Genotype effect on Norway spruce (*Picea abies*) mycobiome and resistance against *Heterobasidion parviporum* under abiotic stress

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I can do all things through Christ who strengthens me

Philippians 4:13

PREFACE

This study monitored the *Heterobasidion parviporum* – Norway spruce (*Picea abies*) pathosystem under abiotic stress (low water availability). The aim was to compare the resistance of different Norway spruce genotypes to *H. parviporum*. To find out if genotype or low water conditions impact the mycobiome composition, Next Generation Sequencing (NGS) technology and bioinformatics were used.

This thesis consists of a general introduction to the research topic and four published research articles, which are presented here as individual chapters. The first chapter (Drought stress described by transcriptional responses of *Picea abies* (L.) H. Karst. under pathogen *Heterobasidion parviporum* attack) discusses the genetic pathways involved in the specific responses to unfavourable environmental stress (drought) with and without the pathogen *H. parviporum*. This chapter also investigates the interplay between *H. parviporum* infection and defence-related gene expression mediated by drought stress.

The second chapter (Genetic variation of *Picea abies* in response to the artificial inoculation of *Heterobasidion parviporum*) compares the variation in resistance of Norway spruce families against *H. parviporum* under abiotic stress. It also assesses the effect of low watering on seedling growth and necrosis among spruce genotypes.

The third chapter (Beyond the surface: exploring the mycobiome of Norway spruce under abiotic stress and with *Heterobasidion parviporum*), focuses on the identification of the mycobiome and certain species within Norway spruce genotypes and their interactions with *H. parviporum* as well as mycobiome stability under drought stress.

Lastly, this thesis ends with the fourth chapter (Leucoanthocyanidin Reductase 3 (*PaLAR3*) Locus in Norway Spruce (*Picea abies*) and its link to resistance against *Heterobasidion parviporum*) which estimates the prevalence of the *Pa*LAR3 allele in Norway spruce and determined whether the trees with homozygotic or heterozygous *Pa*LAR3B genotypes have a different level of resistance against *H. parviporum*.

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SYNOPSIS

Norway spruce (*Picea abies* L. Karst.) holds considerable economic importance as a tree species in Europe. However, it faces significant vulnerability to attacks from the *Heterobasidion annosum* species complex, resulting in estimated annual losses of around 800 € million in Europe. Norway spruce exhibits limited resilience and restorative capabilities under changing climatic conditions, making it susceptible to both biotic and abiotic stresses. As climate patterns, particularly temperature and precipitation, are expected to undergo alterations, the geographic distribution and suitable habitats required for Norway spruce may experience substantial changes. Therefore, comprehending the underlying mechanisms involved in how trees respond to pathogenic attacks could prove beneficial in addressing this issue.

The first chapter described a transcriptional study comparing Norway spruce gene expressions to evaluate the effects of water availability and infection with *Heterobasidion parviporum*. In this study, RNA-seq analysis was conducted on Norway spruce samples to identify the genetic pathways associated with specific responses to adverse environmental stress (drought) both in the presence and absence of the pathogen *H. parviporum*. Additionally, a transcriptional study was implemented to examine the interaction between *H. parviporum* infection and the expression of defense-related genes influenced by drought stress. Eighteen seedlings were randomly assigned to water treatment groups (high or low) and inoculated with *H. parviporum*, mock-inoculated (2% Malt extract agar) or left untreated. Four candidate genes were successfully identified (MA_110169g0010, MA_14707g0010, MA_15852g0010, MA_10427673g0020) to be associated with resistance against *Heterobasidion* sp., especially under drought.

These genes exhibited significant expression changes, either higher or lower, compared to the non-treated control group, exclusively in *H. parviporum*-infected plants. However, we also observed that the defense-related gene response becomes more intricate when additional abiotic stress factors are present. These findings indicate that water availability plays a crucial role in determining the gene regulation strategy (upregulation or downregulation) employed by *H. parviporum*-infected Norway spruce. Moreover, our results suggest that Norway spruce exhibits a preferential defense response to *H. parviporum* infection rather than physical wounding, depending on the prevailing water availability conditions.

The second chapter examined the genetic variation of *Picea abies* in response to the artificial inoculation of *Heterobasidion parviporum* infection. Inoculation experiments were performed to assess how different families of Norway spruce seedlings respond to *Heterobasidion parviporum* infection under abiotic stress. Eight hundred Norway spruce seedlings were categorised based on two water treatments. Four hundred seedlings received the optimum watering required for their growth and survival, while the remaining four hundred received half the optimum watering. Inoculations were done with two strains of *H. parviporum.* The seedlings were inoculated with either *H. parviporum* strains (Hpa 1 and Hpa 2) or 1.5% MEA as a control, others were left untreated. The growth parameters and lesions in both phloem and sapwood were measured. The results show that the seedlings' height, unlike the diameter, was not impacted by water stress nor inoculation treatment but by families and genotypes. Strong positive correlations were also observed between the lesion sizes in both phloem and sapwood. Overall, the study elucidates the influence of Norway spruce genotypes on growth dynamics and that the induced response patterns of *Picea abies* to artificial inoculations by different strains of the same pathogen could be different.

The third chapter explored the mycobiome of Norway spruce and gave insights into the fungal communities and their diversity and stability under abiotic stress and *Heterobasidion parviporum* infection. As part of a continued effort to understand the effects of host genetics and environmental factors on fungal composition and evaluate the "mycobiome-associated-fitness" hypothesis, a comparative analysis was carried out. Sixty-seven Norway spruce seedlings either inoculated with *H. parviporum*, mock-inoculated or left untreated were used for this study. The seedlings were grouped into optimum and low watering categories, and the mycobiome associated with phloem and roots were analysed. The findings underscore the variations in the diversity and abundance of mycobiome genera in Norway spruce genotypes and response to water availability in the phloem. There were varying degrees of necrosis across Norway spruce genotypes, and the presence of one fungus influenced the abundance of another: in the case of *Heterobasidion-Phialocephala*.

Key fungi such as *Phialocephala fortinii* and *Paraphaeosphaeria neglecta* were identified in the root mycobiome to confer inhibitory benefits to Norway spruce against the growth of *Heterobasidion parviporum*. Furthermore, specific endophytes exhibited greater stability under low water availability in the roots than ectomycorrhizal fungi. The study suggests that Norway spruce resistance against pathogens (*Heterobasidion parviporum*) is

shaped by a complex interplay of several factors, including genetic and environmental factors such as soil moisture levels and fungal adaptability.

The fourth chapter assessed the Leucoanthocyanidin Reductase 3 (*PaLAR3*) locus in Norway spruce (*Picea abies*) and its link to resistance against *Heterobasidion parviporum*. The presence of the B allele at the PaLAR3 locus potentially confers enhanced resistance against pathogens in inoculation experiments. This research examined the impact of the PaLAR3 gene on necrosis development induced by *H. parviporum* in the stems of Norway spruce clonal materials under different watering conditions. Seven hundred and fifty-four clonal Norway spruce seedlings used for this study were either inoculated with *H. parviporum* strains, mock-inoculated or left untreated. Designed PaLAR3 locus-specific primers were used to detect PaLAR3 alleles in the Seven hundred and fifty-four seedlings. The outcome shows that the homozygous PaLAR3B allele is present in lower quantities as compared to its heterozygous counterpart or the PaLAR3A, which was the most abundant. Also, there was an interaction between the necrotic area and the homozygous *PaLAR3BB* under conditions of reduced water availability. The findings support previous research that the PaLAR3B allele could serve as one of the valuable markers for identifying resistance in the *Picea abies*-*Heterobasidion* pathosystem. All in all, for Norway spruce, the concept of plants extended genotypic variation against pathogens and inherent mycobiome should be considered as factors to be included in resistance studies.

ZUSAMMENFASSUNG

Die Gemeine Fichte (*Picea abies* L. Karst.) hat als Baumart in Europa eine erhebliche wirtschaftliche Bedeutung. Er ist jedoch sehr anfällig für Infektionen mit dem Artenkomplex *Heterobasidion annosum*, die in Europa zu geschätzten jährlichen Verlusten von rund 800 Mio. EUR führen. Die Gemeine Fichte weist unter wechselnden klimatischen Bedingungen eine begrenzte Widerstandsfähigkeit und Wiederherstellungsfähigkeit auf, was sie sowohl für biotischen als auch für abiotischen Stress anfällig macht. Da sich die Klimamuster, insbesondere die Temperatur und die Niederschläge, voraussichtlich verändern werden, können sich die geografische Verteilung und die geeigneten Lebensräume, die für die Fichte erforderlich sind, erheblich ändern. Daher könnte sich das Verständnis der zugrunde liegenden Mechanismen, die daran beteiligt sind, wie Bäume auf pathogene Angriffe reagieren, als vorteilhaft erweisen, um dieses Problem anzugehen.

Die erste Arbeit beschreibt eine transkriptionelle Studie, in der die Genexpression der Gemeine Fichte verglichen wurde, um die Auswirkungen der Wasserverfügbarkeit und der Infektion mit *Heterobasidion parviporum* zu bewerten. In dieser Studie wurde eine RNA-Seq-Analyse an Fichtenproben durchgeführt, um die genetischen Signalwege zu identifizieren, die mit spezifischen Reaktionen auf widrigen Umweltstress (Trockenheit) sowohl in Anwesenheit als auch in Abwesenheit des Erregers *H. parviporum* verbunden sind. Zusätzlich wurde eine Transkriptionsstudie durchgeführt, um die Interaktion zwischen *H. parviporum*-Infektion und die Expression von Abwehrgenen, die durch Trockenstress beeinflusst werden zu untersuchen. Achtzehn Sämlinge wurden nach dem Zufallsprinzip in Wasserbehandlungsgruppen (hoch oder niedrig) eingeteilt und mit *H. parviporum* beimpft., scheingeimpft (2%iger Malzextrakt-Agar) oder unbehandelt. Vier Kandidatengene (MA_110169g0010, MA_14707g0010, MA_15852g0010, MA_10427673g0020) konnten erfolgreich identifiziert werden, die mit einer Resistenz gegen *Heterobasidion* sp., insbesondere unter Trockenheit, assoziiert sind. Diese Gene zeigten signifikante Expressionsänderungen, entweder höher oder niedriger, im Vergleich zur nicht behandelten Kontrollgruppe, ausschließlich in *H. parviporum*-infizierten Pflanzen. Wir beobachteten jedoch auch, dass die abwehrbezogene Genantwort komplizierter wird, wenn zusätzliche abiotische Stressfaktoren vorhanden sind. Diese Ergebnisse deuten darauf hin, dass die Wasserverfügbarkeit eine entscheidende Rolle bei der Bestimmung der Genregulationsstrategie (Hoch- oder Herunterregulierung) von *H. parviporum*-infizierte

Gemeine Fichte spielt. Darüber hinaus deuten unsere Ergebnisse darauf hin, dass die Gemeine Fichte eine präferentielle Abwehrreaktion auf *H. parviporum*-Infektion, statt physischer Verletzung, zeigt, abhängig von den vorherrschenden Wasserverfügbarkeitsbedingungen.

Die zweite Arbeit untersucht die genetische Variation von *Picea abies* als Reaktion auf die künstliche Inokulation von *Heterobasidion parviporum*-Infektionen. Es wurden Inokulationsexperimente durchgeführt, um zu untersuchen, wie verschiedene Familien von Fichtensämlingen auf eine Infektion mit *H. parviporum* unter abiotischem Stress reagieren. Achthundert Fichtensämlinge wurden anhand von zwei Wasserbehandlungen kategorisiert. Vier hundert Sämlinge erhielten die optimale Bewässerung, die für ihr Wachstum und Überleben erforderlich war, während die restlichen vier hundert die Hälfte der optimalen Bewässerung erhielten. Die Inokulationen wurden mit zwei *H. parviporum*-Stämmen durchgeführt. Die Sämlinge wurden entweder mit *H. parviporum*-Stämmen beimpft (Hpa 1 und Hpa 2) oder 1,5 % MEA als Kontrolle. Andere blieben unbehandelt. Die Wachstumsparameter und Läsionen sowohl im Phloem als auch im Splintholz wurden gemessen. Die Ergebnisse zeigen, dass die Höhe der Sämlinge, im Gegensatz zum Durchmesser, nicht durch Wasserstress oder Impfbehandlung, sondern durch Familien und Genotypen beeinflusst wurde. Starke positive Korrelationen wurden auch zwischen den Läsionsgrößen sowohl im Phloem als auch im Splintholz beobachtet. Insgesamt verdeutlicht die Studie den Einfluss von Fichtengenotypen auf die Wachstumsdynamik und dass die induzierten Reaktionsmuster von *Picea abies* auf künstliche Inokulationen durch verschiedene Stämme desselben Erregers unterschiedlich sein könnten.

Die dritte Arbeit untersucht das Mykobiom der Gemeine Fichte und gibt Einblicke in die Pilzgemeinschaften und ihre Diversität und Stabilität unter abiotischem Stress und *Heterobasidion parviporum*-Infektionen. Im Rahmen der kontinuierlichen Bemühungen, die Auswirkungen der Wirtsgenetik und der Umweltfaktoren auf die Pilzzusammensetzung zu verstehen und die Hypothese der "Mykobiom-assoziierten Fitness" zu bewerten, wurde eine vergleichende Analyse durchgeführt. Siebenundsechzig Fichtensämlinge wurden für diese Studie entweder mit *H. parviporum* beimpft, scheininokuliert oder unbehandelt verwendet. Die Sämlinge wurden in optimale und niedrige Bewässerungskategorien eingeteilt und das Mykobiom mit ihren Phloemen und Wurzeln analysiert. Die Ergebnisse unterstreichen die Unterschiede in der Vielfalt und Häufigkeit von Mykobiom-Gattungen in den Genotypen der Gemeine Fichte und in der Reaktion auf die Wasserverfügbarkeit im Phloem. Es gab

unterschiedliche Grade der Nekrose zwischen den Genotypen der Gemeine Fichte, und das Vorhandensein des einen Pilzes beeinflusste die Häufigkeit des anderen; wie hier im Falle von *Heterobasidion-Phialocephala*.

Schlüsselpilze wie *Phialocephala fortinii* und *Paraphaeosphaeria neglecta* wurden im Wurzelmykobiom identifiziert, um der Fichte hemmende Vorteile gegen das Wachstum von *Heterobasidion parviporum* zu verleihen. Darüber hinaus zeigten bestimmte Endophyten eine höhere Stabilität bei geringer Wasserverfügbarkeit in den Wurzeln als Ektomykorrhizapilze. Die Studie legt nahe, dass die Resistenz der Fichte gegen Krankheitserreger (*Heterobasidion parviporum*) durch ein komplexes Zusammenspiel mehrerer Faktoren geprägt ist, darunter genetische und umweltbedingte Faktoren wie Bodenfeuchte und Anpassungsfähigkeit von Pilzen.

Die vierte Arbeit untersucht den Leucoanthocyanidin Reductase 3 (*PaLAR3*) Locus in der Gemeine Fichte (*Picea abies*) und seinen Zusammenhang mit der Resistenz gegen *H. parviporum*. Das Vorhandensein des B-Allels am PaLAR3-Locus führt möglicherweise zu einer erhöhten Resistenz gegen Krankheitserreger in Inokulationsexperimenten. In dieser Arbeit wurde der Einfluss des PaLAR3-Gens auf die durch *H. parviporum* induzierte Nekroseentwicklung in den Stängeln von Klonmaterialien der Gemeine Fichte unter verschiedenen Bewässerungsbedingungen untersucht. Sieben hundert vier und fünfzig klonale Fichtensämlinge, die für diese Studie verwendet wurden, wurden entweder mit *H. parviporum*-Stämmen inokuliert, die vorgetäuscht oder unbehandelt bleiben. Entworfene PaLAR3-Locus-spezifische Primer wurden verwendet, um PaLAR3-Allele in den Sieben hundert vier und fünfzig Keimlingen nachzuweisen. Das Ergebnis zeigt, dass das homozygote PaLAR3B-Allel in geringeren Mengen vorhanden ist als sein heterozygotes Gegenstück oder das PaLAR3A, das am häufigsten vorkam. Es gab auch eine Interaktion zwischen dem nekrotischen Bereich und dem homozygoten *PaLAR3BB* unter Bedingungen reduzierter Wasserverfügbarkeit. Die Ergebnisse unterstützen das PaLAR3B-Allel als wertvollen Marker für die Identifizierung von Resistenzen im *Heterobasidion*-Pathosystem von *Picea abies*. Alles in allem sollte das Konzept der Pflanzen für die Gemeine Fichte die genotypische Variation gegen Krankheitserreger erweitern, und das inhärente Mykobiom sollte als Faktoren betrachtet werden, die in Resistenzstudien einbezogen werden sollten.

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II **Durodola, B.,** Blumenstein, K., Terhonen, E. Genetic variation of *Picea abies* in response to the artificial inoculation of *Heterobasidion parviporum*. *European Journal of Forest Research* 2023. https://doi.org/10.1007/s10342-023-01534-3.

III **Durodola, B**., Blumenstein, K., Akinbobola, A., Kolehmainen, A., Chano, V., Gailing, O., Terhonen, E. Beyond the surface: exploring the mycobiome of Norway spruce under drought stress and with *Heterobasidion parviporum*. *BMC Microbiology* 23, 350 (2023). https://doi.org/10.1186/s12866-023-03099-y

IV **Durodola**, B., Hanström, N., Blumenstein, K., Haapanen, M., Hantula, J., Kashif, M., Piri, T., Terhonen, E. Leucoanthocyanidin Reductase 3 (*PaLAR3*) Locus in Norway spruce (*Picea abies*) and its link to resistance against *Heterobasidion parviporum*. *Forest Pathology*, 54: e12889 (2024). https://doi.org/10.1111/efp.12889

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Abbreviations

ANOVA	Analysis of Variance
ASV	Amplicon Sequence Variants
cDNA	Complementary Deoxyribonucleic Acid
CTAB	Cetyltrimethyl Ammonium Bromide
DED	Dutch Elm Disease
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
DSE	Dark Septate Endophyte
ECM	Ectomycorrhiza
EDTA	Ethylene-Diamine-Tetraacetic Acid
ET	Ethylene
FDR	False Discovery Rate
gDNA	Genomic DNA
GO	Gene Ontology
Hpa	*Heterobasidion parviporum*
HR	Hypersensitive Response
ITS	Internal Transcribed Spacer
JA	Jasmonic Acid
MEA	Malt Extract Agar
NGS	Next Generation Sequencing
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Analysis of Variance
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
SA	Salicylic Acid
totRNA	Total Ribonucleic Acid
QIIME	Quantitative Insights into Microbial Ecology

General Introduction

1. Introduction

Forest trees have significant importance in terms of their economic and ecological value and their ability to mitigate the impacts of climate change. The forest industry plays a crucial role in the development of a bioeconomy, which aims to build a society that relies on renewable and sustainable biological resources while achieving efficient and sustainable economic growth in an environmentally safe manner (Harfouche et al., 2014). The production of timber and associated products derived from forest trees contributes substantially to the revenue generation of numerous countries worldwide, and there is an anticipated rise in the demand for wood and forest products in the coming years.

Picea abies (L. Karst.), commonly known as Norway spruce, belongs to the *Pinaceae* family. Norway spruce is one of northern Europe's most economically important tree species (Farjon, 2018). It is used for various purposes (such as wood, pulp and paper products), which requires its vigour and stability (Hannrup et al., 2004). Under changing climatic conditions, Norway spruce exhibits limited resistance and capacity to recover from biotic and abiotic stresses (Knoke et al., 2008; Zhang et al., 2009). As temperature and precipitation patterns are projected to change, the geographic distribution/ suitable habitats necessary for Norway spruce may significantly shrink or become restricted over time (Hanewinkel et al., 2013). Due to the species composition in German forests, these stands are very vulnerable to drought, particularly those dominated by spruce (Bolte et al. 2021). Several areas of pure spruce forests have been destroyed by environmental disasters and adverse weather events in Germany (BMEL 2021).

Heterobasidion parviporum Niemelä & Korhonen belongs to *Heterobasidion annosum* species complex, and the species within this complex are the most devastating fungal pathogens in conifers in Europe (Hanewinkel et al., 2013). Most conifer trees are prone to infections by *Heterobasidion annosum* s.l. (Asiegbu et al., 2005) that causes root and stem rot in its hosts. *Heterobasidion parviporum* is practically strictly associated with *Picea abies* (Dalman et al., 2010) but can also infect *Abies* and *Pinus* species (Garbelotto & Gonthier, 2013). *Heterobasidion parviporum* infection makes spruce trees much more prone to windthrow. In fact, studies have shown that Norway spruce is ten times more likely to be uprooted by wind compared to *Quercus robur* and *Quercus petraea* (Matt.) (Knoke et al., 2008). Root rot also

weakens Norway spruce's ability to withstand damage. This includes making them more susceptible to attacks from bark beetles (Netherer et al., 2021).

The primary mode of infection occurs through basidiospores released from the basidiocarp. These spores, dispersed by wind, land on vulnerable sites such as fresh wounds on roots, stems, or recently cut stumps (Swedjemark, 1995). Once established in a stump or any open wound on a tree, *H. parviporum* proliferates by mycelium growth through the root network, thereby facilitating the spread of infection to neighbouring healthy trees (Garbelotto & Gonthier, 2013). The mycelium continues to grow within the roots and stems (Garbelotto & Gonthier, 2013). *Heterobasidion* infection disturbs spruce growth and negatively impacts the value of the logs (Chen et al., 2018). The financial losses caused by this group of fungi were approximated to be around 800 million Euros annually in Europe already two decades ago (Asiegbu et al., 2005; Woodward et al., 1998).

As a result of the long-lasting viability of the inoculum of *Heterobasidion* sp. in conifer stumps (decades) (Greig & Pratt, 1976), planting of tree species susceptible to *Heterobasidion* sp. on infested sites will likely result in high disease incidence which can potentially impact stand productivity in next-generation trees. There is no treatment available for infected trees, and the control strategies are postharvest targeted. Thus, through genetic profiling, favouring trees that may be less susceptible to *Heterobasidion* sp. could bring a new control strategy to diseased sites. Therefore, understanding the mechanisms involved in the response of trees to pathogenic attacks could aid in this process. This research aims to search for differences between genotypes in the resistance or susceptibility of Norway spruce to *Heterobasidion parviporum* infection.

2. Materials and Methods

2.1 Plant materials (chapters II, III and IV)

Plant material used for this project included 1000 three-year-old clonal Norway spruce (*Picea abies* (L.) Karst.) materials received from the Forest Research Institute (LUKE) Finland. Materials consisted of one hundred clones belonging to eight different families (ID: 38, 40, 41, 42, 43, 47, 48, 50, Table 1) with ten ramets per clone. The seedlings were established in the Forest Botany and Plant Physiology greenhouse, Göttingen. They were planted in 3-litre plastic pots filled with 2.5 L fertilized peat (Flora gard, TKS®2 Instant Plus, PERLIGRAN® Extra 2-6 mm, Hermann Meyer KG, Rellingen, Germany). The potted saplings received tap water to maintain moist soil and were monitored and treated for further experiments. Out of the one thousand plants, seven died before the start of the experiment, and the remaining nine hundred and ninety-three plants were used for the study. Each chapter contains details on the exact number of plants used.

2.2 Fungal materials

Two *Heterobasidion parviporum* strains were received from Finland (strain collection of the Natural Resources Institute) and used in the inoculation; *H. parviporum* strain 1 (Hpa 1 – strain number: SB 2005 9.16) and *H. parviporum* strain 2 (Hpa 2 – strain number SB 2014 2.69). The strains were isolated from *Picea abies* stumps (Hpa 1) and infected seedlings (Hpa 2) in Solböle, Finland. The fungal isolates were plated on 1.5% Malt Extract Agar (MEA) and cultured in the growth chamber (Constant climate chamber Memmert HPP 750) for two weeks at 21°C (dark).

2.3 Experimental Design

The seedlings used in this study were grouped into two different watering treatments (optimum and low, Table 1) and randomly arranged in the greenhouse. The plants received regular watering based on these groups, i.e., the saplings grouped under the 50% class received half the quantity of water from the 100% class. The experiments lasted from late July 2020 till early February 2021 under standard ambient lighting. The watering experiment was from late July until November 2020, after which the plants were watered randomly till the end of the experiment in early February 2021. Specific methods employed in this project are further explained in each chapter.

Table 1 Information on plant materials and their watering treatment

S/N	Family	Clone	Number of cuttings	Watering
1	38	3	10	Low
2	38	4	10	Optimum
3	38	5	10	Low
4	38	8	10	Optimum
5	38	9	10	Low
6	38	10	10	Optimum
7	38	12	10	Low
8	38	15	10	Optimum
9	38	17	10	Low
10	38	27	10	Optimum
11	38	28	10	Low
12	38	34	10	Optimum
13	38	35	10	Low
14	38	41	10	Optimum
15	38	44	10	Mixed
16	40	1	10	Low
17	40	3	10	Optimum
18	40	12	10	Low
19	40	15	10	Optimum
20	40	20	10	Low
21	40	24	10	Optimum
22	40	32	10	Low
23	40	34	10	Optimum
24	40	40	10	Low
25	40	41	10	Mixed
26	40	43	10	Low
27	40	47	10	Optimum
28	40	49	10	Low
29	40	50	10	Optimum
30	40	51	10	Optimum
31	41	4	10	Low
32	41	5	10	Optimum
33	41	16	10	Low
34	41	19	10	Optimum
35	41	20	10	Low
36	41	21	10	Mixed
37	41	23	10	Low
38	41	27	10	Optimum
39	41	28	10	Low
40	41	29	10	Optimum
41	41	36	10	Low

42	41	37	10	Optimum
43	41	42	10	Low
44	41	44	10	Optimum
45	41	46	10	Optimum
46	42	1	10	Low
47	42	8	10	Optimum
48	42	9	10	Low
49	42	12	10	Optimum
50	42	13	10	Low
51	42	14	10	Optimum
52	42	16	10	Low
53	42	22	10	Optimum
54	42	24	10	Low
55	42	26	10	Optimum
56	42	29	10	Low
57	42	30	10	Optimum
58	42	34	10	Low
59	42	38	10	Optimum
60	42	43	10	Mixed
61	43	1	10	Low
62	43	8	10	Optimum
63	43	10	10	Low
64	43	11	10	Optimum
65	43	12	10	Low
66	43	15	10	Optimum
67	43	21	10	Low
68	43	22	10	Optimum
69	43	25	10	Low
70	43	26	10	Mixed
71	43	27	10	Optimum
72	47	3	10	Low
73	47	4	10	Optimum
74	47	5	10	Low
75	47	7	10	Optimum
76	47	15	10	Low
77	47	16	10	Optimum
78	47	17	10	Low
79	47	24	10	Optimum
80	47	25	10	Low
81	47	31	10	Optimum
82	47	35	10	Low
83	47	38	10	Optimum
84	47	41	10	Low

85	47	43	10	Optimum
86	47	48	10	Mixed
87	48	3	10	Low
88	48	18	10	Optimum
89	48	19	10	Low
90	48	22	10	Optimum
91	48	23	10	Low
92	48	24	10	Optimum
93	48	29	10	Low
94	48	34	10	Optimum
95	48	40	10	Low
96	48	43	10	Optimum
97	48	44	10	Mixed
98	50	33	10	Low
99	50	34	10	Optimum
100	50	41	10	Mixed

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Chapter I – Drought Stress Described by Transcriptional Responses of *Picea abies* (L.) H. Karst. under Pathogen *Heterobasidion parviporum* Attack

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Drought stress described by transcriptional responses of *Picea abies* (L.) H. Karst. under pathogen *Heterobasidion parviporum* attack

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Abstract

The major threats to the sustainable supply of forest tree products are adverse climate, pests and diseases. Climate change, exemplified by increased drought, poses a unique threat to global forest health. This is attributed to the unpredictable behavior of forest pathosystems, which can favor fungal pathogens over the host under persistent drought stress conditions in the future. Currently, the effects of drought on tree resistance against pathogens are hypothetical, thus, research is needed to identify these correlations. Norway spruce (*Picea abies* (L.) H. Karst.) is one of the most economically important tree species in Europe and is considered highly vulnerable to changes in climate. Dedicated experiments to investigate how disturbances will affect the Norway spruce—*Heterobasidion* sp. pathosystem are important in order to develop different strategies to limit the spread of *H. annosum* s.l. under the predicted climate change. Here, we report a transcriptional study to compare Norway spruce gene expressions to evaluate the effects of water availability and the infection of *Heterobasidion parviporum*. We performed inoculation studies of three-year-old saplings in a greenhouse (purchased from a nursery). Norway spruce saplings were treated in either high (+) or low (−) water groups: high water group received double the water amount than the low water group. RNA was extracted and sequenced. Similarly, we quantified gene expression levels of candidate genes in biotic stress and jasmonic acid (JA) signaling pathways using qRT-PCR, through which we discovered a unique preferential defense response of *H. parviporum*-infected Norway spruce under drought stress at the molecular level. Disturbances related to water availability, especially low water conditions can have negative effects on the tree host and benefit the infection ability of the pathogens in the host. From our RNA-seq analysis, 114 differentially expressed gene regions were identified between high (+) and low (−) water groups under pathogen attack. None of these gene pathways were identified to be differentially expressed from both non-treated and mock-control treatments between high (+) and low (−) water groups. Finally, only four genes were found to be associated with drought in all treatments.

Keywords: drought; Norway spruce; *Heterobasidion*; RNA-seq; qRT-PCR

1. Introduction

Norway spruce (*Picea abies* (L.) H. Karst.) is one of the most economically important forestry tree species in Europe. Currently, massive inputs of anthropogenic greenhouse gases (i.e., CO_2, N_2O, CH_4) into the atmosphere have resulted in increasing atmospheric temperatures in an effect known as 'global warming' [1]. This effect has several consequences; one of which, climate change, is a potential driver in influencing forest health. Under global warming, extreme climate events, e.g., drought, are expected to increase in frequency, duration and intensity [2]. In fact, this has started to take its toll on Norway spruce that is sensitive to abiotic disturbances. Under climate change, there is an increase in drought-associated stress in this particular tree species, rendering them more susceptible to threats, such as pests and pathogens, which can compromise overall tree health [3,4,5]. This phenomenon can be explained by the disease triangle model that shows the relationship between three factors: (1) a susceptible plant host, (2) a pathogen that causes the disease in the plant host, and (3) an environment that favors the pathogen [6,7]. An environment that weakens the defense capability of the plant host and supports the growth and spread of the pathogen can greatly influence the success rate for a pathogen to infect its targeted host. This means that changes in climate do not limit the new challenges of Norway spruce to adjust only to abiotic disturbances, as the projected change in climate will favor certain pathogens in forests [2,5,8,9,10]. The change in tree resistance under global warming against fungal pathogens still remains hypothetical, thus research is needed to identify these correlations. Discriminating responses at the genomic level between biotic and abiotic stressors and understanding the potential trade-offs in plant gene expression will allow for more mechanistic predictions about future scenarios driven by complex shifts in climate change [11,12].

Heterobasidion parviporum Niemelä and Korhonen is one of the native fungal pathogen species (the other two being *H. annosum* s. str. (Fr.:Fr.) Bref. and *H. abietinum* Niemelä and Korhonen) belonging to a root-rot fungus, *H. annosum* sensu lato (s.l.) complex, which is considered to be the most devastating Norway spruce pathogen in Europe [13]. Being necrotrophic in nature, it mainly derives its nutrients by killing its tree host and feeding off its contents. There are two modes in which *H. parviporum* can infect Norway spruce: (1) through the deposition of basidiospores on freshly cut stumps or (2) via root contacts [14]. Detection of *H. parviporum* in Norway spruce is often challenging, as the infected tree might not necessarily show signs of symptoms (stringy white rot, necrosis in sapwood, presence of

fruiting bodies) over decades. This further promotes the spread of *H. parviporum* to neighboring trees via root contacts. In addition, *H. parviporum* mainly colonizes the heartwood of Norway spruce, causing decay and even tree mortality [15].
The induction of a disease by a pathogen in a tree host is often linked to the environmental conditions in the ecosystem as well. In this particular Norway spruce—*H. parviporum* forestry pathosystem, climate plays an important role in promoting the spread of *H. parviporum* in several ways: (1) short winters that provide a longer period of hyphae growth and spore transmission, (2) warmer summers that encourage fungal hyphae to grow faster and produce more spores, and (3) windy days that can promote the dispersion of basidiospores [2,16,17]. Increased frequency and intensity of drought-associated stress in Norway spruce is expected in the following years [18]. Considering how Norway spruce is a valuable tree species to the boreal regions, it is imperative to study the interactions between this tree species and its associated fungal pathogens. This way, appropriate and effective measures can be implemented to mitigate this inevitable climate change.

Rapid advancements in molecular research have provided insights into the Norway spruce—*H. parviporum* forestry pathosystem through various studies, such as pathogen characterization and detection [19], constructing gene expression libraries [20] and screening of resistant trees based on genetic components [21]. Norway spruce resistance against *H. annosum* s.l. is a quantitative trait [21,22,23] and is associated with several known genes (defense responses) with variation [21,24]. Signalling molecules are a group of plant secondary metabolites with a critical role in defense processes [25]. Plant hormones, especially salicylic acid (SA) and jasmonic acid (JA) help mediate information about the attack beyond the point of invasion [25]. By altering the hormonal balance in plants, pathogens use the defensive machinery of plants to their advantage and either induce or suppress the processes relevant for cell death and accumulation of antimicrobial compounds [25,26]. Jasmonic acid (JA)-mediated signalling is the prioritized module in the Norway spruce defence-signalling network against *H. parviporum* [27,28,29]. Similarly, besides JA, the ethylene (ET) signalling pathway probably also plays a central role in the defense response of Norway spruce against *H. parviporum* [28]. In addition, the salicylic acid (SA) signalling pathway can, likewise, have an important role in this pathosystem, as the SA-mediated hypersensitive response (HR) can be facilitated by necrotrophic pathogen infection [30]. Understanding the similarities and differences in transcriptional response between variable environmental conditions can thus provide useful information over how Norway spruce—*H. parviporum* pathosystems adapt to climate change.

We could show previously that *H. parviporum* benefits from host stress (i.e., drought) [18]. In this study, we performed RNA-seq analysis on samples derived from the same trees as in Terhonen et al. [18]. We concentrated on Norway spruce, to find out which genetic pathways are involved in the specific responses to unfavorable environmental stress (drought) with and without the pathogen, *H. parviporum*. In addition, we also built a transcriptional study based on previous studies [27,28,31,32,33] to investigate the interplay between *H. parviporum* infection and defense-related gene expression mediated by drought stress.

2. Materials and Methods

2.1. Plant and Fungal Material

The setting used in this experiment is from Terhonen et al. [18]. Summarily, plant material consisted of 18 three-year-old, apparently healthy and vital Norway spruce saplings purchased from the nursery Schlegel and Co Gartenprodukte (provenience of the saplings: No. 840 11, Thüringer Wald and Frankenwald, montane zone 600 m). Seedlings were potted into 3-L plastic pots filled with fertilized peat (Flora gard, TKS®2 Instant Plus, Hermann Meyer KG, Rellingen, Germany). The potted saplings were then acclimatized to the greenhouse conditions for 16 days prior to the water experiment, during which they received tap water, as required, to maintain moist soil. No additional fertilization was given during the experiment.

