta-alaan. Tilastollisen analyysin perusteella vaikutusta ei havaittu kuivuusstressistä kärsineessä, eikä normaaleissa kasteluoloissa olleessa ryhmässä. Ainoa tilastollisesti merkittävä havainto oli sen, että eri juurikääpäkannat (Hpa1 ja Hpa2) infektoivat eri tavoin. Hpa2 kannan infektiokyky vaikuttaa olevan korkeampi normaaleissa kasteluolosuhteissa. Näiden tulosten valossa metsänjalostusohjelmaan ei tulisi lisätä PaLAR3 geeniä. Tulevaisuudessa tutkimuksen tulisi pyrkiä tutkimaan eri geenien vuorovaikutuksia patogeeni-infektion alaisuudessa. Esimerkiksi kuusella myös toinen geeni (PaLAC5) on jo yhdistetty juurikääpäresistenssiin, ja sen tutkiminen yhdessä PaLAR3-geenin tuottaman entsyymitoiminnan kanssa voisi tuoda ratkaisuja tulevaisuudessa todennäköisesti pahenevaan *Heterobasidion* –suvun juurikääpäinfektioiden torjumiseksi.

Avainsanat Juurikääpä, PaLAR3, *Heterobasidion*, kuivuusstressi

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Heterobasidion genus fungi are the most significant pathogens in Finland causing root rot. These fungi infect Norway spruce (*Picea abies*), causing wood deficit in the forest industry. Leucoanthosyanidin reductase enzyme encoding gene, PaLAR3, has been linked in to the resistance of Norway spruce against *H.parviporum* induced root rot infections. The alleles in this locus can be AA, AB or BB, and the enzyme production and (+)-catechin concentrations have been proven to be higher in inoculation experiments in individuals with B allele present in their PaLAR3 locus. Climate change is predicted to increase disturbances in not only on the hydrological cycles, but also on the weather conditions. The drought is predicted to increase in the Northern hemisphere, increasing the risk of pathogens to spread into new areas. Forest breeding programs aim to make the forests more profitable and productive in the future as well. By studying the genetics of Norway spruce, it is possible to add more resilient individuals into the breeding program, to tackle the climate challenges the future might hold.

In this Master's thesis the spreading of the root rot infection under drought stress was studied. The PaLAR3 alleles were determined, and the area of necrosis caused by the fungal infection was compared against the alleles, watering treatments and the fungal strain used in the inoculations. Regarding the results the alleles in an individual's PaLAR3 locus did not have any effect on the area of the necrosis. There were no statistically significant differences between the watering treatments. The only statistically significant result was that the different fungal strains' (Hpa1 and Hpa2) infectiveness varied between different watering treatments. This is probably due to the different routes of pathogenesis. Hpa2 strain seems to be more infective in normal watering conditions. However, considering these results the PaLAR3 gene should not be added into the forest breeding program as itself. In the future, the research should focus more on gene interactions, since also other genes (e.g., PaLAC5) have been linked in the resistance against root rot infections in Norway spruce. By studying these genes together, the solutions for the deteriorating situation of the spreading of the fungal diseases could be discovered.

Keywords Root rot, PaLAR3, Drought Stress, *Heterobasidion*

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Where deposited

Further information

1. Introduction

Norway spruce (*Picea abies*) is the most economically important species of the forestry in Europe, providing highly valuable raw material for forest industry (Skrøppa 2003). One of the most severe threats to the forest industry is root rot caused by the members of *Heterobasidion* species complex. Two species of *Heterobasidion* are known in Finland: *H. annosum* s.s. and *H. parviporum* (Korhonen 1978, Korhonen et al. 1998). These *Heterobasidion* spp. have partially overlapping, yet different host preferences associated with Sctots pine (*Pinus sylvestris*) and Norway spruce (Korhonen 1978, Carpetti et al. 1990, Carbelotto & Gonthier 2013). The distribution area of *H. parviporum* follows Norway spruce, its main host, to the Northern regions of Finland (Korhonen & Lipponen 2001). *Heterobasidion parviporum* causes root rot in Norway spruce and is predicted to increase in the future, causing more damage to the forests, thus threatening the expected wood productivity increase of the forests (Jansson et al. 2013). Also, the increase of abiotic disturbances, such as wind, snow, flooding, and drought in a forest ecosystem are expected to increase, afflicted by climate change. This could have some major effects on the forest wood production capability in the future, as the wind damages are enhanced by pathogens, such as *H. parviporum*, reducing the stability of the trees and the quality of the wood material (Krisans et al. 2020). As the climate change driven impacts on the hydrology are likely to become more intense in the future, the drought induced stress reactions in vegetation will become more common, making the trees more vulnerable against different pathogens, such as *H. parviporum* and *H. annosum* (Terhonen et al. 2019). As the weather conditions are predicted to become more extreme, the sustainable supply of forest tree products is threatened and the trees are more vulnerable not only against pests, but also diseases, such as root rot (Yeoh et al. 2021).

Under a fungal pathogen attack, the plants have a way of fighting the infection. Among other mechanisms, the plants' defense includes production of different toxic metabolites. To successfully colonize its host, a pathogen must overcome a vast range of different chemicals, including antibacterial compounds and metabolizing enzymes (Hu et al. 2020). Norway spruce genome has been studied in the past and the linkage between root rot resistance and PaLAR3 locus has been discovered (Nemesio-Gorriz et al. 2016). This locus has variability that can be utilized in breeding more resilient saplings for forest regeneration. Variation in one gene, leucoanthocyanidin reductase 3 (PaLAR3), is known to play a role in resistance of Norway spruce against fungal *H. parviporum*

growth in sapwood (Nemezio-Gorriz et al. 2016). The enzyme production, encoded by the PaLAR3 gene, increases under a pathogen attack, as a defense mechanism against the infection (Jyske et al. 2020).

Even though different fungal diseases are predicted to increase in the future, not many of the molecular markers for pathogen resistance have been validated. Nonetheless, in Norway spruce, the leucoanthocyanidin reductase 3 (PaLAR3) gene has been linked in the resistance against *H. parviporum* (Nemesio-Gorriz et al. 2016). Also, other genes, such as laccase gene, PaLAC5, has been linked to *H. parviporum* resistance (Elfstrand et al. 2020), but the importance of the gene interactions in an individual tree is not yet very well known. This knowledge could be utilized in breeding of more resistant forest regeneration material and thereby reduce the loss of the tree material that cannot be utilized due to a fungal infection (Lind et al. 2014).

1.1. Forestry and climate change in Finland

Drought is a complex phenomenon that occurs naturally. However, as climate change alters the hydrological processes, drought periods are likely to become more frequent and more intense than in the past. This not only increases the risk of wildfires (Mukherjee et al. 2018), but also causes changes in the precipitation levels, soil moisture, runoff, and causes stress reactions in the vegetation (Cook et al. 2018). Climate change models suggest globally that the surface temperatures and precipitation will increase in the future, especially in the polar regions (Seo et al. 2015). Therefore, it is vital for the forest industry to find ways to mitigate the damages caused by drought stress induced diseases, such as root rot.

