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The reaction zone as a defence response in Norway spruce (*Picea abies*) when infected by the white-rot fungus *Heterobasidion parviporum*: differentially expressed genes and chemical changes

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Preface

This thesis is written as the final paper of the master program in Forestry at the Norwegian university of Life Sciences.

When first selecting a subject for my master thesis, I wanted to approach obstacles related to root and butt rot in Norway. Forestry is a significant part of the annual income at my parents' farm and there I have witnessed rot degrading large amounts of timber first hand, as many other forest owners have. Contributing to information about this fungus was for me an interesting topic and diving into the chemistry and genetics of this subject has been demanding, but very valuable.

I would like to thank my main supervisor associate professor Line Nybakken for her guidance and help during the thesis. I would also like to thank my co-supervisors senior research scientists Carl Gunnar Fossdal and Halvor Solheim, as well as lead engineer Inger Heldal and post doctoral researcher Elena Carneros for all their help and advice.

I would also like to thank NordGen, the department of ecology and natural resource management at the Norwegian university of Life Sciences and the Norwegian Forest and Landscape Institute for their financial support.

Finally, I want to thank my family and my fellow forestry students for their company and support during the long hours at the forestry study room.

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Abstract

Heterobasidion parviporum (Fr.) is a pathogenic white-rot fungus that causes root and butt rot in Norway spruce (*Picea abies* (L.) Karsten), leading to tremendous economical losses in Norway and the rest of Europe. Knowledge of defense responses to infection of the pathogen will enable us to breed for more resistant plant material. We focused on the reaction zone in sapwood, which occurs as a defense response when sapwood is infected with the pathogen. Sapwood in 60- and 23 year old Norway spruce trees were treated with wounding or inoculation of *H. parviporum* or methyl jasmonate mimicking infection from the heartwood and inducing a reaction zone. Chemical and gene expression analysis was conducted on samples from experimental inductions of chemical reaction zone around the inoculation and wound in sapwood.

The stilbenes E-Astringin and iso-rhaphontin and one chlorogenic acid derivative were identified with HPLC analysis, and decreased in concentration upon pathogen inoculation and wounding in experiment 1 and 2, respectively. Several unidentified phenolic compounds (UPC) decreased upon either wounding or pathogen inoculation, or both. Gene expression was quantified using qRT PCR analysis, verifying an induction of genes connected to the phenylpropanoid pathway. In experiment 1, CCoAOMT1, CCoAOMT2, HCT, DAHP2, ANH2 and PAL2 were up regulated upon pathogen inoculation at different depths. MYB8 showed differences in expression between the inoculation depths. Wounding caused an up regulation of PAL2 and CCoAOMT1 in experiment 2, and down regulation of STS. PAL2 was up regulated upon methyl jasmonate inoculation.

The decrease of stilbenes can be linked to down regulation of STS. We hypothesize that this is a sign of a degradation of the stilbenes, which in turn may be converted to compounds with inhibitory effects on pathogen infection. As we did not detect any increase in any other compounds, we have no good explanation for the observed decreased concentration of chlorogenic acid derivatives. The up regulation of DAPH2 and ANH2 may be connected to the reallocation of carbon to the reaction zone, while the increased expression of PAL2 catalyzes the phenylpropanoid pathway, where HCT, CCoAOMT1 and CCoAOMT2 are involved. This indicates an increase in defense related genes upon infection or wounding in sapwood, and to some degree also by inoculation of methyl jasmonate.

Sammendrag

Heterobasidion parviporum (Fr.) er et hvitråtepatogen som forårsaker rotkjuke i gran (*Picea abies* (L.) Karsten), og fører til enorme økonomiske tap i Norge og Europa. Ved å forstå forsvarsresponser ved infeksjon av patogenet kan vi foredle mer forsvarsdyktig plantemateriale. Vi fokuserte på reaksjonssonen i yteved, som oppstår som en forsvarsrespons når yteved blir infisert med patogen. Yteved i 60- og 23 år gamle grantrær ble enten såret eller inokulert med *H. parviporum* eller metyljasmonat for å etterligne infeksjon fra kjerneveden og indusere en reaksjonssone. Analyse av kjemisk konsentrasjon og genekspresjon ble utført på prøvene fra den eksperimentelle induksjonen av den kjemiske reaksjonssonen rundt inokuleringen og såringen i yteveden.

Stilbenene E-Astringin og iso-rhaphontin og et klorogensyre derivativ ble identifisert ved bruk av HPLC, og sank i konsentrasjon ved patogeninokulering og såring i henholdsvis eksperiment 1 og 2. Uidentifiserte fenolkomponenter (UPC) sank i konsentrasjon enten ved såring eller patogeninokulering, eller begge deler. Genekspresjon ble kvantifisert ved qRT PCR analyse. Dette verifiserte en induksjon av gener som er knyttet til fenylpropanoidreaksjonene. CCoAOMT1, CCoAOMT2, HCT, DAHP2, ANH2 og PAL2 ble oppregulert i eksperiment 1 ved patogeninokulering der inokuleringsdybden var forskjellig. MYB8 hadde forskjellig ekspresjon ved ulik inokuleringsdybde. Såring forårsaket oppregulering av PAL2 og CCoAOMT2 i eksperiment 2, og en nedregulering av STS. PAL2 ble oppregulert av metyljasmonat.

Nedgangen i stilbenene kan være knyttet til nedreguleringen av STS. Vi hypoteserer at denne nedgangen er en degradering av stilbener, som konverteres til komponenter som kan ha inhiberende effekter på patogeninfeksjoner. Siden vi ikke fant oppregulering i noen andre komponenter, har vi ingen god forklaring på den observerte nedgangen av klorogensyre derivativer etter infeksjon eller såring. Oppreguleringen av DAHP2 og ANH2 kan være knyttet til reallokeringen av karbon til reaksjonssonen, mens den økte ekspresjonen av PAL2 katalyserer fenylpropanoid-reaksjoner der HCT, CCoAOMT1 og CCoAOMT2 er involvert. Dette kan indikere en økning i forsvarsgener ved infeksjon eller såring i yteved av gran, og til en viss grad også av inokulering av metyljasmonat.

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1 Introduction

1.1 Background

Heterobasidion parviporum (Fr.) is a pathogenic white-rot fungus that causes root and butt rot in Norway spruce (*Picea abies* (L.) Karsten), and is an extensive problem in Norway and the rest of Europe. The *Heterobasidion* genus was connected to disease of conifers in 1874 by Robert Hartig, and *Heterobasidion parviporum* was described as one of eight taxonomic species within the genus in 1998 by Niemelä and Korhonen (Woodward et al., 1998). This genus is estimated to cause economical losses up to 800 million euros annually in Europe (Asiegbu et al., 2005), and is known for being one of the most economically destructive organisms in forest ecosystems (Oliva et al., 2012).

The forestry sector in Norway relies on the production and harvest of conifer species like Norway spruce, which are prone to this pathogen (Woodward et al., 1998). This has led to extensive research on this necrotrophic pathogen in order to reduce or eliminate this problem by breeding for resistance in the trees.

1.1.1 Infection of Heterobasidion parviporum

H. parviporum can infect trees either by entering wounds directly on the tree, invading the stumps after tree felling or colonizing the root system by transferring the infection to adjacent trees through root-to-root contact (Woodward et al., 1998). The infection degrades lignin and cellulose components in the wood, hence the name white-rot fungus. This will in turn affect the quality of strength, reduces growth, decreases root stability, and lead to a lower timber value (Woodward et al., 1998). The infection can lead to a cylinder of rot within the tree as long as 12 meters (Woodward et al., 1998).

1.1.2 Defense responses and the reaction zone

Defense responses in bark by mimicking infections through wounds have been studied extensively. However, the molecular responses in the sapwood when Norway spruce is infected through root-to-root contact have received much less attention until the later years. The infection triggers the induced defense in sapwood giving rise to the reaction zone, and was first studied in detail by Louis Shain in 1970 (Woodward et al., 1998).