A *H. parviporum* strain was received from the North-West German Forest Research Institute, collected by G. Langer and colleagues. *H. parviporum* (strain NW-FVA 0459) was isolated from *P. abies* in 2010 (Germany, Lower Saxony, forest department Oerrel, Bobenwald). DNA of *H. parviporum* was extracted from 150 mg of the homogenized mycelium sample using the method developed by Keriö et al. [34] and the fungal strain used in this study was tested for the specificity with species-specific primers developed for *H. parviporum* (KJ-F and KJ-R; [19]). In brief, DNA template (100 ng), buffer (KCl extra buffer, 1X), 1.5 mM MgCl2, primers KJ-F and KJ-R (each concentration of 0.5 µM), a dNTP-mix (each deoxynucleotide in a concentration of 200 µM) and 20 U/mL of DNA-polymerase (VWR) was adjusted to 25 µL reaction with autoclaved Milli-Q H_2O. The cycling conditions used were: Initial denaturation 10 min at 95 °C, followed by a 3-step cycling: Denaturation 30 s at 95 °C; annealing 35 s at 67 °C; extension 1 min at 72 °C, for 40 cycles. A final extension step for 7 min at 72 °C was applied. Taq DNA polymerase (Qiagen) was used for PCR amplification of ITS regions with

the primer pair, ITS1-F and ITS4. Briefly, the PCR protocol was as follows: 1X CoralLoad PCR Buffer, 200 µM dNTP, 0.5 µM primer 1, 0.5 µM primer 2, 100ng template DNA, 0.2 U/µL DNA polymerase; the reaction was adjusted to 25 µL with autoclaved Milli-Q H_2O. The PCR conditions used were 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min; a final step at 72 °C for 10 min was applied before storing the PCR products at 4 °C. Possible contaminations were determined with a negative control using sterile water as a template in both PCR protocols. StainIN™ RED Nucleic Acid Stain was used to confirm DNA amplicons on a 2% agarose gel and the visual detection was made by ultraviolet transillumination. The expected band (fragment size around 350 bp) with KJ-F and KJ-R primers was visualized and the ITS region PCR products were purified and sequenced using the ITS4 primer at Microsynth SEQLAB (Göttingen, Germany). The FASTA files thus obtained were checked with BioEdit to confirm that the pathogen was not contaminated with other fungi strains. ITS region sequencing indicated no contamination in the culture of *H. parviporum* (see [18]).

2.2. Experimental Design

The following experiment is described in detail in [18]. In summary, the study was conducted at the Forest Botany and Tree Physiology greenhouses, in Göttingen, Germany (51°33′28.4″ N 9°57′30.5″ E) from early April until early August 2018. From this experiment, we chose 18 seedlings (three per water treatment group) randomly block-assigned to waterproof tables with either high water (high water group = +group) or low water (low water group = −group) availability treatments, which were experimentally inoculated with *H. parviporum*, mock-inoculated controls or left entirely untreated. The water treatment experiment was running for 35 days before the inoculations were performed. *H. parviporum* isolate (NW-FVA 0459) was inoculated using a puncher (Ø5 mm) through the bark to reach the sapwood surface. Equal-sized plugs from a pure culture of *H. parviporum* or control (2% MEA) were placed onto the exposed surface and sealed with Parafilm® [18]. The inoculation experiment was run for 70 days [18]. At the end of the experiment, we collected 30 samples aiming to sequence 18 samples. The stems of Norway spruce saplings (5 cm in both directions: above and below the infection point was collected, around 11 cm) were sampled into liquid nitrogen and stored individually into 15-mL Falcon tubes at −80 °C for long-term storage.

2.3. RNA Extraction

A total of 30 Norway spruce stems were selected, comprising of five sample replicates for each of the six treatment groups for small-scale totRNA extractions; a total of 18 (with three sample replicates for each of the six treatment groups) were selected for upscale totRNA extractions. For the first round of extractions, the surface of the inoculation point (bark, phloem, sapwood) was scraped with a scalpel and the sample was ground into a fine powder (Mixer Mill MM 400 from Retsch GmbH with a set program of 25.0 Hz for 20 s). The samples were handled with liquid nitrogen throughout the entire milling process. The ground product was then stored in 15-mL Falcon tubes at −80 °C for later use in the extractions. The extraction protocols used were modified from Zeng et al. [35] and unfortunately, the first try with 0.1–0.5 g of sample transferred to 2-mL Eppendorf tubes was unsuccessful as the RNA quantity was too low for sequencing. We subsequently re-did the scraping from the infection point and increased the area to collect more material (the sample included bark, phloem and sapwood). The new samples were similarly ground into a fine powder and extracted for totRNA, which was successful. From this, we established the following extraction protocol, which we had modified from Zeng et al. [35] for our study.

Day 1: 2–3 g of sample was transferred to a 50-mL Falcon tube, where 15 mL of Extraction buffer (2% CTAB, 2% PVP, 100 mM Tris-HCl, 25 mM EDTA, 2 M NaCl, DEPC-treated H_2O, pre-heated to 65 °C) was added; 100 µL of β-mercaptoethanol was added to each tube and vigorously vortexed before incubating the tubes at 65 °C for 5 min. An equal volume of chloroform:isoamyl alcohol (24:1) was added to each tube and vortexed vigorously before centrifuging the tubes at 10,000× *g* for 15 min. The upper aqueous phase was transferred to a new sterile 50-mL Falcon tube. Chloroform:isoamyl alcohol was added, then the tubes were centrifuged and subsequently the transfer of the upper aqueous phase was repeated; 1/4 volume of 10 M LiCl was added to each tube and inverted eight times before precipitating the RNA overnight at 4 °C.

Day 2: The tubes were centrifuged using the benchtop Eppendorf 5810R Centrifuge (Rotor A-4-81 with a bucket for 7 × 50 mL conical tubes) with a speed of approximately 6480 rcf at 4 °C for 30 min. The supernatant was collected in a new 50-mL Falcon tube and set aside for gDNA extraction. The remaining pellet (containing totRNA) was briefly dried in a laminar flow hood; 1 mL of SSTE (1 M NaCl, 0.5% SDS, 10 mM Tris-HCl, 1 mM EDTA, DEPC-

treated H_2O, preheated to 65 °C) was then added to dissolve the pellet. Thereafter, an equal volume of chloroform:isoamyl alcohol was added and vortexed vigorously before centrifuging at 10,000× *g* for 15 min. The upper aqueous phase (maximum 500 µL) was transferred to a new 2-mL Eppendorf tube and three volumes of cold 100% absolute EtOH were added to each tube. The resultant mixture is incubated at −20 °C for 1–2 h before transferring to −80 °C overnight for RNA precipitation.

Day 3: totRNA samples were centrifuged at maximum centrifugal speed at 4 °C for 30 min. The supernatant was pipetted out and the remaining totRNA pellet was dried in a sterile hood for approximately 3 min. The pellet was then resuspended in 100 µL of DEPC-treated H_2O, where it could be subsequently used in molecular analyses or stored at −80 °C for long-term storage.

2.4. Quantitation and Purity Analyses of Extracted totRNA

A Qubit 3.0 Fluorometer from Life Technologies (Catalogue number: Q33216) was used to quantitate totRNA. The analysis was performed according to the manufacturer's instructions. Three biological replicates from each treatment (2 µg of total RNA) were sent for RNA sequencing (RNAseq) to Novogene. RNA integrity and quantitation were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Purified RNA was used for library construction using rRNA depletion method (GlobinZero kit) for Illumina® (NEB, Santiago, CA, USA), followed by Illumina paired-end (2 × 150 bp) RNAseq in the facilities of the Novogene (HK) company limited. The raw reads have been submitted to NCBI SRA under Bioproject PRJNA761217.

2.5. RNA-seq Analysis

Quality control was made by Novogene through in-house scripts. Briefly, clean data (clean reads) were obtained by removing reads containing adapter and poly-N sequences and reads with low quality from raw data. The filtering process was as follows: (1) Discard reads with adapter contamination; (2) Discard reads when uncertain nucleotides constitute more than 10% of either read ($N > 10\%$); (3) Discard reads when low quality nucleotides (base quality less than 20) constitute more than 50% of the read. At the same time, Q20, Q30 and GC content of the clean data were calculated. All downstream analyses were based on clean data with high quality. The quality was assessed using FastQC

(http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (accessed on 17 February 2020)). Reference genome and gene model annotation files were downloaded from genome website browser (the processed reads were mapped against the genome assembly of Norway spruce (v 1.0) [36] downloaded from ftp://plantgenie.org/Data/ConGenIE/Picea_abies/ (accessed on 8 March 2020) directly. Paired-end clean reads were mapped to the reference genome using HISAT2 software [37]. The raw read counts table was loaded into R studio version 3.5.1 [38] and differential expression analysis between two conditions (three biological replicates per condition) was performed using DESeq2 R package [39]. Conditions were normal water versus low water in each treatment (treatments: non-treated, mock-inoculated, or *H. parviporum* infection). To track down only drought-related gene pathways, we compared all treatments to the non-treated (+) water treatment. The resulting p values were adjusted using Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with an adjusted p-value < 0.05 and the cutoff value for FDR < 0.05 were assigned as differentially expressed. We searched for statistically expressed genes between different conditions/treatments with Venny 2.1. Results were compared on genes expressed in the study by Chaudhary et al. [40]. When we found interesting genes, we visualized the expression in each treatment. Similarly, we studied how *H. parviporum* resistance candidate gene laccase PaLAC5 (MA_97119g0010 and MA_97119g0020) were expressed in each treatment [24].

2.6. GO Enrichment Test

The GO functions and enrichment analysis were performed using ConGenIE.org (http://congenie.org/ (accessed on 22 July 2020)). Annotate GeneList workflow was created on the ConGenIE platform selecting *P. abies* as the species of interest. The gene IDs were pasted in the search field and annotated. The enrichment analysis was carried out using the functional enrichment tools. The GO slims were run and based on the annotations, the transcripts were categorized in the biological process and molecular function groups with their respective descriptions. The significantly enriched GO terms were then categorized into Biological Process, Molecular Function and Cellular Component.

2.7. Transcriptional Responses Validated with Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

For cDNA preparation, we followed the protocol described by Raffaello and Asiegbu [41]. In this experiment, four genes were tested: LOX [27], ERF1 [28], CHIIV [33] and p/DIR32 [32]. A housekeeping gene, Elongation factor 1α (ELF1α) [31], was used as a reference against the tested genes. To prepare the loading samples for qRT-PCR, gene mastermixes comprising of 0.5 µM each of forward and reverse primers for each selected gene, qPCR Supermix (SsoFast™ EvaGreen® Supermix (BIO-RAD)), and DEPC-H_2O as a diluent were made. A total of 20 µL per qRT-PCR reaction (including the pooled cDNA) were then loaded onto a 96-well V-bottom plate and sealed tight with plate foil. The qRT-PCR (iQ5™ Multicolor Real-Time PCR Detection System (BIO-RAD)) cycling conditions ran were set for an initial 2 min at 95 °C, then 40 cycles of 20 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. According to a primer efficiency test prior to running the qRT-PCR, we decided on a cDNA concentration of 100 ng per pooled cDNA. The relative expressions were subsequently calculated with the method $2^{-\Delta\Delta Ct}$ [42].

3. Results

3.1. RNA-seq

Our analysis of RNA-seq data identified 114 transcripts with a significantly different expression between high (+) and low (−) water groups under pathogen attack (Supplementary File S1). Comparing *H. parviporum* infection under drought stress, the biological processes (in upregulated) significantly enriched were metabolic process, transport, protein folding, and translation. Molecular function GO terms in pathogen-infected seedlings were significant in transferase activity, hydrolase activity, and binding. Cellular component GO terms were significantly related to plant cell organelles (apoplast, chloroplast, Golgi apparatus, mitochondrion, cell wall and plasma membrane) (Supplementary File S1). The roles played by these genes are expressed as GO terms.

Drought substantially increased gene expression under *H. parviporum* inoculation (Table 1). Exclusively 377 genes were downregulated and 129 genes upregulated in the *H. parviporum*-infected (−) group (Supplementary File S1). The upregulated Gene Ontology (GO) terms assigned to the “Biological process” in the (−) water group were, protein folding, response to

stress, oxidation-reduction process, rRNA processing, primary metabolic process, phosphorylation, pentose-phosphate shunt and Golgi organization. The terms assigned to the "Molecular function" were, ATP binding, oxidoreductase activity, nucleotide binding, unfolded protein binding, transferase activity and protein kinase activity. The downregulated genes expressed resulted in different gene functions and the most abundant within this group were metabolic process, oxidation-reduction process, response to wounding, response to fungus, phosphorylation, defense response to fungus, response to water deprivation, and response to oxidative stress. Only the *Heterobasidion*-infected (−) groups were found to be functionally enriched (Supplementary File S1).

Table 1. Statistically differentially expressed genes of each treatment when compared to non-treated (+) water group.

Inoculation	Water	Significant	Up-Regulated	Down-Regulated	Not significant	SUM
Heterobasidion parviporum	–	521	133	388	29,592	30,113
Heterobasidion parviporum	+	184	141	43	29,944	30,128
Non-treated	–	82	27	55	29,715	29,797
Mock-inoculated	–	78	11	67	29,950	30,028
Mock-inoculated	+	202	148	54	29,951	30,153

We found four genes that were expressed significantly as higher or lower amounts (compared to non-treated (+) group) only under drought stress MA_189802g0010, MA_10265000g0010, MA_10435878g0010 and MA_178006g0010 (Figure 1, Table 2). These genes were not observed to be significantly expressed (up or downregulated) in normal water treatments. Only gene DEG MA_10435878g0010 had a role in the metabolic process (GO:0008152) (Table 2).

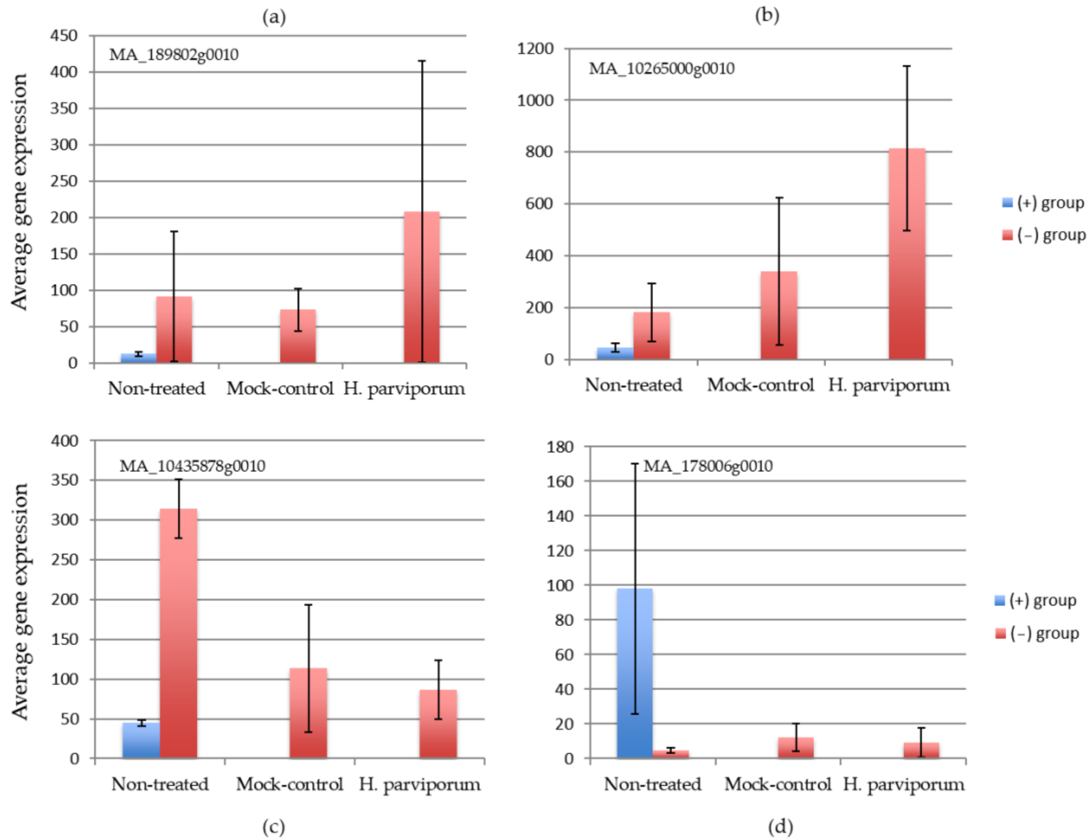

Figure 1. The gene expression levels of the four genes ((**a**) MA_189802g0010, (**b**) MA_10265000g0010, (**c**) MA_10435878g0010 and (**d**) MA_178006g0010) found exclusively to be significantly expressed only under drought (compared to (+) group non-treated).

Table 2. Four Drought-related candidate genes, their description and GO ID.

Gene	Description	Chromosome	Source	Confidence	Trinity	GO	GO Description
MA_10265000g0010	PR10	MA_10265000	AUGUSTUS	High	comp87387_c0_seq1		
MA_10435878g0010	ACC	MA_10435878	AUGUSTUS	High	comp94559_c0_seq1	GO:0008152	GO:0008152-metabolic process
MA_178006g0010	BURP domain RD22-like	MA_178006	AUGUSTUS	High	comp75759_c0_seq1		
MA_189802g0010	Thaumatin	MA_189802	AUGUSTUS	High	comp87856_c1_seq1		

After comparing to dataset of Chaudhary et al. [40], we identified four candidate genes related to *Heterobasidion* sp. resistance (MA_110169g0010, MA_14707g0010, MA_15852g0010, MA_10427673g0020) that were significantly expressed (as higher or lower amounts compared to non-treated (+) group) only in *H. parviporum* infected plants (only in -group:

MA_14707g0010, MA_15852g0010, MA_10427673g0020). Their expression levels in each treatment are presented in Figure 2. Palac5 genes MA_97119g0010 (Figure 3a) and MA_97119g0020 (Figure 3b) were not significantly expressed (compared to the non-treated (+) group) in *H. parviporum* infected plants, but these genes accumulated more under *H. parviporum* infection (Figure 3a,b).

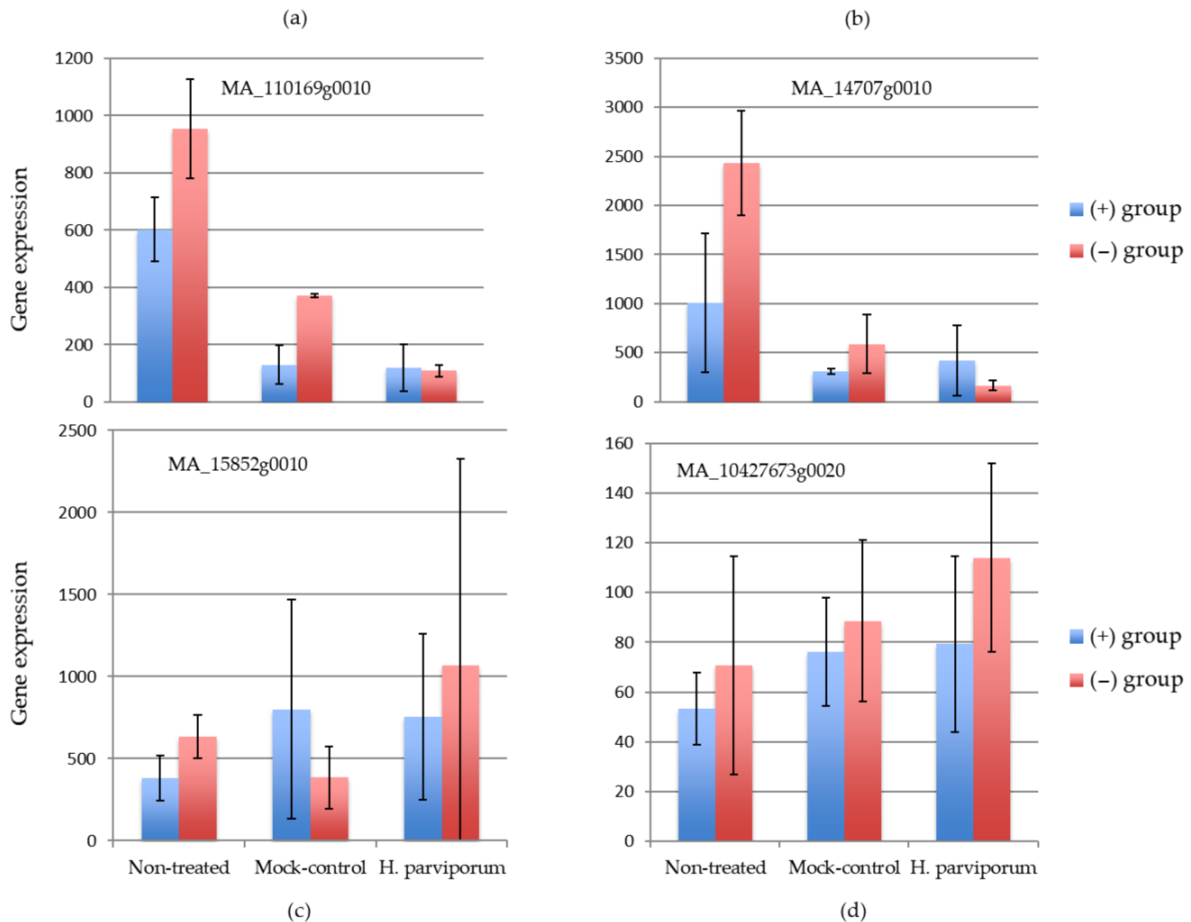

Figure 2. *Heterobasidion* resistance-related genes and their expressions in each treatment (compared to (+) group non-treated). (**a**) MA_110169g0010 and (**b**) MA_14707g0010 were downregulated, while (**c**) MA_15852g0010 and (**d**) MA_10427673g0020 were upregulated.

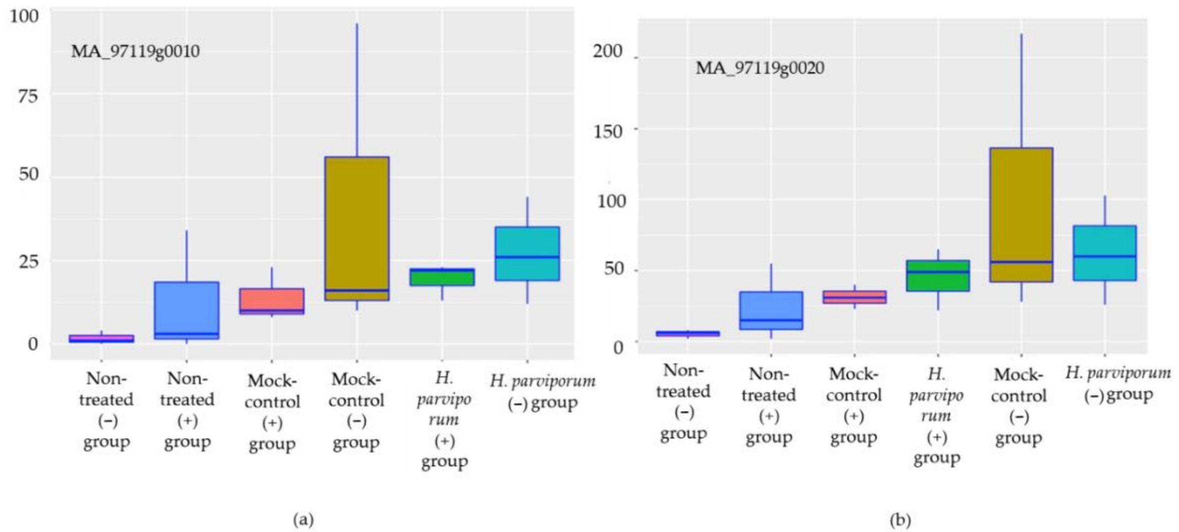

Figure 3. Palac5 (**a**) MA_97119g0010 and (**b**) MA_97119g0020 gene expression levels in each treatment.

3.2. qRT-PCR

Normalization between technical triplicate samples was achieved by determining the mean fold change of each gene (i.e., LOX, ERF1, CHIIV, p/DIR32) against the housekeeping gene, ELF1, for each treatment scheme (Table 3). The difference in gene expression between technical triplicates was mostly with a mean fold change value ($2-\Delta\Delta Ct$) of 1, irrespective of water treatment groups and inoculation status, indicating little non-biological variation. This is further illustrated with Figure 4, which generally demonstrated consistency between triplicates except for two treatment groups in p/DIR32 (SD: *H. parviporum*, normal = 0.788; mock control, low = 0.822) and one group in ERF1 (SD: mock-control, low = 0.565).

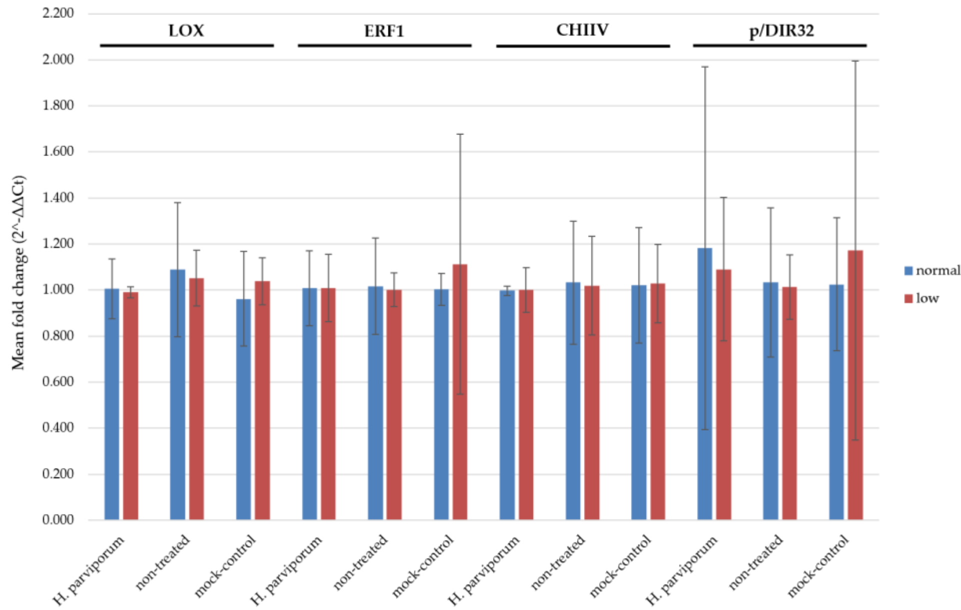

Figure 4. Overview of qRT-PCR mean fold change for each tested gene against ELF1. Variation between triplicate samples of each treatment group was represented by error bars (+/− SD of each triplicate) respectively.

Table 3. Mean fold change of each candidate gene in qRT-PCR for each treatment scheme.

Gene	Mean fold change ($2^{-\Delta\Delta Ct}$)	SD	Inoculation type	Water treatment
LOX	1.006	0.129	*H. parviporum*	normal
	1.088	0.291	non-treated	normal
	0.962	0.206	mock-control	normal
	0.990	0.024	*H. parviporum*	low
	1.052	0.122	non-treated	low
	1.038	0.102	mock-control	low
ERF1	1.009	0.162	*H. parviporum*	normal
	1.017	0.210	non-treated	normal
	1.004	0.069	mock-control	normal
	1.009	0.145	*H. parviporum*	low
	1.002	0.073	non-treated	low
	1.112	0.565	mock-control	low
CHIIV	0.998	0.020	*H. parviporum*	normal
	1.033	0.267	non-treated	normal
	1.020	0.251	mock-control	normal
	1.001	0.098	*H. parviporum*	low
	1.019	0.214	non-treated	low
	1.028	0.171	mock-control	low
p/DIR32	1.182	0.788	*H. parviporum*	normal
	1.034	0.324	non-treated	normal
	1.025	0.288	mock-control	normal
	1.090	0.311	*H. parviporum*	low
	1.013	0.139	non-treated	low
	1.172	0.822	mock-control	low

To obtain a meaningful interpretation of our results, we also compared the fold change in gene expression mediated by *H. parviporum* and physical wounding as singular and combinatorial factors to examine how Norway spruce responds differently under drought conditions (Table 4).

Table 4. Comparative quantification determining mean fold change ($2^{-\Delta\Delta Ct}$) in gene expression mediated by *H. parviporum* infection only, physical wounding only, and the combination of both *H. parviporum* infection and physical wounding caused by inoculation

Gene	Water treatment	$2^{-\Delta\Delta Ct}$		
		H. parviporum	**Physical wound**	***H. parviporum* + Physical wound**
LOX	normal	1.60	0.17	0.27
	low	1.27	6.92	8.82
ERF1	normal	2.81	0.31	0.87
	low	0.46	2.31	1.06
CHIIV	normal	0.19	10.85	2.03
	low	7.16	0.91	6.50
p/DIR32	normal	0.25	0.33	0.08
	low	2.64	17.03	44.94

LOX (1.60) and ERF1 (2.81) were upregulated while CHIIV (0.19) and p/DIR32 (0.25) were downregulated when mediated solely by *H. parviporum* infection under normal water conditions. With the additional stress induced by low water conditions, CHIIV (7.16) and p/DIR32 (2.64) had an increase in transcription levels and were both upregulated as a result. The sole influence of a physical wound led to an increase in gene expression of LOX (6.92), ERF1 (2.31) and p/DIR32 (17.03) when subjected to drought stress. This was the opposite observation in CHIIV (0.91) where it is downregulated instead. With sufficient water supply, CHIIV (10.85) was abundantly expressed. Combinatorically, LOX, CHIIV and p/DIR32 were upregulated when wounded *H. parviporum*-infected plants were subjected to drought stress (LOX = 8.82, CHIIV = 6.50, p/DIR32 = 44.94). On the other hand, with the exception of CHIIV, the tested genes were downregulated when the plants received normal water treatment (LOX = 0.27, ERF1 = 0.87, p/DIR32 = 0.08). No change was observed for ERF1 even at low water treatment conditions (ERF1 = 1.06). These observations are further represented in Figure 5.

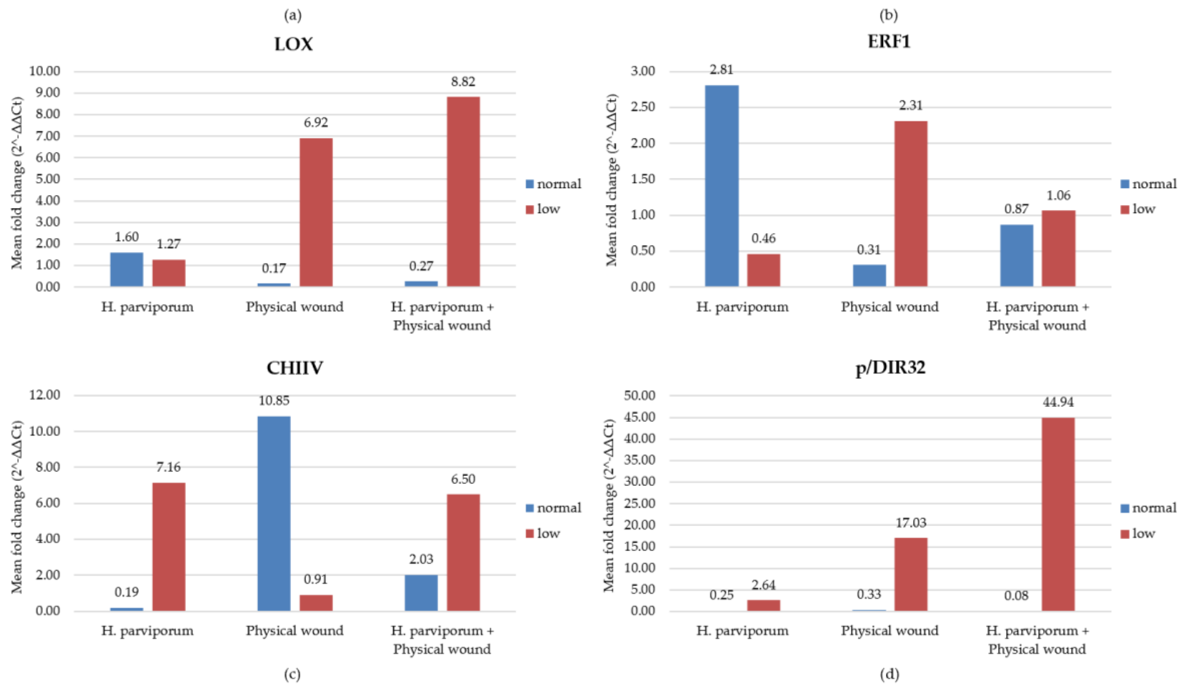

Figure 5. Influence of *H. parviporum* infection and physical wounding as singular and combinatorial factors in Norway spruce defense responses under drought stress conditions, as reflected by gene regulation in (**a**) LOX, (**b**) ERF1, (**c**) CHIIV, and (**d**) p/DIR32.

In general, LOX and p/DIR32 showed an increase in gene expression levels under drought stress when combinatorically influenced by *H. parviporum* infection and physical wounding in comparison to their respective singular factors that are independent of each other. Under normal water conditions, ERF1 is dominantly expressed in *H. parviporum*-infected Norway spruce; this is the same for CHIIV in Norway spruce that are physically wounded. This observation is flipped under low water conditions (Figure 5).

4. Discussion

4.1. Heterobasidion Resistance Related Genes

Drought induced 114 DEGs between *H. parviporum* treatments (−/+), similarly exclusively 377 DEGs were observed in (−) group of *H. parviporum*. Chaudhary et al. [40] identified several candidate genes related to *Heterobasidion* sp. resistance from which we found only four (MA_110169g0010, MA_14707g0010, MA_15852g0010, MA_10427673g0020) in *H. parviporum* infected plants to be statistically expressed. The accumulation of these genes was induced in drought treatment in almost all cases. The same trend could be found for Palac5 genes (MA_97119g0010 and MA_97119g0020) [24] as they accumulated more in *H.*

parviporum infected plants under drought, and in non-treated (−) group plants these genes were at the minimum level. These results highlight the need for combined factor studies, abiotic and biotic stress in plant gene expression studies to resolve this combination effect as some pathways can be induced by the environment.

4.2. Changing Environment: Drought as a New Factor in Forest Pathosystems

Climatic extremes, such as drought affect trees directly, by inducing water deficiency, and indirectly, by making trees more susceptible to fungal pathogens [10,18]. This leads to unknown behavior of host-fungi interactions, resulting in increased disease outbreaks caused by native fungal pathogens [43]. Prolonged water and nutrient deficiencies are causing severe damage to Norway spruce and the drought disturbances are expected to increase in following years [2]. Norway spruce provenances react differentially to drought [44]. Trujillo-Moya et al. [44] found drought response indicators (24 genes, 29 SNPs) of Norway spruce genotypes. One of these 24 genes, MA_588952g0010 (no similarity to any known gene), was found exclusively to be significantly expressed in *H. parviporum* (-) treatment. Our analysis of RNA-seq data identified only four genes (MA_189802g0010, MA_10265000g0010, MA_10435878g0010 and MA_178006g0010) significantly expressed in drought stressed trees. The gene (MA_178006g0010) is a member of the BURP domain family (RD22-like). Members of this family have been described to be associated with several plant species subjected to stress conditions [45]. In *Arabidopsis thaliana* (L.) Heynh., the AtRD22 and AtUSPL1 (both members of the *Arabidopsis thaliana* BURP) are upregulated as part of the abscisic acid (ABA)-mediated moisture stress response [45]. ABA is a phytohormone that adjusts the drought stress response in plants. Additional ABA application enhances the tolerance of wheat [46] and tomato [47] seedlings to drought and ABA is accumulating under drought in maize [48].