Finland is the most forested country in EU, as approximately 80% of the land is covered by forests. Improved forest management activities have increased the volume of the growing stock past years, but on the other hand, the biodiversity of the Finnish forests has decreased. As the management of the forest has been targeted mainly on increasing the wood production, Norway spruce has become the most common conifer, over Scots pine (*Pinus sylvestris*). In addition to the biodiversity loss, loss of ecosystem service provision, leaching of nutrients, and increased carbon emissions due to ditching have become more common (Lehtonen et al. 2021). During the last decades, monocultures have been favored in managed forests, over mixed forests. According to the Finnish National Forest Inventory (NFI) the dominance of one tree species in a pure stand is more than 95% (Korhonen et

al. 2017). The pure stand areas are more vulnerable to pathogens and diseases, as they are usually species-specific. Especially Norway spruce has become more common in pure stand monocultures, leading to several problems. Even though spruce is not as likely eaten by moose, as pine is, the drought stress combined with e.g., bark beetle invasions and fungal pathogens has already created vast destruction in the managed spruce forests (Tanin et al. 2021, Öhrn et al. 2021).

The trees in Finland have genetically adapted to the current environmental conditions and the natural growth rate varies between the Southern and Northern light and temperature conditions. The mechanisms that the trees use to distinguish seasonal changes, can act as a buffer for adaptation for the long-term environmental change as well. However, the current warming rate is rapid, and even though the trees have adaptive mechanisms, the natural adaptation to new environmental conditions might not be fast enough. Therefore, to maintain the wood production, forest breeding programs are necessary. By choosing the individuals for breeding that are most resilient in the future conditions, might help to secure the forest industry's future (Haapanen & Mikola 2008).

In the Finnish forest breeding program, the used procedures are determined in a long-term forest breeding program that is carried out by Natural Resources Institute Finland, Luke. The aim is to secure wood production and economically sustainable forest industry in Finland. Some of the key factors in the breeding program is genetical variation and how to benefit from it in the future, while avoiding the possible problems caused by inbreeding. The breeding material is carefully tested in different climatic conditions and field experiments and only the most genetically adjustable genotypes are chosen for further breeding (Haapanen & Mikola 2008).

2. *Heterobasidion parviporum* in Norway Spruce

Heterobasidion parviporum is one of the native fungal pathogen species that belongs to a root-rot fungus complex and is considered the most destructive pathogen in Norway spruce in Finland (Asiegbu et al. 2005). Primary infections by *Heterobasidion* species are established when airborne basidiospores land and germinate on freshly exposed wood substrate (Redfern & Stenlid 1998), followed by secondary spread to neighboring trees via root contacts (Redfern & Stenlid 1998, Piri et al. 2021). This necrotrophic fungus derives its nutrients mainly by eating the wood material, thus

killing its tree host. The fungal infection causes e.g., stringy white rot, necrosis in sapwood or presence of fruiting bodies in the tree. *H. parviporum* often colonizes the heartwood of Norway spruce, causing decay and tree mortality. However, an infected tree might not show any symptoms in decades, which makes the detection of the infection difficult (Allikmäe et al. 2017). Furthermore, this promotes the spreading of the fungi to neighboring trees via root contacts (Gonthier & Garbelotto 2013, Piri 1996, Piri 2003, Piri & Hamberg 2015). Therefore, mitigating the spreading of the fungi by planting more resilient trees is important. By doing so the forests could become more resilient, and the wood deficit would possibly decrease, as the trees' ability to fight the infection becomes more effective. Also, by adding different species of trees could prevent the infections from spreading (Huuskonen et al. 2021).

Heterobasidion parviporum naturally infects and causes not only root, but also stem rot in Norway spruce (Wen et al. 2021). The pathogen causes necrosis on the infected individuals in the northern hemisphere, and is therefore, an important pathogen in Finland, spreading from the South to North as the climate change driven warming creates more suitable habitats for the fungi and its host. Different *Heterobasidion* species cause trees to fall more easily in storms, as the wood inside the tree is getting rotten. Also, this affects the quality of the wood, and the material cannot be utilized to its full extent by the forest industry, if the infection has spread in the wood. The concern about the fact that the forests are monocultures, dominated by only one species, has risen recently. All forests consisting only one tree species are vulnerable to both biotic and abiotic stress and damage. In addition, especially spruce is susceptible to many pathogens and pests, e.g., *Heterobasidion* spp. and bark beetle, that are more likely to spread to new areas due to climate change (Huuskonen et al. 2021).

Pathogen and pest attacks on trees have negative impacts on not only the health, but also the biodiversity of natural forests as well as managed forests. This has major socio-economic impacts globally and can have large economic and ecological consequences. Therefore, the value of increasing the understanding of interactions between the pathogens and the trees is extremely important. In addition, this knowledge enables the possibilities to incorporate the increased resistance into forest tree breeding programs (Elfstrand et al. 2020).

2.1. Root rot resistance marker PaLAR3

PaLAR3 is a gene that encodes leucoanthocyanidin reductase (LAR) enzyme, and it catalyzes the flavonoid biosynthetic pathway. It belongs to the short-chain dehydrogenase/reductase protein family and is a catalyzer in the synthesis of 3-flavanol (+)-catechin, that is a precursor of the proanthcyanidins from leucoanthocyanidin (Maugé et al. 2010). It has been associated with increased resistance against pests and pathogens (Hammerbacker et al. 2014) In addition, the presence of PaLAR3B allele has been associated with higher (+)-catechin concentrations, reducing the *H. parviporum* growth in Norway spruce sapwood (Nemezio-Gorriz et al. 2016). Increased levels, especially in the local (+)-catechin concentrations, both in phloem and in sapwood have been detected under a pathogen attack (Jyske et al. 2020) as a defense mechanism against the infection (Hu et al. 2020). However, the primary location of the (+)-catechin production and storage is in the phloem parenchyma cells (Jyske et al. 2020).

3. Aim of the study

The aim of this Master's thesis is to test the effects of drought in Norway spruce and the linkage to resistance against *H. parviporum* infection between the different allele combinations in an individual tree's PaLAR3 locus. The goal is to see if one of the alleles (A or B) gives the trees more resilience, and whether a particular allele combination can be more advantageous over the others, against inoculated *H. parviporum* induced fungal infection in tree trunk under drought stress. Nemezio-Gorriz et al. (2016) suggest, that the individuals with allele B in their PaLAR3 locus, even the heterozygous individuals, are more resistant against *H. parviporum* infection, since the leucoanthocyanidin reductase enzyme production and (+)-catechin concentrations are higher. However, increasing drought conditions were not included in their study, and therefore, this Master's thesis focuses on the PaLAR3 locus and its link to root rot infection resistance in Norway spruce against *H. parviporum* attack in drought stress. Therefore, the hypothesis in this thesis is:

The resistance against *H. parviporum* infection (here defined as necrotic tissue) under drought stress is higher in the Norway spruce that have PaLAR3B allele present in their PaLAR3 locus.