When spruce is attacked from the inside, a reaction zone is formed between the sapwood and the central core of the wood that is infected (Woodward et al., 1998). The heartwood consists of non-living cells and will not induce responses, but contains large amounts of phenols in many trees (Shain, 1979). The resistance towards infection varies between individual trees (Nagy et al., 2005, Fossdal et al., 2012, Bodles et al., 2006, Franceschi et al., 1998).

The formation of the reaction zone starts when the sapwood is invaded, and induces production of secondary metabolites (Arnerup, 2011, Shain, 1979). These are compounds produced by plants, but that are not essential for growth (Lawrence, 2005). The fungal infection of *H. parviporum* will activate this defense in the tree to prevent further damage (Krokene et al., 2008, Shain, 1970, Franceschi et al., 2005, Shain, 1979). It has been hypothesized that the formation of the reaction zone involves a reallocation of carbohydrates and that these affect the production of compounds with antifungal properties (Shain and Hillis, 1971, Rommel, 2012). Knowledge about these chemical and transcriptional changes in sapwood when the reaction zone is formed will enable breeding for resistance that will in turn reduce the economic loss that *Heterobasidion* infection represents.

1.1.3 Methyl jasmonate as defense induction

Resistance to fungal infection can be increased by pretreatment of methyl jasmonate to the stem (Krokene et al., 2008). This application induces up-regulation of defense responses similar to responses upon pathogen infection (Krokene et al., 2008), and the effect of this treatment on gene expression and phenolic concentration in Norway spruce upon wounding or infection may also be of importance in breeding programs.

1.2 Phenolic compounds and genes expression

The metabolic pathways within cells describe the biochemical reactions catalyzed by enzymes (Lawrence, 2005). Shain (1970) first reported the induction of enzyme activity connected to the formation of the reaction zone in sapwood in response to white-rot fungus. He also concludes that certain substances in the reaction zone have a crucial part in the host defense. More recent studies have reviewed the role of specific defense

related genes upon pathogen infection (Yaqoob et al., 2012, Rommel, 2012, Oliva et al., unpublished) and their relation with chemical compounds in the reaction zone is of great interest. The changes in chemical compounds connected to the changes in gene expression level in the reaction zone will provide relevant information about this line of defense.

The chemical group of phenols is involved in both constitutive and induced defense responses, and has shown inhibitory effects on pathogen infection (Nagy et al., 2004, Lindberg et al., 1992, Woodward and Pearce, 1988). Phenols are comprised by hydroxybenzoic acids and the phenylpropanoids, including the compounds derived from them (Lea and Leegood, 1998). They are synthesized and stored in polyphenolic parenchyma cells (PP cells), which are found in the phloem and resin canal network in the xylem (Franceschi et al., 1998). Changes in phenolic concentration have been observed in response to pathogen attack (Danielsson et al., 2011) and studies on *Heterobasidion* species has shown that trees that are inoculated with fungi increase their phenol content (Johansson et al., 2004).

The production of phenols happens mainly via the phenylpropanoid pathway, and genes connected to this pathway, as well as signaling pathways, have in previous studies been explored to elucidate defense responses in Norway spruce and other conifer species. These studies have shown responses to both wounding, inoculation of *H. parviporum* and methyl jasmonate. However, there are contradictory finds in these studies, which leads to questioning the genes role in this pathway. Bark and sapwood have in some studies shown difference in defense responses within the same experiment, where bark had a stronger resistance towards attack than sapwood (Deflorio et al., 2011, Bodles et al., 2006). Others have found similarities in gene expression in both tissues (Yaqoob et al., 2012).

Regarding signaling pathways, jasmonates have been reported as important inducers or signalers of defense responses as trees are infected with pathogens (Rommel, 2012, Arnerup et al., 2013). Previous studies have shown responses in bark regarding jasmonate genes, where this signaling mediator accumulated in response to *H. parviporum* inoculation (Arnerup, 2011, Arnerup et al., 2013). This signaling response

will affect the pathways that lead to production of antifungal components found in wood, which in turn form the reaction zone.

One of these components is lignin. Lignin has been observed in high amounts in the reaction zone (Woodward et al., 1998, Shain, 1979), so genes connected to the lignin biosynthesis, such as phenylalanine ammonia-lyase-like proteins (PAL1 and PAL2), caffeoyl-CoA 3-O-methyltransferase (CCoAOMT1 and CCoAOMT2) and hydroxycinnamoyl-coenzyme (HCT), are also interesting target points. These genes are involved in the phenylpropanoid pathway, and have defense related properties or have been found as resistance markers (Yaqoob et al., 2012, Koutaniemi et al., 2007, Porth et al., 2012). The latter CCoAOMT-genes and HCT-gene have shown connection to the production of phenols with inhibitory effects, and this connection is of relevance (Lepelley et al., 2007, Kojima and Uritani, 1973, Lallemand et al., 2012).

The regulation of lignin in the reaction zone has not been explored in large degree in Norway spruce. However, it has been reported that R2R3-MYB transcription factor regulate parts of the phenylpropanoid metabolism and lignin biosynthesis, which in turn affects the PAL family (Bedon et al., 2007). The phenylpropanoid pathway also leads to the synthesis of phenolic stilbenes, and the role of the stilbene synthase gene STS in sapwood is therefore interesting to study.

The formation of the reaction zone occurs after the death of host parenchyma (Shain, 1979). This cell death has been closely linked to the regulation of the chitinase gene PaChi4, which have been known to mediate programmed cell death (Fossdal et al., 2006, Yaqoob et al., 2012, Nagy et al., 2014). This genes' response to wood decaying fungi has been thoroughly studied, and has shown some differences between the tissues when comparing bark and sapwood (Deflorio et al., 2011, Rommel, 2012).

As the reaction zone is formed, the pH value decreases compared to the surrounding tissue, which may be caused by an accumulation of carbonates (Shain, 1979). This increase of carbonates has been hypothesized to be a reallocation from adjacent tissue to the reaction zone (Shain and Hillis, 1971). This encourages the analysis of deoxy-D-arabinoheptulosonate 7-phosphate synthase (DAHP1 and DAHP2), as well as the

carbonic anhydrase genes (ANH1, ANH2 and ANH3), which have been linked to defense mechanisms. Here these genes may be involved in an allocation of carbon towards production of secondary metabolites (Arnerup et al., 2011, Porth et al., 2012, Rommel, 2012).

1.3 Thesis aim

Knowledge of the changes in both gene expression and chemical concentration will increase our understanding of the reaction zone as a defense mechanism. The aim of the present study was to identify changes in extractable phenolic compounds and the transcriptional changes of defense related genes close to the reaction zone following either wounding, inoculation of *H. parviporum* or inoculation of methyl jasmonate in Norway spruce. The differentially expressed genes were measured through quantitative real-time polymerase chain reaction (qRT PCR) and the phenolic concentrations through HPLC-analyses. In contrast to previous studies we mimicked infection from within the tree to look at defense responses in the absence of damage to the bark and cambium. Results will contribute to further understanding of the induced defense mechanisms in Norway spruce sapwood.

2 Material and methods

2.1 Study area and species

2.1.1 Localization

The study was an induction of defense responses in Norway spruce (*Picea abies* (L.) Karsten) by inoculation of the pathogenic white-rot fungus *H. parviporum*.

The study comprised of two inoculation experiments in the Akershus County in southeast Norway (figure 1). The first inoculation was conducted on July 6th 2007 in a forest adjacent to the Norwegian University of Life Sciences, Ås, Norway. The samples were harvested on September 19th 2007 (table 1). The second inoculation was conducted on June 13th 2014 in a clonal stand at the Hogsmark Plantation of the Norwegian Forest Research Institute, Ås, Norway. We used clones of several genotypes to get a better description of the within and between genotype response to the inoculations and to obtain material better suited for statistical analysis than the initial inoculation experiment. The samples were harvested on September 16th and 23rd 2014 (table 2).