Gene MA_10265000g0010 is highly homologous to intracellular pathogenesis-related PR10 proteins. These genes produce proteins that are toxic to invading fungal pathogens and act in defense signaling. PR10 gene down-regulation was observed in maize early in drought-induced plants [49]. PR-10 protein was also noted to accumulate in the needles of drought-stressed *Pinus pinaster* Ait. seedlings [50]. The sequence of gene MA_10265000g0010 has a 100% query coverage and 98.97% identity to *Picea glauca* (Moench) Voss putative intracellular pathogenesis-related protein (picg4) [51]. Ekramoddoullah [51] showed that the

expression of PR-10 (including picg4) genes is induced by both biotic (white pine blister rust, *Cronartium ribicola* J.C.Fischer) and abiotic (cold) stress. Therefore, we suggest that the gene MA_10265000g0010 could be a possible marker of abiotic and biotic factors (i.e., drought stress and pathogen attack) for transcriptional response studies in conifers [51] as the expression seems to accumulate in the combination of these stressors.

Gene MA_10435878g0010 plays a role in metabolism and it belongs to the 2OG-Fe (II) oxygenase superfamily. It has been found to be significantly expressed in the biosynthesis of secondary metabolites and in metabolic pathways under different light spectra in Norway spruce [52]. It appears that this gene reacts to environmental change and is accumulates more when we combine biotic stress (*H. parviporum*) with abiotic stress (drought). MA_189802g0010 belongs to the thaumatin family and it responds to fungal organisms and salinity; the association of this gene with *P. abies* was previously unknown.
In addition to the RNA-seq analysis, another aim of this study was to uncover preliminary insights into the defense responses of *H. parviporum*-infected Norway spruce under drought stress. LOX [31], ERF1 [28], CHIIV [33], and p/DIR32 [32] have previously been demonstrated to be activated and expressed when Norway spruce is infected with *H. parviporum*; thus far, our observations from LOX and ERF1 through qRT-PCR concur with these studies. On the other hand, although we had similar observations [32] from p/DIR32 showing greater expression in wounded Norway spruce than *H. parviporum*-infected ones, they were only induced under drought stress conditions. This similar observation was also the same in CHIIV [33]. Plausible reasons for these findings are unfortunately unknown to date.

Nevertheless, we discover that this defense-related gene response is more complex when additional abiotic stress factors are present. Summarily, we deduce that water availability plays a critical role in the gene switch between up-/down-regulation strategies employed by *H. parviporum*-infected Norway spruce. This is evident when p/DIR32 regulation is heavily influenced by water supply. Although CHIIV is more responsive to the presence of physical wounding under normal water conditions, this response can be easily overtaken by *H. parviporum* infection when subjected to drought stress. This suggests a preferential defense response of Norway spruce to *H. parviporum* infection over physical wounding, depending on water availability conditions. Likewise, this preferential response can also be influenced by the type of damage and infection present in the plant. For example, when affected by physical wounding, CHIIV is expressed under normal water conditions while ERF1 is expressed under

drought stress conditions; this is the other way around when Norway spruce is infected with *H. parviporum*. Through these observations, the complexity of defense-related gene response in *H. parviporum*-infected Norway spruce is demonstrated and piques new research questions: within the Norway spruce—*H. parviporum* forest pathosystem, how does the host make its choice in activating a specific defense-related gene, and how does it relate back to the various abiotic signaling pathways involved? Currently, due to the explorative nature of our study, the RNA-seq and qRT-PCR dataset we have gathered thus far is, unfortunately, not sufficiently comprehensive to make sensible inferences to the above-mentioned questions. Therefore, opportunities for further in-depth research in this direction may be prompted.

4.3. Norway Spruce Resistance in a Changing Climate

Norway spruce is not only highly economic but also possesses ecological value in Europe, particularly in Northern and Central European countries. The current breeding activities concentrate on developing more productive and climate resilient trees in Scandinavia. Similarly, several markers are suggested for *Heterobasidion annosum* s.l. resistance [24,40,53,54]. These traits should all be studied for suitability in breeding programs as due to long tree generations, Norway spruce might not keep up with the changes in the local environment, making them more susceptible to infection by *Heterobasidion* species. In addition, with the new discovery of a preferential defense gene response in Norway spruce mediated by water availability, understanding how specific defense-related genes are regulated in the midst of a changing climate will be pivotal to support the successful breeding of these trees. The expression of these gene regions would be good to be tested in multifactorial analysis. Necrosis caused by fungal pathogens is increasing under host stress [3,10,18], and revealing the molecular determinants of resistance/virulence in a changing environment of forest pathosystems would be needed to mitigate this detrimental response. The major threats to the sustainable supply of forest tree products are adverse climates that benefit the pests and diseases at the cost of the host. Changes in the environment claim for the inclusion of new traits in breeding programs. Increasing the availability of Norway spruce and *H. parviporum* transcriptome data thus creates new opportunities to discover genes that underlie this tree-pathogen interaction on the projected climate change continuum.

Supplementary Materials

The following are available online at https://www.mdpi.com/article/10.3390/f12101379/s1.

Author Contributions

Conceptualization, K.B. and E.T.; Data curation, X.H.-Y.Y., K.B. and E.T.; Formal analysis, X.H.-Y.Y., B.D. and E.T.; Methodology, X.H.-Y.Y. and E.T.; Project administration, E.T.; Supervision, K.B. and E.T.; Writing—original draft, X.H.-Y.Y., B.D. and E.T.; Writing—Review & editing, X.H.-Y.Y., K.B. and E.T. All authors have read and agreed to the published version of the manuscript.

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Data is contained within the article, the Supplementary Material and at the NCBI SRA under Bioproject PRJNA761217.

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Conflicts of Interests

The authors declare no conflict of interest.

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6. Supplementary materials

All supporting information is contained in this link - https://www.mdpi.com/article/10.3390/f12101379/s1

Chapter II – Genetic variation of *Picea abies* in response to the artificial inoculation of *Heterobasidion parviporum*

Blessing Durodola, Kathrin Blumenstein and Eeva Terhonen

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Genetic variation of *Picea abies* in response to the artificial inoculation of *Heterobasidion parviporum*

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Abstract

Norway spruce *Picea abies* is one of Europe's most economically important tree species. However, it is highly susceptible to the root rot fungus *Heterobasidion parviporum*. Climate change will benefit the pathogen as the tree host is weakened by, e.g., extended drought. Breeding can improve forest health, and several root rot genetic markers are suggested to improve the resistance of Norway spruce. This study aimed to compare the resistance (here defined as necrosis length) of Norway spruce families and genotypes against two strains of *H. parviporum* under different water availabilities. Our results show that the family and the genotype within the family have an impact on the necrosis length that is related to the aggressiveness of the fungal strains. Under low water conditions, the necrosis increased only in horizontal directions in phloem and sapwood. Similarly, the growth (seedling height) was not disturbed by abiotic stress (less water), indicating that the stress level (drought) was too low in this setting. The knowledge gained in this study could improve forest health in the changing climate by understanding the response of Norway spruce to pathogenic attacks under additional stress at the family level. This knowledge could be strategically used in forest breeding to improve the resistance of Norway spruce trees against root rot.

Keywords: Drought stress; Fungi-host relationship; Genotypic variation; Necrosis; Norway spruce; Root rot.

1. Introduction

Forest trees do not only have important roles in their economic and ecological value; they also mitigate the effects of climate change by storing carbon. However, fungal pathogens endanger the health of forests (Wingfield et al. 2015) and thus threaten these stocks. Norway spruce (*Picea abies*) is one of the highly valued tree species in Europe, but it is the main host of the pathogen *Heterobasidion parviporum* (Garbelotto and Gonthier 2013). Infections from this fungus make the trees highly susceptible to windthrow and lower their resistance, e.g., against bark beetles (Netherer et al. 2021). The continued growth of Norway spruce is threatened by root rot caused by members of the *Heterobasidion annosum* sensu lato species complex (Oliva et al. 2011; Piri 1996; Piri and Korhonen 2001, 2007). The economic loss due to this fungal complex was estimated to be ~ 800 million euros per year in Europe already 20 years ago (Asiegbu et al. 2005, Woodward et al. 1998), which should amount to about 1.2 billion euros as of 2020, given the present-day monetary value.

Diseased Norway spruce are difficult to detect, as the infected tree might not necessarily show any visible signs of symptoms (Allikmäe et al. 2017). Clearcutting is vastly creating new propagation sites for *H. parviporum*. The primary infection route is through basidiospores produced by the basidiocarp. These spores, carried by the wind, land on newly exposed surfaces, such as wounds on roots and stems or recently cut stumps (Garbelotto and Gonthier 2013; Redfern and Stenlid 1998; Swedjemark 1995). The fungus spreads to healthy trees via mycelium growth through the root network (Garbelotto and Gonthier 2013).
As the environment changes, there is an increasing possibility of extreme drought increasing in frequency and magnitude, posing even more significant threats to the forest (Allen et al. 2010, IPCC 2014, Senf and Seidl 2021). For example, in Germany, Norway spruce-dominated stands are particularly sensitive to drought (Bolte et al. 2021; Terhonen et al. 2019), and trees in Germany are declining due to extreme weather conditions, especially in areas with poor water supplies (BMEL 2021). For these reasons, research to test the environmental impact of this pathosystem can provide new strategies to limit the root rot disease. Selecting more adaptive and resistant trees through breeding can give solutions for this. Norway spruce has an inherent resistance to pathogenic attacks, including the *Heterobasidion* species. Several studies have proven that this intrinsic component plays a role in the susceptibility of Norway spruce to this pathogen.

The resistance of Norway spruce against *H. parviporum* is a quantitative trait (Arnerup et al. 2010, Capador-Barreto et al. 2021, Lind et al. 2014). Lind et al. (2014) could map four quantitative trait loci in the *P. abies* genome for four distinct resistance traits against *H. parviporum*. These resistance traits included, e.g., lesion length at the inoculation site. For resistance breeding, inoculations trials in different families are needed to define if specific genotypes are suitable in (able to restrict the necrosis development) breeding programs. These traits are associated with several known defense responses with variation depending on the environment (Capador-Barreto et al. 2021, Elfstrand et al. 2020; Yeoh et al. 2021). Therefore, understanding the family variation in tree response to pathogenic attacks under abiotic stress could aid in this process. Our objectives were to compare the variation in resistance to *H. parviporum* infection among Norway spruce families under abiotic stress, assess the effect of drought on seedling growth and estimate correlations between lesion size and genotypes.

2. Materials and Methods

Plant materials

Plant material included 800 3-year-old Norway spruce (*P. abies*) seedlings received from the Haapastensyrjä field unit (60° 37′ 34.9″ N 24° 27′ 34.9″ E) of the Natural Resources Institute Finland (Luke). Materials consisted of eight families (ID: 38, 40, 41, 42, 43, 47, 48, 50) with 82 genotypes, each genotype having 3–10 ramets per clone. Seventy-three (73) genotypes had ten ramets, four genotypes had nine ramets, three had eight ramets, one had seven ramets, and one genotype had three ramets. The seedlings were established in the Forest Botany and Tree physiology greenhouse in Göttingen, Germany (51° 33′ 28.4″ N 9° 57′ 30.5″ E). Seedlings were planted (March 5th, 2020) in 3-L plastic pots filled with 2.5 L fertilized peat (Floragard, TKS®2 Instant Plus, PERLIGRAN® Extra 2–6 mm, Hermann Meyer KG, Rellingen, Germany). The potted seedlings received tap water to maintain moist soil.

Fungal material

Two heterokaryotic *H. parviporum* strains, received from the strain collection of Natural Resources Institute Finland (collected by Dr. Tuula Piri), were used for the inoculation; *H. parviporum* strain 1 (Hpa 1 – strain number: SB 2005 9.16, isolated from a Norway spruce stump Solböle, Finland) and *H. parviporum* strain 2 (Hpa 2—strain number SB 2014 2.69, isolated from an infected Norway spruce seedling, Solböle, Finland) (Terhonen et

al. 2022). The fungal isolates were plated on 1.5% Malt Extract Agar (MEA) and cultured in the growth chamber (Constant climate chamber Memmert HPP 750) for 2 weeks at 21 °C (in darkness) before inoculation.

Experimental design

The experiment was run from July (July 22nd, 2020) until February (February 3rd, 2021). According to watering treatment, the seedlings were grouped into two (normal and low) groups of 400 plants per category. The plants received watering based on these groups, i.e., the seedlings in the 50% group received half the quantity of water received by the seedlings in the 100% class. The watering was adjusted according to the observed temperatures and soil moisture content (aiming for constantly moist soil for the 100% group). The soil moisture was measured before watering using a tensiometer—HH2 device equipped with the ML2x sensor (Delta-T Devices Ltd., Cambridge, UK). The temperature and humidity were recorded every watering day (Monday and Friday) from August until November with the digital thermometer. The experiment was carried out under ambient light conditions. The average monthly temperatures in the greenhouse were 31.8 °C (July), 25.9 °C (August), 23.2 °C (September), 15 °C (October) and 11.8 °C (November).

The normal water treatment group received 576 mL × 2 times per week, while the low water treatment received 288 mL × 2 times per week. The water amount was later adjusted to 384 mL/192 mL twice a week. The watering amount was further reduced to 192/96 mL (18.08.2020). The drought experiment lasted 16 weeks (22.07.2020–10.11.2020), and the seedlings were watered normally until the end of the experiment (03.02.2021). Due to Covid-19 restrictions, the watering treatment started in July 2020 instead of an anticipated earlier start of treatments.

Inoculation

The bark of the seedlings was punched through with a sterile 5-mm cork borer to reach the sapwood surface; this was done at the height of ~ 10 cm from the base of the stem. The trees were artificially inoculated by placing equal-sized plugs of either *H. parviporum* strains (Hpa 1 and Hpa 2) or 1.5% malt extract agar (MEA) as mock control and sealed with Parafilm® (inoculations done on July 22, 2020) placing the fungal hyphae directly on the sapwood. Two hundred twenty-two (222) plants were inoculated with the first strain of *H. parviporum* and 222 with the second strain. Two hundred twenty-two (222) plants served as the mock control

group inoculated with 1.5% MEA, while 134 plants remained non-treated. The plants in each inoculation group were divided into watering groups based on family numbers resulting in equal amounts in 50% and 100% water availability.

Material collection and measurement

The growth data were measured after planting (diameter, starting height) and end of the watering experiment (final height, current year growth). The diameter was measured at ~ 5 cm from the stem base. Seedlings were harvested 28 weeks after inoculations; the stems were cut from ~ 10 cm above and below the inoculation point and stored at −20 °C before measurements. The necrosis in the phloem was measured by gently scraping off the bark with a scalpel and measuring the surrounding lesion in both vertical and horizontal directions. The stem was then further scraped to assess the extent of damage at the sapwood level. The lesion length and width in phloem and sapwood were measured using a digital caliper.

Statistical analysis

Growth

The soil moisture content between 50 and 100% was compared with t test at each sampling point. Data were analyzed using the SPSS version 28.0 (IBM Corporation, New York, NY, USA) and R (R Core Team 2021). Sixty-five (65) plants died during the experiment; one-way ANOVA (aov) was run to see if the death was due to a specific inoculation treatment. The remaining 735 plants were used for the growth analysis. One hundred twenty-three (123) out of 134 trees were analyzed as non-treated; 60 were grouped to normal water treatment and 63 to low water treatment. We constructed a generalized linear model to evaluate the non-treated seedling growth. The model included the water treatment group and family as initial fixed explanatory variables for the growth. Seven genotypes from these non-treated trees, each consisting of seven to ten ramets, were divided equally between different water treatments (each genotype ramets in low and normal water treatment). The growth of these 56 plants between two water treatments was further analyzed with a t test. Similarly, a generalized linear model was constructed to evaluate all seedling growth; this model included all the seedlings used in the experiment (*H. parviporum* strains 1 and 2, mock-inoculated and non-treated). This model had water treatment, inoculation treatment and family as initial fixed explanatory variables for the growth.

For the growth of plants, data distribution was assessed by means of the Shapiro–Wilk test in R (Royston 1982). Due to a deviation from normally distributed data, Levene's test was used to test for homogeneity of variances. The Kruskal–Wallis test was done for homoscedastic data, and for heteroscedastic data, a Welch's ANOVA was used. Lastly, when a dependent variable differed between treatments, post hoc analyses were performed using Dunn–Bonferroni for the analysis carried out with the Kruskal–Wallis's test and Games–Howell multiple comparison test for the analysis carried out with Welch's ANOVA test. Spearman's correlation analysis was used to determine correlations between seedling growth ($height_{start}$) and family. The impact and correlations were considered statistically significant if the p value was below the threshold of 0.01. The smaller p value was chosen to gain stronger evidence that a certain effect (family, inoculation, water availability, $height_{start}$) was significant.

Necrosis

For the necrosis analysis, the non-treated plants (123) were excluded. A generalized linear model was also constructed to evaluate the fixed effects of the different treatments (mock-inoculated-control, *H. parviporum* strains 1 and 2) under different water treatments (high, low) on necrosis (length/width) in phloem and sapwood. Initial fixed explanatory variables in the necrosis length model included family, inoculation, water treatment, seedling growth and seedling start height.

The size of necrosis caused by *H. parviporum* strains (length/width) in phloem and sapwood per family (within genotype) was further evaluated. The data distribution was similar to the above assessed by employing the Shapiro–Wilk test in R. Due to a deviation from normally distributed data, Levene's test was used to test for homogeneity of variances. The Kruskal–Wallis's test was done for homoscedastic data, and for heteroscedastic data, a Welch's ANOVA was used. Lastly, when a dependent variable differed between treatments, post hoc analyses were performed using Dunn–Bonferroni for the analysis carried out with the Kruskal–Wallis's test and Games–Howell multiple comparison test for the analysis carried out with Welch's ANOVA test. The Wald Chi-square test was used to assess the interactions between variables. Spearman's correlation analysis was used to determine correlations of seedling growth ($height_{start}$) with the sizes of necrotic lesions (phloem/sapwood and length/width) caused by *Heterobasidion* strains. In this study, correlations were considered statistically significant if the p value was below the threshold of 0.01. Same as above, the smaller p value

was chosen to gain stronger evidence that certain effect (family, genotype, inoculation, water availability, $height_{start}$) has importance on necrosis size.

3. Results

Growth analysis

The soil moisture was statistically different ($p = 2.79e{-}06$) from the fifth week (Fig S1 supplementary data). The death of trees was not affected by the different treatments ($p = 0.948$). The death of seedlings was assumed to be random (the death of each seedling was observed during the experiment, not at the end of the experiment), and they were not used in data analysis.

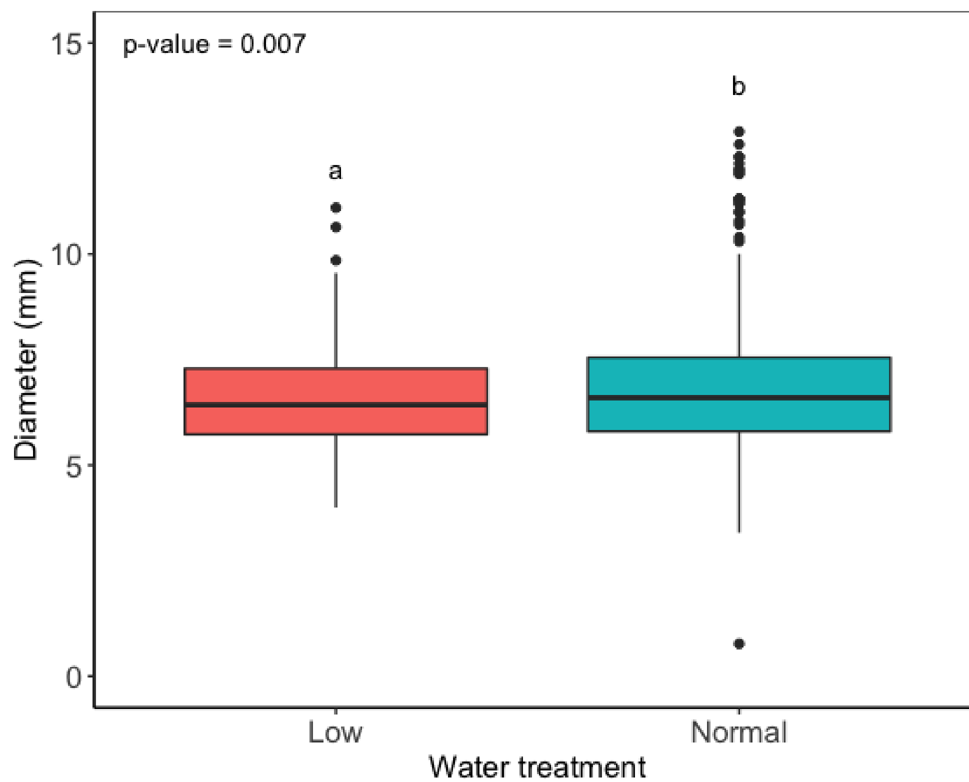

Fig. 1 Effects of water treatment on plant diametric growth. Different letters above plots denote significantly different groups after post hoc test

The height growth between non-treated ramets (56 plants) was not statistically different ($p = 0.875$) between the water treatments. Furthermore, the growth model for all non-treated plants (123 trees) showed that the water treatment did not impact the height ($p = 0.665$). Similarly, for all trees in the experiment, the growth model showed that the water treatment did not impact height growth ($p = 0.974$). However, a significant effect of water treatment was observed in the diametric growth ($p = 0.007$) (Fig. 1). Further differences observed were due to the family ($p < 0.001$). The different treatments (*H. parviporum* 1 or 2, mock control or non-treated) did not impact the growth ($p = 0.526$). The diameter, family and genotypes were the fixed explanatory for all trees for the differences between growth ($p < 0.001$). Welch's analysis of variance showed a significant difference in height growth among the different families (Fig. 2) and genotypes (Fig S2 supplementary data). Games–Howell test revealed that plants of family 48 grew significantly taller than families 42 ($p < 0.001$), 47 ($p < 0.001$) and 50 ($p < 0.001$). Family 42 grew statistically less compared to families 38 ($p = 0.009$), 40 ($p = 6.78e{-}07$) and family 48 ($p = 6.5e{-}10$). Similarly, the variation in the diameter of plants was statistically significant in relation to the families ($p = 5.01e{-}13$, Fig S3 supplementary data). Family 43 had the lowest diameter statistically compared to families 38, 40, 41, 42 and 47.

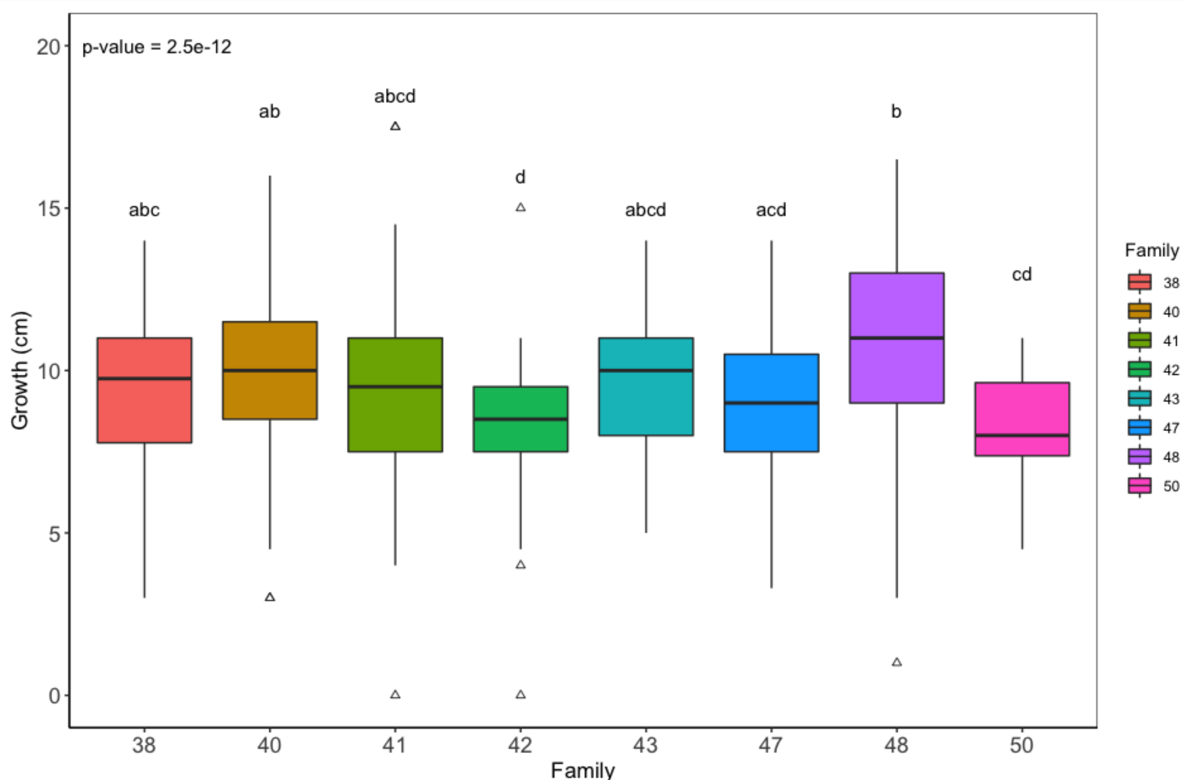

Fig. 2 Effects of Norway spruce family on the height growth (median height growth for each family). Different letters above plots denote significantly different groups after post hoc test

Necrosis analysis

The necroses in phloem and sapwood (length/width) were statistically different ($p < 0.001$) due to the family and different treatments (Table 1, Fig. 3). The lesion width was also influenced by starting height (Table 1). Necrotic lesions (all treatments together) in the phloem were significantly wider ($p < 0.01$) in the horizontal direction for the low water treated plants when compared to the optimally watered plants (Fig. 4). The analysis of the impact of water treatment on lesion size shows no significant differences in lesion length (Table 1).

Table 1 A generalized linear model for response variables. Models included interactions between the dependent and response variables

	Variable	Fixed effect	*p*-value
Sapwood	Necrosis, length	Family	>0.01
		Water	0.053
		Inoculation	<0.01
		Starting height	0.169
		$Growth_{height}$	0.958
	Necrosis, width	Family	<0.01
		Water	0.098
		Inoculation	<0.01
		Starting height	<0.01
		$Growth_{height}$	0.015
Phloem	Necrosis, length	Family	<0.01
		Water	0.169
		Inoculation	<0.01
		Starting height	0.366
		$Growth_{height}$	0.29
	Necrosis, width	Family	<0.01
		Water	<0.01
		Inoculation	<0.01
		Starting height	<0.01
		$Growth_{height}$	0.419

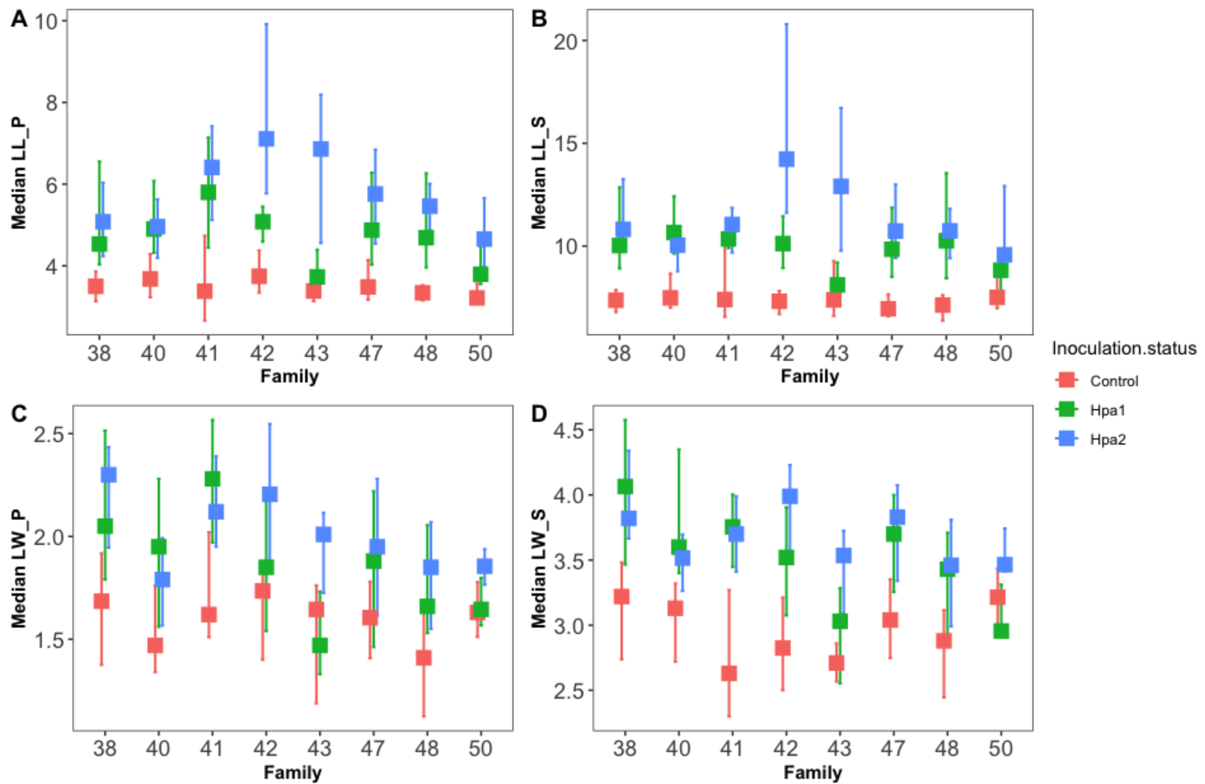

Fig. 3 Interaction between families and different inoculation treatments (median values) **a)** *LL_P* lesion length in the phloem, **b)** *LL_S* lesion length in sapwood, **c)** *LW_P* lesion width in phloem **d)** *LW_S* lesion width in sapwood

The lesion length and width in the sapwood are larger than those in the phloem, and *H. parviporum* strains caused more necrosis than in the mock control. The lesion lengths in the phloem and sapwood were significantly different between the strains (Fig. 5a, b).

The strain Hpa 2 caused statistically higher necrosis (lesion length) in both phloem and sapwood than the other strain (Hpa 1) ($p = 0.002$). The only significant difference in the lesion width for phloem and sapwood was between the *Heterobasidion* inoculated and the mock control plants (Fig. 5). The lesion width is not statistically different between *H. parviporum* strains (Fig. 5c, d). There were significant differences in lesion length and width in the phloem among genotypes (Supplementary data; Table 2). In the sapwood, there were significant differences in the lesion length ($p = 0.001$) and width ($p = 1.9\text{e}{-}04$) among genotypes (Supplementary data; Fig S4).

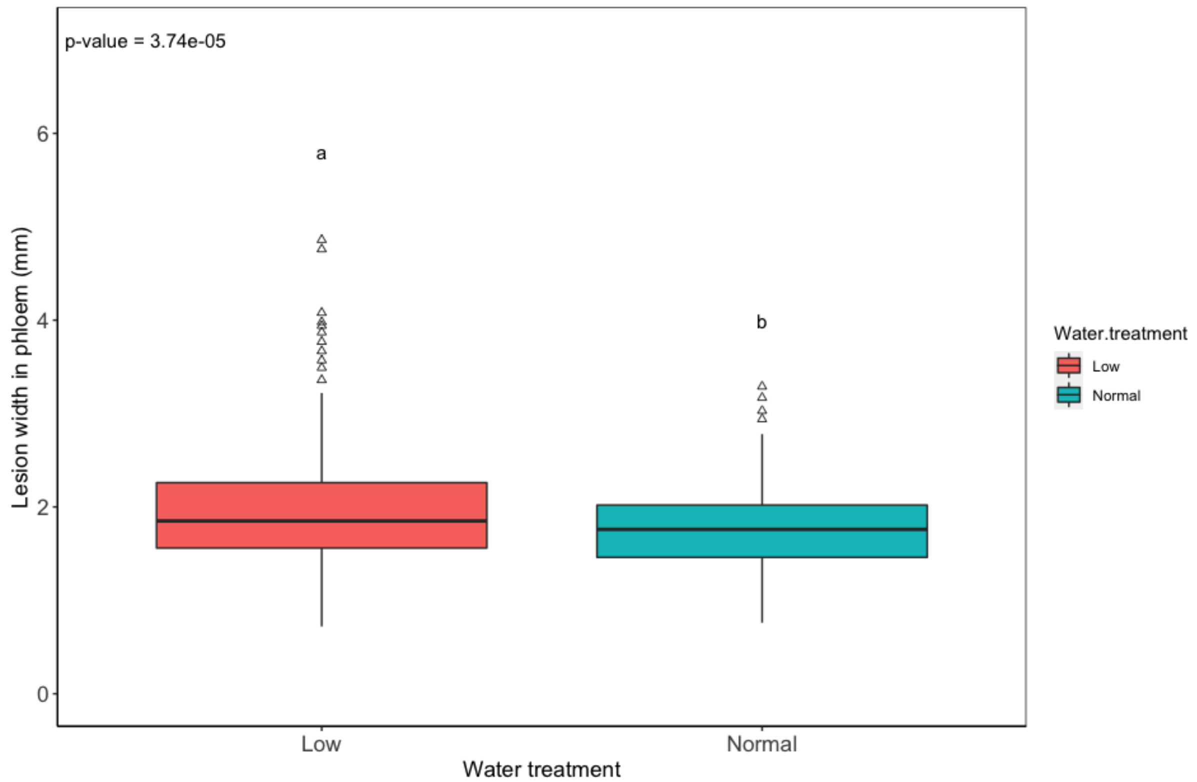

Fig. 4 Impact of water treatment on lesion width in the phloem. Different letters above plots denote significantly different groups after post hoc test.

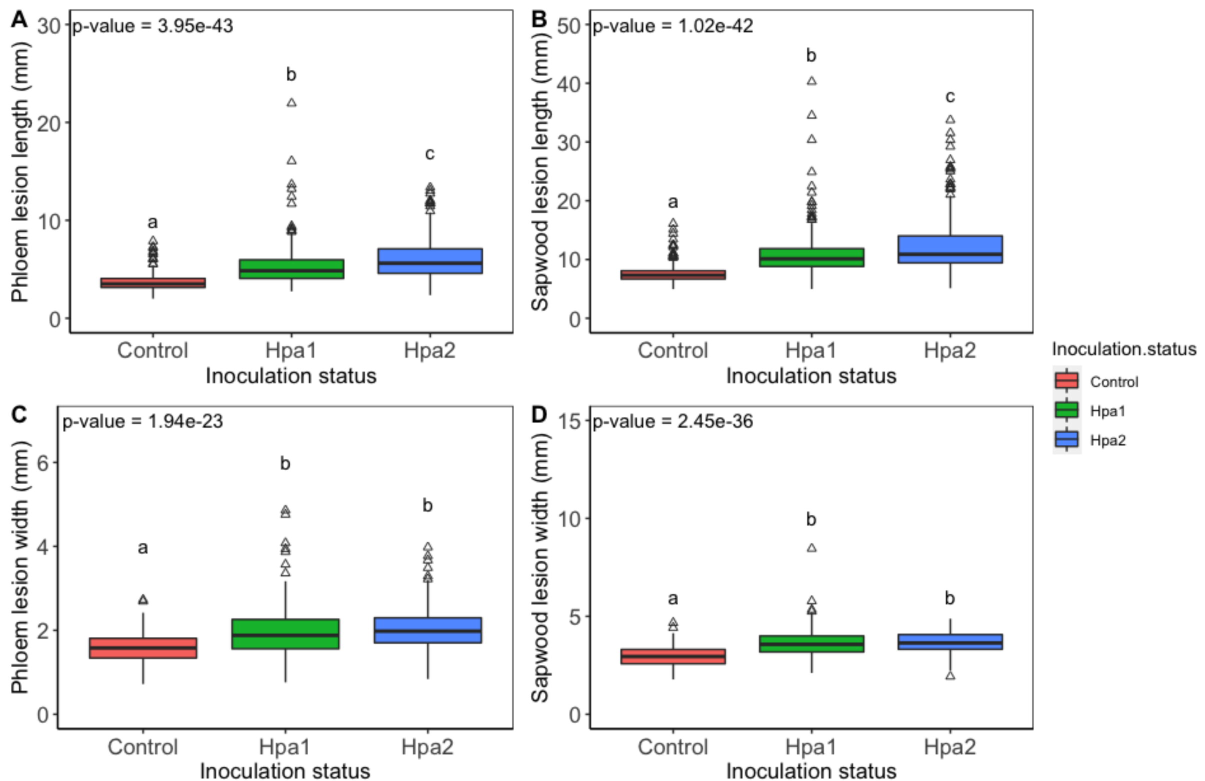

Fig. 5 Inoculation effect on lesion size. **a** Lesion length in the phloem, **b** lesion length in sapwood, **c** lesion width in the phloem, **d** lesion width in sapwood

Correlation analysis

There is a strong positive correlation between the lesion sizes in both phloem and sapwood ($p < 0.01$) (Fig. 6a, b). The higher the lesion in the phloem, the higher it is in the sapwood. There were no significant correlations between lesion length and starting height, but there are significant positive correlations between the lesion width and starting height (Fig. 6c, d). Correlations between diameter and lesion width in sapwood also resulted in a significant moderate positive correlation (R; 0.35, $p < 001$) (Fig. 7). A negative correlation was revealed between growth (height) and lesion length in the phloem ($R = -0.096$, $p = 0.016$) and the sapwood ($R = -0.048$, $p = 0.23$) (Fig S5 supplementary data).