4. Materials and methods

4.1. Plant and fungal material

The experiment was carried out ex-situ in order to control the environmental aspects during the experiment. The three-year-old Norway spruce test saplings were received from the Natural Resources Institute Finland (Luke), and then established in the Forest Botany and Tree physiology greenhouse in Göttingen, Germany (51°33'28.4'' N 9°57'30.5'' E). The saplings were planted in 3-liter plastic pots, containing 2,5 liters fertilized peat soil (Flora gard, TKS®2 Instant Plus, Hermann Meyer KG, Rellingen, Germany). After potting, the saplings received tap water to maintain the soil moisture, and they were treated and monitored for further experiment. No fertilizers were added during the experiment.

Two different *Heterobasidion parviporum* strains were received from Luke and used for the inoculation. The strains were *H. parviporum* strain 1 (Hpa1: strain number: SB2005 9.16, isolated from Norway spruce seedling) and *H. parviporum* strain 2 (Hpa2: strain number: SB 201401 2.69, isolated from Norway spruce seedling). The fungal isolates were cultured in the dark, on 1,5% Malt Extract Agar (MEA) for two weeks at +21°C prior to the inoculations. The inoculation was done with a sterile 5mm cork borer to punch through the bark to reach the sapwood surface. Equal sized plugs from pure culture of *Heterobasidion* sp. (Hpa1 or Hpa2) or control (1,5% MEA) were placed onto the exposed surface and sealed with Parafilm®, approximately 5 cm above the stem base. Some of the trees were treated with mock-inoculation, in which the inoculated agarose did not contain any fungi. Two different *H. parviporum* strains (Hpa 1 and Hpa 2) were inoculated in different specimens. Trees that were not inoculated were also included in both watering treatments as control specimens.

The watering experiment started for the inoculated saplings in July 2020 at the Forest Botany and Tree physiology greenhouse Göttingen and it lasted until February 2021. The saplings were grouped to low and normal watering conditions for the experiment, and the saplings received watering accordingly to the group, in which low watering group received only 50% of the amount that was given to the normal watering group. The watering amount was adjusted to the temperatures, humidity, and soil moisture, that were monitored closely throughout the experiment period, as the plant's water intake varies as the environmental conditions change. Measurements were recorded

every watering day from August to November by using a digital thermometer and a tensiometer (HH2 device Quipped with the ML2x sensor (Delta-T Devices Ltd., Cambridge, U.K.)).

Total amount of specimens (Table 1) in the experiment was 754, of which 212 were inoculated with *H. parviporum* strain 1 (Hpa1) and 209 individuals with *H. parviporum* strain 2 (Hpa2). Also, mock-inoculated trees and non-treated control trees were added into the experiment. The trees were divided into two watering groups. The low watering group received only 50% of the amount of water compared to the normal watering group. The height of the saplings was measured before and after the treatment to the nearest 0,1 cm and after the experiment ended, needle samples were collected from the specimens in 1,5 mL Eppendorf tubes and they were kept in a freezer (-20°C) before the DNA extractions.

Table1. The division of the specimens into different treatment groups. The Control stands for non-treated specimens.

Watering treatment	Hpa1	Hpa2	Mock-Inoculation	Control NT	Total
Low	110	108	110	63	391
Normal	102	101	99	61	363
Total	212	209	209	124	754

4.2. DNA extraction

Before the DNA extraction the samples had to be prepared by transforming a piece (70–100 µg) of needle material into a Tissue disruption tube, following the addition of lysis buffers according to the protocol provided by QiaGen (Appendix 1). The samples were incubated at +65°C for 15 minutes before they were put into Tissuelyser. The lysis of the samples was done in Tissuelyser, in 24Hz for 2 x 2 minutes. After the lysis, the samples were centrifuged in 14 000 rfc for 5 minutes, and 400 µL of the supernatant was pipetted for the DNA extraction.

DNA was extracted from spruce needles by using a QiaGen DNeasy® Plant Pro Kit and Qiacube DNA extraction automate, that is intended for fully automated DNA processing. The extraction was carried out by using the protocol on the DNeasy® Plant Pro Kit Handbook (Appendix 1). After the

DNA extraction, the concentration and purity of the DNA was measured by using NanoDrop™ One/One Microvolume UV-Vis Spectrophotometer in Luke's forest pathology laboratory.

4.3. PCR and gelelectrophoresis for genotyping PaLAR3 alleles

After the DNA extraction, the PCR was carried out by using a ThermoFisher Scientific™ DreamTaq Green DNA Polymerase (5U/µL) kit. PaLAR3 locus specific primers (Table 2) were designed against the genomic sequence of PaLAR3 (KX574230.1 and KX574229.1) for one locus and two alleles. For the detection of PaLAR3 alleles 10–40 ng genomic DNA per sample was used in each 12,5 µL PCR reaction (Table 3). In each reaction, 0,4 µM of locus-specific LAR_ComA primer and 0,2 µM of each allele-specific primer were included. The PCR conditions were 95°C for 5 min; followed by 35 cycles of 30 s at 95°C, 30 s at 54°C, 30 s at 72°C; and final elongation for 10 min at 72°C, according to the protocol presented in Table 4.

Table 2. Primers used for determination of PaLAR3 alleles (Edesi et al. 2021).

Primer ID	Name	Sequence 5'-3'	Specification	Conc. (µM)
Primer 1	LAR_ComA	GAAATCTGCAGCCAATGGA	allele A and B	0,5
Primer 2	LAR_B2	CTGTATAACCGTAACATCTACTG	B allele specific	0,25
Primer 3	LAR_A	GAACGGGTATAAACTCCGT	A allele specific	0,25

Table 3. The protocol for the PRC mastermix. The ingredients for the mastermix are presented for one PCR reaction.

Component	Volume (µL)
Buffer	1,25
dNTP	0,5
Com_A primer	0,5
B2 primer	0,25
A primer	0,25
H2O	7,15
Polymerase	0,1
Template	2,5
Total volume	12,5

Table 4. The protocol for the PCR conditions for PaLAR3 gene replication.

Step	Temperature °C	Time	Cycles
Initial Denaturation	95	5 min	1
Denaturation	95	30 s	35
Annealing	54	30 s	
Extension	72	1 min	
Final Extension	72	10 min	1
Finish	4	∞	∞

An agarose gel electrophoresis (120V / 1h 15 min) was carried out in 1,5% agarose gel to distinguish the PCR products accordingly. The alleles were determined after the electrophoresis, based on the absence and/or presence of a 110 bp (PaLAR3A) and 201 bp (PaLAR3B) band.