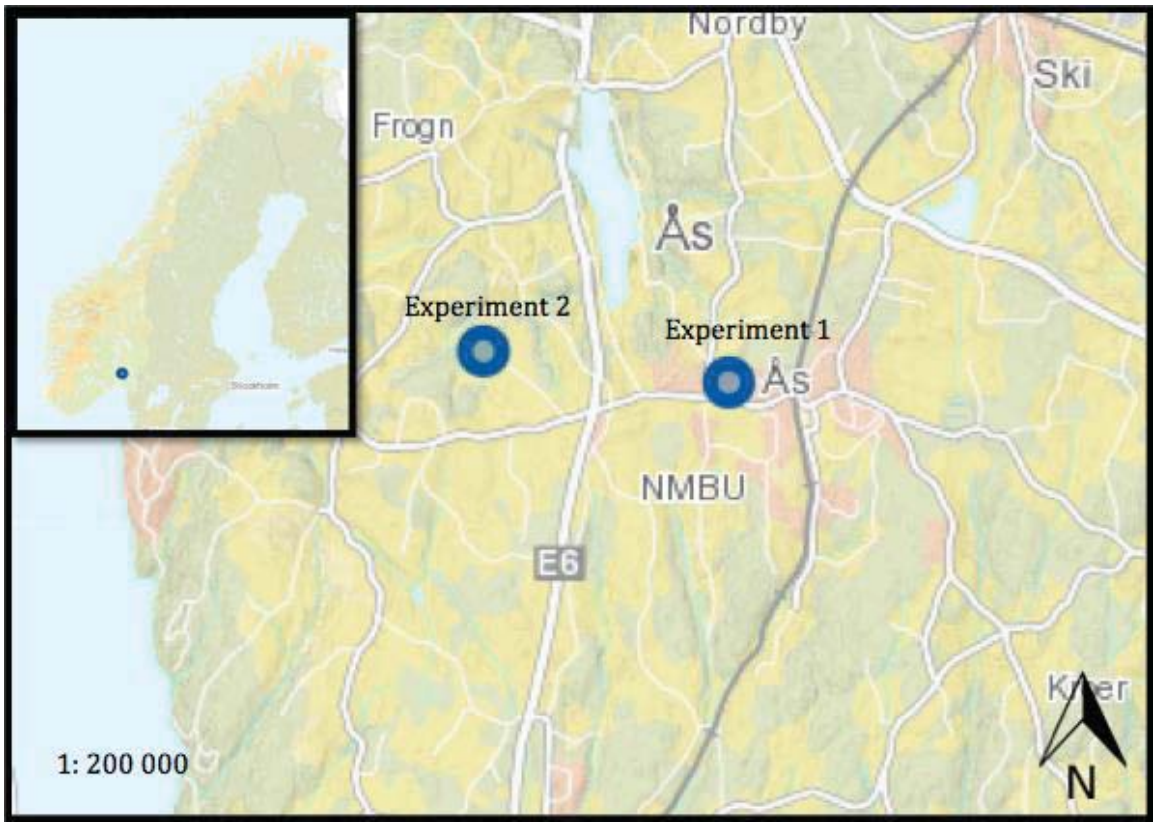

Figure 1: Map showing the position of the two selected study areas in southeast Norway. Experiment 1 was located close to Sørhelinga, and experiment 2 was located at Hogsmark. Map projection EU89, UTM zone 33. Maps from Geonorge (2015).

The trees close to Sørhellinga (experiment 1) were planted and had not been managed during the time before the experiment. Trees were estimated to be 60 years of age based on number of growth rings, and were found in an area were root-rot was present. However, the trees selected for the inoculation experiment were healthy and an increment borer at 30 cm above ground level was used to verify the absence of rot in the stem.

The clones in experiment 2 was planted as rooted cuttings in August 1992, according to Skrøppa (2015). Cuttings were taken from two families, which were generated after controlled crossings in spring 1983, and formed in summer 1988. Clone 275 and 288 were from the same crossing, and therefore related and full-siblings. Clone 335 was a crossing between two parent trees from Latvia and Poland, respectively. The area was chosen because of the knowledge about the plant material, but was in other ways connected to the first study area at Sørhellinga.

2.1.2 Inoculations

The trees were examined by making a bore sample to verify if they were healthy and to determine the location of the sapwood and heartwood. Old branches were removed up to a height of 1,5-2 meters without causing wounds or damage to the stem. To expose the sapwood for inoculations, we drilled into the heartwood from the opposite side of the tree (figure 2), and repeated this at different heights and angles in the same tree to avoid eliciting a reaction impacting on the next or previous inoculation, directly above or below, respectively (figure 3).

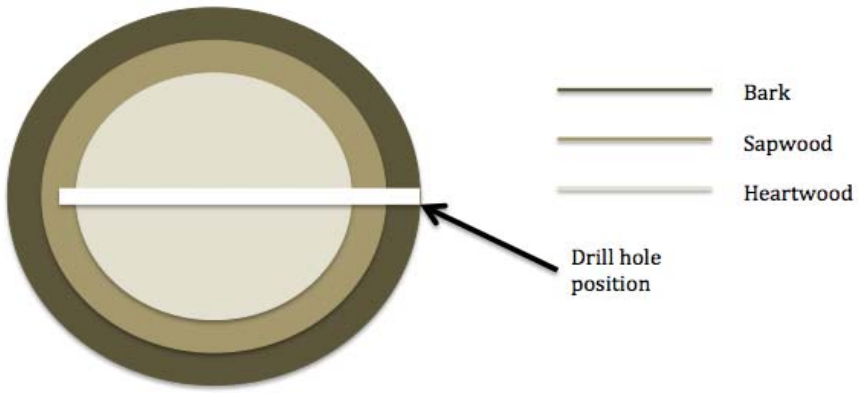

Figure 2: Example of a cut surface in the sample trees. Drilling started at the opposite side of the stem until sapwood was reached. The depth of these drillings differed between and within the two experiments.

The trees in experiment 1 were inoculated at eight different heights. These were four inoculations of *H. parviporum* grown on sawdust (Nagy et al., 2004) and four inoculations of pure sawdust, which imitated wounding. The depth of the drilling varied between each inoculation to determine at what distance to living tissue *H. parviporum* or wounding could induce defense responses in the tree, including drilling into the sapwood (proper inoculation), close to the sapwood and just into the heartwood (table 1). Wounded sample with the longest distance from bark was later selected as local controls since these were only drilled into the dead heartwood and at many centimeters distance from any living sapwood therefore could not induce a reaction zone within the short timeframe of this experiment as the fungus is known to colonize only a few millimeters in the radial direction per year.

Table 1: Data from experiment 1 done in 2007 with their respective sampling numbers.