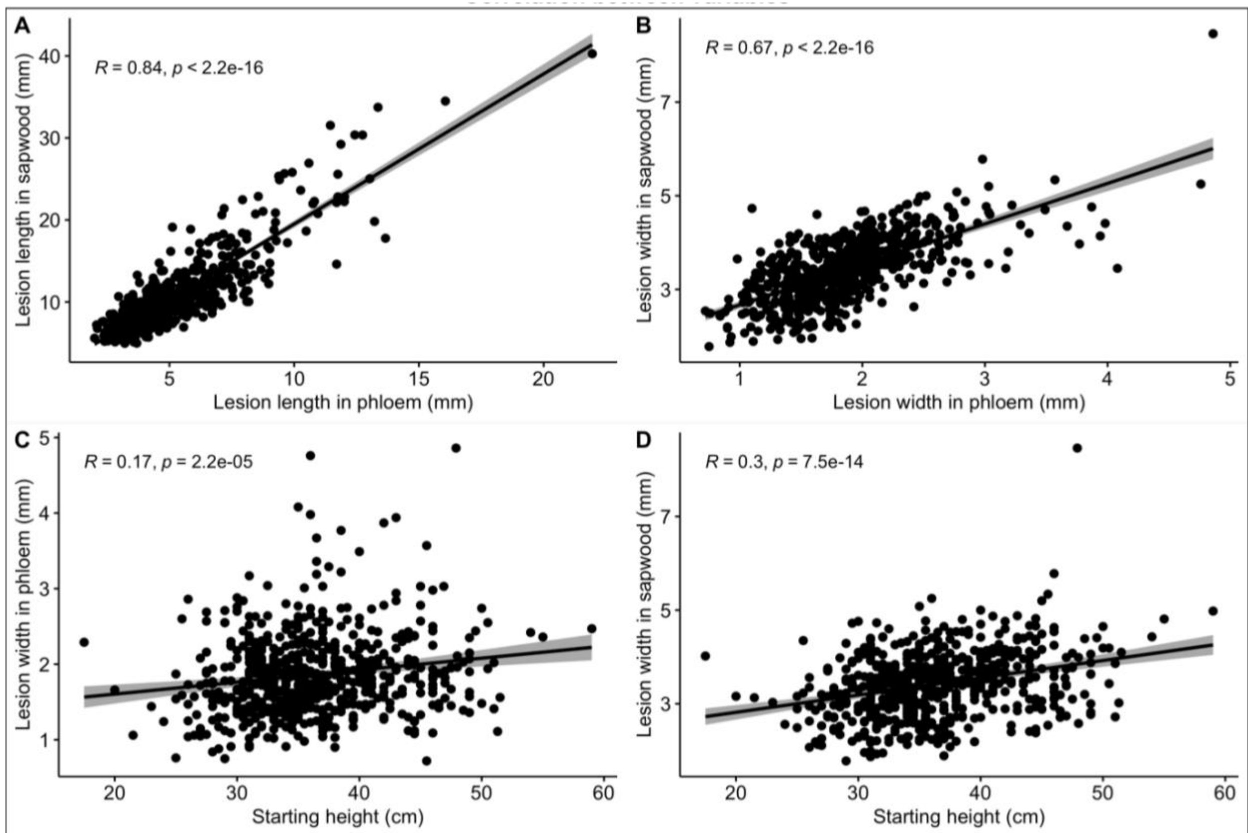

Fig. 6 Correlation analysis between **a** lesion length in phloem and sapwood, **b** lesion width in phloem and sapwood, **c** lesion width in phloem and starting height, **d** lesion width in sapwood and starting height

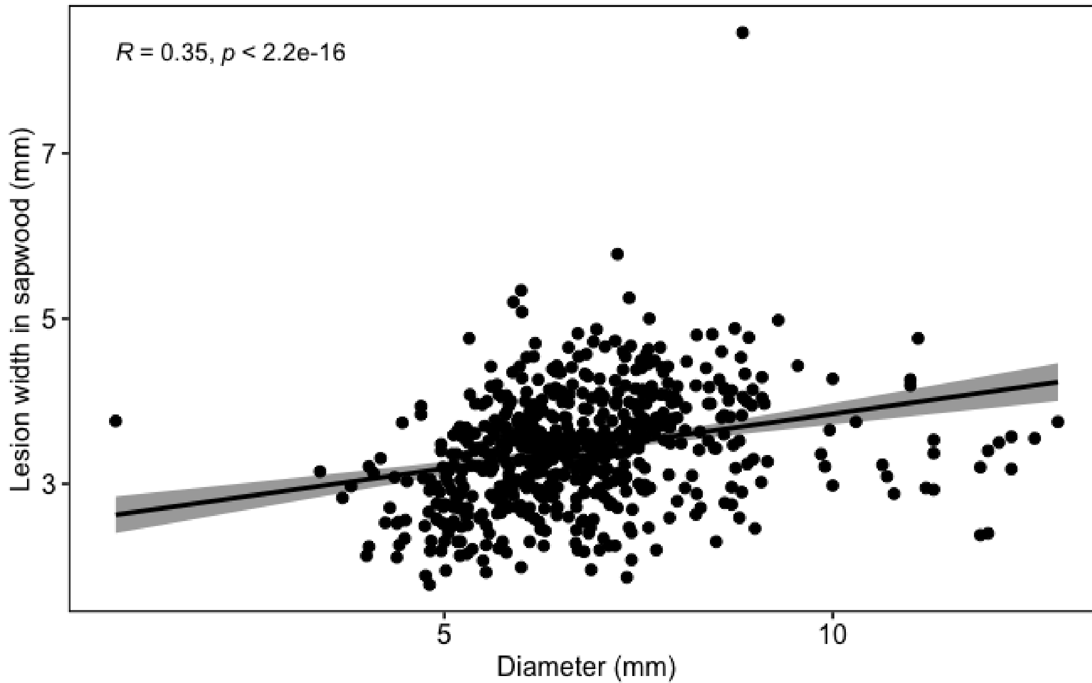

Fig. 7 Correlation analysis between stem diameter and lesion width in the sapwood

4. Discussion

Growth

In this study, the height growth of Norway spruce seedlings was not affected by water availability; rather, the differences were due to the stem diameter, families and genotypes. Water stress impedes plant growth (Hsiao 1973; Bigler et al. 2006, Rötzer 2017), and understanding the response of trees to drought is important for predicting how they will react to a changing climate (McDowell 2011). Taeger et al. (2013) investigated the effects of drought on two different populations of *Pinus sylvestris* seedlings (Germany and Spain) in a greenhouse experiment. Overall, drought led to reductions in growth, while differences were observed between populations (Taeger et al. 2013). Similarly, higher drought reduced the growth of *Pinus halepensis* pine in the semi-arid forest (Klein et al. 2014). Limited water availability can negatively influence Norway spruce seedling growth (Linnakoski et al. 2017; Terhonen et al. 2019). In contrast to our expectations, the height growth of the seedlings was not significantly impacted by the different treatments (water treatment nor inoculation) but by the genetic variation.

Terhonen et al. (2019) could show that 50% of water availability was enough to disturb the growth of 3-year-old Norway spruce seedlings in a 15-week interval. However, in our setting (due to COVID-19 restrictions), we started the water treatment rather late (in late July) compared to Terhonen et al. (2019) (early April). The abiotic stress was not critical anymore for the Norway spruce growth after this time (even though the terminal growth period was still ongoing). The height growth most likely had ended when the watering experiment started and cannot be considered a marker for stress level in this study. Although the height growth was not impacted by low water availability, the diametric growth was. Host–pathogen interaction, however, may have been affected by the drought treatment as lesion width in phloem was larger in low-watered plants compared to optimally watered plants. As a result, plants that received optimum watering grew better (larger in diameter) than those that received low watering.

Similarly, Rötzer et al. (2017) found that drought significantly decreases the diameter increment of Norway spruce. Drought stress has also been reported to negatively impact the tree ring width of some Norway spruce clones as a result of a reduction in the cambial activity (Gryc et al. 2012). Due to limited water availability, cells necessary for the formation of a new ring are formed in fewer quantities in comparison with other clones that were not subjected to water stress (Gryc et al. 2012).

However, it was clear that the growth also varied between families and genotypes. This is in line with several studies showing differences in the growth of Norway spruce clones/genotypes (Hannerz et al. 1999; Jansone et al. 2020). Liu et al. (2022) could also show significant growth differences among 3-year-old Norway spruce clones inoculated with *H. parviporum*.

Similarly, in a study carried out on Norway spruce plantation, clonal and genotypic differences were evident in the growth rate of the Norway spruce (Zeltiņš et al. 2022). Additionally, Chen et al. (2018) found that Norway spruce genotypes in a Swedish breeding program differed widely in growth traits. Besides the variation in height (growth), the genotypes also differed in diameter. This is in line with the study by Zeltinš et al. (2022), where the diameter growth differed significantly among the genotypes.

Necrosis

Here, we show resistance (necrosis length) phenotyping results based on 3-year-old *Picea abies* families originating from controlled crosses between plus trees of Norway spruce, Finland. Necrosis length was significantly different among Norway spruce genotypes. This is consistent with results showing variability in Norway spruce reaction to *H. parviporum* between genotypes (Swedjemark 1995; Skrøppa et al. 2015). Although there

was no impact of water treatment on plant height, there was a significant difference in lesion width in the drought-treated plants compared to the optimally watered plants. The lesion width has been shown to increase under drought in Norway spruce after inoculation with *H. annosum* s.s. (Terhonen et al. 2019). At the same time, inoculation with *H. parviporum* increased lesion length in drought-treated plants compared to normally watered plants (Terhonen et al. 2019). Madmony et al. (2018) inoculated 2-year-old branches of different Norway spruce clonal ramets (4-year-old) with *H. parviporum* under well-watered and drought environments, which led to increased pathogen growth in well-watered seedlings. In our experiment, the water treatment did not have an impact on height growth and necrosis; the effect of the water treatment was only shown in the diametric growth and lesion width in the phloem. Controversial results have been found as necrosis length has been shown to increase due to lower water availability (Terhonen et al. 2019). Reasons for this might be the different experimental settings, as both genotype and environment can impact the defense (Potts and Hunter 2021). It is evident that the resistance (necrosis width/length) varies between families and genotypes.

In our study, the death of trees was considered random. Pathogenic fungi induce defensive responses in trees, which aim to restrict the growth of the pathogen and foster recovery (Berryman 1972). This defense uses a lot of the host resources. Therefore, plant growth can be negatively impacted by allocating resources to defense instead (Walters and Heil 2007). Several studies have reported negative (Mukrimin et al. 2018; Liu et al. 2022), positive (Karlsson et al. 2008; Chen et al. 2018; Mukrimin et al. 2019) and negligible correlations (Lamara et al. 2018; Camisón et al. 2019) between defense and growth in different tree species. The results of our study did not support this hypothesis between growth and resistance (lesion length) in phloem and sapwood of Norway spruce because there was no correlation, although the values are very close to a weak negative correlation. Seedlings' growth was not affected by lesion length in phloem and sapwood.

Similarly, Steffenrem et al. (2016) did not find a genetic correlation between growth and resistance. However, a significant positive correlation was found between lesion width and seedlings' starting height. The same observations have been made before (Karlsson et al. 2008; Terhonen et al. 2019). This indicates (the higher starting height equals higher lesion width) that seedlings with higher values of growth parameters (in this case, taller seedlings) are not unquestionably less sensitive to *Heterobasidion* inoculation.

Studies have focused on the defense response of *Picea abies* to different pathogens (Skrøppa et al. 2015; Terhonen et al. 2019; Axelsson et al. 2020). They have shown differences in the defense response of Norway spruce clones/genotypes to fungal infection. Still, little focus has been placed on the degree of virulence between different strains of the same pathogen. Axelsson et al. (2020) showed variabilities in defense response among Norway spruce clones after inoculation with *Endoconidiophora polonica* and *Heterobasidion parviporum*. Inoculation with *H. parviporum* strongly induced terpenes in all clones as a response to defense from pathogenic attack, but the quantity of terpenes induced varied among clones. One of the important results of our study is that the virulence (lesion length) varied between the fungal strains of *Heterobasidion* used. *H. parviporum* strain 2 showed to be more pathogenic (caused longer necrosis) than the first strain. This could be due to the isolation source (stump versus seedling), as there may be a trade-off between saprobic and pathogenic competence (Olson et al. 2012). Similarly, Linnakoski et al. (2017) showed that the virulence of *Endoconidiophora polonica* varied among the different strains. The differences observed in the virulence (necrosis length) among pathogenic strains in this and previous studies highlight the importance of using more than one strain in inoculation studies.

Tree breeding has been shown to increase the growth performance of forest trees (Jansson et al. 2017), particularly Norway spruce, by 8–20%. From our study, considering the reaction of plant hosts to different strains is essential for selecting resistant genotypes to be used in reforestation and breeding, as there could be variations in resistance to various strains.
Climate change is expected to cause warmer temperatures and drought (IPCC 2014), which will have unpredicted effects on pathogen behavior, hosts' vitality and their ability to fight infection. As a result, there are several risks facing future generations of Norway spruce forests. Further research is needed with stricter water stress taking different genotypes and fungal strains into account to assess the physiological and genetic factors influencing host resistance and pathogen virulence in the face of climate change. Results from this study contribute to the body of research on host resistance, and the plant–pathogen interactions can serve as a foundation for further research.

Conclusion

This study highlights that the genotypes of Norway spruce have an impact on the growth and response to artificial infections for different *H. parviporum* strains. Therefore, further research is suggested to assess Norway spruce susceptibility against several pathogenic strains. The transcriptomics studies for these genotypes could reveal traits that control lesion length at the inoculation site. These could be used to find new molecular markers for resistance breeding and will be the focus of our future studies.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10342-023-01534-3.

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Author contributions Maintenance of the greenhouse experiment, necrosis measurement and analysis of the results were performed by BD. BD and ET designed the experimental and watering protocol. ET conceived and supervised the experiment and analyzed parts of the data. KB helped with the greenhouse work and co-supervised the experiments. BD wrote the first draft of the manuscript, and all authors commented on subsequent versions. All authors read and approved the final manuscript.

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Declarations

Conflicts of interest The authors declare no conflict of interest.

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6. Supplementary materials

Supplementary figures

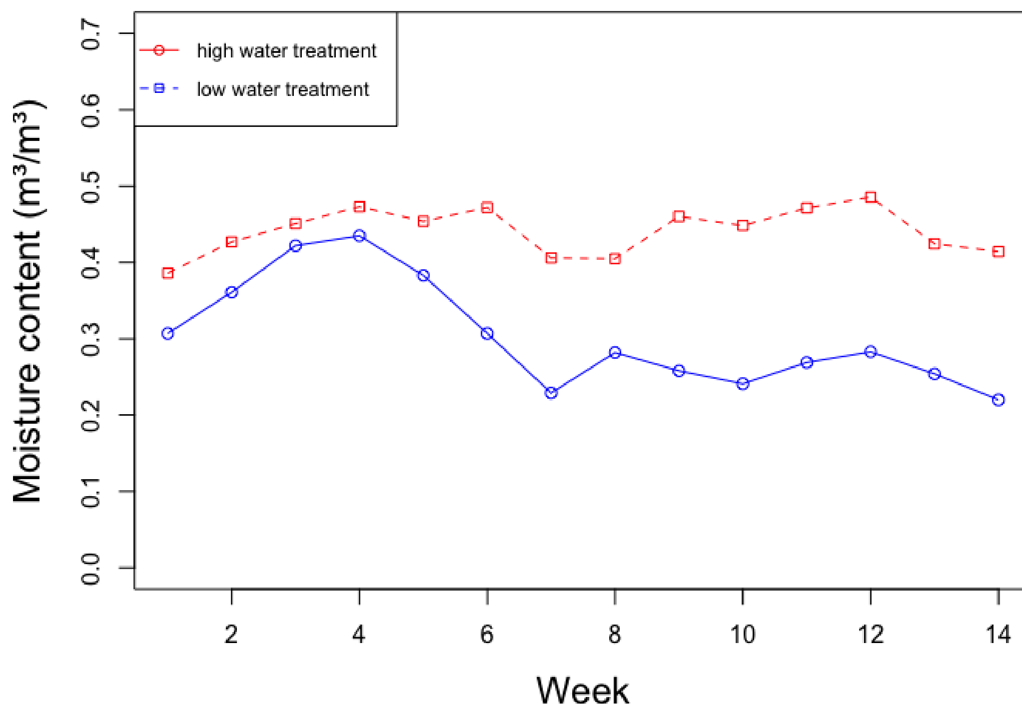

Fig S1 Soil moisture content between low and well-watered plants (significance level; p = 2.79e-06**).** There are fourteen weeks because there were no measurements taken for two weeks in the middle of the experiment.

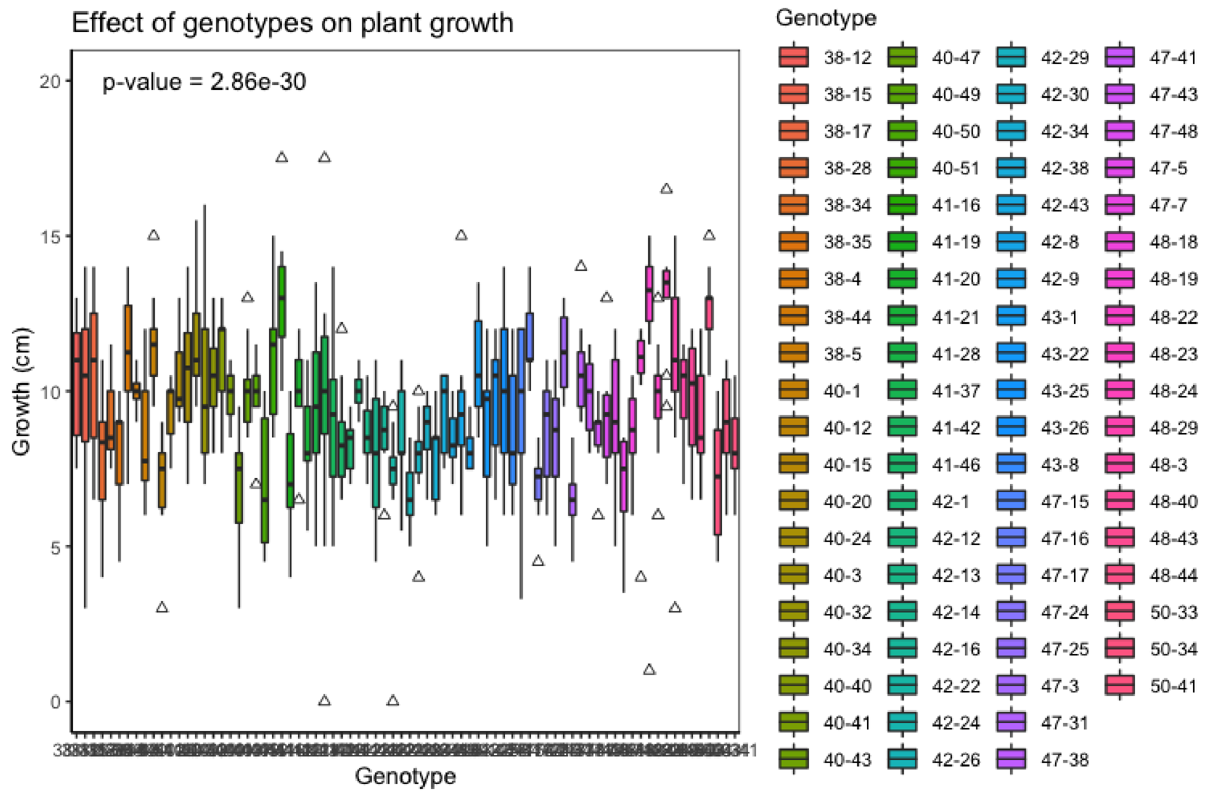

Fig S2 Effects of Norway spruce genotypes on plant growth

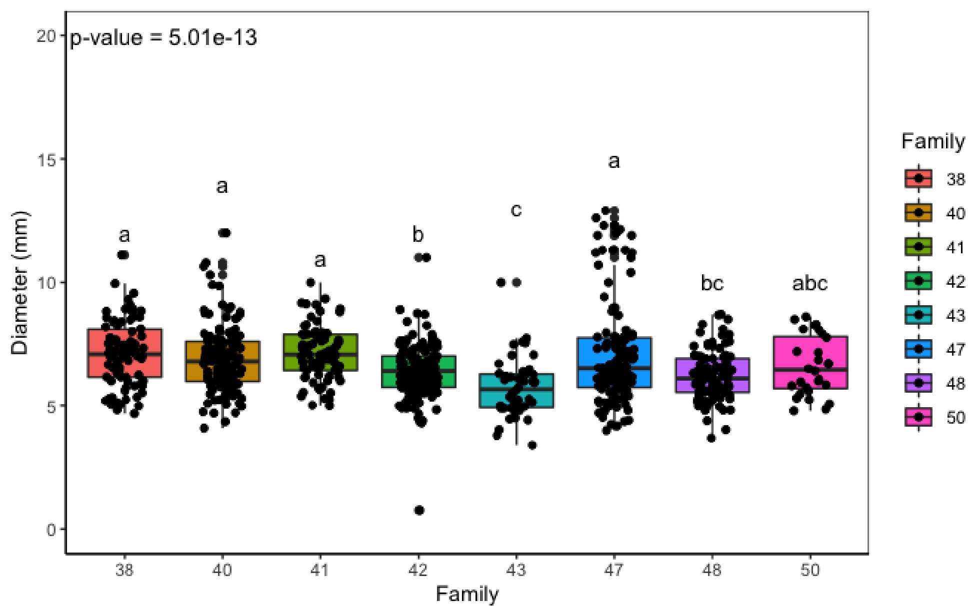

Fig S3 Diameter distribution among families (median diameter for each family. Different letters above plots denote significantly different groups after posthoc test)

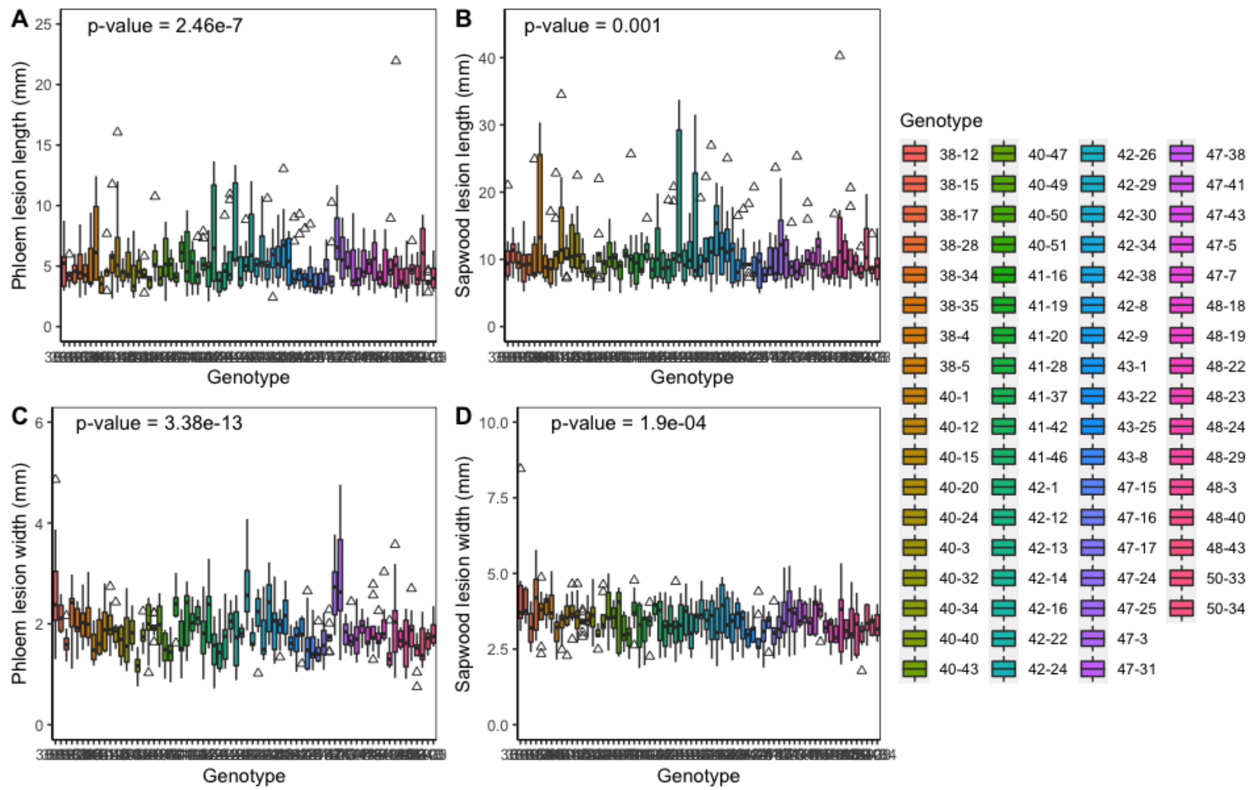

Fig S4 Genotype effect on lesion size. **a)** lesion length in phloem **b)** lesion length in sapwood **c)** lesion width in phloem **d)** lesion width in sapwood

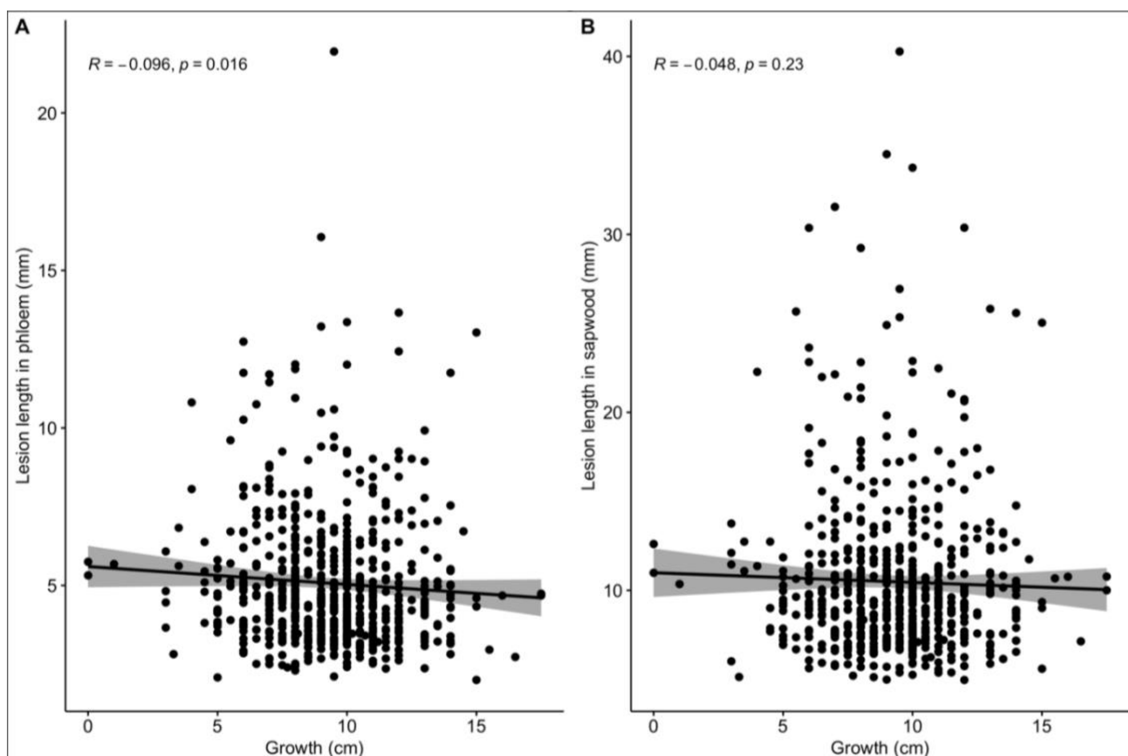

Fig S5 Correlation analysis between growth and lesion length in **a)** phloem, **b)** sapwood

Supplementary tables

Table 1 Significant differences in lesion in phloem and sapwood among families. LL_P denotes lesion length in Phloem, LW_P denotes lesion width in the phloem, LW_S denotes lesion width in sapwood

.y.	group1	group2	n1	n2	statistic	p	p.adj	p.adj.signif
LL_P	42	50	117	18	-3.41117522645604	0.000646835082042906	0.0181113822972014	*
LL_P	42	43	117	36	-3.13058744425898	0.00174457052102484	0.0488479745886954	*
LW_P	41	48	62	79	-4.64871939945031	3.34002222311134E-06	9.35206222471175E-05	****
LW_P	38	48	71	79	-4.2810701998121	1.85996668309342E-05	0.000520790671266158	***
LW_P	40	41	122	62	3.98438112342891	6.7656214845916E-05	0.00189437401568565	**
LW_P	38	40	71	122	-3.5692981213	0.000357938921086483	0.0100222897904215	*
LW_P	41	43	62	36	-3.53088967739828	0.000414164453295332	0.0115966046922693	*
LW_P	42	48	117	79	-3.4114812541816	0.000646109438076217	0.0180910642661341	*
LW_P	38	43	71	36	-3.1828205049313	0.00145847998306918	0.0408374395259371	*
LW_S	38	43	71	36	-4.78451891157609	1.71397360125504E-06	4.79912608351412E-05	****
LW_S	38	48	71	79	-4.59826885197763	4.26016018909622E-06	0.000119284485294694	***
LW_S	43	47	36	107	3.27269117149149	0.00106528804654984	0.0298280653033955	*

Table 2 Significant differences in lesion in phloem and sapwood among genotypes. LL_P denotes lesion length in Phloem, LL_S denotes lesion length in sapwood, and LW_P denotes lesion width in the phloem

.y.	group1	group2	estimate	conf.low	conf.high	p.adj	p.adj.signif
LL_P	40-40	41-16	2.38333333333333	0.40441813167361	4.36224853499306	0.013	*
LL_P	41-16	47-15	-2.51444444444444	-4.76795261271894	-0.260936276169949	0.019	*
LL_P	41-16	47-16	-2.49541666666667	-4.75194785384373	-0.238885479489606	0.021	*
LL_P	41-16	50-33	-2.38555555555556	-4.40143557178511	-0.369675539326002	0.013	*
LL_P	38-5	41-16	2.54444444444444	0.445984322771353	4.64290456611754	0.009	**
LL_S	40-34	41-16	2.55222222222222	0.197250041100992	4.90719440334345	0.025	*
LW_P	38-15	48-22	-0.75	-1.49533759243925	-0.00466240756075009	0.048	*
LW_P	38-34	40-34	-0.808888888888889	-1.55428420526788	-0.0634935725098975	0.026	*
LW_P	38-35	40-34	-0.845555555555555	-1.65744196114143	-0.0336691499696823	0.037	*
LW_P	40-20	40-34	-0.675555555555556	-1.28275735576863	-0.068353755342481	0.02	*
LW_P	40-34	40-40	0.603333333333333	0.0206863388794454	1.18598032778722	0.037	*
LW_P	40-34	42-8	0.737777777777778	0.0537784006720795	1.42177715488348	0.027	*
LW_P	40-34	47-25	1.49111111111111	0.184120773319692	2.79810144890253	0.02	*
LW_P	40-34	47-31	0.634444444444445	0.0352644615719299	1.23362442731696	0.031	*
LW_P	40-34	48-24	0.511222222222222	0.00504410213066753	1.01740034231378	0.046	*
LW_P	40-34	50-34	0.666666666666667	0.0527267863195551	1.28060654701378	0.025	*
LW_P	40-50	41-16	0.858888888888889	0.00264135849415015	1.71513641928363	0.049	*
LW_P	40-51	41-16	0.971111111111111	0.10086609268272	1.8413561295395	0.019	*
LW_P	40-51	41-37	0.764444444444444	0.0415034049420123	1.48738548394688	0.032	*
LW_P	41-16	47-16	-0.881944444444444	-1.71062003163479	-0.0532688572541017	0.03	*
LW_P	41-16	48-22	-0.954444444444444	-1.79029306518129	-0.118595823707595	0.016	*
LW_P	41-16	48-40	-0.947777777777778	-1.79482685840363	-0.100728697151925	0.019	*
LW_P	41-37	43-1	-0.522222222222222	-1.03292241613538	-0.0115220283090601	0.042	*
LW_P	41-37	47-16	-0.675277777777778	-1.33031688267533	-0.0202386728802275	0.04	*
LW_P	41-37	48-22	-0.747777777777778	-1.41373498273514	-0.0818205728204136	0.019	*
LW_P	41-37	48-40	-0.741111111111111	-1.42625113421544	-0.055971088006785	0.026	*
LW_P	40-34	41-28	0.86	0.252312089847911	1.46768791015209	0.002	**
LW_P	40-34	42-22	0.624444444444445	0.140777545783701	1.10811134310519	0.006	**
LW_P	40-34	47-43	0.743333333333333	0.141142582999115	1.34552408366755	0.007	**
LW_P	38-15	40-34	-0.974444444444444	-1.64308088866347	-0.305808000225418	0.001	***
LW_P	40-34	40-47	0.76	0.248895989718388	1.27110401028161	0.001	***
LW_P	40-34	41-16	1.17888888888889	0.397777487078828	1.96000029069895	0.001	***
LW_P	40-34	41-37	0.972222222222222	0.415218434666576	1.52922600977787	0.000127	***
LW_P	40-34	42-14	0.865555555555556	0.271742446560321	1.45936866455079	0.001	***

Chapter III – Beyond the surface: exploring the mycobiome of Norway spruce under drought stress and with *Heterobasidion parviporum*

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Beyond the surface: exploring the mycobiome of Norway spruce under drought stress and with *Heterobasidion parviporum*

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Abstract:

The mycobiome, comprising fungi inhabiting plants, potentially plays a crucial role in tree health and survival amidst environmental stressors like climate change and pathogenic fungi. Understanding the intricate relationships between trees and their microbial communities is essential for developing effective strategies to bolster the resilience and well-being of forest ecosystems as we adopt more sustainable forest management practices. The mycobiome can be considered an integral aspect of a tree's biology, closely linked to its genotype. To explore the influence of host genetics and environmental factors on fungal composition, we examined the mycobiome associated with phloem and roots of Norway spruce (*Picea abies* (L.) Karst.) cuttings under varying watering conditions. To test the "mycobiome-associated-fitness" hypothesis, we compared seedlings artificially inoculated with *Heterobasidion parviporum* and control plants to evaluate mycobiome interaction on necrosis development. We aimed to 1) identify specific mycobiome species for the Norway spruce genotypes/families within the phloem and root tissues and their interactions with *H. parviporum* and 2) assess stability in the mycobiome species composition under abiotic disturbances (reduced water availability). The mycobiome was analyzed by sequencing the ribosomal ITS2 region. Our results revealed significant variations in the diversity and prevalence of the phloem mycobiome among different Norway spruce genotypes, highlighting the considerable impact of genetic variation on the composition and diversity of the phloem mycobiome. Additionally, specific mycobiome genera in the phloem showed variations in response to water availability, indicating the influence of environmental conditions on the relative proportion of certain fungal genera in Norway spruce trees. In the root mycobiome, key fungi such as *Phialocephala fortinii* and *Paraphaeosphaeria neglecta* were identified as conferring inhibitory effects against *H. parviporum* growth in Norway spruce genotypes. Furthermore, certain endophytes demonstrated greater stability in root ecosystems under low water conditions than ectomycorrhizal fungi. This knowledge can contribute to developing sustainable forest management practices that enhance the well-being of trees and their ecosystems, ultimately bolstering forest resilience.