4.4. Necrose data

After the experiment had ended, the bark was gently peeled off to expose the necrosis in phloem for measuring. The peeling continued further, until sapwood was exposed and measured. The lesion length was measured in horizontal and vertical directions and the lengths and widths of the lesions were measured both in phloem and in sapwood (Terhonen et al. 2019). In order to determine the area of the lesion, an area of an ellipse was calculated with the measured lengths and widths.

The area of the necrosis was calculated by using an equilibrium to count the area of an ellipse:

$$A = \pi r r$$

in which the value of r was determined by dividing the total lengths and widths of the lesion in two. The area of necrosis was calculated both in phloem and in sapwood. This area was used to distinguish differences of the necrosis in the specimens between the different treatments and between the different alleles. All the inoculated samples were pealed and measured, including the mock-inoculation group.

4.5. Statistical methods

The statistical analysis was done in RStudio and SPSS version 25.0 (IBM Corporation, New York, NY, USA). Kruskal-Wallis test was used to determine the statistical significance of each test that was not normally distributed. The result is shown as a *p*-value, and if $p < 0,01$, there is a statistical significance between the variables. Often the limit of a statistically significant *p*-value is set to <0,05, but since <0,01 is stricter, it increases the confidence in the determination of significance. In this research, the value was set to be stricter since genetic interactions in this field are not yet very well known. By choosing this limit, the number of false positives is minimized and the conclusions regarding the significance are more likely to be accurate. This *p*-value has also been used in the field of forest genetical studies before (Terhonen et al. 2019, Edesi et al. 2021). In addition, the experimental setting of this research did not include a sufficient number of watering treatments or different *H. parviporum* strains to produce reliable enough results. Therefore, if $p > 0,01$, there is no statistical significance between the variables. A generalized linear model (GLM) was constructed to evaluate the fixed effects on the necrosis area in phloem and in sapwood. Initial fixed explanatory variables in the necrosis area model included alleles, watering treatment, inoculation status and starting height of the saplings. GLM was constructed for growth: initial fixed explanatory variables in the growth model included alleles, watering treatment, and inoculation status of the saplings.

5. Results

The allele distribution is shown in Table 5. Homozygous PaLAR3A specimens are the most well-presented group with 500 individuals and 66% of the saplings. There were 185 individuals with AB alleles (25%), and 69 with BB alleles, representing only 9% of the specimens. In total 754 specimens were included in the experiment (Table 5).

Table 5. The number of specimens and the number of individuals in each allele group in the study. The % indicates the percentage of each allele combination in the study.

Allele	Number of saplings	%
AA	500	66
AB	185	25
BB	69	9
Total	754	100

5.1. Growth

At the beginning the height of the saplings was measured to the closest 0,1 cm. The starting height of the trees was significantly different (p = 0,0028) due to homozygous PaLAR3B. I.e., the saplings with BB alleles in their PaLAR3 locus have grown significantly more than the others before the experiment started (Figure 1). The growth of the trees during the experiment was measured and the current year growth was assessed to see if the watering treatment had had an impact on the growth in the individuals with different alleles. The growth is shown in Figure 2 and in low watering treatment the p = 0,56 and in normal watering p = 0,67, showing no signs of statistical significance.

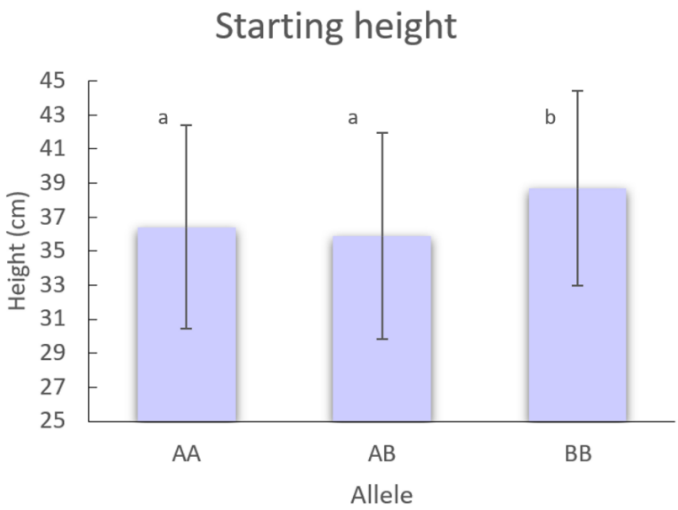

Figure 1. The average starting height of the trees before the experiment. The letter a indicates similarity between the groups. Letter b indicates the group that is statistically different from the other groups.

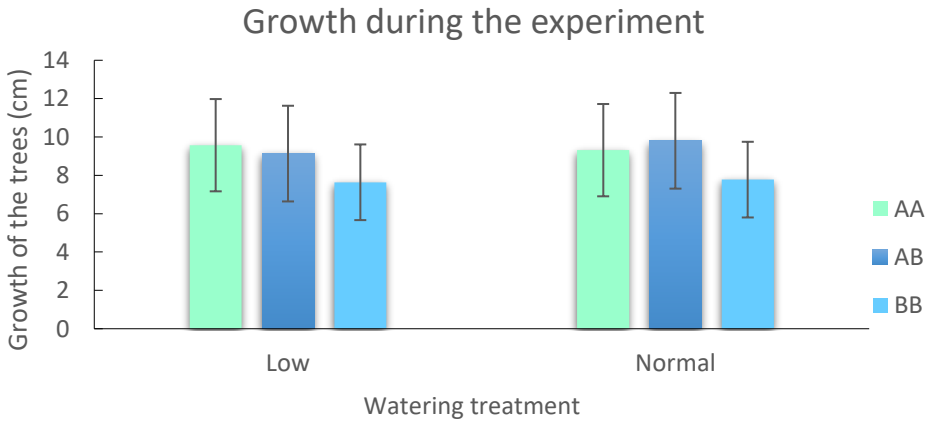

Figure 2. The average current year growth of the trees with different alleles, within different watering conditions. The error bar indicates SD.

5.2. Necrosis

The necrosis length and width in phloem and in sapwood was measured, and the area of necrosis was calculated both in phloem and in sapwood (Table 6, Figure 3).

Table 6. The area of the necrosis in phloem and sapwood in the different allele groups. Inoculation or watering treatment are not considered.