Tree number	Section	Treatment	Distance from bark (cm)	Sample number
31		Distal control	6	4
31	1	*H. parviporum* infection	2,5	11
31	2	*H. parviporum* infection	2	12
31	3	*H. parviporum* infection	4	13
31	4	*H. parviporum* infection	6	14
31	6	Wounded	4	1
31	7	Wounded	6	2
31	8	Local control	8	3
32		Distal control	5	15
32	1	*H. parviporum* infection	2,5	17
32	2	*H. parviporum* infection	2	8
32	3	*H. parviporum* infection	3	16
32	4	*H. parviporum* infection	4	5
32	5	Wounded	2,5	6
32	6	Wounded	3	7
32	7	Wounded	5	9
32	8	Local control	6,5	10

Experiment 2 included 4 ramets (individuals in a clonal population) from each of three clones (275, 288 and 335). The choice of clones was based on available material at the research field in 2014 as well as the clones' age. There is no previous information about

difference in the clones' resistance to fungal attack or other stresses. The clones were located in two separate blocks within 5 minutes walking distance. Within each block, the ramets for each clone were located next to each other to minimize the microclimatic and edaphic variation between them. Six different holes were drilled into each ramet (figure 3 and table 2), where two holes were inoculated with a minimum of 1 cm^3 *H. parviporum* grown on malt agar (Solheim, 2015), two holes initiated wounding, one hole was inoculated with 50 µl of 100 mM methyl jasmonate diluted in 0,1% 500 µl Tween® 20 (Sigma-Aldrich, Oslo, Norway) on cotton. The surfactant Tween® 20 (0,1%) was used to solubilize methyl jasmonate (Sigma-Aldrich, 2015). The methyl jasmonate was added to examine if it would induce a defense response in sapwood resulting in a defensive reaction zone forming.

The last hole worked as a water control for methyl jasmonate where we added 550 µl water with 0,1% Tween® 20 on cotton. Three of the four ramets in each clone were treated by this procedure, while the remaining fourth was left untouched and used as a control. After the inoculations, the boreholes were closed with cotton.

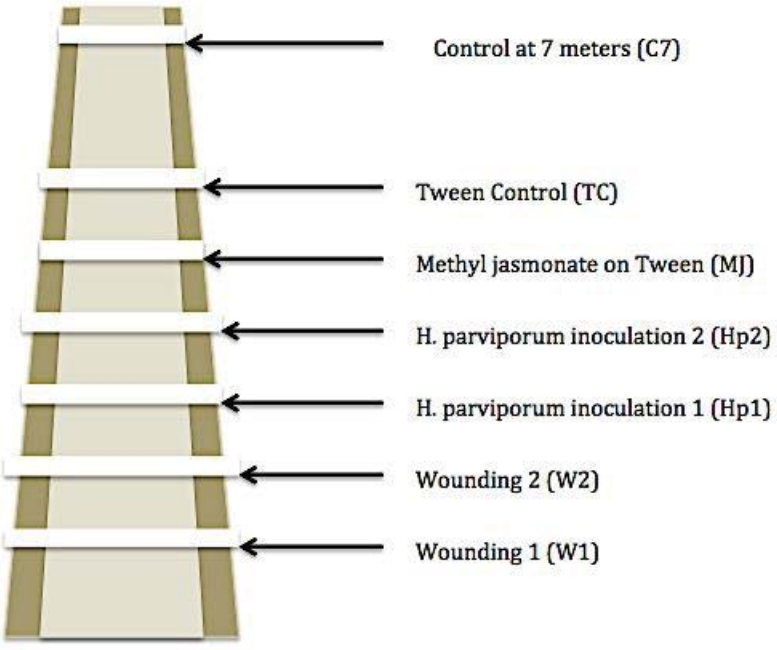

Figure 3: Illustration of the inoculations in clone 275, 288 and 335. The same procedure was used for tree 31 and 32, where *H. parviporum* inoculation, wounding, distal control and unwounded control were used.

Table 2: Data from experiment 2 in 2014. Samples were collected from three different clones (275, 288 and 335) and from four ramets from each clone (Hp1, Hp2, Hp3, 4C). Three of the ramets (Hp1, Hp2 and Hp3) were inoculated with the different treatments as shown in figure 3, and the last ramet (4C) was used as a control.

Clone	Tree	Treatment	Sample name
335	Hp1	Control at 7 m	18
335	Hp1	Wounding 1	19
335	Hp2	Wounding 1	20
335	Hp2	Control at 7 m	21
335	Hp2	Tween Control	22
275	Hp1	Wounding 3	23
275	Hp1	H.parviporum inoculation 2	24
275	Hp2	Wounding 2	25
275	Hp2	Wounding 3	26
275	Hp2	Control at 7 m	27
275	Hp1	Control at 7 m	28
275	Hp2	Tween Control	29
275	Hp2	Wounding 1	30
275	Hp3	Methyl jasmonate	31
275	Hp1	Wounding 1	32
275	Hp3	H.parviporum inoculation 2	33
275	Hp3	Wounding 1	34
275	Hp3	Tween Control	35
275	4C	Control at 7 m	36
275	Hp2	H.parviporum inoculation1	37
275	Hp1	Methyl jasmonate	38
275	4C	Control at 1 m	39
275	Hp2	H.parviporum inoculation 2	40
275	Hp1	H.parviporum inoculation 1	41
275	Hp2	Methyl jasmonate	42
275	Hp1	Tween Control	43
275	Hp3	H.parviporum inoculation 1	44
275	Hp1	Wounding 2	45
335	Hp3	Control at 7 m	46
335	Hp2	Methyl jasmonate	47
335	Hp2	H.parviporum inoculation 2	48
335	Hp2	Wounding 2	49
335	Hp1	Methyl jasmonate	50
335	Hp1	Tween Control	51

335	Hp2	H.parviporum inoculation 1	52
335	Hp1	H.parviporum inoculation 2	53
335	Hp1	Wounding 2	54
335	Hp1	H.parviporum inoculation 1	55
288	Hp1	Wounding 2	56
288	Hp2	H.parviporum inoculation 2	57
288	Hp2	Methyl jasmonate	58
288	Hp1	Wounding 1	59
288	Hp1	Methyl jasmonate	60
288	Hp2	Tween Control	61
288	4C	Control	62
288	Hp3	Methyl jasmonate	63
288	Hp3	Control at 7 m	64
288	Hp1	H.parviporum 2	65
288	Hp1	Tween Control	66
288	Hp2	Wounding 2	67
288	Hp1	H.parviporum inoculation 1	68
288	4C	Control at 7 m	69
288	Hp3	Wounding 2	70
288	Hp3	H.parviporum inoculation 1	71
288	Hp2	Control at 7 m	72
288	Hp3	Wounding 1	73
288	Hp1	Control at 6 m	74
288	Hp3	Tween Control	75
288	Hp2	Wounding 1	76
288	Hp2	H.parviporum inoculation 1	77
288	Hp2	Wounding 3	78
288	Hp3	H.parviporum 2	79
335	Hp3	Wounding	80
335	Hp3	H.parviporum 2	81
335	Hp3	Tween Control	82
335	4C	Control	83
335	Hp3	Methyl jasmonate	84
335	Hp3	H.parviporum inoculation 1	85
335	Hp3	Tween Control	86

2.1.3 Sample collection

We felled the trees from the experiment 1 and 2, at 78 and 102 days after inoculation, respectively. The entire treated section (disk) of the stem was cut out with a chainsaw,

transported to the lab, and further divided to separate the treatments with fine blade saw. The samples were chosen based on being in the prolongation of the borehole. Samples were stored in a freezer of -80°C until sawdust was collected for chemical and transcriptional analysis. This method was used for both experiments. However, experiment 2 included an additional control that was taken at 7 meters tree height.

2.2 Laboratory work

Chemical and gene expression analysis was conducted on samples from the experimental inductions of chemical reaction zone around the inoculation and wounding in the sapwood of Norway spruce. The following methods were conducted for all samples from both experiments.

2.2.1 Test material

Samples were collected by using an Einhell SB 401/1 (Einhell, United Kingdom) bench drill to perform both gene expression analysis and chemical analysis. The amount of tissue used for gene expression analysis was 30-40 mg. Sample tissue was immediately transferred the sample to 2.5 ml eppendorf vials and into liquid N_2. The samples were stored in a freezer at -80°C until further use.