Keywords Picea abies, Plant-host relationship, Drought stress, Genotypic variation, Microbes, Fungal community, Environmental conditions

1. Introduction

The hidden mycobiome of ecosystems may represent one of the key solutions for increasing resilience in forest trees during climate change. In the field of evolutionary ecology, the "insurance hypothesis" proposes that a wide variety of species maintains the cohesion of an ecosystem while there are alterations in both biotic and abiotic environmental conditions [1–5]. Similarly, competitive exclusion ensures that beneficial fungi have the potential to overcome pathogens in the same habitat [6, 7]. In the plant roots, dark septate endophytes (DSE) can improve water balance and increase resistance to drought [8], and foliar endophytes have been shown to protect their host against fungal pathogens [9] and pests [10–12]. In that sense, diverse mycobiomes can act as part of tree resistance, enabling trees to respond to new stress [13]. We hypothesize that the hidden mycobiome biodiversity provides a link between more diverse mycobiomes and certain biological processes (function) needed to increase tree resilience (fewer disease symptoms/more adapted to environmental stress). We term this the "mycobiome-associated-fitness" hypothesis. This kind of extended tree resistance can be crucial in the near future as latent pathogens/saprotrophs can switch from symptomatic to a pathogenic lifestyle under abiotic disturbance, for instance, drought [14, 15].

One of the important pathogens of Norway spruce (*Picea abies* (L.) Karst.) is the species complex *H. anno*sum sensu lato, a group of fungi that cause root rot and stem decay [16, 17]. *Heterobasidion parviporum* Niemelä & Korhonen belongs to the *H. annosum* species complex recognized in Europe and is associated with Norway spruce. However, it can also be associated with some other conifers, such as Abies and Pinus species [16]. The primary infection by *Heterobasidion* species takes place when the windborne basidiospores are deposited onto fresh stump surfaces or wounds on the tree stem and roots. *H. parviporum* can continue spreading through root networks and infect healthy neighbouring trees [16]. The continuous threat of root and stem rot to Norway spruce leads to severe economic losses to the forest industry. There is currently no treatment available for infected trees, and the control strategies for this fungus can only be implemented after the harvest, and there are no measures in place for dealing with it in living trees. Further, selecting more tolerant trees through breeding could improve resistance as Norway spruce genotypes vary in their susceptibility to *H. parviporum* [18]. The host plant genotype has been noted to be also one factor defining the fungal endophytic composition [19–22]. In that sense, the mycobiome is an integral part of a tree's overall biology and can be seen as an extension of its genotype. Similarly, a few Norway spruce root fungal endophytes, such

as *Phialocephala sphaeroides*, have been noted to suppress *H. parviporum* growth [23, 24] and *Phytophthora citricola* s.l. [25] under in vitro conditions. It can be hypothesized that choosing specific tree genotypes with preferred mycobiome could lead to prolonged resistance against *H. parviporum* in diseased sites.

Norway spruce is an essential tree species for ecological and economic reasons. The mycobiome associated with Norway spruce includes various groups of fungi, such as mutualistic, saprotrophic, and pathogenic fungi. The composition of these fungal communities can vary depending on factors such as soil properties [26], tree genotypes [21, 27, 28] and environmental conditions [20]. Mycorrhizal fungi form a well-known mutualistic symbiotic relationship with the tree host. Part of trees' mycobiome are also fungal endophytes that live non-symptomatically within plant tissues throughout their whole life cycle or at substantial period of time without inflicting any visible detrimental effects to the host [29, 30]. The extensive range of fungal endophytes found within individual hosts has prompted a surge in research focused on investigating the beneficial roles of fungal
endophytes in forest trees [13].

In Norway spruce roots, *Phialocephala fortinii* – Acephala applanata species complex (PAC), which are dark septate endophytes (DSE), are the most abundant fungal endophytes [23, 31, 32]. DSE have high melanin concentrations and microsclerotia in their hyphae, which could be why they are able to offer protection to the host roots in extreme habitats [8, 33], as they are hydrophobic, drought-resistant, and capable of withstanding repeated freeze-thaw cycles [34]. Previous studies have demonstrated the increased positive effects of endophytes on the growth and protection against pathogens in forest trees [35–37]. One DSE, *Phialocephala sphareoides*, has been shown to enhance the Norway spruce root and seedling growth [24] and disturb the infection capability through roots of the pathogen in Norway spruce [37].

Trees are directly impacted by climatic extremes such as higher temperatures and drought, leading to water scarcity. These extremes indirectly render trees more vulnerable to pathogens [38, 39]. This affects host-microbiome (pathogens included) interactions, resulting in increased occurrence of disease outbreaks caused by previously harmless fungi [40]. The warming climate also allows fungi to emerge in more northern regions, resulting in unexpected new outbreaks [41, 42]. Overall, the distribution and evolvement of forest pests and pathogens are

being changed as a result of climate change. There is particular concern about the effects of a warmer climate and drought on the interactions between plants and pathogens [43, 44]
Besides the increased interest in mycobiomes and their impact on tree health, understanding the relationship between plants and mycobiome remains challenging. It is hypothesized that the phloem endophytic mycobiome is mainly horizontally transmitted [45] and that the root mycobiome may change after seedlings are planted in field [46]. Overall, the fungal community associated with Norway spruce is complex and dynamic. Elucidating the specific fungal species and their interactions that con- tribute to the resistance of Norway spruce can deepen the understanding of the multiple interactions between plants and fungi. Further studies are required to fully understand mycobiome's role in tree health and development. Taken together, the composition of the mycobiome is shaped by factors such as host plant genotype, environmental conditions, and interactions with other microorganisms. By studying how these factors contribute to the mycobiome's makeup, we can better understand the intricate relationships between plants and their microbial communities. This knowledge can then be applied to develop more effective strategies to promote tree health and combat forest diseases.

To test the "mycobiome-associated-fitness" hypothesis, we compared control seedlings and seedlings artificially inoculated with *H. parviporum* for necrosis development and mycobiome composition under well-watered and drought-stressed conditions. Our aims were to determine the phloem and root tissue-specific mycobiomes and core species at Norway spruce genotype/family levels and the changes in mycobiome composition upon *H. parviporum* challenge and drought stress.

2. Materials and methods

Fungal and plant material

Fungal material consisting of two heterokaryotic strains of *H. parviporum* was obtained from the strain collection of Natural Resources Institute Finland. The strains, collected by Dr. Tuula Piri, include Hpa 1 (strain num- ber: SB 2005 9.16), isolated from a Norway spruce stump in Solböle, Finland, and Hpa 2 (strain number: SB 2014 2.69), isolated from a Norway spruce seedling in Solböle, Finland [18]. Prior to the inoculations, the strains were cultured for 2 weeks at 21 °C, in darkness, on 1.5% Malt Extract Agar (MEA) in a growth chamber (Memmert HPP 750 constant climate chamber).

Three-year-old Norway spruce rooted cuttings used in this study originated from the Haapastensyrjä field unit (60°37′34.9“ N 24°27’34.9” E) of the Natural Resources Institute Finland (Luke). The rooted cuttings were initially grown outside in the field of Haapastensyrjä field unit before being collected and sent to Göttingen, Ger- many. On March 5, 2020, the plants were planted in 3-l plastic pots filled with 2.5 L of fertilized peat (Floragard, TKS®2 Instant Plus, PERLIGRAN® Extra 2–6 mm, Hermann Meyer KG, Rellingen, Germany) and placed in the greenhouse facilities at the University of Göttingen, Germany (51°33′28.4“ N 9°57’30.5”E). Plant material consisted of seven genotypes from four half-sib families: genotypes 38–3 and 38–8 (from family ID 38); genotype 40–41 (from family ID 40); genotypes 41–36 and 41–44 (from family ID 41); and genotypes 43–12 and 43–15 (from family ID 43) (Table 1).

Half of the genotypes (38–8, 41–44 and 43–15) were optimally watered for 16 weeks, while the other half (genotypes 38–3, 41–36 and 43–12) were subjected to drought stress by receiving half of the optimal watering amount (details on the watering amount are described in [18]). The watering quantity varied based on the soil moisture content (see [18]), which was constantly measured using a tensiometer (Supplementary Fig. S1A). Moreover, 10 ramets per genotype were used as biological replicates for inoculation treatments: three replicates were inoculated with the *H. parviporum* strain 1 (Hpa 1), another three replicates with strain 2 (Hpa 2), and, finally, another three replicates were mock inoculated with 1.5% Malt Extract Agar. The remaining ramet for each genotype was non-treated. Moreover, a seventh genotype (40– 41, from family ID 40) was used as internal control, with three ramets being optimally watered and four subjected to drought stress, and no inoculation treatment was applied. The experiment was conducted under standard ambient lighting, and temperatures in the greenhouse averaged 31.8 °C in July, 25.9 °C in August, 23.2 °C in September, 15 °C in October, and 11.8 °C in November (Supplementary Fig. S1B).

Table 1 Family ID, genotype ID, average starting height, average diameter, number of cuttings in each genotype and treatments for Norway spruce. Hpa 1 presents the strain *H. parviporum* no 1, and Hpa 2 *H. parviporum* no 2. Control refers to mock control (Malt), and NT refers to non-treated.

Family ID	Genotype ID	Average starting height (cm)	Average diameter (mm)	No. of cuttings per genotype	Watering treatment	Number of cuttings per inoculation
F38	38-3	44	7	10	Lower	Hpa1 (3), Hpa2 (3), Control (3), NT (1)
F38	38-8	37	7	10	Optimum	Hpa1 (3), Hpa2 (3), Control (3), NT (1)
F40	40-41O	40	6	3	Optimum	Non-treated
F40	40-41D	40	8	4	Lower	Non-treated
F41	41-36	44	7	10	Lower	Hpa1 (3), Hpa2 (3), Control (3), NT (1)
F41	41-44	42	7	10	Optimum	Hpa1 (3), Hpa2 (3), Control (3), NT (1)
F43	43-12	34	5	10	Lower	Hpa1 (3), Hpa2 (3), Control (3), NT (1)
F43	43-15	38	7	10	Optimum	Hpa1 (3), Hpa2 (3), Control (3), NT (1)

Inoculation and sampling

Inoculation was done by boring with a 5 mm cork borer through the bark of the seedlings (at the lower stem region) to reach the sapwood surface and placing equal-sized 1.5% MEA plugs of the inoculum (*H. parviporum*) on the exposed surface before wrapping it with parafilm to prevent falling off or drying out. The exact process was carried out also for the mock control with the inoculum replaced with sterile 1.5% MEA plugs. For each of the 67 trees, the phloem tissue was harvested in tubes, immediately frozen in liquid nitrogen, and transferred to − 80 °C for storage. The bark was scraped with a scalpel (after freezing) to measure the necrosis. The vertical and horizontal lesions in the phloem and sapwood were measured with a digital caliper. Additionally, from the root tissues, twelve samples were collected from mock-inoculated control seedlings of families 38 and 41 and six from the non-treated seed- lings (Family 40) (Table 1).

DNA extraction, amplification and sequencing

The root samples were washed to remove the soil and other debris, the bark was removed from the stem, and the phloem was collected from and around the inoculation point. One hundred

fifty (150) mg of the phloem or root material was ground into powder in liquid nitro- gen with a sterile mortar and pestle. The genomic DNA (gDNA) was extracted using the modified cetyltrimethylammonium bromide (CTAB) method [47], and the gDNA concentration was measured using Qubit fluorimeter (Life Technologies) quantification. The PCR amplification of the internal transcribed spacer 2 (ITS2) region was performed with primer pair ITS3F and ITS4R. The amplicons were sequenced with the Illumina Sequencing platform to generate paired-end raw reads of 250 bp length. The PCR, library preparation and sequencing were carried out by Novogene (Cambridge Science Park, United Kingdom). The DNA amplicons were run on a 1.5% agarose gel at 60 V for 90 min.

Raw data processing

Paired-end reads were allotted to the samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. The reads were merged using FLASH (V1.2.7) [48] and spliced where there was an overlap between the reads and those generated from the opposite ends of the same DNA fragment. The raw sequences pre-processed by Novogene with the barcodes and primers removed were used for further analysis. The bioinformatic platform Quantitative Insights into Microbial Ecology (QIIME2–2021.8) [49] was used to analyze the mycobiome composition and diversity associated with the samples. The raw sequences were imported into QIIME2 and processed using the DADA2 pipeline to denoise and infer the exact amplicon sequence vari- ants (ASVs). To study the microbial community com- position in each sample, the ASVs were aligned and annotated using the UNITE QIIME release for fungi (Version 10.05.2021) [50] database. Aligning sequences with 99% similarity were assigned to the same ASV. The sampling depth was normalized using the sample with the minimum sequencing depth, and the normalized sampling depth was sufficient enough for the subsequent analysis. Alpha rarefaction curves were generated using QIIME2 to assess sample coverage and whether the normalized sampling depth was enough to capture fungal diversity. The datasets produced and/or examined in the present research are accessible at NCBI under BioProject PRJNA990335; SRA number SUB13564759 (SRR25109452 - SRR25109534).

Statistical analysis

Necrosis analysis was carried out for samples across families and genotypes. The data distribution was assessed employing the Shapiro–Wilk test [51]. The Bartlett test was employed to evaluate the homogeneity of variances for normally distributed data. Analysis of variance (ANOVA) analysis was carried out, followed by a Tukey HSD post hoc. Homogeneity of

variance for not-normally distributed data was assessed using Levene's test; due to the homoscedasticity of the data, a Kruskal-Wallis analysis was carried out.

Mycobiome analysis was carried out separately for phloem and root samples, and this was assessed against family/genotypes and water treatments. Analysis was performed, excluding ASV values from inoculated *Heterobasidion*. The normal distribution of our data was evaluated using the Shapiro-Wilks normality test [51]. To analyze the non-normally distributed data, the non-parametric Kruskal-Wallis test was employed to identify and compare the differences in diversity and prevalence among fungal communities with variables greater than two. Post hoc analysis was carried out using Dunn Bonferroni. The non-parametric Wilcoxon rank sum test was used for factors with two variables. The p-value adjustment method employed was Bonferroni. For normally distributed data, the standard t-test was used for factors with two variables, while the ANOVA was used for factors with more than two variables. This was followed by a Tukey HSD when factors significantly varied.
Alpha diversity was assessed using the observed features/ASVs, Simpson and Shannon-Wiener diversity indices with the R package vegan [52, 53] and using the adonis function to perform a permutational analysis of variance (PERMANOVA) based on Bray-Curtis with 999 permutations. Bonferroni post hoc analysis was done when the fungal taxa significantly differed. Principal coordinate analysis (PCoA) was used to visualize the fungal community structure. This was performed using vegan [52] and ggplot2 [54] packages in R. A correlation analysis was carried out between the necrosis and growth variables and the most abundant identified fungal genera. All reads for each genus were combined, and the 50 most abundant identified genera were used for the correlation using Spearman's rank correlation. The taxonomic classification / fungal diversity for the top 50 species was visualized in a tree format using the heat tree function in the Metacoder package in R [55]. Functional annotation of the resulting fungal communities in roots was assessed using the FungalTrait database [56]. We analyzed the stability between dark septate endophytes (DSEs) and ectomycorrhiza fungi (ECM) between water treatments using the frequency of the major DSEs and ECMs present in our samples. Indicator species for each family were identified with the R package indicspecies [57]. A p-value below 0.05 was considered statistically significant in this study.

Correlation between genetic distance and distance among taxa

Using genomic information of the seven genotypes used in this study (data not shown; Chano et al., in preparation), we performed a Mantel test [58] to infer the correlation between pairwise

genetic distance and the calculated pairwise distances from taxa abundance. As the genotypes were split into two sets subjected to different water regimes (genotypes 38–8, 40–41, 41–44 and 43–15 for optimum watering and genotypes 38–3, 40–41, 41–36 and 43–12 for drought condition), two separate Mantel tests were performed to avoid the effect due to watering. In addition, just three of the four genotypes from each set were used for inoculation experiments, so just untreated plants were considered. Distance matrices were generated with the function dist() in R [53] and Mantel test was performed by using the function mantel.rtest() from the R package ade4 [59], including 9999 permutations.

Fungal isolation, identification, and dual-culture testing

Based on data analysis, fungal endophytes were recovered from the three Norway spruce families (38, 41, 43). The soil was removed from the roots under running tap water. The roots were cut into small pieces (1 cm) and surface sterilized (70% ethanol 1 min, 2% sodium hypochlorite (NaOCl) 30 sec, rinsed in sterile ddH2O). The surface- sterilized roots were plated on 1.5% malt extract (MEA). The plates were stored in darkness at 19 °C with 75% relative humidity. Based on morphology, we chose one endophyte from each family for dual-culture-inoculation against *H. parviporum*. The DNA was extracted as described in [60], and species of endophytes were con- firmed with ITS regions with primer pair ITS1-F [61] and ITS4 [62]. Purified PCR products were sequenced using the ITS4 at Microsynth SEQLAB (Germany).
The chosen three endophytes and *H. parviporum* were plated on a 1.5% MEA at 0.5 cm from the edge of 8.5 cm Petri plates for a dual culture antagonisms assay (paired growth assay). The growth of *H. parviporum* was measured on days 7 and 10. The sequences for the isolates are available under the ascension numbers: OR167041, OR167042, and OR167043.

3. Results

After denoising and quality filtering of the phloem samples, 7629 features/ASVs were obtained with a total frequency of 7,973,147. Frequency per sample ranged from 64,507 to 167,468. Normalization was carried out using the sample with the minimum sampling depth (64,507) to include all our samples in the analysis - such that all samples have the same sequencing depth. The 7629 features/ amplicon sequence variants (ASVs) were obtained from phloem samples and clustered into 1181 identified ASVs. From root materials, two samples were excluded due to bad quality. From the remaining 16 samples, 1793 features/ASVs were obtained with a total

frequency of 2,290,039. Frequency per sample ranged from 109,538 to 165,187. Sample normalization was done with a mini- mum sampling depth of 109,538. The total of 1793 features obtained was clustered into 454 identified ASVs.

The clustered ASVs were used for further analysis. The data were explored to see whether specific taxa differed between the genotypes and water treatment. The soil moisture content differed significantly between the treatments from the fifth week ($p = 2.79e-06$, Supplementary Fig. S1A). In phloem, the highest percentage of the phloem taxa belongs to the Ascomycota phylum with 72 % (72%), followed by Basidiomycota with 24 (24%) percent. Mortierellomycota had 2 %, while Chytridiomycota and Mucoromycota each had 1% of the total fungal com- position. Other negligible taxa include Aphelidiomycota, Basidiobolomycota, Glomeromycota, and Rozellomycota. In roots, Ascomycota also occupied the largest percentage of the mycobiome (75%), followed by Basidiomycota (18%). The other taxa included Mortierellomycota (3%), Mucoromycota (2%) and Chytridiomycota (1%). Other negligible root taxa include Aphelidiomycota, Basidiobolomycota, Glomeromycota, and Rozellomycota. A comparison of the most abundant fungi in the phloem and the root shows some uniqueness and overlap, as some fungi are found both in the stems and roots but with differences in their abundances/expression levels (Table 2).

There were variations among relative proportions of taxa across all samples; certain species or genera were more abundant in some genotypes and treatments than others. Overall, *Amphinema* sp. were the most abundant in roots (Table 2). *Paraphaeosphaeria neglecta*, followed by *Setomelanomma holmii*, were the most abundant fungi in phloem samples (Table 2, Fig. 1). *Paraphaeosphaeria* and *Setomelanomma* genera displayed a similar pattern in their response to water treatment in the phloem. There was a higher abundance in plants that were optimally watered than in plants with low water availability (Fig. 2A). This pattern was consistent across the different families and genotypes, except for family 40, where *Setomelanomma holmii* showed the opposite trend between the different water treatments, with higher presence in low-watered plants (Fig. 2B). Genotype 40–41 (low-watered) had a higher presence of *Paraphaeosphaeria neglecta* than the optimally watered plants with the same genotype (Fig. 2B). *Phialocephala fortinii* has the lowest abundance in family 40, which was non-treated (Fig. 2C).

Table 2 Top 12 identified Amplicon Sequence Variants in phloem and root tissues

ASV_ID	Phylum	Species	Total reads	Relative abundance (%)
Phloem				
ASV_1	Ascomycota	*Paraphaeosphaeria neglecta*	1,584,628	19.9
ASV_2	Ascomycota	*Setomelanomma holmii*	1,466,026	18.4
ASV_3	Ascomycota	*Lachnum virgineum*	626,774	7.9
ASV_4	Ascomycota	*Angustimassarina acerina*	493,135	6.2
ASV_5	Ascomycota	*Phialocephala fortinii*	272,659	3.4
ASV_6	Ascomycota	*Lachnum* sp.	216,555	2.7
ASV_7	Basidiomycota	*Heterobasidion parviporum*	208,438	2.6
ASV_8	Ascomycota	*Phialocephala* sp.	168,709	2.1
ASV_9	Ascomycota	*Brunnipila fuscescens*	98,498	1.2
ASV_10	Ascomycota	*Cadophora* sp.	78,448	1.0
ASV_11	Basidiomycota	*Amphinema* sp.	76,332	1.0
ASV_12	Ascomycota	*Xenochalara* sp.	44,594	0.6
Roots				
ASV_1	Basidiomycota	*Amphinema* sp.	312,469	13.6
ASV_2	Ascomycota	*Trichophaea* sp.	282,120	12.3
ASV_3	Basidiomycota	*Amphinema byssoides*	253,470	11.1
ASV_4	Ascomycota	*Phialocephala fortinii*	224,494	9.8
ASV_5	Basidiomycota	*Thelephora terrestris*	222,772	9.7
ASV_6	Ascomycota	*Wilcoxina* sp.	174,489	7.6
ASV_7	Ascomycota	*Hyaloscypha finlandica*	92,689	4.0
ASV_8	Ascomycota	*Dactylonectria macrodidyma*	65,356	2.9
ASV_9	Ascomycota	*Dactylonectria anthuriicola*	58,814	2.6
ASV_10	Ascomycota	*Ilyonectria mors*-panacis	30,738	1.3
ASV_11	Ascomycota	*Hyaloscypha_variabilis*	24,039	1.0
ASV_12	Ascomycota	*Lecanicillium_primulinum*	16,814	0.7

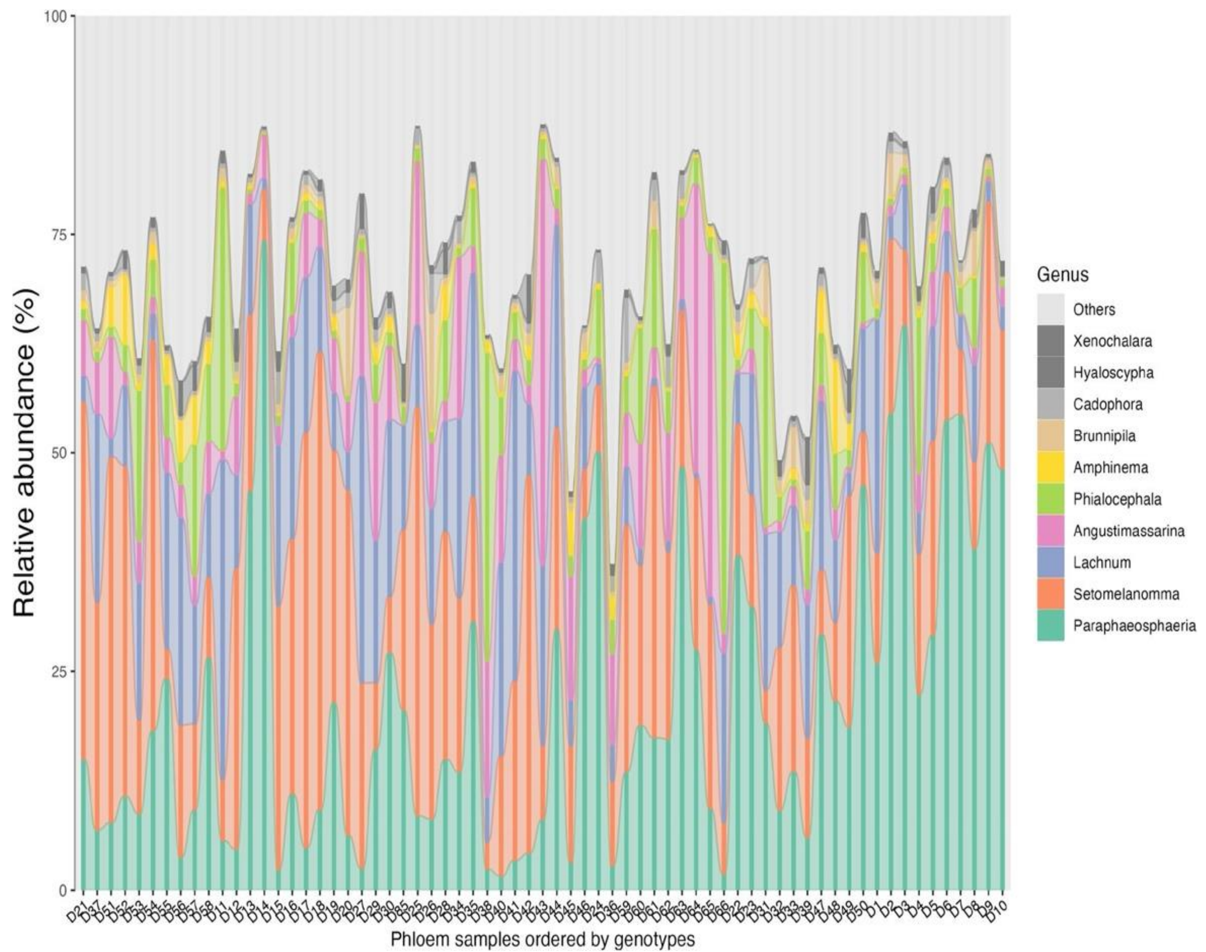

Fig. 1 The relative abundance of the top 10 taxa of the 67 phloem samples from different genotypes labelled D1-D67, ordered according to genotypes (38–3, 38–8, 40-41D, 40-41O, 41–36, 41–44, 43–12, 43–15. D1-D67; Supplementary table S1)

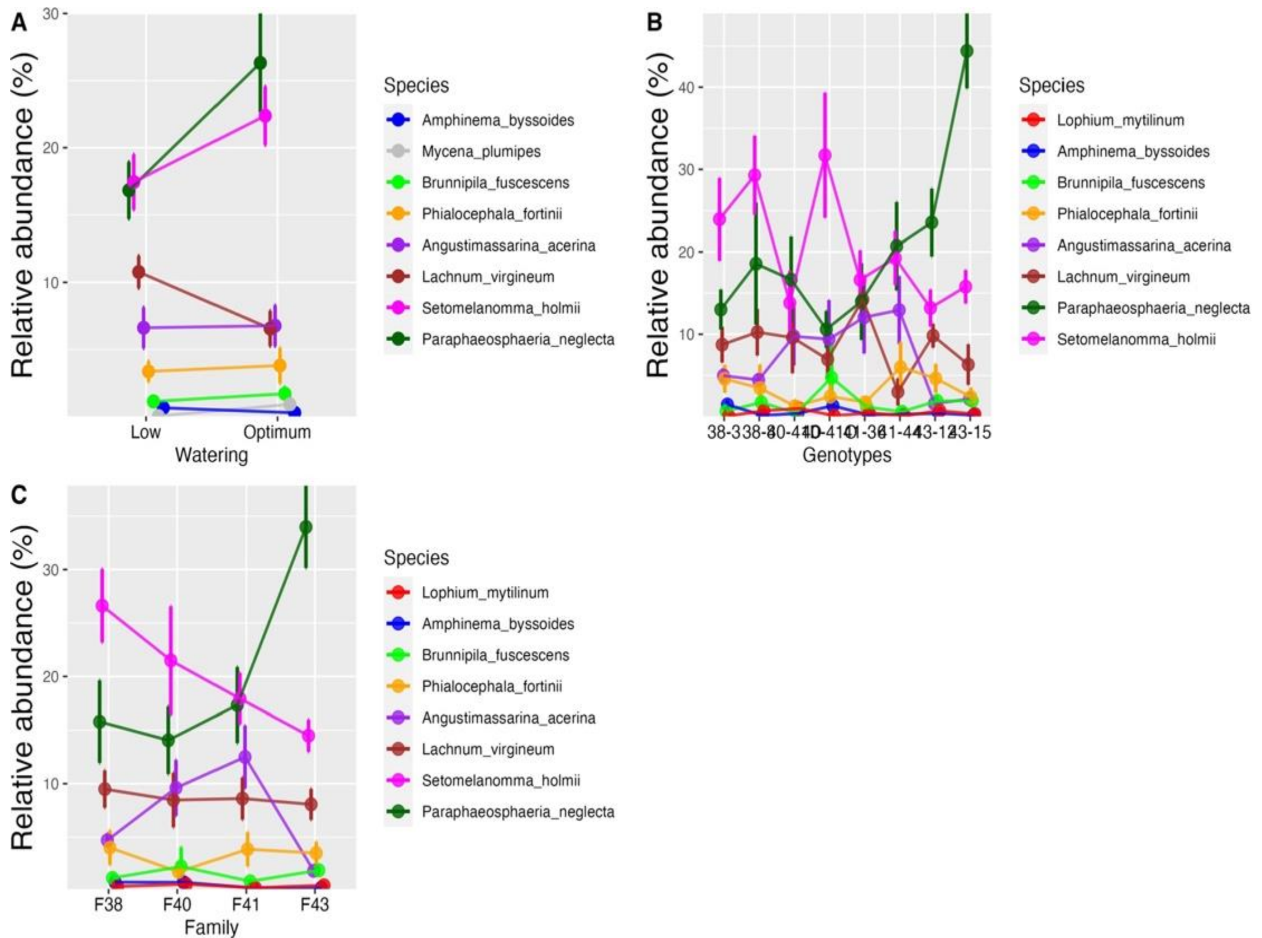

Fig. 2 Relative abundance differences of the top eight taxa in the phloem along **A** water treatment, **B** genotypes, **C** family. 40-41D are with low-water treated genotypes in family 40, while 40-41O are optimally watered (Connecting lines are included to visualize common patterns)

Results for phloem mycobiome

Genotype variation

Alpha diversity differed significantly ($p < 0.05$) among genotypes in phloem samples (Fig. 3). There were significant differences in the alpha diversity among genotypes based on Kruskal-Wallis tests, observed ASVs ($p = 0.02$), Shannon ($p = 0.004$) and Simpson indices ($p = 0.005$) (Fig. 3). Based on observed ASVs, there were significant differences between genotypes 40-41O and 38–3 ($p = 0.02$) (Fig. 3A). Simpsons and Shannon's diversity indices showed significant differences only between genotypes 43–12 and 43–15 ($p = 0.02$) (Fig. 3B, C).

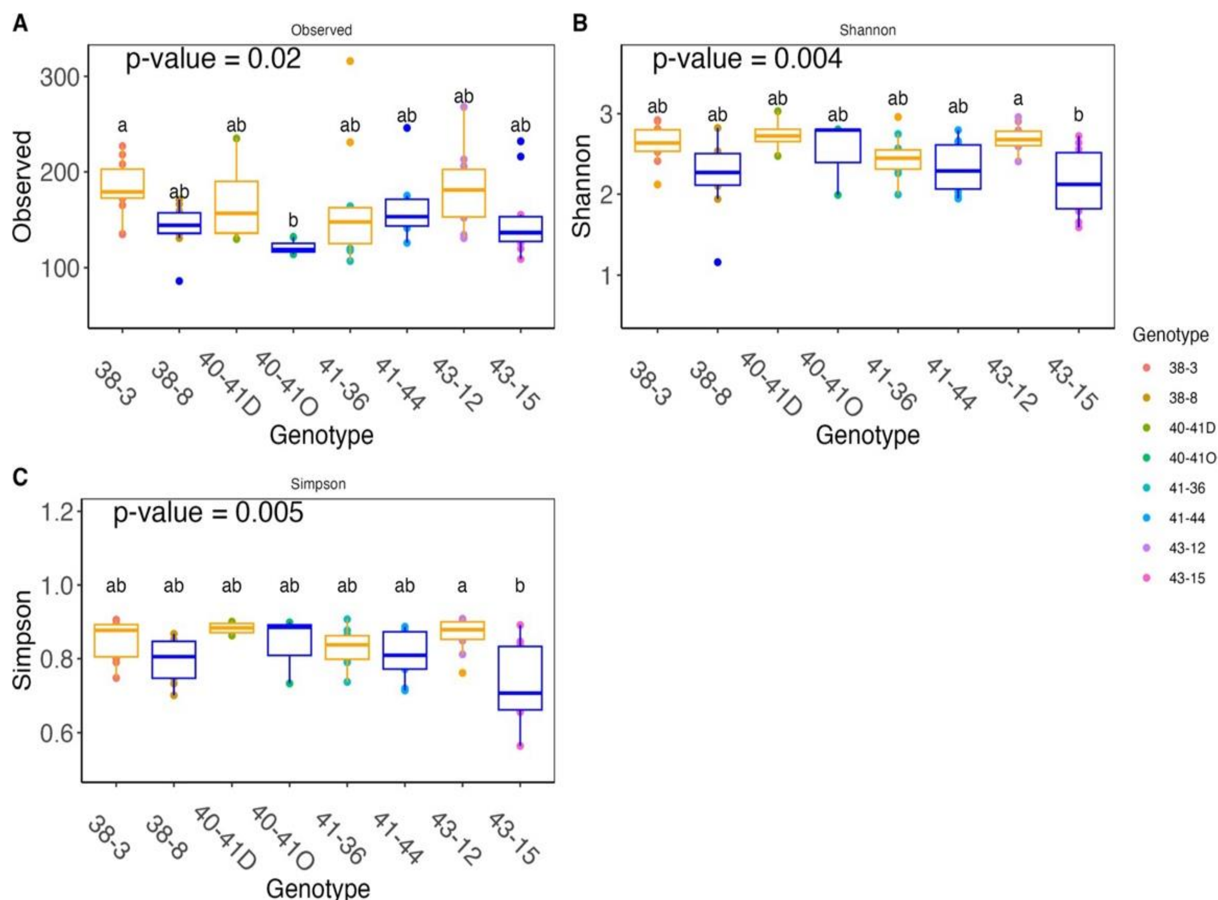

Fig. 3 Alpha diversity among phloem genotypes. **A** observed amplicon sequence variants, **B** Shannon diversity index, **C** Simpson index. Different letters above plots denote significantly different groups after the post hoc test. Low-watered genotypes (orange colour) are 38–3, 40-41D, 41–36 and 43–12. Optimally watered plants (blue colour) are 38–8, 40-41O, 41–44, and 43–15

Results from the PERMANOVA analysis showed significant differences ($R2 = 0.23$, $F = 2.52$, $p = 0.001$) among the genotypes in the phloem mycobiome (Supplementary Fig. S2). To test the impact of the water treatment, we compared the dispersion of fungal communities (dispersion genotypes) inside the corresponding group. Based on the permutational analysis of variance (PERMANOVA) of dispersion genotypes for each water treatment, meaningful differences were found between dispersion genotypes for lower water conditions ($p = 0.001$), but not for the optimally watered plants ($p = 0.07$) (Fig. 4). Genotype 38–3 seems located further away from the other genotypes (Fig. 4a), and genotypes 41–36 and 43–12 also appeared to cluster further apart from each other.