Alleles	Necrosis area (mm^2) in phloem	Necrosis area (mm^2) in sapwood
AA	8,17	29,85
AB	7,61	29,06
BB	6,75	26,45

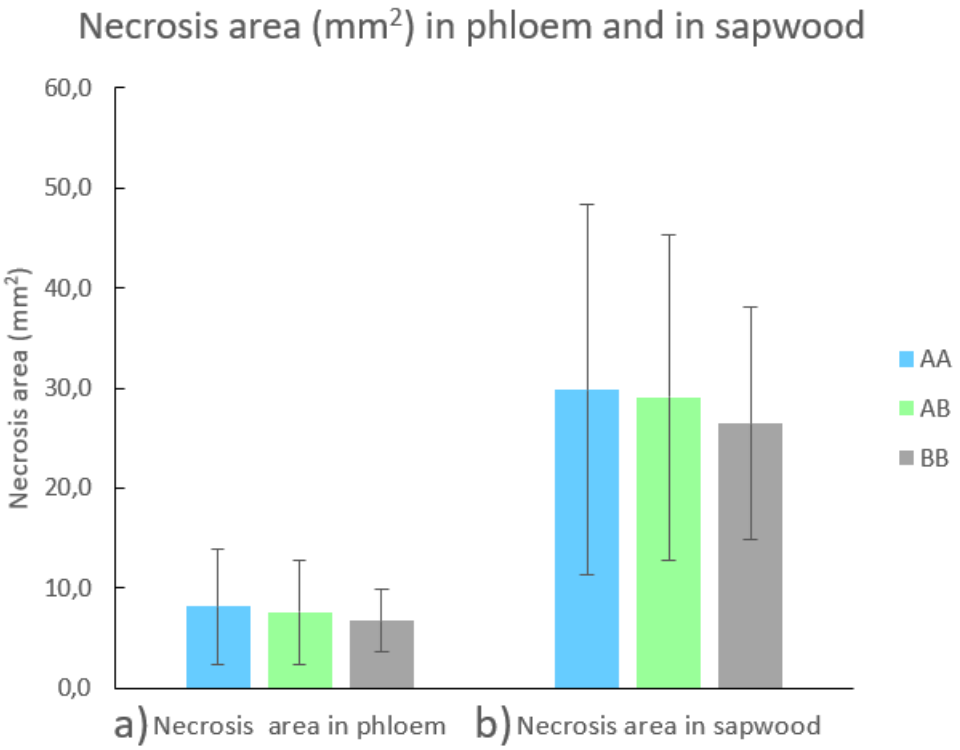

Figure 3. The area of the necrosis in phloem (a) and in sapwood (b). The error bar indicates SD of the necrosis area within each allele group. Inoculation treatment or watering conditions are not considered.

The necrosis area between the alleles was studied both in phloem and in sapwood, and the statistical analysis was carried out with Kruskal-Wallis test. No statistical significance in the necrosis area was discovered between the allele groups (p = 0,48 in phloem, and in sapwood p = 0,52) (Figure 3).

The general linear model suggests that the watering treatment has no significant impact on the necrosis area neither in phloem nor in sapwood (Table 7). Also, no statistical significance in the necrosis area between the different allele composition in an individual sapling's PaLAR3 locus were observed, neither in phloem (p = 0,06) nor in sapwood (p = 0,09). However, statistical significance was discovered between the necrosis area and the inoculation status (p < 0,01), both in phloem and in sapwood, and between the necrosis area in the sapwood and the starting height of the sapling (p < 0,01).

Table 7. Estimates from the generalized linear model (GLM). Statistically significant (p < 0,01) differences are indicated by the asterisks (*). Statistical analysis on the necrosis areas in phloem and in sapwood.

Variable	Fixed Effects	Std. Error	F	Sig
Necrosis area in Phloem	Alleles	1,211	2,91	0,06
	Watering treatment	0,335	1,88	0,17
	Inoculation status	1,211	156,88	<0,01*
	Starting height	0,028	5,12	0,02
Necrosis area in Sapwood	Alleles	1,908	2,43	0,09
	Watering treatment	1,029	0,67	0,41
	Inoculation status	1,602	225,12	<0,01*
	Starting height	0,086	13,57	<0,01*

In addition, the correlation between the starting height and necrosis area in sapwood was studied, and the starting height correlated positively with the necrosis area in Hpa1 treatment (Figure 4), with 0,336 correlation value. This positive correlation indicates that the Hpa1 afflicted necrosis area in sapwood is larger in taller saplings, i.e., the taller the sapling, the larger the Hpa1 induced necrosis area in sapwood is. No other correlations were discovered.

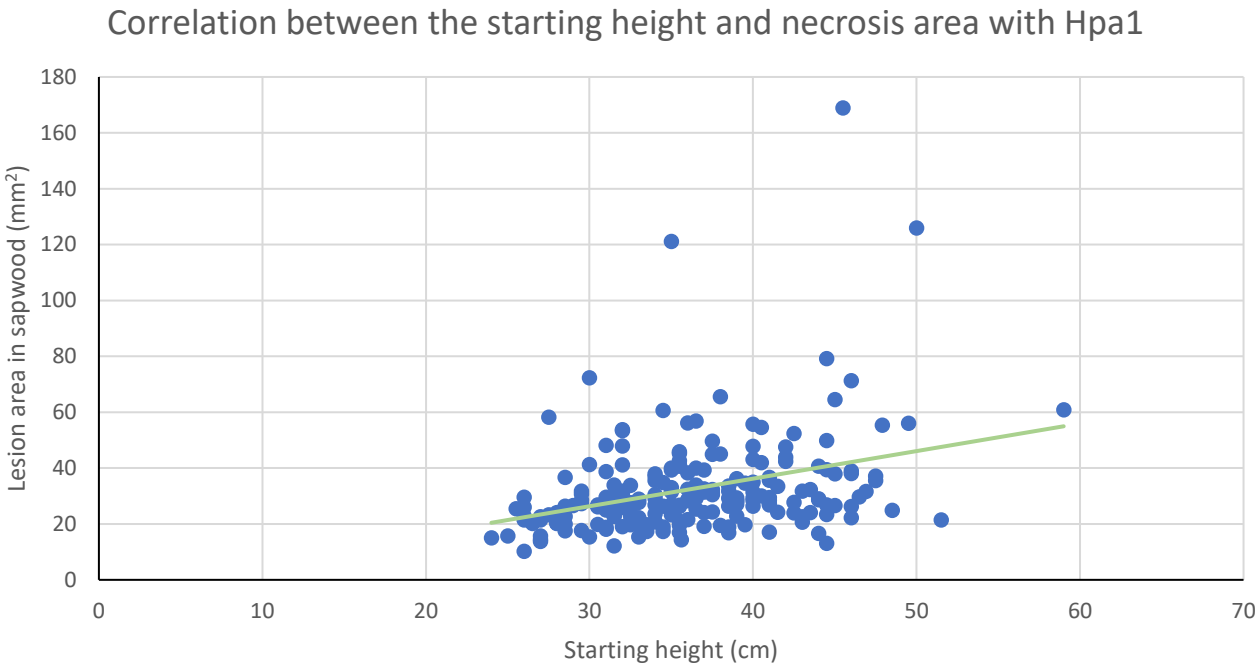

Figure 4. Correlation between the necrosis lesion area (mm^2) in sapwood after the experiment, and the starting height (cm) of the saplings at the beginning of the experiment. Green line indicates the correlation curve, and the correlation value is 0,336.