2.3 Chemical analysis (HPLC)

Samples were freeze-dried in a Heto DryWinner (Fisher Scientific, USA) for 24 hours. When completely dry, samples were placed in room temperature with the sample lids opened for approximately 17-24 hours. To extract phenolic compounds, the material from each sample was first weighed on a Mettler Toledo UMX2 (Mettler Toledo, Oslo, Norway) scale. 30 mg from each sample was placed in a Precellys-vial with 4-5 Teflon-beads to enhance the extraction. 600 µl methanol (MeOH) was then added to each vial, homogenized for 30 seconds at 6500 rpm in a Precellys 24 homogenizer (Bertin Technologies, USA), and placed on ice for 15 minutes. The vials were centrifuged for 3 minutes at 15000 rpm in an Effendorf Centrifuge 5417C (Eppendorf, USA). After the centrifugation, the supernatant was pipetted with a Pasteur pipette into a test tube. 600 µl MeOH was again added to the precipitation in the vials, samples were homogenized for 30 seconds and centrifuged for 3 minutes to pipette the supernatant into the test

tubes along with the first pipetting. This step was repeated two more times. Test tubes were placed in an Eppendorf Concentrator plus (Eppendorf, USA) to evaporate the MeOH from the combined extractions. Test tubes were then stored in a freezer at -20°C.

To analyse the samples, they were removed from the freezer and placed in room temperature for 20-30 minutes. The material in each sample was re-dissolved in 200 µl MeOH, which was added to each test tube. To further dissolve the material, each test tube was placed in an ultrasonic cleaner (VWR International, USA). 200 µl of ultrapure H_2O was added to each test tube, and the content of the test tubes were poured into 2.5 ml eppendorf-vials. The vials were centrifuged for 3 minutes at 15000 rpm in a Centrifuge 5417C (Eppendorf, USA) and the supernatant was poured into a BD Plastipak 2 ml syringe (BD, USA) with an added GHP Acrodisc® 13 mm Syringe Filter (Pall Corporation, USA) to remove any leftover material. The liquid in the syringe was then transferred into HPLC-vials and a lid was added.

We analyzed the samples with high-performance liquid chromatography (HPLC), which consisted of a binary pump (G1312A), a thermostated autosampler (G1329A), a thermostated column oven (G1316A) and a diode array detector (G1315B). The phenolic compounds were separated on a ODS Hypersil (50 x 4.6 mm) HPLC coloum (Thermo Scientific, USA). Samples were eluted using a methanol:water gradient (table 3) with a flow rate of 2 ml min^{-1}, which consisted of a methanol B-solution of 100% MeOH, and an A-solution consisting of 5 ml ortho-Phosphoric acid (85%), 30 ml Tetrahydrofuran for liquid chromatography and 1965 ml of ultrapure water.

Table 3: Gradient of B-solution during HPLC-run of each sample. Continuant gradient were the first 5 minutes have a pure MeOH-solution, but increases the percentage of B-solution up to 15% after the 5 minutes are up.

Time	%B
5	0
10	15
20	30
40	50
45	50
46	100
58	100
60	0

The injected volume in the HPLC was 40 µl. For some samples, the injected volume was adjusted lower if the peaks were unusually high. These samples were run again. We identified peaks comparing the retention time and UV spectrum with those of commercial standards (table 4) at 220 nm and 320 nm.

Table 4: List of commercial standards used to identify and compute concentrations of phenolic compounds. Standard 1 = Sigma-Aldrich (St. Louis, MO, USA). Standard 2 = Polyphenols (Sandnes, Norway).

Standards	Applied to the following compounds	Supplier
Neochlorogenic acid	Chlorogenic acid and derivatives	1
E-Astringin	E-Astringin	2
Iso-rhaphontin	Iso-rhaphontin	2

2.4 Gene expression analysis (qRT PCR)

2.4.1 RNA extraction

For the genetic analysis, we extracted RNA from each treatment. The samples were grounded twice with beads in liquid N_2-chilled containers for 1,5 min each time at 20 hertz in an MM 300 mill (Retsch Gmbh, Haan, Germany). Between grounding, the samples were chilled in liquid N_2 to avoid an increase in the samples temperature. Masterpure™ Plant RNA Purification Kit (Epicentre, USA) was used to isolate RNA according to the manufacturer's instructions, except from the exchange of 6 µl of 100 mM DTT with 3 µl of 2-mercaploethanol. Total RNA was quantified with NanoDrop (Thermo scientific, USA), a spectrophotometer that shows the concentration of RNA in each sample, as well as the purity.

2.4.2 cDNA synthesis

The total RNA was normalized between samples to obtain 300 ng RNA from each sample for cDNA synthesis. We reverse transcribed the total RNA to cDNA with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions. 10 µl of RNA and nuclein free water (table 4) and 2,5 µM of 50 µM Oligo $d(T)_{16}$ was incubated in a GeneAmp® PCR System 9700 thermo cycler (Applied Biosystems, USA) for 5 min at 65°C and 2 min at 4°C. The mastermix was then added, and the final concentration included 11,5 µl of NFW, 1X of 10X Buffer, 1,75

mM of 25 mM $MgCl_2$, 0,5 mM each of 10 mM dNTP Mix, 5,0 mM of 100 mM DTT, 1,0 U/µl of RNase Inhibitor (20 U/µl) and 2,5 U/µl of Multiscribe™ RT (50 U/µl). The reverse transcription procedure was used for a single 50-µl reaction. Three controls where also included (No template control). The reverse transcription proceeded for 30 min at 37°C, followed by 5 min at 95°C to denature and inactivate the RT enzyme and at last lowered to 4°C until the samples were stored in a freezer at -80°C.

2.4.3 Standard curves

The standard curves were created for the qRT PCR to have a known series of input, which the change in unknown amount of gene transcript (cDNA) target could be quantified from. The standard curve contained known concentrations of cDNA that all samples were compared to and enabled us to convert cycle threshold values into quantified values. For samples in experiment 1, we combined 5 µl of each resulting cDNA sample and diluted it into 6 different concentrations (100%, 50%, 25%, 5%, 1% and 0,5%) to create the standard curve. For samples in experiment 2, 3 µl was used in the same manner to create a standard curve.

2.4.4 Primer design

We selected genes (table 5) for the analysis of genetic expression in treatments in the two experiments. The selections of genes were based on literature and previous studies that were relevant for this thesis (Yaqoob et al., 2012, Porth et al., 2012, Arnerup et al., 2011, Hammerbacher et al., 2011).

Primers for the following genes, ZIM1, ZIM2, ZIM3, ANH1, ANH2, ANH3 and MYB8 were designed based on the mRNA sequence found in the NCBI BLASTN database (NCBI, Maryland, USA). To design the primers, Genscript PCR Primer Design Tool (Genscript, USA) and Primer 3 (Simgene, Indore, India) were used. We compared sequences in NCBI BLASTN database to find matches for the entered sequence with an already discovered sequence. Some of these sequences were only registered for other species in the same genus, and were tested for specificity through their amplification and melting curve in order to determine their specificity. We obtained primers from Invitrogen™ by Life Technologies (Thermo Fisher Technologies, USA).

The jasmonate ZIM-domain genes ZIM1, ZIM2 and ZIM3 showed 100% similarity to CO210382, CO210689 and CO210862 GenBank accession numbers from Engelmann spruce x white spruce (*Picea engelmannii x Picea glauca*) cDNA clone, respectively. The carbonic anhydrase genes ANH1, ANH2 and ANH3 showed 100% similarity to CO229804, CO213444 and DR465621 GenBank accession numbers from white spruce cDNA clone and Engelmann spruce x white spruce cDNA clone, respectively. The transcription factor MYB8 based primer showed 100% similarity to GenBank EF601071 accession number from white spruce.

Table 5: Details for the primer sequences used in the qRT-PCR study. Primer sequences obtained from 1 Yaqoob et al. (2012); 2 Porth et al. (2012); 3 Arnerup et al. (2011); 4 Hammerbacher et al. (2011).