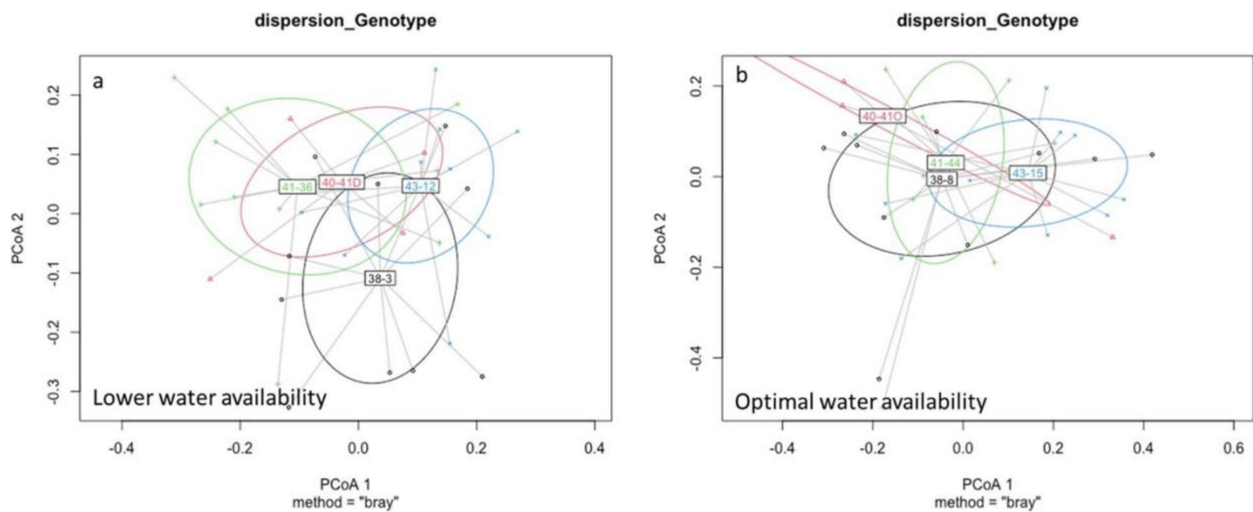

Fig. 4 Dispersion of fungal communities among genotypes based on Bray-Curtis **A** low-water treated plants **B** optimally watered

The results of the taxonomic classification of the phloem mycobiome for genotypes were visualized using the heat tree matrix (Supplementary Fig. S3). Taxa are represented as nodes, and sizes and colours indicate their abundance. Each taxon is coloured by the log-2 ratio of the median phloem ASV counts observed at each genotype. The colouring also shows significant differences between the median proportions of ASV counts for samples from the different genotypes. The colour intensity is proportional to the log2 ratio of the differences. For instance, differentially expressed taxa were different between water treatments between genotypes of family 43. There were more abundant species specific to Genotype 43–12 than 43–15. Family *Venturiaceae* and *Paraphaeosphaeria neglecta* were more abundant in genotype 43–15, while some of the taxa more abundant in genotype 43–12 included *Amphinema* sp., *Thelephora terrestris*, *Mycena plunipes*, *Neonectria tsugae* and *Spirophaera floriformis* (Supplementary Fig. S3).

Water treatments

Alpha diversity also differed significantly between watering treatments across all indices in the phloem mycobiome (Supplementary Fig. S4). Observed features had a p-value of = 0.007, while both Shannon and Simpson indices had p-values of = 0.0002 (Supplementary Fig. S4). There was higher fungal diversity in the drought-treated plants than in the optimally watered plants. The permutation analysis of variance among the phloem mycobiome also showed slightly significant differences ($R2 = 0.04$, $F = 2.60$, $p = 0.01$) among the watering treatments

(Fig. 5). Using the heat tree function in the Metacoder package, the taxonomic classification of the phloem mycobiome (water treatment) was visualized (Fig. 6). The taxa are also represented here as the nodes, and the sizes and colours represent the abundances associated with the taxa. Taxa-coloured tan are more abundant in optimally watered plants, while those coloured cyan were more abundant in low-watered plants, although the significance level is not considered here. Low-watered plants are generally associated with more taxa than in the optimally watered plants. They contain genera such as *Phialocephala*, *Thelephora*, *Mycena*, *Tylospora*, *Amphinema*, *Neonectria*, and *Hyaloscypha*. The largest genera in the optimally watered group include; *Paraphaeosphaeria*, *Setomelanomma*, *Diaporthe*, *Xenochalara*, and *Brunnipila* (Fig. 6).

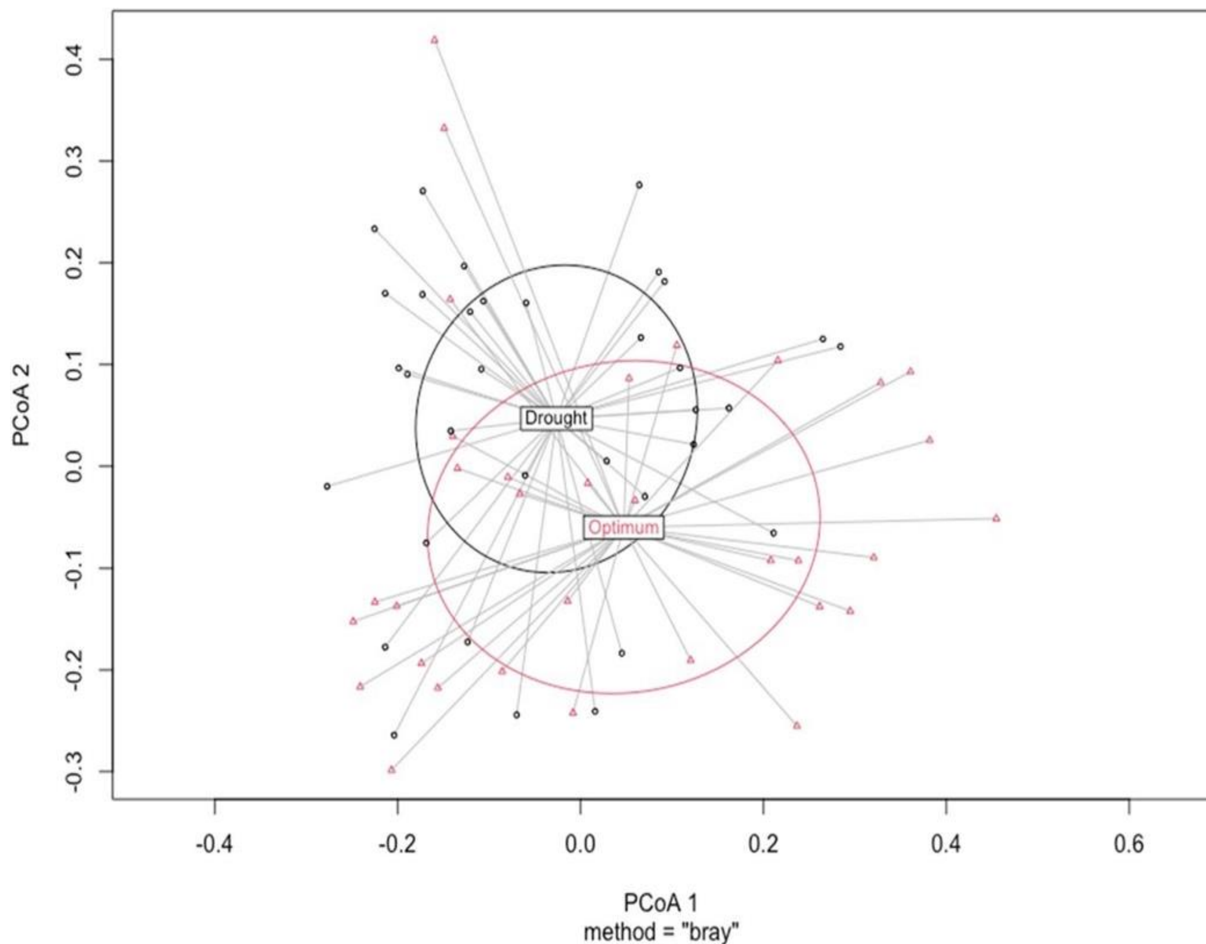

Fig. 5 Fungal communities' dispersion among watering treatments based on Bray-Curtis distances

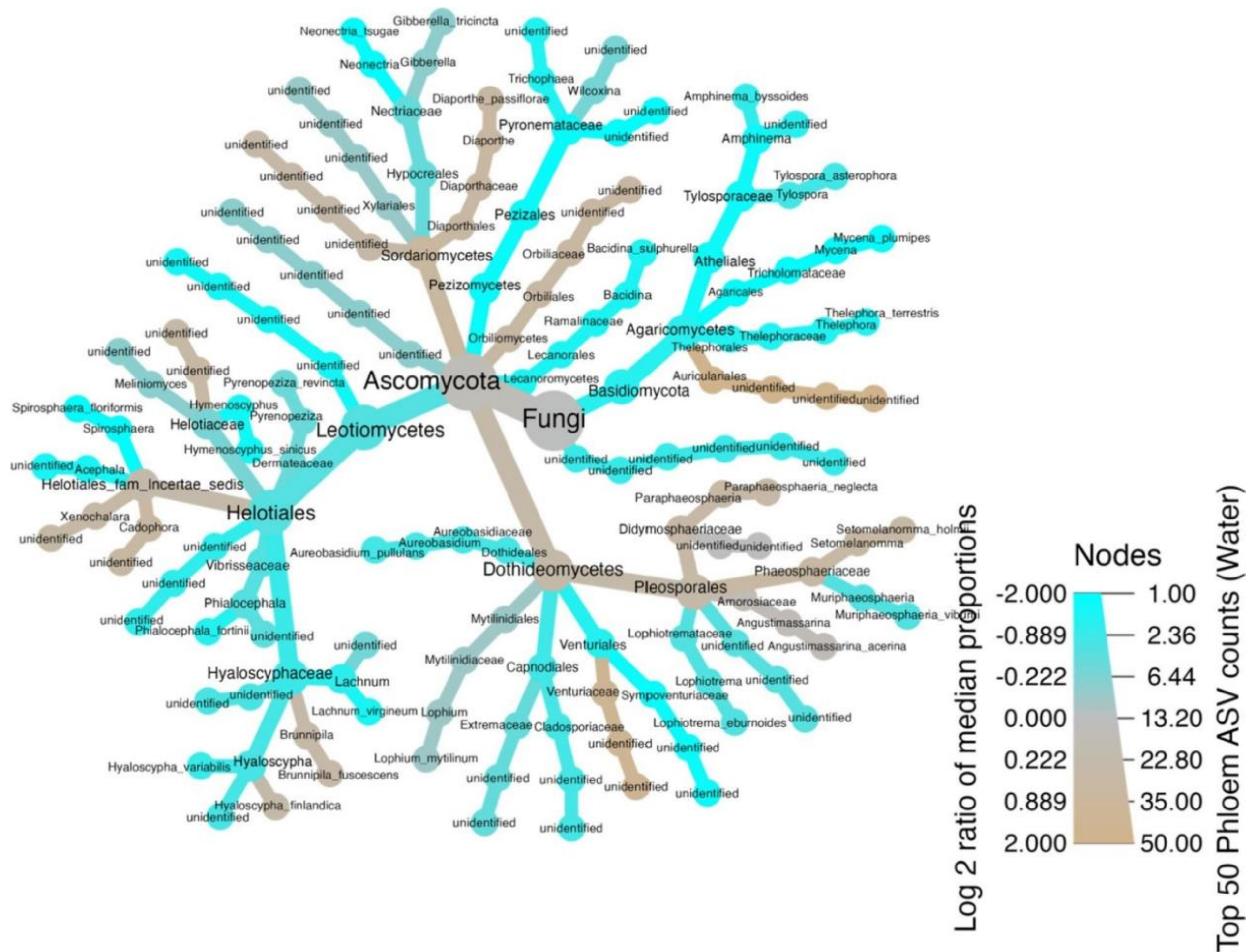

Fig. 6 Phloem mycobiome abundance according to water treatment groups. The colour of each taxon represents the log-2 ratio of median proportions of phloem ASV counts observed at each water treatment category. Taxa-coloured tan have a higher relative abundance in the optimal watering category, while taxa-coloured cyan have a higher relative abundance in low-water treated plants

Results for roots

Roots (genotype and water treatment)

No significant differences were observed in the mycobiome alpha diversity with the indices used (Shannon, Simpson and observed ASVs) for any factors (water treatment, family and genotypes). Also, no significant differences were observed in the permutation analysis of variance among the root mycobiomes (Supplementary Fig. S5). Taxa-coloured including *Thelephora*, *Trichophaea*, *Cadophora*, and *Angustimassarina* genera showed higher relative abundance in well-watered plants, while taxa such as *Amphinema*, *Phialocephala*, and *Mortierella*, among others, showed higher relative abundance in the drought-treated plants (Supplementary Fig. S5). Major dark septate endophytes (DSEs) genera in our samples included *Acephala*, *Cadophora*, *Cladophialophora*, *Exophiala*, *Gyoerffyella*, and

Phialocephala. According to the FungalTraits database used for this study, the *Cadophora* genus was not stated to have DSE capabilities. We included it because it was identified based on other studies as a DSE. Ectomycorrhizal (ECM) genera included *Amphinema*, *Inocybe*, *Thelephora*, *Trichophaea*, *Tylospora*, and *Wilcoxina* (Supplementary table S2).

ECMs and DSEs frequency varied between water treatments. The *Amphinema* genus was the most abundant among ECM fungi, followed by *Thelephora* and *Trichophaea*. *Thelephora*, *Trichophaea* and *Tylospora* seemed more unstable in low water conditions as their quantity was reduced (Fig. 7, Supplementary table S2). Under the low water treatment, the genera *Amphinema* and *Thelephora* had the highest presence, with 341,543 and 11,165 sequences, respectively. In contrast, these genera were less abundant under the optimum water treatment, with 224,396 and 211,607 sequences, respectively.

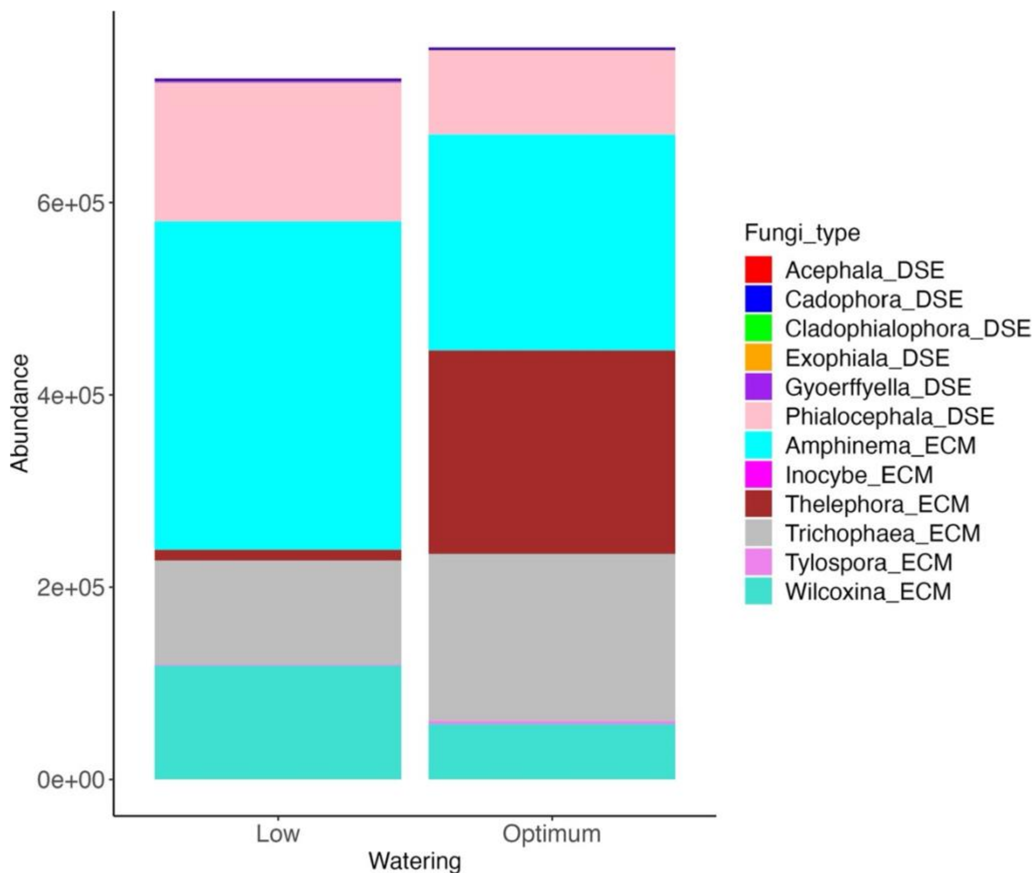

Fig. 7 Relative abundances of dark septate endophytes (DSE) and ectomycorrhiza fungi (ECM) in the Norway spruce root tissues under different watering groups

The genera *Trichophaea* and *Phialocephala* also exhibited higher abundances under the low water treatment, with 108,305 and 144,730 sequences, respectively, compared to the optimum water treatment, where they had 173,817 and 87,491 sequences, respectively (Supplementary

table S2). For the DSEs, the *Phialocephala* genus was the most abundant and more stable than most ECM fungi in low-water conditions. Other genera that exhibited higher abundances under the low water treatment include *Wilcoxina*, *Tylospora*, and *Cadophora*. In contrast, genera such as *Amanita*, *Capronia*, and *Phialophora* were not detected in the optimum water treatment, while they had low prevalence under the low water treatment.

Interactions of phloem mycobiome with H. Parviporum

The top eight genera in the phloem showed different patterns in their relative abundance across families (Fig. 8). *Paraphaeosphaeria* was present in similar quantities in families 38, 40 and 41, with lower abundance than in family 43. Among the top eight taxa, there were only significant differences between *Paraphaosphaeria* and *Angustimassarina* (Fig. 8). Family 43 showed a significantly higher relative abundance of the *Paraphaosphaeria* genus than families 38 and 41 ($p < 0.01$), while the relative abundance of the *Angustimassarina* genus differed significantly between families 43 and families 38 ($p < 0.03$) and 41 ($p < 0.0002$). *Setomelanomma* showed a decreasing trend from family 38 to family 43 (Fig. 8). More striking is the relationship between the inoculated *Heterobasidion* and DSE *Phialocephala*, which followed the same pattern. *Phialocephala fortinii* seemed to react to the presence of *H. parviporum*, as its abundance increased in concert with *Heterobasidion* (Supplementary Fig. S6).

There were no significant differences observed between the lesion length in sapwood and lesion width in both phloem and sapwood. The only significant differences observed among families were in the phloem lesion length ($p = 0.04$, Fig. 9). Family 41 has the highest lesion length but not significantly different from family 38. Family 43 has the lowest lesion length, significantly different from family 41 (Fig. 9). Family 41 has the highest amount of *Phialocephala fortinii* (Fig. 8), and also the highest lesion length (Fig. 9). Family 43 had the lowest lesion length compared to other families.

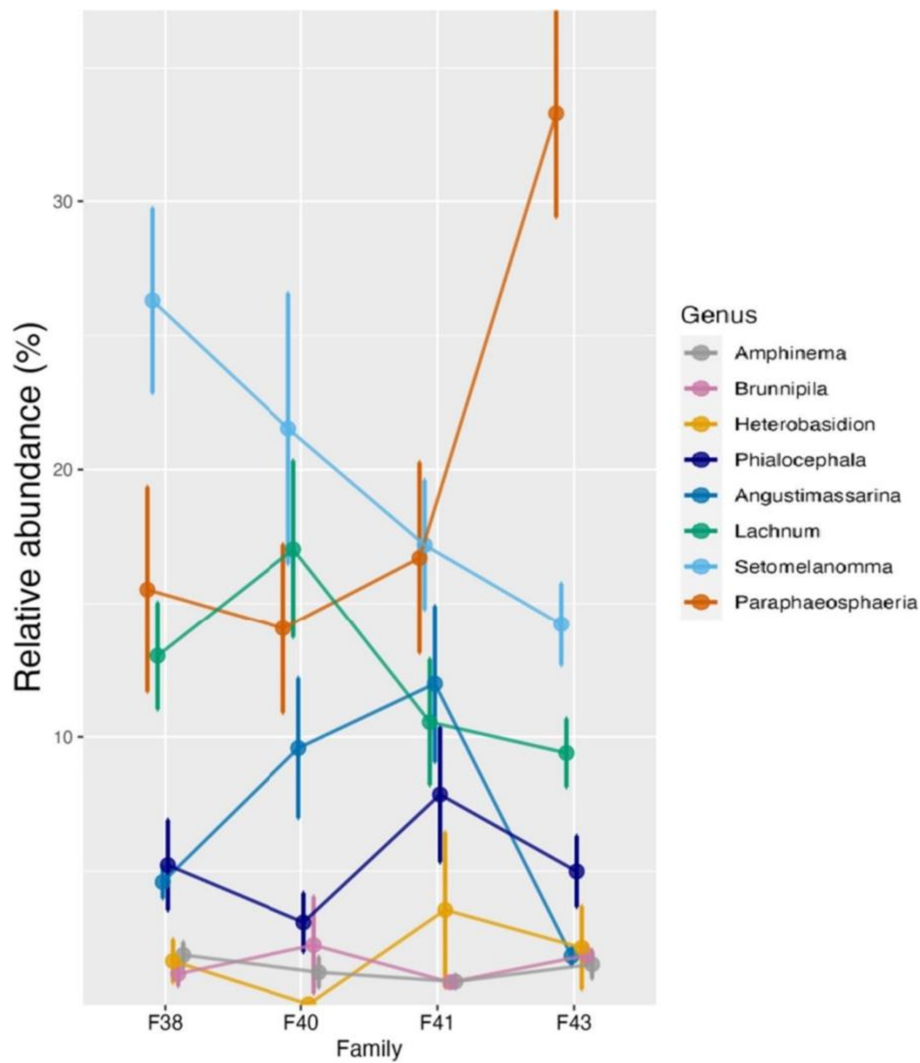

Fig. 8 Relative abundance of taxa of the top eight fungi (phloem mycobiome) among families in the presence of *H. parviporum*. Connecting lines are included to visualize common patterns

Family 43 had 10 indicator species in the phloem (*Paraphaeosphaeria neglecta*, *Myxocephala albida*, *Metapochonia suchlasporia*, *Solicoccozyma terrea*, *Coniothyrium aleuritis*, *Mariannaea punicea*, *Mortierella cystojenkinii*) and three unidentified fungi. The correlation analysis was conducted between the measured parameters (growth and lesion) and the top 50 fungal genera. There were strong positive correlations between the lesion length and lesion width in both phloem and sapwood (Fig. 10). The starting and final heights were also strongly positively correlated with each other.

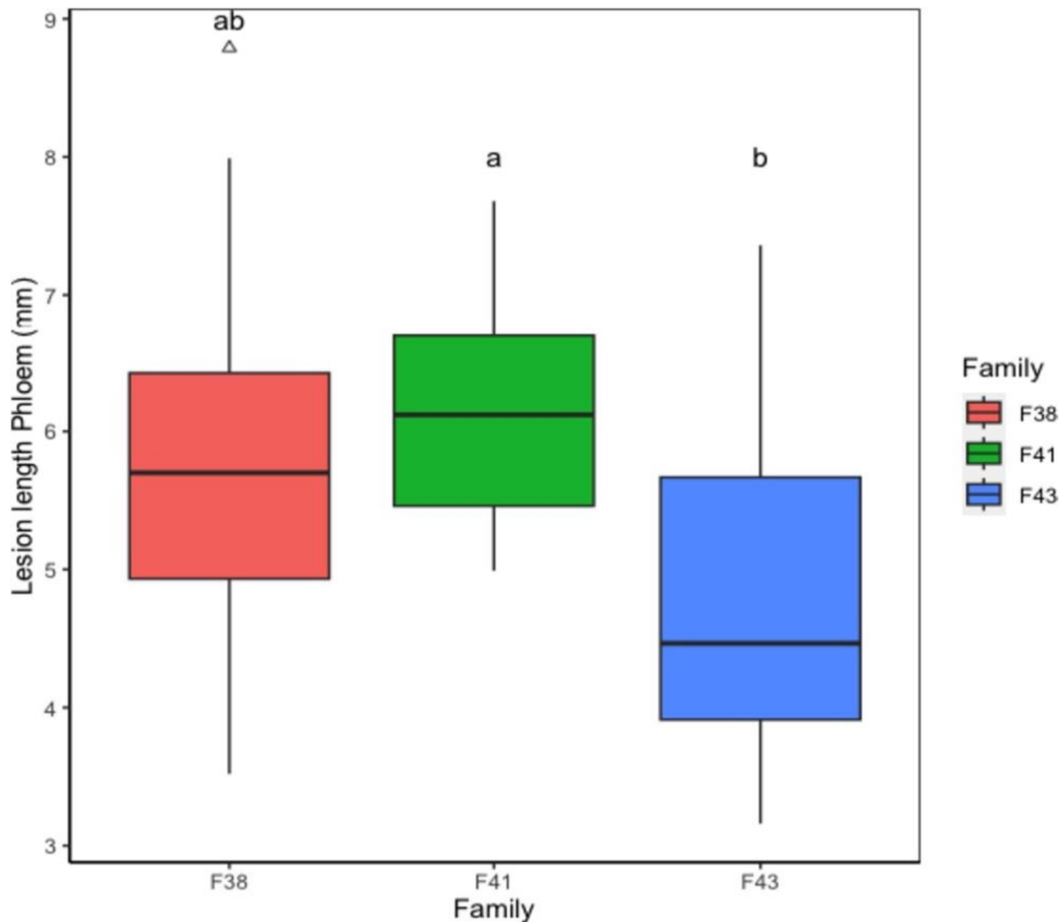

Fig. 9 The effect of families on the lesion length in the phloem. Different letters above plots denote significantly different groups after post hoc test

The diameter had moderate to strong negative correlations with almost all the fungal communities, e.g. *Amphinema*, *Wilcoxina*, *Tylospora*, *Thelephora*, and *Alternaria*. The starting height had weak to moderate negative correlations with *Paraphaeospharia*, *Meliniomyces*, *Penicillium*, *Phacidium*, *Devriesia*, and *Pezicula*. Positive weak to moderate correlations existed between starting height and *Angustinassarina*, *Lophiotrema*, *Acephala*, *Saccharomyces*, and *Coniochaeta*. *Phialocephala* genus had a weak negative correlation with the lesion measurements (lesion length in phloem and sap- wood and lesion width in phloem) except lesion width in the sapwood. As expected, there were moderate to strong positive correlations between *Heterobasidion* abundance and lesion length in phloem and sapwood (Fig. 10). *Heterobasidion* abundance had a weak negative correlation to the growth variables. It was weakly to moderately negatively correlated to *Tylospora*, *Spirosphaera*, and *Chalara* genera. *Heterobasidion* was moderately to strongly positively correlated with fungi such as *Paraphaeosphaeria*, *Trichophaea*, *Mortierella*, *Gibberella*, *Fusarium* and *Coniochaeta* (Fig. 10).

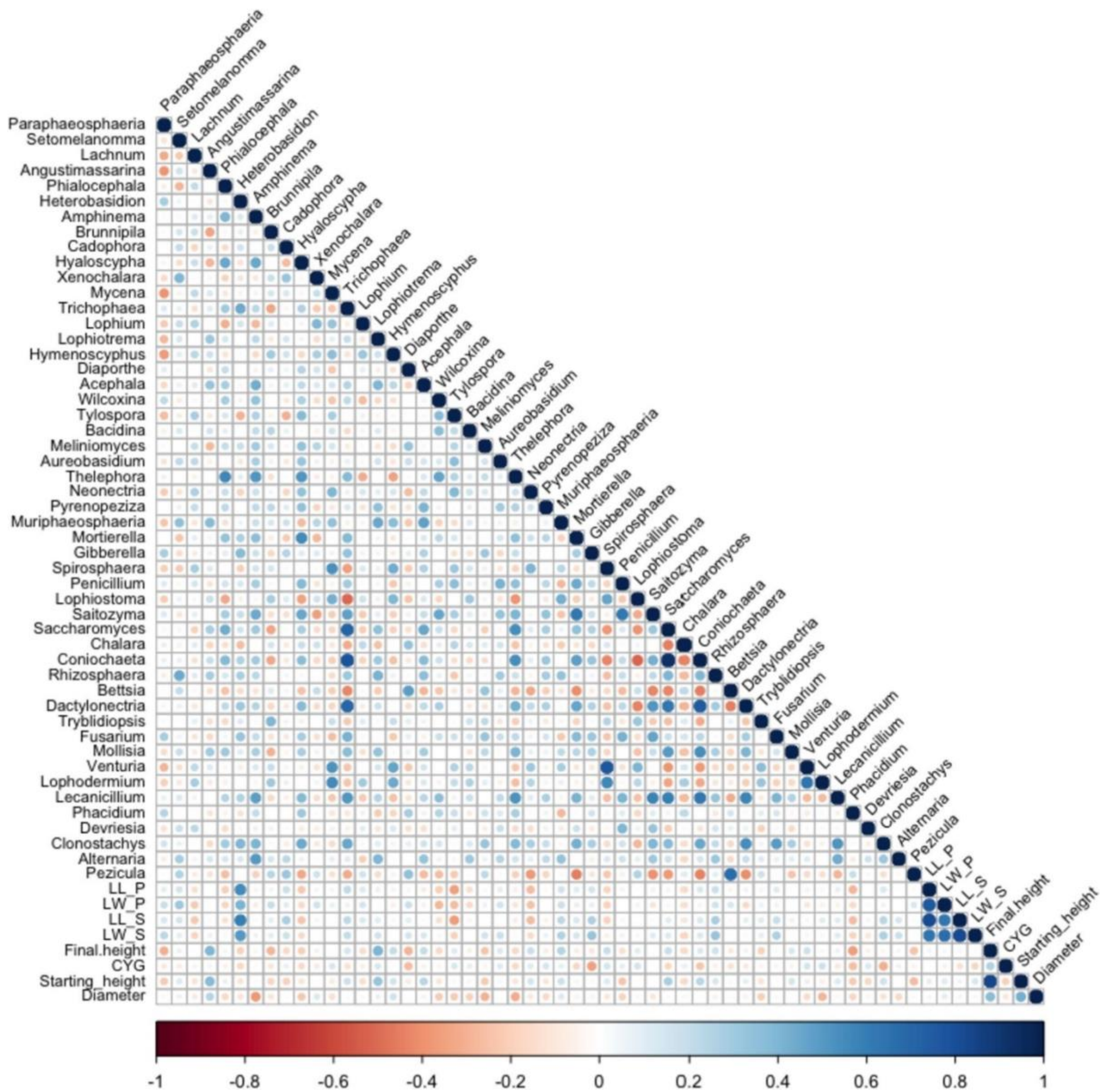

Fig. 10 Correlogram representing the matrices of Spearman's rank order correlation coefficient between the 50 most abundant identified different fungal communities and the Lesion length in the phloem (LL_P) and the sapwood (LL_S), Lesion width in the phloem (LW_P) and the sapwood (LW_S), final height of seedlings, current year growth (CYG), starting height and diameter measurements at the genus level. Positive (blue) and negative (red) correlations are only shown in the graph ($p < 0.05$)

Isolated root endophytes and their interaction with H. parviporum

Two isolates were identified as *Paraphaeosphaeria neglecta* and one as *Phialocephala fortinii* (*Phialocephala fortinii*-Acephala applanata species complex). All three fungi inhibited the growth of *H. parviporum* after seven and 10 days (Fig. 11). However, the 2 *P. neglecta* strains were able to stop the growth of *H. parviporum* (Fig. 11B) as it could not grow further after 7 days. Endophytes can restrict the pathogen growth after 7 days (Fig. 11A), and *P. neglecta* strains have stronger inhibition compared to *P. fortinii* after 10 days.

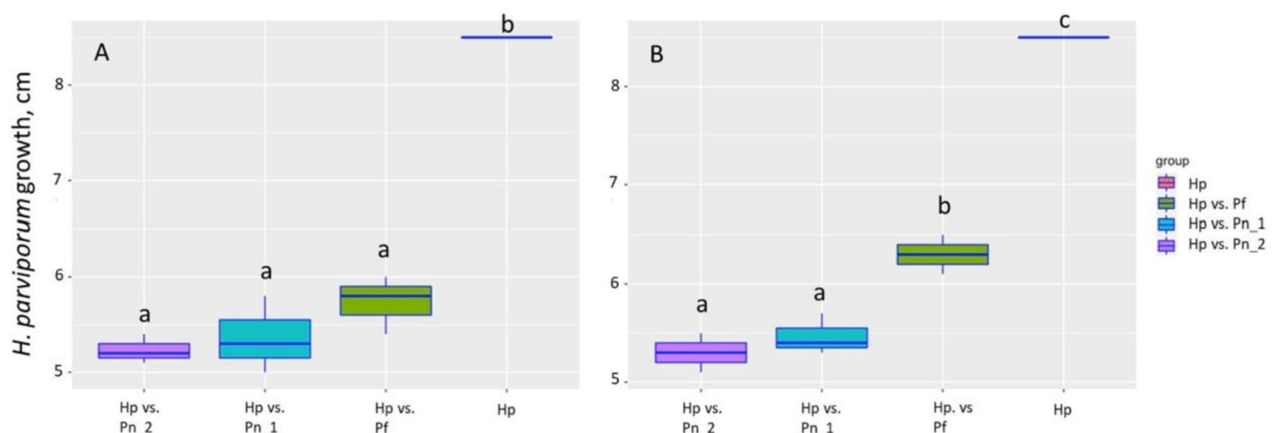

Fig. 11 The growth of *H. parviporum* in dual culture with *Paraphaeosphaeria neglecta* strain 1 (Pn_1) and 2 (Pn_2) and Phialocephala fortinii (Pf) after 7 days (A) and 10 days (B) *H. parviporum* (Hp) growing alone has reached the end of a Petri dish in 7 days (8.5 cm)

Correlation between genetic and mycobiome distances

No correlation was found in Mantel tests of pairwise comparisons of genetic distance and distances calculated from taxa abundance using genotypes used in both optimum watering and low-watered plants (simulated p-values of 0.5027 and 0.2538, respectively, based on 9999 permutations; Supplementary table S3).

4. Discussion

Understanding the basis of plant fitness is vital for developing sustainable management strategies for forest ecosystems. Competitive fungal strains in the same niche would establish a new approach to forest disease research (or a novel paradigm). They could also form a basis for applied research using these principles to control forest and other plant pathogens. To get more insights into the possibility that the mycobiome is linked to increased tree resilience ("mycobiome-associated-fitness"), we monitored the mycobiome associated with different

genotypes of Norway spruce under different watering conditions. To test the "mycobiome-associated-fitness" hypothesis, we also analyzed the interaction of artificially inoculated *H. parviporum* and the mycobiome. Our aims were to: 1) identify the specific mycobiome and core fungi within Norway spruce genotype/family level and changes in their relative abundance in relation to that of *H. parviporum* and 2) assess mycobiome stability under abiotic disturbance (lower water availability).