The GLM suggested that there is statistical significance between the inoculation status and the alleles (Table 7). Therefore, the area of necrosis in each allele group was tested between the two different watering treatments and each inoculation treatment. Statistical significance between the two watering treatments in BB allele group was discovered under Hpa2 inoculation treatment (Figure 5). This is also seen in the GLM, as the statistical significance ($p < 0,01$) was observed between the alleles and inoculation status (Table 7). No other statistical significance was found. In addition, no statistical significance in the necrosis area was discovered either in phloem or in sapwood of the different allele groups. Furthermore, no statistical significance in the necrosis area is shown between the alleles in either low or normal watering conditions. Finally, no statistical significance between the alleles in different inoculation treatments was shown as the watering treatment was not considered in the statistical analysis (Figure 6).

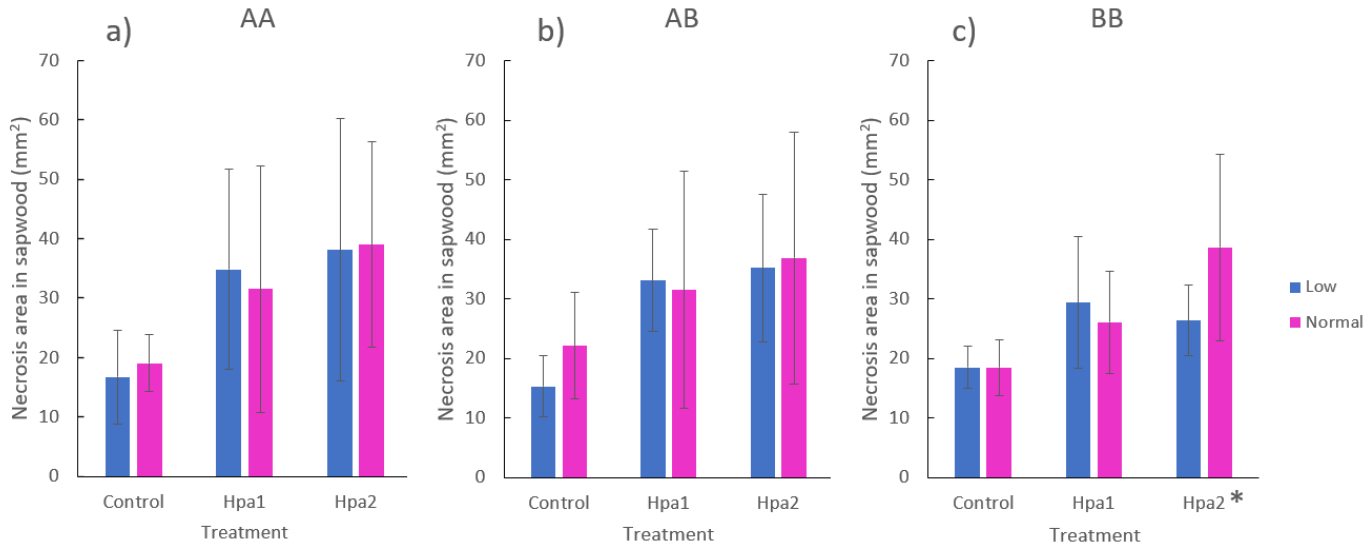

Figure 5. The effect of the watering treatment to the necrosis area (mm^2) in sapwood between a treatment within each PaLAR3 allele (AA, AB, BB). a) The necrosis in each inoculation treatment between low and normal water availability in *PaLAR3A* homozygotes (AA); b) heterozygotes (AB). c) *PaLAR3B* homozygotes (BB). Statistical significance is indicated with an asterisks (*) symbol. The error bar indicates SD of the necrosis area inside a certain watering treatment group, within an inoculation treatment.

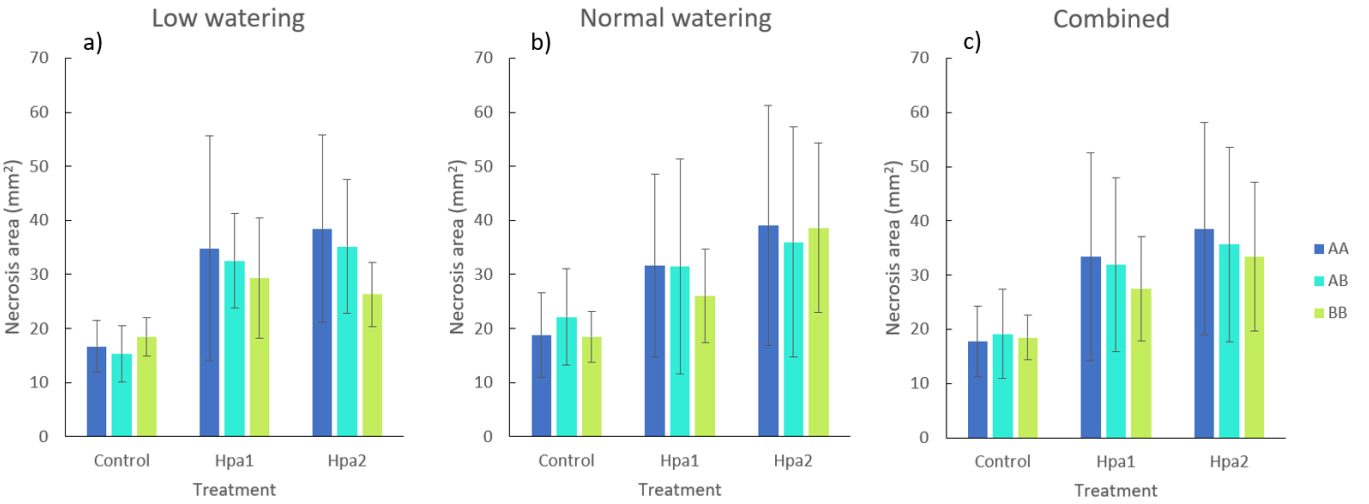

Figure 6. The average necrosis area (mm^2) in sapwood between different alleles (AA, AB, BB) within an inoculation treatment, presented in each watering treatment. a) Low watering; b) Normal watering; c) Both watering treatments combined. The error bar indicates SD in the necrosis area inside an allele group within an inoculation treatment. No statistically meaningful significance was found in necrosis area between alleles in any of the treatments.