Gene	Forward primer	Reverse primer	GenBank accession no.
Actin	TGAGCTCCCTGATGGGCAGGTGA	TGGATACCAGCAGCTTCCATCCCAAT	FJ869868
PaαTub[1]	GGCATACCGGCAGCTCTTC	AAGTTGTTGGCGGCGTCTT	X57980
PaPAL1[1]	CAGCCCTCTGCCCAACAG	AGCTGGGTTCTCACGAATTCA	AY952468
PaPAL2[1]	TTGCTCGTAGGCACCAATAGC	GCCTTGCCTTCGTTGATAGC	AM293549
PaChi4[1]	GCGAGGGCAAGGGATTCTAC	GTGGTGCCAAATCCAGAA	FJ423771
CCoAOMT1[2]	CCGATCCCCGCATTGAG	AATAGTTACGCCTGCAAAGAGTGA	AM175255
CCoAOMT2[2]	GTGAGCCCGAGCCATTGA	AGTCATGAGGTTCCAGGGATGT	AM174215
PaHCT1[1]	TGCGAGTGTAATCCATGAAGCTT	GGCTGCAATCCCAAATAGTCTAAA	AM173211
PaDAHP1[3]	AGATCCCCAAAGGATGGTTC	TGAAATCCAAGTTCCACTGAA	HQ441161
PaDAHP2[3]	TGCAACAGGAGGTTATGCT	ATGAATCCCAGTGCCTCAT	HQ441162
STS[4]	GTGGCGAGCAGAACACAGACTT	CAGCGATGGTACCTCCATGAAC	JN400069
MYB8	ATCACCTCCGAAAATGAACG	ATTGGCAATGTCTCCTCCTG	EF601071
JAZ[3]	TACGATGTGCCTGCTGAGAA	TGTTCGATCTTACCGCTGGT	HS032497
ZIM1	ATCGTGGTGTAAGGAGCCAC	CAACCTGAAAGACATCCCGT	CO210382
ZIM2	GCGCCGAGTCTGAAGTAATC	GCATAGTGGGTCTCCGTCAT	CO210689
ZIM3	GGGCCTAAAACTCGACACAA	TCTCCTCTGTTGGGAGCTGT	CO210862
ANH1	TGGAAGAATTGTTCGCCTAT	CACTTTGCTAATCTATGAAATGTT	CO229804
ANH2	ATGCAAGACCTATCCAACCCT	TTATCAAACTCAAGCATAACGATCA	CO213444
ANH3	GATGCATGTTGAGAACTGAGC	TCCTCTCCCATTGATAGATCCT	DR465621

2.4.5 Gene expression with quantitative real time polymerase chain reaction (qRT PCR)
For the qRT PCR-reactions, we made a mastermix of 5 µl of the fluorescent dye 2 x SYBR® Green Fast MM, 0,2 µl of the forward and 0,2 µl of the reverse primer and 2,6 µl of nuclein free water for each sample, which was added to a MicroAmp®, Fast optical 96-Well Reaction Plate with Barcode (Applied Biosystems®, Life Technologies, USA). The final concentration of each primer was 10 mM. 2 µl of diluted cDNA from each sample was then added to each well containing the mastermix.

A film was then placed over the wells to avoid evaporation and contamination between the wells. The plate was then centrifuged in an Avanti™ J-25 Centrifuge (Beckman, USA) for 2 minutes at 1500 x G and then placed in the ViiA™ 7 Real Time PCR System (Life Technologies, USA). The PCR cycle included a hold stage of 95°C for 20 seconds, and a PCR stage of an initial phase of 1 seconds at 95°C, then a phase of 20 seconds at 60°C for 40 cycles, and a melt curve stage of 15 seconds at 95°C, 1 minute at 60°C, and a final stage of 15 seconds at 95°C. The procedure would amplify the amount of DNA in the samples to enable measurement of gene expression for the specific gene.

A melting curve analysis was performed after each run, and the slope of the standard curve, of each target, gives the amplification efficiency of each run. With a specific PCR reaction, the melting curve should only generate one sharp peak (Matz, 2013). As some of the amplifications showed signs of primer-dimer formations as additional low melting temperature peak was observed, the concentration of the primer was diluted to 0,1 µl. This applied to the following genes; JAZ, ANH3, MYB8 and PAL2. The reaction was run again with the new primer concentration to examine if this would avoid primer-dimer formation.

2.5 Statistical analysis

In order to analyze the results, we used Minitab Express™ (Minitab inc., version 1.2.0 (410997), United Kingdom). A One-Way Analysis of variance (ANOVA) was used to test if there were a significant difference between the treatments. One-way ANOVA was used to compare two or more populations. For the analysis, the null hypothesis stated that all means were equal (H_0: $\mu_1 = \mu_2 = ... = \mu_l$) where l was the treatments being compared

(Devore and Berk, 2012). The alternative hypothesis stated that at least one mean was different (H_i: $\mu_1 \neq \mu_2 \neq .... \neq \mu_l$) with a significance level of $\alpha = 0,05$.

In experiment 1, treatments were divided into group based on their distance from bark (table 6), in order to see if this affected the response. Samples further away from bark than 4 cm were excluded, because of lack of influence on the sapwood.

Table 6: Statistical groups for experiment 1 to analyze if there were differences between the distance from bark. Each tree was tested separately.

Tree	Control	Wounding group 1	*H. Parviporum* group 1	*H. Parviporum* group 2
31	Distal control	Wounded at 4 cm	H.p. 2 cm	H.p. 4 cm
31	Local control		H.p. 2,5 cm	
32	Distal control	Wounding 2,5 cm	H.p. 2 cm	H.p. 3 cm
32	Local control	Wounding 3 cm	H.p. 2,5 cm	

3 Results

3.1 Phenolic compound concentrations (HPLC)

3.1.1 Identification of peaks

We identified iso-rhaphontin, E-Astringin and one chlorogenic acid derivative in most wood samples (figure 4). In addition, a number of unidentified peaks were registered, that generally had lower peak areas. We were unable to identify any peaks in three of the samples in experiment 1. For all treatments, iso-rhaphontin, E-Astringin and the chlorogenic acid derivative were the most abundant compounds.