Family and genotype impact on the mycobiome

We focused on the mycobiome within the stems and roots of Norway spruce. We observed that the mycobiome diversity was higher in stems than in roots. This is consistent with results on Norway spruce by [63], whose study also reveals that roots had the lowest diversity among all the tissue types tested. Some of the fungal genera found in shoots and roots of the study by [63] were also found in ours and include *Amphinema*, *Cadophora*, *Inocybe*, *Phialocephala*, and *Tylospora*. Several research studies have specifically focused on analyzing the mycobiome of needles in coniferous trees, particularly Norway spruce and pine species [21, 64, 65] and limited research on the phloem/stem mycobiome was conducted. Fungal species associated with Norway spruce needles include *Phoma herbarum*, *Alternaria alternata*, *Aureobasidium pullulans*, *Phialophora sessilis*, *Setomelanomma holmii*, *Sydowia polyspora*, *Aureobasidium pullulans*, *Cladosporium cladosporioides*, and *Rhizosphaera kalkhoffii* [21, 66, 67]. It has been shown that foliar endophyte communities strongly depend on the plant species they inhabit [68–71]. The needle [21] and bud [28] mycobiomes in Norway spruce vary largely in Norway spruce.

In our study, the diversity of the phloem mycobiome was found to differ significantly among the different genotypes of Norway spruce. Our genetic distance analysis shows no correlation between the genotypes and taxa communities, indicating no significant relationship between the genetic differentiation of the spruce genotypes used in this study and the composition of their fungal communities. The lack of correlation suggests that the genetic differences in our spruce seedlings are not the major drivers of mycobiome diversity. Other factors, such as environmental conditions (soil composition, moisture levels, temperature, and local microclimate, e.g. mycobiome present in the geographic location/origin of the cuttings), may have a greater impact on fungal community composition than the trees' genetic makeup. Another implication could be fungi's high adaptability and plasticity [72, 73], allowing them to respond to changing environmental conditions and establish associations with various host genotypes. Overall, the lack of correlation between genetic distance and mycobiome-based

distances in Norway spruce genotypes implies that the mycobiome is shaped by a complex interplay of environmental factors and fungal adaptability, highlighting the need for further research to unravel the specific mechanisms driving these interactions. Multiple research studies have provided evidence indicating that the fungal communities are influenced by the genetic makeup of the host organism [19–21, 74–77]. A study by [65] demonstrated that the host genotype played a primary role in shaping the composition of the needle mycobiome in Norway spruce clones. It was observed that clones with a higher degree of genetic similarity exhibited a greater resemblance in their mycobiome profiles. In contrast, [64] could show no correlation between genotypic traits and mycobiome community in Picea glauca, but instead, the composition of the mycobiome showed a strong and positive correlation with the location of the trees that were sampled. Specifically, when two trees were close to each other, their needle mycobiome exhibited greater similarity.

Our study shows no genotype or drought effect on the root fungal communities, which is supported by a study from [78], which shows no variations in mycobiome across different populations of Pinus pinaster. In contrast, [79] revealed a host and drought effect on the Ectomycorrhizal fungi composition in Pinus edulis. In the course of our study, we encountered a fundamental limitation that warrants consideration. Specifically, our sample size comprised only seven genotypes from four families. This limited genetic diversity, while reflective of the available resources, may have constrained our ability to draw comprehensive conclusions, particularly in the context of our primary research aim. This limitation is further highlighted by the results of Mantel tests, which failed to reveal significant correlations between genetic distance and species abundance. While our findings provide valuable insights, it is essential to acknowledge the potential impact of this limitation. A larger and more diverse sample size could have offered a broader perspective on the relationships we explored. Further studies with a wider array of genotypes and families are needed to verify and expand on our findings.

Mycobiome stability under abiotic disturbance (lower water availability)

In our study, most of the DSEs were more stable in their relative proportion in the low-watered plants than in the optimally watered plants as compared to the ECM fungi. *Thelephora terrestris* exhibited higher prevalence in our root samples but mainly in optimally watered plants. This fungus is one of the most common ectomycorrhizal fungi and has been suggested to be a strong competitor and capable of thriving in environments with limited species diversity [80]. The reduction in prevalence of ectomycorrhizal fungi in our study is consistent with results from [81], which also show that ectomycorrhizal fungi are more susceptible to drought

than dark septate endophytes. Castaño et al. [78] could also show reductions in ECM fungi under drought stress. Drought associated with a warming climate will undoubtedly continue to impact (negatively) the future prevalence and functions of ectomycorrhizal fungi. Ectomycorrhiza (ECM) and dark septate endophytes (DSEs) are important fungi in plant growth and ecological functioning by forming mutualistic associations with tree roots, contributing to nutrient uptake and cycling [82] and helping to cope with stress [37, 83], and pathogen attack [84]. Changes in water availability, such as drought, can alter the abundance and functioning of fungal communities in ecosystems, which can, in turn, affect plant health, nutrient cycling, and ecosystem productivity [79, 85]. Based on the review by [86], drought has been suggested to lower mycorrhizal abundance. In a recent work by [76], members of the Ascomycota phylum were increased under flood conditions in roots of *Ulmus minor* genotypes resistant to Dutch elm disease (DED), while the presence of Basidiomycota members was reduced. However, the impact of drought caused the opposite effect, slightly increasing the presence of Basidiomycota members while Ascomycota decreased. Persistent low water conditions also limit ectomycorrhizal fungi abundance [87].

Interestingly, in our study the response of specific mycobiome genera to water availability differed across the genotypes and families (in the phloem mycobiome), indicating a complex interplay between genetic factors and environmental conditions. The results of the study suggest that the abundance of certain fungal genera in the phloem of Norway spruce trees is influenced by water availability. Generally, the genera *Paraphaeosphaeria* and *Setomelanomma* showed a higher abundance in plants that were optimally watered compared to plants with low water availability. This trend was consistent across most of the different families and genotypes studied, indicating a general response of these fungi to changes in water availability. But in contrast, the low-watered genotype of family 40 had a higher abundance of *Paraphaeosphaeria neglecta* than in optimally watered plants in the same genotype. This exception suggests that the relationship between fungal abundance and water availability may be more complex than a simple positive or negative correlation.

Water availability may be a driving factor in shaping the diversity and abundance of fungi within the phloem mycobiome. Water availability might also be triggering a reaction in the plant cells, causing shifts in the abundance of these species such that the low-watered plants are subjected to a more competitive environment and, therefore, are more likely to harbour diverse microbial communities that enable them to survive under such conditions. The effects of climate change with lower precipitation are shown to increase the frequency of drought and

disease incidence rates [88]. Low water availability also alters the microbiome, particularly the bacterial composition in the soil [89]. Host mycobiome, depending on its composition and functional diversity among the co-inhabiting fungal species, can influence several processes responsible for plant growth [80]. These systems can, in turn, be affected by various factors, including environmental conditions and genetic variations within the host plant [70, 79].

The mycobiome as antagonist towards pathogens and Heterobasidion

Our findings show varying degrees of necrosis and relative fungal abundances across different families. It is important to note that metabarcoding data provides only relative abundance of species. An increase or decrease in the relative abundance of a species between treatments does not necessarily imply a corresponding change in the biomass of the species, but can also be caused by variation in the prevalence of co-existing species. Family 43 had the lowest necrosis and stood out from the other families studied as having the highest number of indicator species in the phloem. This suggests that the phloem of trees in family 43 is particularly conducive to the growth of these fungi and that these fungi may have a closer relationship with these trees compared to the other families studied. Of particular note is *Paraphaeosphaeria neglecta*, which was found in similar quantities across families 38, 40, and 41 but was much more abundant in family 43. This indicates that this fungus may have a particularly close association with the trees in family 43. These results may have implications for understanding the interactions between fungal species and their impact on plant health in forest ecosystems. Fungi are known to enhance plant growth [37], protect and increase plants' tolerance against stress, e.g. drought, high temperature, salinity, and pathogens [90–93]. The mycorrhizal fungus *Tricholoma vaccinum*'s protective function against pathogenic fungi was validated using dual cultures that included both *Botrytis cinerea* and *H. annosum* [84]. This experiment demonstrated decreased pathogen growth and increased survival rates of spruce trees. The symptoms on needles were also mitigated when the trees formed a symbiotic relationship with *T. vaccinum*.

Our antagonism assay shows *Phialocephala fortinii* and *Paraphaeosphaeria neglecta* to inhibit the growth of *H. parviporum*. Other species in the *Phialocephala* genus have been shown to inhibit the growth of *H. parviporum* in Norway spruce [24, 37]. Furthermore, our results also show that certain fungi, including *Setomelanomma holmii*, could significantly inhibit the growth of *Hymenoscyphus fraxineus* [94]. The function of *Setomelanomma holmii* in our study is, however, unknown. The ability of dark septate endophyte to suppress the growth of *H. annosum* was tested in a study by [95]; *Cadophora* sp. and *Phialophora mustea* were able to

substantially reduce the growth of *H. annosum*. The inhibitory effect of fungal endophytes has also been reported with other tree species. A study by [96] could show several fungi with inhibitory potential against *Sphaeropsis sapinea*. Arnold et al. [97] also demonstrated the role of endophytes in plant defense in a tropical tree species. *Theobroma cacao* seedlings were pre-inoculated with endophytes before inoculating with *Phytophthora*. *T. cacao* leaves not treated with endophytes showed higher leaf area damage when inoculated with *Phytophthora* pathogen than leaves pre-inoculated with endophytes. This shows that the endophytes confer some form of protection against the pathogen, thus limiting pathogen infection. In *U. minor*, endophytes primed the plant immune system against the DED pathogen [98], and the inoculation with endophytes showing activity against DED promoted root growth and photosynthetic rates as well as resistance to the pathogen [99, 100]. Kosawang et al. [94] hypothesized that fungal endophytes associated with *Fraxinus* sp. could protect them from ash dieback and possibly act as biocontrol agents.

Higher fungal diversity in stressed plants

Our results reveal greater fungal diversity in plants subjected to drought stress than in optimally watered plants. The mycobiome's response to drought can vary depending on the initial fungal diversity within the ecosystem. In some instances, environmental stress can reduce fungal diversity, particularly if sensitive species decline [101]. Conversely, it could favour either drought tolerant fungal species that enhance the tree's ability to cope with water stress by improving water and nutrient acquisition or benefit opportunistic pathogens that take advantage of the weakened host condition [101]. This diversification may be beneficial to the host, as in our case, there was an increased relative sequence abundance of fungal taxa with antagonistic effects on pathogenic fungi such as *Paraphaeosphaeria* and *Phialocephala* genera. These fungi were also shown to inhibit the growth of *Heterobasidion* in vitro in our study, thus they could potentially provide resistance against *Heterobasidion* infected trees in nature. However, it is worth noting that diversification may also have negative implications, such as the introduction of pathogenic fungi that could harm the tree host or compete with beneficial mycorrhizal fungi, reducing the tree's access to water and nutrients [102].

Conclusion

The results of this study highlight the importance of considering the genetic diversity of host plants when assessing the diversity and composition of fungal communities associated with them. *Paraphaeosphaeria neglecta* was an indicator species in family 43 that had the lowest

necrosis, and it also seemed to interact with *H. parviporum*. Not much is known about this fungus, and we cannot fully ascertain whether it competes in the same niche or performs completely different functions. It was found as an endophyte, but could it also be an opportunistic saprophyte? Is it a beneficial or opportunistic fungus? The findings of this study shed light on the intricate relationships between fungal communities and water availability in Norway spruce genotypes. While the general trend is that fungal diversity decreases under water stress, the exceptions found in this study suggest that further research is needed to understand the factors influencing fungal communities in forest ecosystems fully.
These findings suggest that the phloem mycobiome of Norway spruce is shaped by a combination of genetic and environmental factors and that specific mycobiome genera may have adaptive responses to abiotic stress. Most ectomycorrhiza fungi were more susceptible to low water availability than dark septate endophytes. Few endophytes were able to restrict the pathogen growth. So, can "mycobiome-associated-fitness" be real? It is clear, however, that trees have both beneficial and opportunistic fungi. The mycobiome should be included in resistance studies and could be considered one factor in plants' extended genotype variation against pathogens. How climate change will impact these fungi and their roles needs to be considered. Further research is required to explore the mechanisms underlying the observed differences in mycobiome diversity and composition among the genotypes and to determine the functional significance of these differences. These could have important implications for understanding the ecology and evolution of Norway spruce species and developing strategies for managing forest ecosystems under changing environ- mental conditions.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-023-03099-y.

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Authors' contributions

Blessing Durodola was responsible for maintaining the greenhouse experi- ment, measuring necrosis, and analyzing the results. Blessing Durodola and Eeva Terhonen developed the experimental design and watering protocol. Eeva Terhonen conceived the experiment, supervised its implementation, and analyzed some data. Blessing Durodola, Adedolapo Akinbobola, and Anna Kolehmainen carried out molecular experiments. Kathrin Blumenstein assisted with the greenhouse work and provided co-supervision for the experiments. Victor Chano calculated the genetic and mycobiome-based distances and performed the Mantel tests. Oliver Gailing offered technical advice. Blessing Durodola wrote the first draft of the manuscript, and all authors commented on and edited subsequent versions. All authors reviewed and approved the final manuscript. Blessing Durodola, Eeva Terhonen and Adedolapo Akinbobola edited the review.

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Availability of data and materials

The datasets produced and/or examined in the present research are accessible at NCBI under BioProject PRJNA990335; ascension numbers SRR25109452 - SRR25109534. The sequences for the isolates are available under the ascension numbers OR167041, OR167042, and OR167043.

Declaration

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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6. Supplementary Materials

Supplementary figures

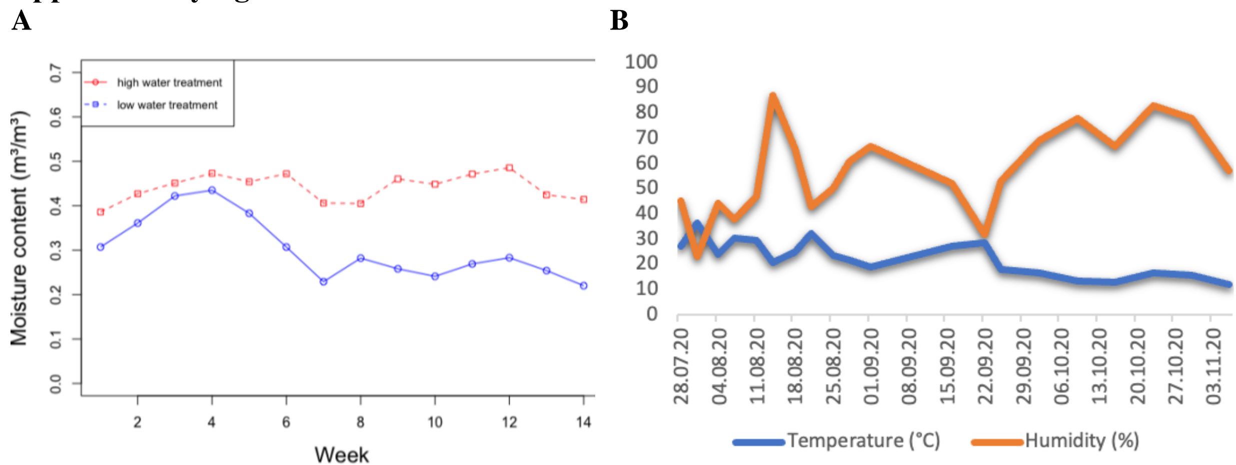

Fig. S1 Abiotic parameters measured during the experiment. **A)** soil moisture, **B)** atmospheric temperature and humidity.

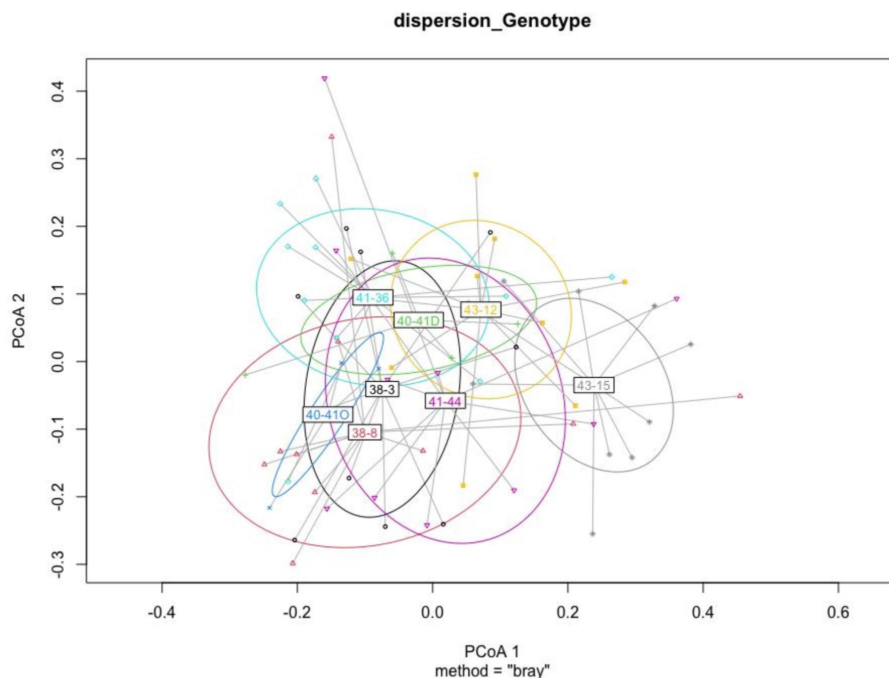

Fig. S2 Fungal communities among phloem genotypes based on Bray-Curtis.

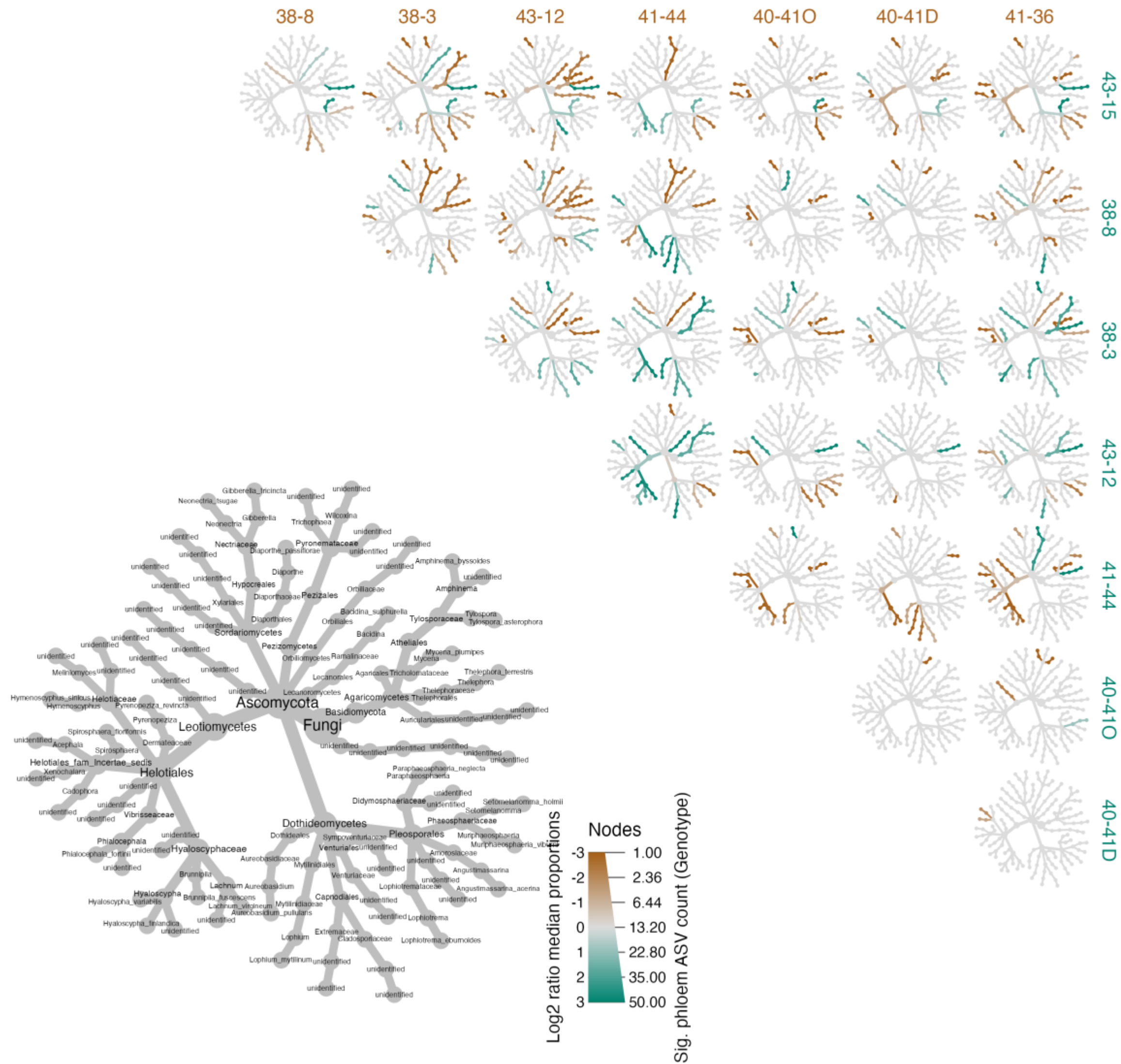

Fig. S3 Pairwise comparisons of the phloem mycobiome between the different genotypes. The colour of each taxon represents the log-2 ratio of median proportions of phloem ASV counts observed at each genotype. Taxa-coloured green are enriched in the genotypes shown in the rows, and those coloured cyan are enriched in the genotypes shown in the columns. The grey tree on the lower left serves as a key for the smaller unlabelled trees.

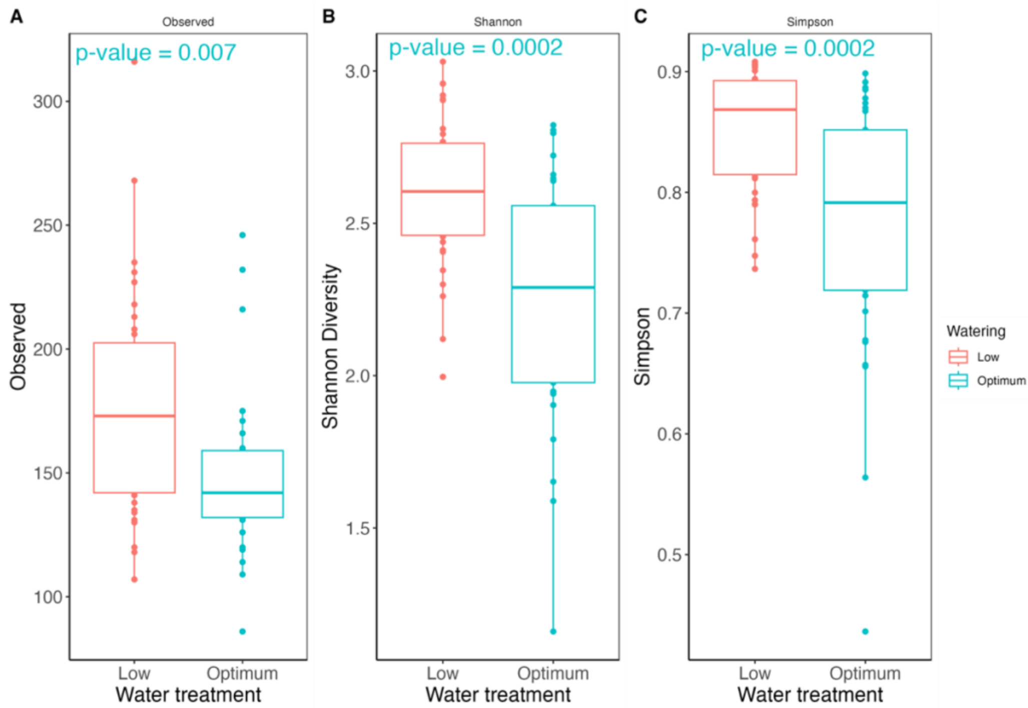

Fig. S4 Alpha diversity for water treatments. **A)** Observed amplicon sequence variants, B**)** Shannon diversity index, **C)** Simpson index.

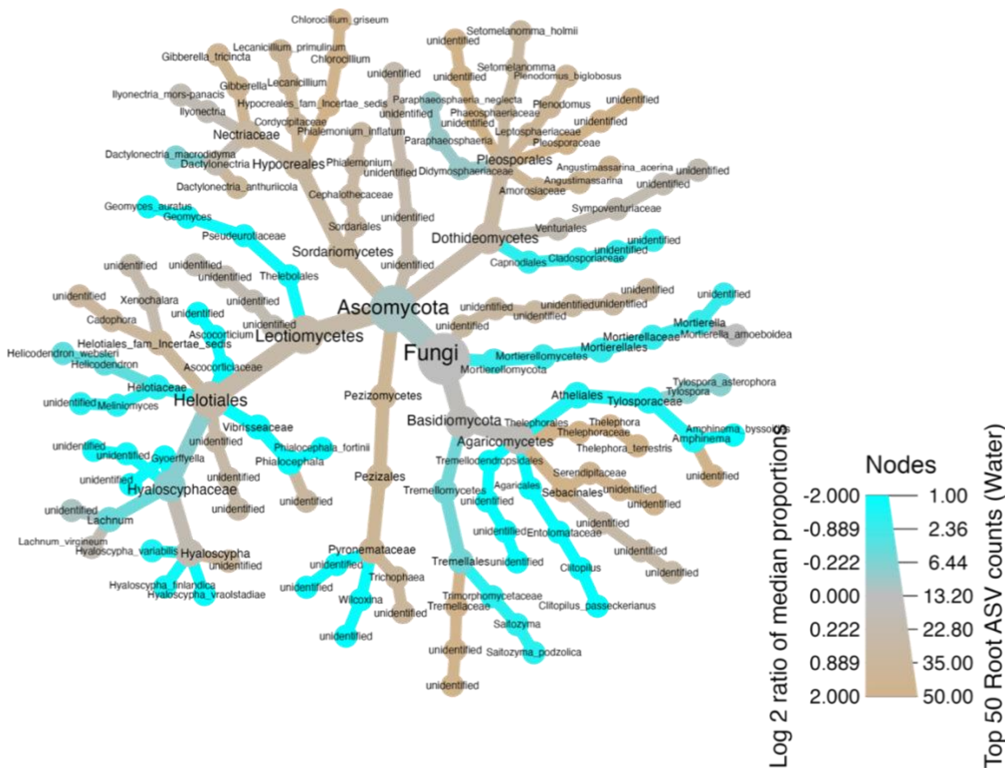

Fig. S5 Root mycobiome abundance according to watering groups. The colour of each taxon represents the log-2 ratio of median proportions of phloem ASV counts observed at each water treatment category. Taxa-coloured tan are more abundant in the optimum watering category, while taxa-coloured cyan are more abundant in low-water treated plants.

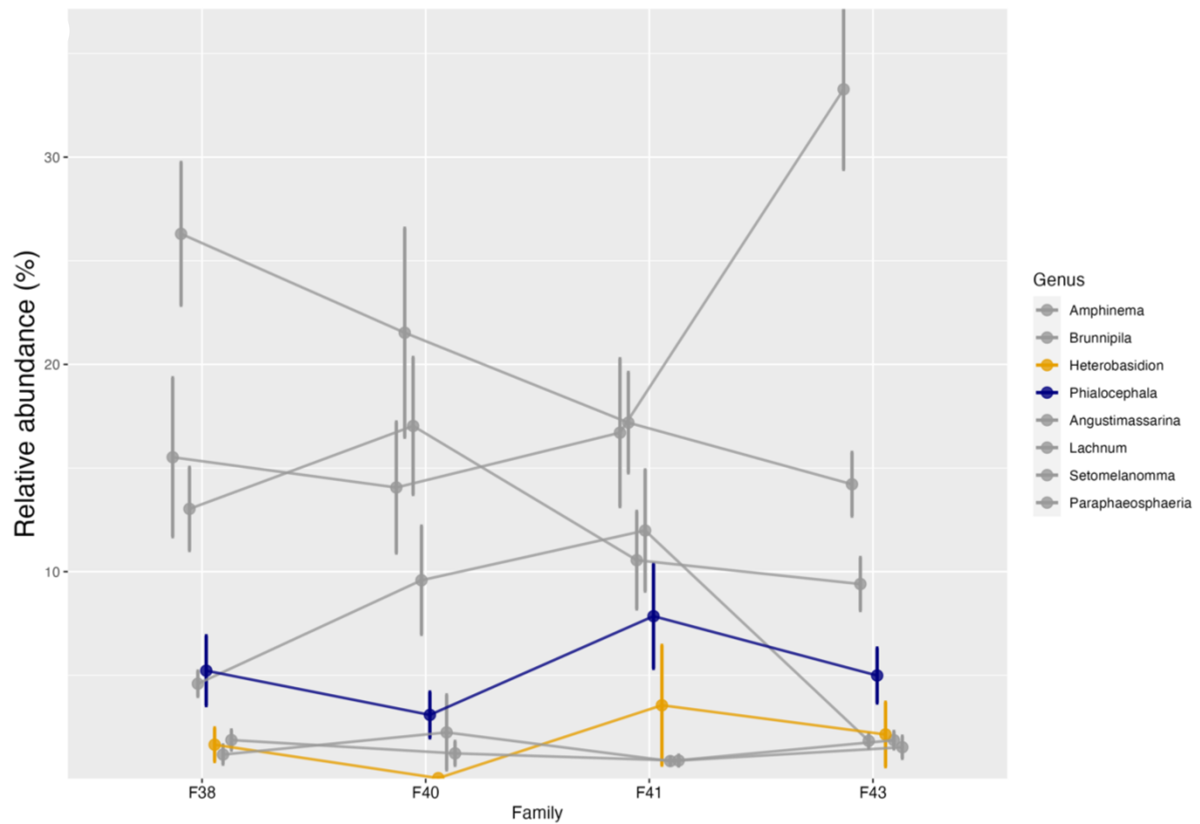

Fig. S6 Relative abundance of taxa of the top eight fungi among families in the presence of *H. parviporum* highlighting only the pattern between *Phialocephala* and *Heterobasidion* genera. Connecting lines are included to visualize common patterns.

Supplementary tables

Table S1 Information on phloem plant materials genotype and treatment.

Sample ID	Family	Genotype	Water-treatment	Inoculation treatment
D21	F38	38-3A	Drought	Hpa2
D37	F38	38-3B	Drought	Hpa1
D51	F38	38-3J	Drought	Non-treated
D52	F38	38-3D	Drought	Hpa1
D53	F38	38-3F	Drought	Hpa2
D54	F38	38-3G	Drought	Hpa2
D55	F38	38-3E	Drought	Hpa1
D56	F38	38-3C	Drought	Control
D57	F38	38-3I	Drought	Control
D58	F38	38-3H	Drought	Control
D11	F38	38-8J	Optimum	Non-treated
D12	F38	38-8F	Optimum	Hpa2
D13	F38	38-8G	Optimum	Hpa1
D14	F38	38-8C	Optimum	Control
D15	F38	38-8E	Optimum	Hpa1
D16	F38	38-8A	Optimum	Control
D17	F38	38-8I	Optimum	Hpa1
D18	F38	38-8H	Optimum	Control
D19	F38	38-8B	Optimum	Hpa2
D20	F38	38-8D	Optimum	Hpa2
D25	F40	40-41G	Optimum	Non-treated
D26	F40	40-41A	Optimum	Non-treated
D27	F40	40-41H	Drought	Non-treated
D28	F40	40-41J	Optimum	Non-treated
D29	F40	40-41B	Drought	Non-treated
D30	F40	40-41E	Drought	Non-treated
D85	F40	40-41I	Drought	Non-treated
D34	F41	41-36D	Drought	Hpa2
D35	F41	41-36B	Drought	Hpa1
D38	F41	41-36F	Drought	Control
D40	F41	41-36I	Drought	Non-treated
D41	F41	41-36C	Drought	Hpa1
D42	F41	41-36G	Drought	Control
D43	F41	41-36A	Drought	Control
D44	F41	41-36H	Drought	Hpa1
D45	F41	41-36E	Drought	Hpa2
D46	F41	41-36J	Drought	Hpa2
D24	F41	41-44I	Optimum	Control
D36	F41	41-44C	Optimum	Hpa2

D59	F41	41-44H	Optimum	Control
D60	F41	41-44A	Optimum	Non-treated
D61	F41	41-44D	Optimum	Hpa1
D62	F41	41-44J	Optimum	Hpa1
D63	F41	41-44B	Optimum	Control
D64	F41	41-44G	Optimum	Hpa2
D65	F41	41-44F	Optimum	Hpa2
D66	F41	41-44E	Optimum	Hpa1
D22	F43	43-12A	Drought	Hpa2
D23	F43	43-12F	Drought	Control
D31	F43	43-12J	Drought	Non-treated
D32	F43	43-12E	Drought	Control
D33	F43	43-12D	Drought	Hpa2
D39	F43	43-12H	Drought	Control
D47	F43	43-12C	Drought	Hpa1
D48	F43	43-12B	Drought	Hpa1
D49	F43	43-12G	Drought	Hpa2
D50	F43	43-12I	Drought	Hpa1
D10	F43	43-15H	Optimum	Hpa2
D1	F43	43-15F	Optimum	Hpa2
D2	F43	43-15E	Optimum	Control
D3	F43	43-15G	Optimum	Hpa1
D4	F43	43-15A	Optimum	Non-treated
D5	F43	43-15C	Optimum	Control
D6	F43	43-15I	Optimum	Hpa1
D7	F43	43-15B	Optimum	Hpa1
D8	F43	43-15D	Optimum	Control
D9	F43	43-15J	Optimum	Hpa2

Table S2 Abundance of dark septate endophyte (DSE) and Ectomycorrhizal (ECM) fungi in different root water treatments.

Phylum	Genera	Optimum	Low	Relative Abundance (%)	Fungi type
Ascomycota	*Phialocephala*	87491	144730	10.1	DSE
Ascomycota	*Cadophora*	2164	2015	0.2	DSE
Ascomycota	*Exophiala*	696	485	0.1	DSE
Ascomycota	*Acephala*	397	812	0.1	DSE
Ascomycota	*Gyoerffyella*	101	1029	0.0493	DSE
Ascomycota	*Cladophialophora*	36	34	0.0031	DSE
Ascomycota	*Capronia*	0	63	0.0028	DSE
Ascomycota	*Phialophora*	0	3	0.0001	DSE
Basidiomycota	*Amphinema*	224396	341543	24.7	ECM
Basidiomycota	*Thelephora*	211607	11165	9.7	ECM
Ascomycota	*Trichophaea*	173817	108305	12.3	ECM
Ascomycota	*Wilcoxina*	57384	118050	7.7	ECM
Basidiomycota	*Tylospora*	3430	1244	0.2	ECM
Basidiomycota	*Inocybe*	79	5	0.0037	ECM
Basidiomycota	*Hygrophorus*	10	0	0.0004	ECM
Basidiomycota	*Tomentella*	9	3	0.0005	ECM
Basidiomycota	*Laccaria*	5	0	0.0002	ECM
Ascomycota	*Geopora*	3	0	0.0001	ECM
Ascomycota	*Cenococcum*	0	24	0.0010	ECM
Basidiomycota	*Rhizopogon*	0	10	0.0004	ECM
Basidiomycota	*Amanita*	0	5	0.0002	ECM

Table S3 Pairwise genetic distance (below the diagonal) and pairwise distance based on taxa abundance (above the diagonal) for the genotypes used in both water regimes (optimum watering and drought stress).