6. Discussion

6.1. Growth

The statistical analysis of this research shows that the starting height of the trees with homozygous B alleles was significantly higher than the height of the trees with AA or AB alleles in their PaLAR3 locus. However, the year-growth of the trees with different alleles did not have any statistical significance, regardless the watering treatment (Figure 2). At the beginning, the trees with BB alleles were taller, even though all the saplings had been in similar conditions prior to the experiment. The reason is unknown, and perhaps more research on the connection between tree growth rate and PaLAR3 gene should be studied in the future. However, during the experiment no differences between the growth rate of any of the allele combinations were discovered, which indicates that the growing conditions have been equally beneficial (Jevšenak et al. 2021), regardless to their watering treatment or inoculation group. This might indicate that the watering treatments in this study were not different enough, i.e., the low watering treatment was not low enough to affect the growth rate of the trees. Perhaps in more drastic water treatment, the allele composition would start to show statistical significance, as the drought induced stress reactions are activated in the saplings. Similarly, due to the COVID-19 restrictions the timing of the experiment was not ideal, and it might have impacted the experiment. The water availability treatment started in late July (22.7.2020) and even if the terminal growth period lasted still for multiple weeks, the growth of *Picea abies* might have already started to slow down, approaching the end of the growing period. By adding more watering treatments (e.g., 25 %, 60 % and 100 %) in the future experiments, the actual drought conditions might have more impact on the growth rate (Blumenstein et al. 2021). This could also provide valuable knowledge on the behavior and pathogenic pathways of different *H. parviporum* strains, and their infectiveness, in different environmental conditions.

6.2. Necrosis

The necrosis areas in sapwood between the alleles (AA. AB, BB) were not statistically different between the inoculation- or watering treatments (Table 7). This indicates that the PaLAR3 allele does not influence the necrosis development. The BB allele, however, showed less necrosis (except

in normal watering conditions), but it was not statistically meaningful when compared to other alleles (Figures 5 & 6). In this study the PaLAR3 allele does not have influence on the necrosis development.

Positive correlation was discovered between the starting height and the *H. parviporum* (Hpa1) inoculated necrosis area. This indicates that the height of the trees is related to the necrosis area in the sapwood (the higher seedling has vertically longer necrosis). Similarly, higher seedling height has been associated with longer vertical necrosis length in previous research as well (Terhonen et al. 2019). No correlation between the other *H. parviporum* strain (Hpa2) and the starting height was observed, indicating that there are differences in the virulence between the Hpa1 and Hpa2 strains. Differences between virulence of variable fungal strains of the same species have also been observed earlier (Linnakoski et al. 2017). These results highlight the fact that inoculation studies should be done with several different strains. In this study drought conditions did not impact the necrosis area in sapwood, on the contrary, in normal watering conditions the *H. parviporum*, Hpa2, strain was causing larger necrosis areas than in drought conditions (Figure 5). The question is, if the low necrosis under drought conditions in saplings (inoculated with Hpa2) is due to homozygous B allele. The only statistical significance was between the watering treatments in saplings that had two B alleles (Figure 5). Could in some pathosystems, with certain fungal strains, the trees with BB alleles restrict the necrosis under abiotic stress? This is still a hypothesis and future research is needed over this topic.

Since the pathogenic pathways vary between the strains, more different strains of *H. parviporum* need to be added to the research in the future experiments. Also, the differences in the infectiveness could be more unambiguous if the watering treatments in the experiment were more drastic. These include also more severe drought and flooding, during the experiment, and depending on the strains' pathogenesis, the fungal infections might be able to overcome the trees' defense mechanisms more easily, leading to larger necrosis areas.

6.3. Reliability of the study

This research was carried out in a greenhouse, and the environmental parameters (temperature, humidity) were monitored and adjusted (watering). The environmental conditions in this

experiment were not similar to a natural environment of the Norway spruce, and many of the biotic and abiotic environmental factors were not affecting the saplings. The conditions in which the trees were kept during the experiment were optimal for tree growth regarding light and temperature. In a natural environment shading would usually play a key role especially in the early life of the tree saplings that inhabit the forest floor.

The distribution of the alleles in the sapling specimens in this study was not equally distributed. The lack of homozygous B allele in the dataset raises questions about the reliability of the results, but the homozygous PaLAR3B genotype seems well enough presented regarding to the statistics. In further research, more equal allele distribution could provide more statistically significant results, and the differences between the alleles might show more clearly.

6.4. PaLAR3 in forest breeding

In Sweden, plans for establishing the homozygous PaLAR3B genotype into their forest breeding program are proceeding (Elfstrand 2022). Genetic biodiversity is a key factor in evolutionary change, and it helps species to adapt to ever changing environmental conditions, climate change and habitat change (Haapanen & Mikola 2008). Also, it has been proven to help species fight novel diseases (Hoban et al. 2020). Climate change driven impacts, especially on the hydrological cycles and more severe drought conditions in the North, are likely to raise issues in the future for the forest industry (Mukherjee et al. 2018). Climate models suggest that both the temperatures and the precipitation levels will rise in the future (Seo et al. 2015), increasing the risk of pest and parasite attack induced wood deficit. Pathogen related wood deficit is likely to increase, as the pests and fungi species migrate to new areas, due to more suitable environmental conditions. Therefore, discovering the solutions for more resilient and more adaptive forests is vital to ensure the productivity of the forest industry. In order to do so, the forest breeding program is trying to breed more resilient tree individuals for the future. The goal is to secure wood production and economically sustainable forest industry in Finland in the future as well, even though the climate conditions change rapidly. The trees cannot keep up with the pace of the change just by natural adaptation, and therefore, more advanced methods, like genetical studies, are important tools in finding the most resilient individuals for future conditions (Haapanen & Mikola 2008).

More research is needed to be able to know all the aspects the PaLAR3 locus might hold within it. If different allele combinations make the forests more resilient or adaptive against pest invasions, diseases or changing climate conditions, genetic biodiversity loss might bring problems in the monoculture industrialized forests. Therefore, the genetical variation in the trees that are planted in the forests after a clear cut, might provide more resilience. However, all the genetical factors have not yet been studied, and the contribution a single gene can have for the resilience is not yet known. Also, adding the genes that help vegetation overcome the symptoms of the drought stress into the research, could help finding the most resilient individuals for the futures' climate conditions and requirements. Mixed forests should be studied more as an option to replace monoculture, to find out if they can provide solutions for mitigating pests and pathogens by increasing the genetic biodiversity in the forest (Huuskonen et al. 2021). The *Heterobasidion* fungi does not infect all tree species (Tanin et al. 2021), and therefore, planting a mixed forest could possibly prevent the fungi from spreading to new individuals via root contact.