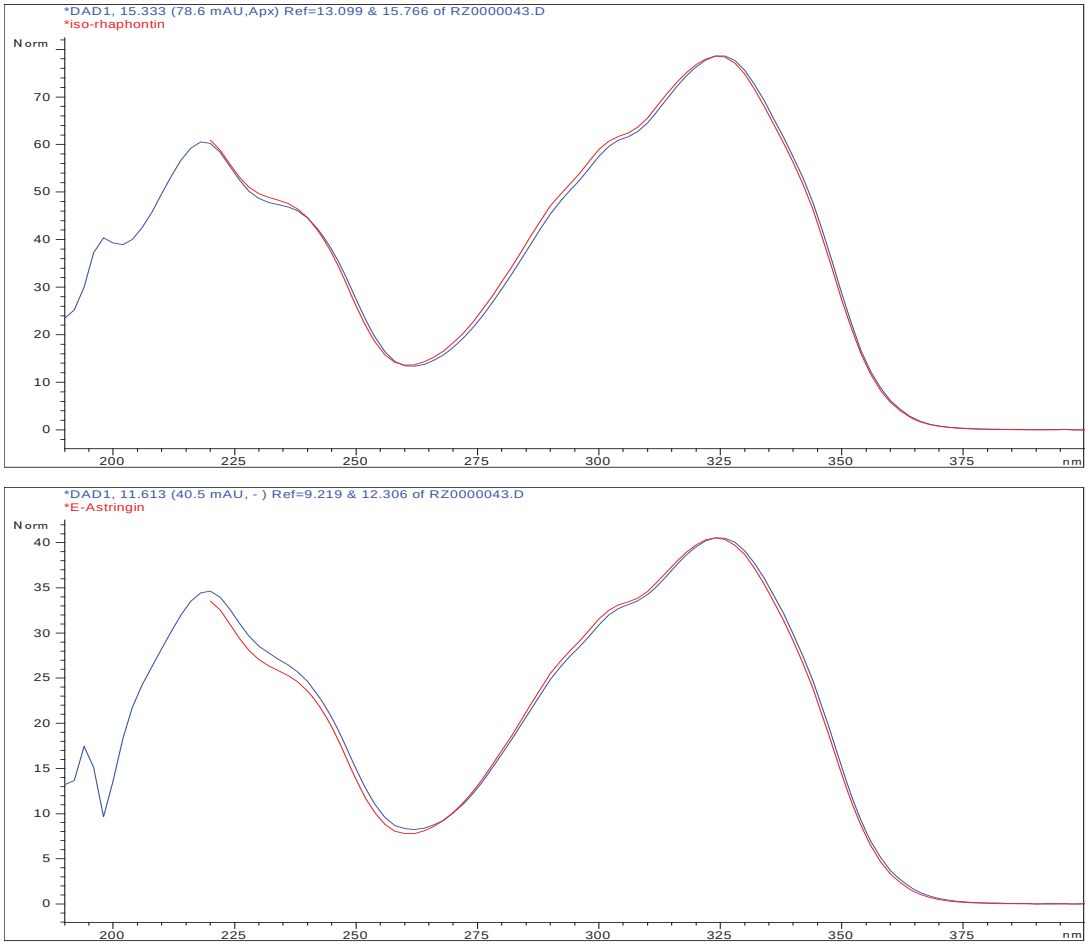

Figure 4: UV-spectra of iso-rhaphontin (above) and E-Astringin (below), which were identified in most wood samples.

3.1.2 Significant effects of treatments on phenolic compound concentrations

In experiment 1 (table 7), the only phenolic compound that was affected by the treatments in tree 31, was the unidentified phenolic compound (UPC) 27. Wounding and inoculation of *H. parviporum,* when compared with the distal control, caused a decreased

phenolic concentration of UPC 27. In tree 32, both wounding and *H. parviporum* inoculation decreased the concentration of UPC 4, regardless of which control we compared the treatments with. For the UPC 9, inoculation of pathogen resulted in a decrease in the concentration. Iso-rhaphontin, the chlorogenic acid derivative and E-Astringin were all decreased by the pathogen inoculation. Because of undetectable peaks in many samples and a low amount of replicas, we were unable to provide reliable statistical results regarding differences between the inoculation- and wounding depths.

In experiment 2 (table 7), we had statistically significant effects of treatments in clone 335 (figure 5). The wounding treatment caused a decrease in UPC 4, UPC 27, iso-rhaphontin, the chlorogenic acid derivative and E-Astringin.

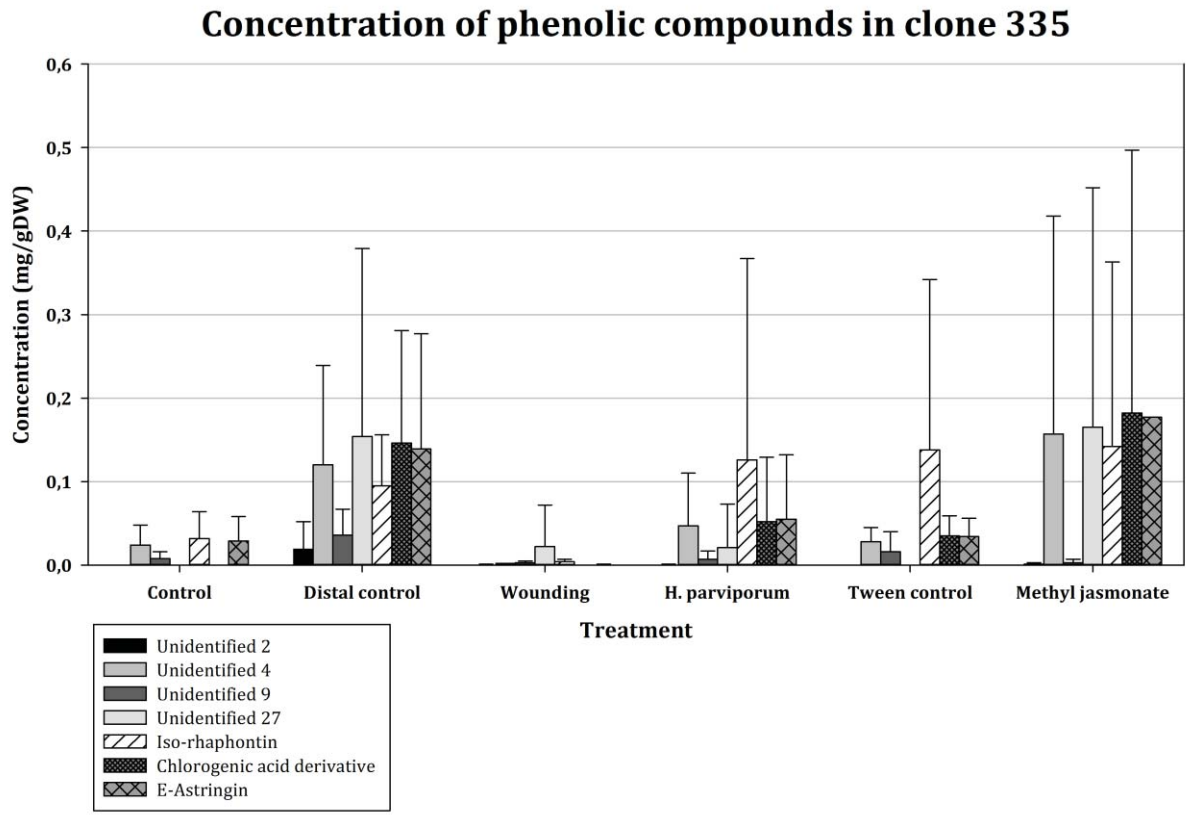

Figure 5: Mean concentration with its respective standard deviation of the selected phenolic compounds in clone 335.

Table 7: Statistical results for the selected phenolic compounds. Treatments with significant concentration changes (p < 0,05) are marked in red. W = Wounding; LC = Local control; DC = Distal Control; Hp = *H. parviporum* inoculation; MJ = Methyl jasmonate inoculation; TC = Tween control; C = Control tree; * = Unable to calculate.