	Optimum watering			
	38-8	**40-41**	**41-44**	**43-15**
38-8	--	43225.39	46753.1	41050.17
40-41	217.01	--	28012.91	20060.94
41-44	214.51	199.49	--	22450.02
43-15	215.6	218.11	215.47	--
	Low watering			
	38-3	**40-41**	**41-36**	**43-12**
38-3	--	31795.45	40825.93	50635.47
40-41	220.26	--	29683.51	31918.36
41-36	216.59	197.34	--	38605.93
43-12	216.66	217.4	214.25	--

Chapter IV – Leucoanthocyanidin Reductase 3 (*PaLAR3*) Locus in Norway Spruce (*Picea abies*) and Its Link to Resistance Against *Heterobasidion parviporum*

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Leucoanthocyanidin reductase 3 (*PaLAR3*) locus in Norway spruce (*Picea abies*) and its link to resistance against *Heterobasidion parviporum*

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Abstract

Heterobasidion parviporum is a fungal pathogen that is drastically damaging Norway spruce (*Picea abies*) in Europe. The infections will result in root and stem rot, causing significant economic losses for forest owners. Previous studies have shown that the *PaLAR3* gene, which encodes the leucoanthocyanidin reductase enzyme, can increase resistance to *H. parviporum* in Norway spruce. The presence of the B allele at the *PaLAR3* locus has been associated with higher (+)-catechin concentrations and increased enzyme production in inoculation experiments, resulting in inhibited pathogen growth. The control of *H. parviporum* involves a multifaceted approach, including silvicultural and sustainable forestry practices, genetic resistance, and chemical/biological control. In this study, we determined the *PaLAR3* genotypes in a representative sample of Norway spruce breeding materials from southern Finland and examined their effect on necrosis caused by *H. parviporum* in spruce stems. The results showed that the homozygous *PaLAR3BB* genotype was present in only 9% of the trees. However, the necrotic area interacted with homozygous *PaLAR3BB* under low-water treatment. These findings support the idea that the *PaLAR3* locus may be a valuable marker for identifying *P. abies* resistance to different strains of *Heterobasidion parviporum.*

Keywords: molecular marker, root rot, tree breeding

1. Introduction

Norway spruce (*Picea abies* (L.) Karst.) is one of the most economically significant tree species in Europe, playing a crucial role in the forest industry as a source of raw material (Skrøppa, 2003). This species contributes to mitigating the effects of climate change through carbon storage and conserving biological diversity, and it is a crucial part of Europe's ecosystem (Niinimäki et al., 2013). *Heterobasidion* root rot, caused by fungi of the genus *Heterobasidion*, is among the most severe threats to the economic use of spruce trees in boreal forests, as the decay can reach up to 12 meters in the stem of the infected individuals (Stenlid & Wästerlund, 1986). In Finland, two species of *Heterobasidion* are known to exist: *H. annosum* s.s. (Fr.) Bref. and *H. parviporum* Niemelä and Korhonen (Korhonen, 1978). These species have distinct host preferences, with Norway spruce being the primary host for *H. parviporum* and Scots pine (*Pinus sylvestris*) being the primary host for *H. annosum* s.s. (Korhonen, 1978; Capretti et al., 1990; Gonthier & Garbelotto, 2013). The distribution area of *H. parviporum* is assumed to follow Norway spruce to its northernmost habitats in the future (Korhonen et al., 1998; Korhonen & Lipponen, 2001).

The primary mode of infection is through spores entering wounds in the tree (Gonthier & Garbelotto, 2013), often facilitated by logging activities or natural factors. As the disease progresses, the fungus causes decay in the heartwood, weakening the tree and making it susceptible to wind throw (Netherer et al. 2021). Treating freshly cut stumps with chemical or biological control reduces the risk of spore production and further infections. However, eradication becomes impractical when the pathogen has been established in the host. This, in turn, increases the risk of secondary infections through root contacts. For this reason, we require additional control measures, e.g., increased host tree resistance.

As the climate changes, damages caused by fungal pathogens are expected to increase (Seidl et al., 2017), thus threatening the anticipated growth of wood productivity in forests. In addition, more abiotic disturbances, such as wind, snow, and drought in forest ecosystems, are expected to rise (Seidl et al., 2017; Senf & Seidl, 2021; Patacca et al., 2022). This could have some significant effects on forest health, e.g., the wind damage is enhanced by pathogens, such as *H. parviporum*, creating a positive feedback loop and reducing the stability of the trees and quality of the wood material (Krisans et al., 2020). Residuals of Norway spruce colonised by *H. parviporum* can serve as a persistent source of inoculum for multiple years, leading to

potential infections in nearby spruce seedlings of the subsequent tree generation (Piri & Hamberg, 2015). Also, the climate change-driven impacts on the water supply are likely to worsen (Senf & Seidl, 2021) by increasing drought-induced stress reactions in vegetation and making the trees more vulnerable to different pests and pathogens, including *H. parviporum* and *H. annosum* s.s. (Terhonen et al., 2019; Yeoh et al., 2021). However, under a pathogen attack, plants can fight the infection by, e.g., producing different toxic metabolites (Franceschi et al., 2005; Keeling & Bohlman, 2006). Therefore, enhancing tree tolerance to pathogens in the changing climate through tree breeding is essential.

An earlier study (Nemesio-Gorriz et al., 2016) discovered an association between *H. parviporum* resistance and Norway spruce's leucoanthocyanidin reductase 3 (*PaLAR3*) gene. A group of variants in this gene, designated as group B alleles, have been reported to improve the resistance of Norway spruce trees as they can limit the fungal *H. parviporum* growth in sapwood (Nemesio-Gorriz et al., 2016). The enzyme production, encoded by the *PaLAR3* gene, increases under a pathogen attack as a defence mechanism against the infection (Jyske et al., 2020). Nemesio-Gorriz et al. (2016) inoculated one fungal strain in one-year-old Norway spruce twigs and followed the fungal growth in the sapwood. One of the measures of susceptibility of Norway spruce trees against *H. parviporum* is the lesion length around the inoculation point (Lind et al., 2014). Understanding the potential of the *PaLAR3B* allele in the locus to the *Heterobasidion* resistance of Norway spruce would require further inoculation experiments. This study aimed to estimate the prevalence of the *PaLAR3* genotypes in a breeding population of Norway spruce and to determine whether the trees with a homozygotic *PaLAR3BB* or heterozygous *PaLAR3AB* genotype differ in their ability to resist an infection (as measured by the area of necrosis in phloem/sapwood) of two different strains of *H. parviporum*. We hypothesise that in *PaLAR3B* allele-containing genotypes, the necrosis caused by *H. parviporum* is smaller.

2. Materials and methods

2.1 Greenhouse experiment for necrosis assessment

The study material included 754 3-year-old rooted cuttings from 80 Norway spruce clones provided by the Natural Resources Institute Finland (Luke) on March 5, 2020 (Table 1). Each clone had 8–10 ramets. The clones are second-generation breeding material from southern

Finland, representing eight full-sib families produced through controlled crossing. The same material was also growing in Luke's Haapastensyrjä field unit (60°37.581′N, 24°27.581′E) as 10-year-old saplings from which the young ramets were previously derived.

Table 1: Plants used in the experiment and their respective treatments. Hpa1 refers to *Heterobasidion parviporum* strain 1, Hpa2 represents *Heterobasidion parviporum* strain 2, control indicates wounded plants (1.5% Malt Extract Agar), and NT signifies non-treated plants.

Treatment	Number of plants
Hpa1	212
Hpa2	209
Control	209
NT	124
Total	754

The experiment was carried out ex-situ at the Forest Botany and Tree Physiology greenhouse in Göttingen, Germany, to monitor the environmental parameters during the experiment (as described in Durodola, Blumenstein and Terhonen 2023). The rooted cuttings were transplanted into 3-litre plastic pots filled with 2.5 litres of fertilised peat soil (Flora Gard, TKS®2 Instant Plus, Hermann Meyer KG, Rellingen, Germany). Before and after the watering experiment (see below), the height and diameter of each sapling were measured to the nearest 0.1 cm, with the height measured again once the growth period had ended (Durodola, Blumenstein and Terhonen 2023).).

Two different fungal strains were used: *H. parviporum* strain 1 (Hpa1: strain number: SB2005 9.16, isolated from a Norway spruce stump) and *H. parviporum* strain 2 (Hpa2: strain number: SB 2014 2.69, isolated from a Norway spruce seedling) (collected by Dr. Tuula Piri). The non-treated plants were removed from the necrosis analysis as the aim was focused on the effect of *PaLAR3* genotypes on necrosis caused by *H. parviporum* infection. Therefore, 630 plants were used for the necrosis analysis, categorised into 73 clones and 8 families (ID: 38, 40, 41, 42, 43, 47, 48, 50) (Table 2). Prior to the inoculations, the fungal isolates were cultured on 1.5% Malt Extract Agar (MEA) for two weeks in the dark at +21°C. The inoculation holes were made approximately 10 cm above the stem base by pushing a sterile 5mm cork borer through the bark to the sapwood surface. Equal-sized plugs from pure cultures of *H. parviporum* (Hpa1 or Hpa2) or control (1.5% MEA) were placed onto the exposed surface and sealed with Parafilm®. Mock-inoculated trees with agar inoculation without fungi and non-inoculated trees were added to the experiment as control specimens. The watering treatments started in July

2020 (2020/7/22) and lasted 16 weeks, after which the plants were watered optimally until February 2021. The plants were divided into groups subjected to lower (50% of the optimal) and optimal watering. The amount of watering was adjusted according to the temperature and soil moisture (HH2 device equipped with the ML2x sensor [Delta-T Devices Ltd., Cambridge, UK]) that were monitored and recorded throughout the experiment.

Table 2: *PaLAR3* genotype prevalence (*PaLAR3AA*, *PaLAR3AB* and *PaLAR3BB*) among plant genotypes in necrosis analysis

Family	Genotype	Number of clones	Percentage (%)
38	AA	5	6.85
38	AB	3	4.11
38	BB	0	0.00
40	AA	10	13.70
40	AB	4	5.48
40	BB	0	0.00
41	AA	5	6.85
41	AB	2	2.74
41	BB	0	0.00
42	AA	14	19.18
42	AB	0	0.00
42	BB	0	0.00
43	AA	4	5.48
43	AB	0	0.00
43	BB	0	0.00
47	AA	2	2.74
47	AB	7	9.59
47	BB	5	6.85
48	AA	9	12.33
48	AB	0	0.00
48	BB	0	0.00
50	AA	0	0.00
50	AB	1	1.37
50	BB	2	2.74
Total 8		**73**	**100.00**
Summary of *PaLAR3* prevalence among samples			
	Genotype	**Number of samples**	**Percentage (%)**
	AA	424	67.30
	AB	143	22.70
	BB	63	10.00
Total		**630**	**100.00**

The optimally watered group initially received 576 mL twice weekly, while the low water treatment group received 288 mL twice weekly. Subsequently, the water amounts were adjusted to 384 mL and 192 mL twice weekly. On August 18, 2020, the watering quantity was further reduced to 192 mL and 96 mL twice weekly for both optimal and low watered groups respectively. After the experiment, the bark of each specimen was peeled off to reveal the necrotic tissue. The length and width of the necrotic area on each tree was measured from the phloem and the sapwood (Durodola, Blumenstein and Terhonen 2023).). The area of the necrotic lesion was determined by using the equation to count the area of an ellipse ($A = \pi r_1 r_2$), in which the values of the two radii were determined by dividing the total lengths (r_1) and widths (r_2) of the lesion into two. The necrotic areas were calculated both for phloem and sapwood.

2.2 DNA extraction, PCR, and gel electrophoresis for genotyping PaLAR3

Needle samples were collected from each cutting's original specimen (10-year-old tree) in the Haapastensyrjä field unit as described in Terhonen et al. (2022). The needles were stored in 1.5mL Eppendorf tubes and kept in a -20°C freezer before the DNA extractions. DNA was extracted using the Qiagen DNeasy® Plant Pro Kit (Qiagen, USA). Briefly, 70-100 µg of needle material was transferred into a ready-to-use tissue disruption tube, following the addition of lysis buffers. The samples were then incubated at +65°C for 15 minutes. The lysis of the samples was done in Tissuelyser (Tissuelyser II, Qiagen, USA) at 24Hz twice for two minutes. After the lysis, the samples were centrifuged in 14 000 RFC (relative centrifugal force, g-force) for 5 min, and 400 µL of the supernatant was pipetted for DNA extraction. DNA was extracted using a Qiagen DNeasy® Plant Pro Kit and Qiacube DNA extraction automate, intended for fully automated DNA processing. After the DNA extraction, the concentration and purity of the DNA were measured using NanoDrop™ One/One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific™, USA).

After the DNA extraction, PCR was carried out using a ThermoFisher Scientific™ (USA) DreamTaq Green DNA Polymerase (5U/µL) kit. *PaLAR3* locus-specific primers were designed (Edesi et al., 2021) against the genomic sequence of *PaLAR3* (KX574230.1 and KX574229.1) for one locus and two alleles. To detect *PaLAR3* alleles, 10–40 ng genomic DNA per sample was used in each 12.5 µL PCR reaction. In each reaction, 0.4 µM of locus-specific LAR_ComA primer (5' GAAATCTGCAGCCAATGGA 3') (Edesi et al., 2021) and 0.2 µM of each allele-specific primer (LAR_B2: 5' CTGTATAACCGTAACATCTACTG 3', and LAR_A: 5' GAACGGGTATAAACTCCGT 3') (Edesi et al., 2021) were included. For each reaction

200 μM dNTP and 0.2 U/μl DNA polymerase (DreamTaq DNA Polymerase, Thermo Scientific™, USA) were used. The total reaction volume was adjusted for 12.5 μL with autoclaved MQ water. 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. Isolates with a known *PaLAR3AB* genotype were used as positive controls (Terhonen et al., 2022). The presence of *PaLAR3A* and *PaLAR3B* alleles or both was determined by the visual detection made by ultraviolet transillumination of DNA amplicons on a 1.5% agarose gel, stained with ethidium bromide (1-1.5 hours, 120V) (Terhonen et al., 2022). The *PaLAR3A* allele formed a band with a size of 110 bp and *PaLAR3B*, a size of 200 bp (Edesi et al., 2021; Terhonen et al., 2022).

2.3 Additional DNA isolation and qPCR-based validation of PaLAR3 genotypes

The DNA extraction process was conducted following the method described by Pirttilä et al. (2001) using fresh needles (50-100 mg) ground in liquid nitrogen. The DNA quality was tested using a nanodrop spectrophotometer. The extraction process involved the addition of prewarmed LiCl and CTAB extraction buffer to the sample, followed by adding chloroform and isoamyl alcohol and centrifugation. The DNA was then precipitated with isopropanol and absolute ethanol, washed in 70% ethanol, and dissolved in water. The DNA templates were diluted to half of their volume with water before being used in quantitative PCR (qPCR) using EvaGreen dye (Solis BioDyne, Estonia) on a Rotor-GeneQ device (Qiagen, USA). The validation of *PaLAR3* alleles was performed using allele-specific primers, as described by Nemesio-Gorriz et al. (2016). The qPCR program consisted of an initial denaturation step of 15 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 60°C, and 30 seconds at 72°C. The melting curves were also analysed, ranging from 62°C to 99°C.

2.4 Statistical methods

First, we assessed the distribution of datasets in the R statistical software (R Core Team, 2023) by employing the Shapiro-Wilk test. Since the data did not follow a normal distribution, we utilised the Levene test to examine the homogeneity of variances. The Kruskal-Wallis test was used for homoscedastic data (PaLAR3 genotypes and watering treatments). In instances where the data exhibited heteroscedasticity (unequal variance), we applied Welch's ANOVA (inoculation treatment). Subsequently, post-hoc analyses were conducted when there were variations in a dependent variable among treatments. We utilised Dunn-Bonferroni or Games-Howell multiple comparison tests depending on the specific assessment method (Kruskal-

Wallis or Welch-ANOVA, respectively). To analyse necrosis, we studied how certain factors (*PaLAR3* genotypes – *PaLAR3AA*, *PaLAR3AB*, *PaLAR3BB*; inoculation types - two strains of *H. parviporum* and control; water treatment - low and optimal) affect the lesion area in the sapwood. For this analysis, we used generalised linear mixed models with the lme4 package (Bates et al. 2015) in R. Additionally, for plant growth, we looked at how the *PaLAR3* genotypes and water treatment influenced plant diameter and starting height. In our models, we considered and included the origin of Norway spruce saplings (family) as a random factor, as only one family had all *PaLAR3* genotypes. Therefore, we could not use 'family' as an explanatory variable.

3. Results

3.1 Genotype

The most prevalent genotype among the samples was the homozygous *PaLAR3AA* genotype, which was found in 424 samples (67.3%). The heterozygous genotype *PaLAR3AB* was found in 143 samples (22.7%), while 63 samples had the homozygous *PaLAR3BB* genotype (10%) (Table 2).

3.2 Necrosis

The necrosis model shows significant differences between the inoculation treatments (p = <2e-16). Also, significant differences in the lesion area in sapwood based on the individual effects of *PaLAR3* genotypes and water treatment were shown (Table 3). The interaction effects suggest that the influence of certain factors such as *Heterobasidion* strains, *PaLAR3* genotypes, and water treatments on the lesion area in sapwood can vary depending on the presence of other factors. Some of these interactions are significant, showing that the effect of one variable depends on the levels of others. The interaction effect between factors Hpa2 (*H. parviporum* strain 2) and Genotypes BB (*PaLAR3BB*) is significant (p = 0.0167) (Figure 1A), indicating that the smaller necrotic areas caused in the sapwood by *H. parviporum* strain two may be influenced by the presence of genotype *PaLAR3BB*. Similarly, the interaction effect between *H. parviporum* strain 1 (Hpa1) in the optimal watered plants was significant (p = 0.0160), suggesting that the impact of Hpa1 on the lesion area is significantly affected by optimum watering compared to the low watering treatment (Figure 1B). Overall, regardless of the water

treatments, a consistent pattern of decline in lesion area is evident across *PaLAR3* genotypes (from *PaLAR3AA* to *PaLAR3BB*) due to *H. parviporum* inoculation (Figure 1C).

3.3 Growth

Similar to the necrosis results, the most prevalent genotype among the samples was the homozygous *PaLAR3AA* found in 500 samples (66%). The heterozygous *PaLAR3AB* genotype was found in 185 samples (25%), while 69 samples had the homozygous *PaLAR3BB* genotype (9%) (Table 4).

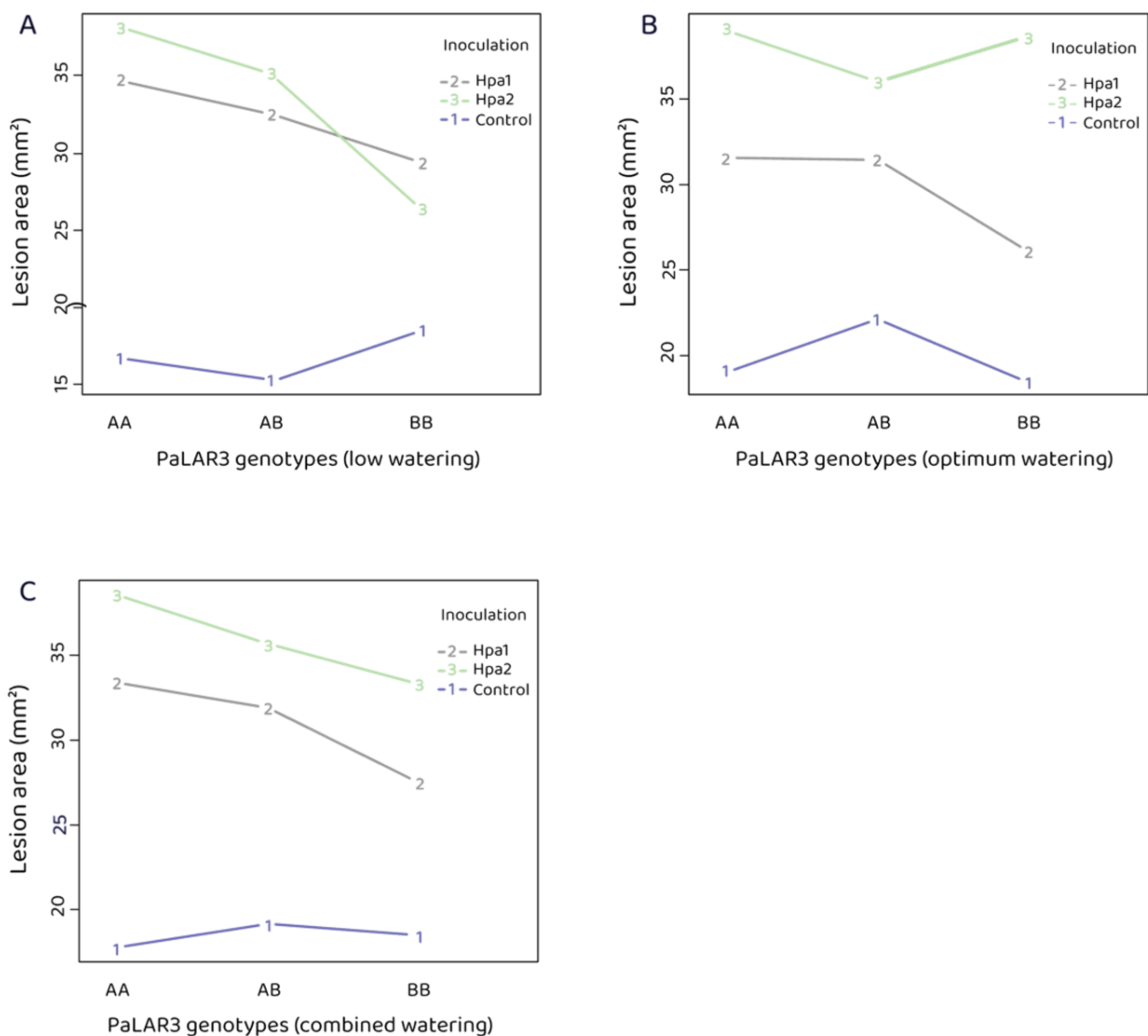

FIGURE 1: Lesion area in sapwood between Norway spruce *PaLAR3* genotypes due to *H. parviporum* strain 1 (Hpa1), *H. parviporum* strain 2 (Hpa2), and the mock-control in A) low-watered plants, B) optimally watered plants and C) combined, i.e. all plants.

Table 3: Variations in the lesion area within the sapwood of Norway spruce plants, which were subjected to inoculation with two *H. parviporum* strains (including a mock control), *PaLAR3* genotypes (AA, AB, and BB) under different water treatments (low and optimum). The data analysis employed generalised mixed linear models, with statistical significance at $p < 0.05$.

	Estimate	Std. Error	t-value	P-value
Intercept	2.79100	0.05877	47.487	**<2e-16**
Inoculation Hpa1 [a]	0.73045	0.06179	11.822	**<2e-16**
Inoculation Hpa2 [b]	0.81263	0.06210	13.085	**<2e-16**
Genotypes AB [c]	-0.09148	0.10110	-0.905	0.3655
Genotypes BB [d]	0.14381	0.14539	0.989	0.3226
Watering Optimum [e]	0.13031	0.06643	1.962	**0.0498**
Inoculation Hpa1: Genotypes AB [f]	0.03265	0.13673	0.239	0.8113
Inoculation Hpa2: Genotypes AB [g]	0.03129	0.13817	0.226	0.8209
Inoculation Hpa1: Genotypes BB [h]	-0.27439	0.19406	-1.414	0.1574
Inoculation Hpa2: Genotypes BB [i]	-0.46471	0.19418	-2.393	**0.0167**
Inoculation Hpa1: Watering Optimum [j]	-0.22518	0.09343	-2.410	**0.0160**
Inoculation Hpa2: Watering Optimum [k]	-0.10206	0.09380	-1.088	0.2766
Genotypes AB: Watering Optimum [l]	0.23060	0.13427	1.717	0.0859
Genotypes BB: Watering Optimum [m]	-0.14050	0.18482	-0.760	0.4472
Inoculation Hpa1: Genotypes AB: Watering Optimum [n]	-0.19418	0.18709	-1.038	0.2993
Inoculation Hpa2: Genotypes AB: Watering Optimum [o]	-0.26206	0.18814	-1.393	0.1637
Inoculation Hpa1: Genotypes BB: Watering Optimum [p]	0.11115	0.26054	0.427	0.6697
Inoculation Hpa2: Genotypes BB: Watering Optimum [q]	0.49251	0.26076	1.889	0.0589

The intercept represents the baseline, i.e., mock control, AA genotypes, and low-watering genotypes. The superscript letters denote the differences/interaction with reference to the intercept.

[a] Interaction between intercept/mock control and Hpa1
[b] Interaction between control and Hpa2
[c] Difference between genotypes AA and AB
[d] Difference between genotypes AA and BB
[e] Difference between low and optimal watering
[f] Interaction effect between Hpa1and genotypes AB
[g] Interaction effect between Hpa2 and genotypes AB
[h] Interaction effect between Hpa1and genotypes BB
[i] The interaction effect between Hpa2 and genotypes BB
[j] Interaction effect between Hpa1and optimum watering
[k] Interaction effect between Hpa2 and optimum watering
[l] Interaction effect between genotype AA and optimum watering
[m] Interaction effect between genotype BB and optimum watering
[n] Interaction effect between Hpa1, genotype AB and optimum watering
[o] Interaction effect between Hpa2, genotype AB and optimum watering
[p] Interaction effect between Hpa1, genotype BB and optimum watering
[q] Interaction effect between Hpa2, genotype BB and optimum watering

Table 4: *PaLAR3* genotype prevalence among plant genotypes in growth analysis

***PaLAR3* genotype**	**Number of samples**	**Percentage (%)**
PaLAR3AA	500	66
PaLAR3AB	185	25
PaLAR3BB	69	9
Total	**754**	**100**

3.4 Starting height

The initial heights of plants significantly differed among the *PaLAR3* genotypes (p = 0.002). These findings indicate that plants carrying the *PaLAR3BB* genotypes demonstrated a notably greater height than those with the *PaLAR3AA* (p = 3.91e-3) and AB (p = 1.86e-3) genotypes

(Figure 2). No statistically significant differences were observed between the plants with *PaLAR3AA* and AB genotypes (Figure 2), and no significant differences occurred in the diameter among *PaLAR3* genotypes (p = 0.61).

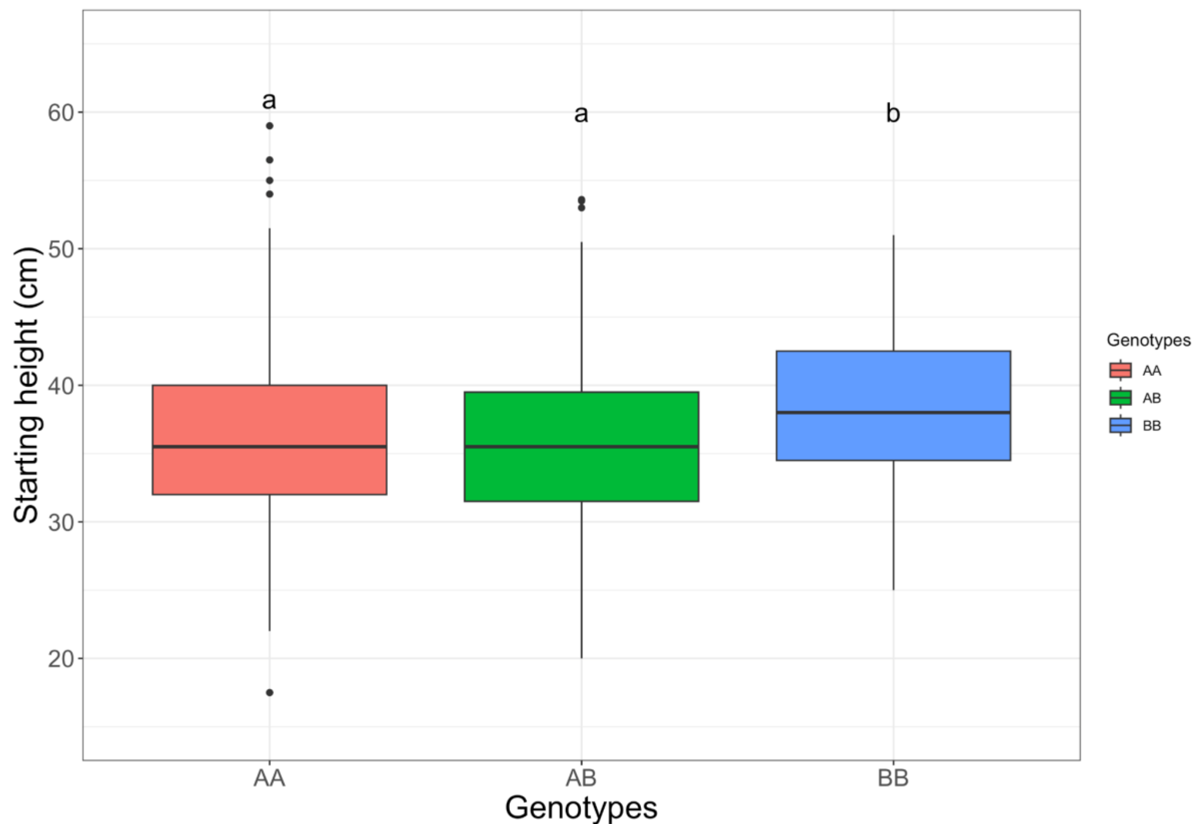

FIGURE 2: A boxplot comparing the starting height (cm) of the Norway spruce saplings at the beginning of the experiment. Each box represents a different *PaLAR3* genotype (AA in red, AB in green and BB in blue). The letters above the boxes determine the significantly different groups, where group "a" is significantly different from group "b" (p = 0.002). The line in each box represents the median and the whiskers show the distribution of the individual samplings.

4. Discussion

We investigated the prevalence of *PaLAR3* genotypes in clonally propagated Norway spruce saplings and discovered a predominant presence of the homozygous *PaLAR3AA* genotype, constituting 66% of the samples. *PaLAR3AB* and *PaLAR3BB* genotypes accounted for 25% and 9%, respectively. We observed a significant decrease in sapwood lesion area only for the *PaLAR3BB* genotype under reduced watering. This contradicts our hypothesis that homozygous and heterozygous *PaLAR3B* genotypes differ in their resistance to *H. parviporum*. The results of the necrosis model reveal substantial differences among the inoculation treatments, indicating an evident influence of the strain's impact on the observed necrosis area

(Simonen, 2023). However, we found marginally significant differences when assessing the individual effects of *PaLAR3* genotypes and water treatment on the lesion area in the sapwood. This implies that the specific *PaLAR3* genotypes and water treatment levels may contribute meaningfully to the observed variations in the lesion area in the sapwood. In addition, *PaLAR3* might be a factor contributing to drought tolerance in the trees as well. Moreover, examining interaction effects underscores the complexity of factors influencing the lesion area. Some of these interaction effects were statistically significant, indicating that the impact of one variable is contingent upon the levels of others. For instance, the interaction effect between *H. parviporum* strain 2 (Hpa2) and genotype *PaLAR3BB* is noteworthy, suggesting that the reduced necrosis caused by *H. parviporum* strain 2 in the sapwood may be influenced by the presence of the *PaLAR3BB* genotype under limited water treatment. Simonen (2023) also noted that *H. parviporum* exhibited similar growth across all *PaLAR3* genotypes. These findings emphasise the intricate interplay of multiple factors in determining the extent of necrosis in the sapwood. Understanding these interactions is crucial for a more comprehensive grasp of the dynamics involved in response to *H. parviporum* strains, the influence of specific plant genotypes, and environmental conditions such as water availability.

The observed height differences among genotypes highlight the potential role of the *PaLAR3* gene in modulating plant growth. The specific attributes associated with the *PaLAR3BB* genotype seem to contribute to enhancement in plant height. As demonstrated by Simonen in 2023, plants carrying the *PaLAR3BB* genotype exhibited greater growth in height compared to plants with alternative genotypes. This finding aligns with the notion that specific genetic variations can significantly influence observable characteristics or phenotypic traits, such as the height of plants (Zeltiņš et al., 2022; Chen et al., 2018). These results may have implications for plant breeding and genetic selection strategies. If the *PaLAR3BB* genotype consistently results in taller plants, this genetic trait could be targeted for cultivation in breeding programs aimed at enhancing plant height. However, further research is necessary to explore the underlying mechanisms by which the *PaLAR3* gene influences plant growth and to ascertain whether other factors may interact with these genotypic effects.

Gene interactions in terrestrial plants are complex, and efforts to investigate them have been made during the past decade (Zhou & Zhang, 2020). Since the *P. abies* genome has a comparatively high fraction of repetitive sequences in its overall huge and complex genome, as typical for conifers (Bernhardsson et al., 2019), it is a challenging species in which to explore

expressed genes and to map their functional traits. Two-thirds of the total *P. abies* genome size (12 Gbp out of 20 Gbp) have been assembled, containing the majority of expressed genes (Nystedt et al., 2013; De La Torre et al., 2014). Nevertheless, discovering other candidate genes related to the defence against fungal pathogens is a fairly unexplored field, and the potential for discovering other genes is high in the future. So far, in addition to *PaLAR3*, other candidate genes have been associated with resistance against *H. parviporum* infections in Norway spruce (Elfstrand et al., 2020; Chaudhary et al., 2020), and it has become evident that the root rot resistance in Norway spruce is a quantitative trait (Lind et al., 2014; Capador-Barreto et al., 2021).

As climate change not only supports the further spread of fungal pathogens but also increases abiotic stress, such as drought, the challenges for spruce trees will become manifold. In addition to genes resistant to pathogens such as *Heterobasidion* spp., the genetic adaptation to drought as a physiological response will also strengthen the trees (Yeoh et al., 2021). Studies by Depardieu et al. (2021; 2020) have shown that 285 genes in spruce are linked to adaptation to climate, and 110 genes can be connected to drought response. Further, *P. abies* has a strong production of terpenes and phenolics for defence against bark beetles and pathogens (Danielsson et al., 2011; Keeling & Bohlmann, 2006). Those diterpene acids inhibit the growth of fungal pathogens and may even kill them (Kusumoto et al., 2014). Studies by Schiebe et al. (2012) and Zhao et al. (2011) have shown that differences in Norway spruce genotypes with a higher defence against fungal infections are correlated with a higher terpene response. Mainly (+)-3-carene was observed to be highly induced by *H. parviporum* inoculation (Danielsson et al., 2011; Zhao et al., 2010).

Conclusion

In homozygous *PaLAR3BB* genotypes, the necrotic area was smaller (under limited water) than those with the homozygous *PaLAR3AA* and heterozygous *PaLAR3AB* genotypes, even though they were present in fewer quantities than their counterparts. The observed patterns in growth parameters emphasise the intricate interplay between genetic factors and tree physiology. These findings enhance our understanding of the genetic basis of resistance to *H. parviporum*. It suggests that just having the *PaLAR3BB* genotype alone does not automatically make Norway spruce more resistant to this infection, and there could be other factors involved that make it more complicated. Our findings, however, provide a foundation for further exploration of the molecular mechanisms underlying these interactions in the resilience of the trees towards biotic and abiotic stress factors, giving valuable information for breeding.

Authors Contributions

ET, BD, and JH designed the study. BD, KM and NH performed the experiments. BD analysed the data, and BD and NH wrote the first draft of the manuscript. MH provided the sapling material used in this study. TP provided the fungal strains used in this study. ET, JH and KB conceived the study. BD, ET, KB, MH, MK, and NH, edited the manuscript. BD and NH revised the manuscript.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting the findings of this study are available from the authors upon reasonable request.

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