Other genes than PaLAR3 have also been linked to resistance against *H. parviporum* infections in Norway spruce. These genes, such as laccase gene, PaLAC5 (Elfstrand et al. 2020), should also be added to the research before any hasty decisions are made for the forest breeding programs. Gene interactions are very complex, and not yet much is known about the genes that are responsible for resistance against pathogen attack (Lind et al. 2014). Nonetheless, studying PaLAC5 and PaLAR3 interactions in severe drought treatments, combined with inoculation treatments with multiple different *Heterobasidion* strains could provide significant results. By combining these genetical factors in research, the most suitable outcome to reduce the root rot infections in forest industry could be possibly determined. In addition, the different endophytic species that mitigate the pathogenic effects of *Heterobasidion* on Norway spruce, such as *Phialocephala sphaeroides*, (Wen et al. 2021) could be added to the discussion and research for more resilient forest breeding program. Furthermore, low soil pH is a likely factor to increase the resilience of the trees against *Heterobasidion* infections in the North (Müller et al. 2018). Soil pH can therefore play an important role in the future as well, as different pathogens are most likely more abundant and more infective in these areas compared to *Heterobasidion*. In conclusion, in the future the research should focus on more wide range of factors that affect the resilience of Norway spruce against fungal attack, and not only focus on one gene.

7. Conclusions

This Master's thesis studied the resilience of Norway spruce against *H. parviporum* infection under drought stress. In previous studies, the PaLAR3 locus of Norway spruce has been linked with higher enzyme production and higher (+)-catechin concentrations in presence of the B allele, indicating higher resilience against a pathogen attack (Nemezio-Gorriz et al. 2016). However, the effect of the stress reaction in the drought conditions was not included in the research, and therefore, this research focused on the drought stress induced rot infections.

Considering the results of this thesis, the area of the necrosis in the saplings was not different, regardless of the alleles in their PaLAR3 locus. The infectiveness and route of pathogenesis of the fungal strains, however, is different. The height of the saplings with BB alleles in their PaLAR3 locus was significantly higher compared to others, despite similar environmental conditions prior to the experiment. Therefore, the effects the PaLAR3 allele has to the growth rate of the saplings should be studied more in the future. Similarly, as in one combination (drought + Hpa2) the necrosis area was significantly lower, PaLAR3B impact to susceptibility of the host under abiotic stress should be studied. Also, adding more watering treatments and increasing the number of the different *H. parviporum* strains in the experiment might bring more clear and more significant results.

For future forest industry, finding solutions for the problems climate change driven impacts cause is extremely important. Extreme weather conditions, such as drought and floods, are expected to become more common in the future, creating huge challenges (Krisans et al. 2020). By studying the resilience of the trees, the most resistant individuals can be chosen for the forest breeding program, creating a solution to the problem. In order to do so, the genetic interactions of Norway spruce should me studied more to gain understanding of the resistance mechanisms and gene functioning more thoroughly (Elfstrand et al. 2020). More precise understanding becomes possible by adding multiple genes and the end products that they encode into the research. Also, by adding more watering treatments and fungal strains to the experiments to come, the results might become more significant and area specific.

Even though according to this research, the allele combination in the Norway spruce PaLAR3 locus doesn't affect the resistance against *H. parviporum*, the production of leucoanthocyanidin 3 enzyme is suggested to be higher in the individuals with presence of B allele in their PaLAR3 locus (Nemerio-

Gorriz et al. 2016 & Jyske et al. 2020). Therefore, studying the linkage and interactions between the resistance genes, e.g., PaLAC5 and PaLAR3, could provide the answers the forest industry desperately needs.

8. Acknowledgements

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10. Appendix

Appendix 1. The protocol for the DNA extraction, provided by QiaGen.

Quick-Start Protocol August 2019

DNeasy® Plant Pro Kit

Solution CD2 should be stored at 2–8°C upon arrival. All other reagents and kit components should be stored at room temperature (15–25°C), until the expiry date printed on the box label.

Further information

- *DNeasy Plant Pro Handbook*: **www.qiagen.com/HB-2552**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- If Buffer APP contains precipitates, heat at 60°C until precipitate dissolves.
- Perform all centrifugation steps at room temperature (15–25°C).
- Refer to kit handbook for optimal homogenization method in step 2.

1. Add 5–100 mg of fresh or frozen plant tissue and 500 µl of Solution CD1 to a 2 ml tissue disruption tube. Vortex briefly to mix.
   Note: If your sample is high in phenolic compounds, add 450 µl Solution CD1 and 50 µl Solution PS.
2. Homogenize using one of these methods:
   - 2a. **Vortex**: Secure tissue disruption tubes to a Vortex Adapter (cat. no. 13000-V1-24) and vortex at maximum speed for 10 min.
   - 2b. **TissueLyser II**: Most plant samples can be lysed with the TissueLyser II, using the TissueLyser Adapter Set 2 x 24: Place samples in the TissueLyser II and run at 24 Hz for 2 min. Reorient the adapter so the side closest to the machine body becomes furthest from it, and then run the TissueLyser again at 24 Hz for another 2 min.
   - 2c. **PowerLyzer® 24 Homogenizer**: Tissue disruption tubes must be properly balanced in the tube holder of the PowerLyzer 24 Homogenizer. Homogenize the tissue for 1 cycle at the appropriate speed depending on sample type for 2 min.
3. Centrifuge the tissue disruption tubes at 12,000 x *g* for 2 min.

Sample to Insight QIAGEN

4. Transfer the supernatant to a clean 1.5 ml microcentrifuge tube (provided).
   Note: Expect 350–450 µl. The supernatant may still contain some plant particles.
5. Add 200 µl Solution CD2 and vortex for 5 s.
   Note: For problematic samples, add 250 µl Solution CD2.
6. Centrifuge at 12,000 x *g* for 1 min at room temperature. Avoiding the pellet, transfer the supernatant to a clean 1.5 ml microcentrifuge tube (provided).
   Note: Expect 400–500 µl.
7. Add 500 µl of Buffer APP and vortex for 5 s.
8. Load 600 µl lysate onto an MB Spin Column. Centrifuge at 12,000 x *g* for 1 min.
9. Discard the flow-through and repeat step 8 to ensure that all of the lysate has passed through the MB spin column.
10. Place the MB spin column into a clean 2 ml collection tube (provided).
11. Add 650 µl Buffer AW1 to the MB spin column. Centrifuge at 12,000 x *g* for 1 min. Discard the flow-through and place the MB spin column back into the same 2 ml collection tube.
12. Add 650 µl of Buffer AW2 to the MB spin column. Centrifuge at 12,000 x *g* for 1 min. Discard the flow-through and place the MB spin column into the same 2 ml collection tube.
13. Centrifuge at up to 16,000 x *g* for 2 min. Place the MB spin column into a new 1.5 ml elution tube (provided).
14. Add 50–100 µl of Buffer EB to the center of the white filter membrane.
15. Centrifuge at 12,000 x *g* for 1 min. Discard the MB spin column. The DNA is now ready for downstream applications.

Revision History

Date	Changes
August 2019	Updated storage conditions. Rephrased homogenization instructions for TissueLyser 2, for clarity.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.