		Unidentified 2		Unidentified 4		Unidentified 9		Unidentified 27		Iso-rhaphontin		Chl. acid der.		E-Astringin	
Treatment	Tree	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value
W vs LC	31	0.667	0.33	0.667	0.33	0.667	0.33	0.667	0.33	0.664	0.34	0.667	0.33	0.666	0.34
W vs DC	31	0.667	0.33	0.687	0.29	0.667	0.33	0.018	1267.88	0.703	0.25	0.691	0.28	0.691	0.28
Hp vs LC	31	0.685	0.20	0.605	0.33	0.562	0.42	0.506	0.57	0.635	0.28	0.604	0.33	0.605	0.33
Hp vs DC	31	0.685	0.20	0.940	0.01	0.562	0.42	0.010	35.00	0.819	0.06	0.938	0.01	0.988	0.00
W vs Hp	31	0.907	0.02	0.215	2.17	0.226	2.04	0.391	0.92	0.345	1.14	0.223	2.08	0.218	2.14
W vs LC	32	*	*	0.003	313.58	0.963	0.00	0.977	0.00	0.786	0.10	0.575	0.44	0.529	0.57
W vs DC	32	*	*	0.000	5045.59	0.143	5.54	0.667	0.25	0.198	3.60	0.132	6.08	0.053	17.34
Hp vs LC	32	0.504	0.57	0.015	25.66	0.218	2.41	0.833	0.05	0.917	0.01	0.003	85.84	0.058	8.95
Hp vs DC	32	0.504	0.57	0.000	449.86	0.002	117.28	0.516	0.54	0.001	137.36	0.000	590.07	0.001	180.80
W vs Hp	32	0.212	2.04	0.610	0.30	0.481	0.58	0.711	0.15	0.476	0.59	0.340	1.11	0.645	0.24
W vs DC	275	*	*	0.584	0.32	0.217	1.79	*	*	0.764	0.10	0.611	0.28	0.978	0.00
W vs C	275	*	*	0.438	0,68	0.317	1.16	*	*	0.471	0.58	0.386	0.85	0.436	0.68
Hp vs DC	275	0.516	0.47	0.484	0.55	0.356	0.98	0.316	1.17	0.459	0.61	0.510	0.48	0.490	0.53
Hp vs C	275	0.604	0.30	0.567	0.37	0.458	0.63	0.420	0.75	0.492	0.54	0.596	0.36	0.564	0.37
W vs Hp	275	0.300	1.18	0.275	1.32	0.163	2.24	0.113	2.96	0.221	1.68	0.280	1.29	0.271	1.34
MJ vs TC	275	0.374	1.00	0.455	0.68	0.142	3.32	0.125	3.75	0.556	41.00	0.456	0.68	0.438	0.74
W vs DC	288	0.545	0.40	0.482	0.54	0.331	1.07	0.252	1.52	0.812	0.06	0.546	0.40	0.476	0.56
W vs C	288	0.626	0.26	0.379	0.88	0.792	0.08	0.353	0.99	0.291	1.30	0.389	0.84	0.338	1.06
Hp vs DC	288	0.411	0.76	0.436	0.68	0.290	1.31	0.349	1.01	0.334	1.08	0.591	0.32	0.433	0.69
Hp vs C	288	0.510	0.49	0.566	0.37	0.638	0.25	0.452	0.65	0.743	0.12	0.722	0.14	0.573	0.36
W vs Hp	288	0.392	0.79	0.147	2.44	0.280	1.29	0.175	2.10	0.158	2.29	0.294	1.22	0.141	2.52
MJ vs TC	288	0.374	1.00	0.484	0.59	0.406	0.86	0.965	0.00	0.447	0.71	0.447	0.71	0.502	0.54
W vs DC	335	0.229	1.79	0.059	5.43	0.048	6.11	0.233	1.76	0.012	12.71	0.043	6.51	0.054	5.69
W vs C	335	0.704	0.17	0.000	5054.73	0.109	4.22	0.704	0.17	0.002	57.24	*	*	0.000	1526.21
Hp vs DC	335	0.176	2.27	0.254	1.54	0.063	4.88	0.184	2.17	0.839	0.04	0.215	1.86	0.264	1.48
Hp vs C	335	0.721	0.14	0.754	0.11	0.928	0.01	0.721	0.14	0.733	0.13	0.559	0.39	0.770	0.09
W vs Hp	335	0.738	0.12	0.155	2.41	0.479	0.54	0.965	0.00	0.288	1.27	0.169	2.24	0.152	2.45
MJ vs TC	335	0.374	1.00	0.442	0.73	0.414	0.83	0.374	1.00	0.983	0.00	0.465	0.65	0.444	0.72

3.2 Gene expression (qRT PCR)

In experiment 1 (table 8) the target gene CCoAOMT1 in tree 31 was significantly up regulated by *H. parviporum* inoculation at both 2-2.5 cm and 4 cm from the bark (table 5). HCT was up regulated when pathogen inoculation was 4 cm from the bark, while MYB8 was significantly different between the two inoculation depths, where the inoculation at 4 cm from bark had the highest expression. Finally, expression of PAL2 in samples closest to the bark was significantly different from the control.

In tree 32 (figure 6), CCoAOMT1 was significantly up regulated by pathogen inoculation 3 cm from bark, as was CCoAOMT2, DAHP2 and HCT. The target genes ANH2 and PAL2 were significantly up regulated in inoculated samples closest to the bark and at both depths, respectively.

Table 8: Statistical values for Experiment 1 where each target genes has been tested with ANOVA. Significant values ($p < 0.05$) are marked in red. W = Wounding; C = Control; Hp 1= Group 1 of *H. parviporum* inoculated samples; Hp 2 = Group 2 of *H. parviporum* inoculated samples; * = Too few samples for statistical analysis.

		Tree 31		Tree 32	
Treatment	Target gene	P-value	F-value	P-value	F-value
C vs W	STS	0.365	2.41	0.141	5.65
C vs Hp 1	STS	0.512	0.63	0.302	1.90
C vs Hp 2	STS	0.340	2.87	0.667	0.33
Hp 1 vs Hp 2	STS	0.221	7.65	0.319	3.32
C vs W	CCoAOMT1	0.154	16.38	0.591	0.40
C vs Hp 1	CCoAOMT1	0.002	497.63	0.164	4.64
C vs Hp 2	CCoAOMT1	0.038	277.73	0.013	2378.41
Hp 1 vs Hp 2	CCoAOMT1	0.355	2.57	0.361	2.47
C vs W	CCoAOMT2	0.317	3.37	0.556	0.49
C vs Hp 1	CCoAOMT2	0.123	6.69	0.207	3.39
C vs Hp 2	CCoAOMT2	0.088	51.81	0.005	13810.92
Hp 1 vs Hp 2	CCoAOMT2	0.546	0.75	0.402	1.86
C vs W	DAHP2	0.668	0.33	0.255	2.50
C vs Hp 1	DAHP2	0.405	1.09	0.277	2.19
C vs Hp 2	DAHP2	0.865	0.05	0.027	563.08
Hp 1 vs Hp 2	DAHP2	0.660	0.35	0.493	1.05
C vs W	ZIM2	*	*	0.408	1.08
C vs Hp 1	ZIM2	*	*	0.356	1.41
C vs Hp 2	ZIM2	*	*	0.618	0.47
Hp 1 vs Hp 2	ZIM2	*	*	0.947	0.01
C vs W	ANH2	0.314	3.46	0.303	1.89
C vs Hp 1	ANH2	0.428	0.97	0.031	31.08
C vs Hp 2	ANH2	0.492	1.05	0.085	55.10
Hp 1 vs Hp 2	ANH2	0.833	0.07	0.672	0.32
C vs W	MYB8	0.510	0.94	0.401	1.12

C vs Hp 1	MYB8	0.829	0.06	0.719	0.17
C vs Hp 2	MYB8	0.059	114.83	0.408	1.80
Hp 1 vs Hp 2	MYB8	0.043	220.01	0.614	0.48
C vs W	HCT	0.062	103.80	0.747	0.14
C vs Hp 1	HCT	0.361	1.38	0.299	1.94
C vs Hp 2	HCT	0.029	489.52	0.003	38632.86
Hp 1 vs Hp 2	HCT	0.657	0.36	0.436	1.50
C vs W	PAL1	0.389	2.04	0.872	0.03
C vs Hp 1	PAL1	0.467	0.79	0.090	9.69
C vs Hp 2	PAL1	0.717	0.23	0.533	0.81
Hp 1 vs Hp 2	PAL1	0.772	0.14	0.246	6.03
C vs W	PAL2	0.080	0.82	0.614	0.35
C vs Hp 1	PAL2	0.007	138.97	0.042	22.04
C vs Hp 2	PAL2	0.148	17.93	0.014	2067.19
Hp 1 vs Hp 2	PAL2	0.049	170.25	0.049	167.56
C vs W	PaChi4	0.302	3.80	0.872	0.03
C vs Hp 1	PaChi4	0.438	0.92	0.090	9.69
C vs Hp 2	PaChi4	0.604	0.52	0.533	0.81
Hp 1 vs Hp 2	PaChi4	0.772	0.14	0.246	6.03

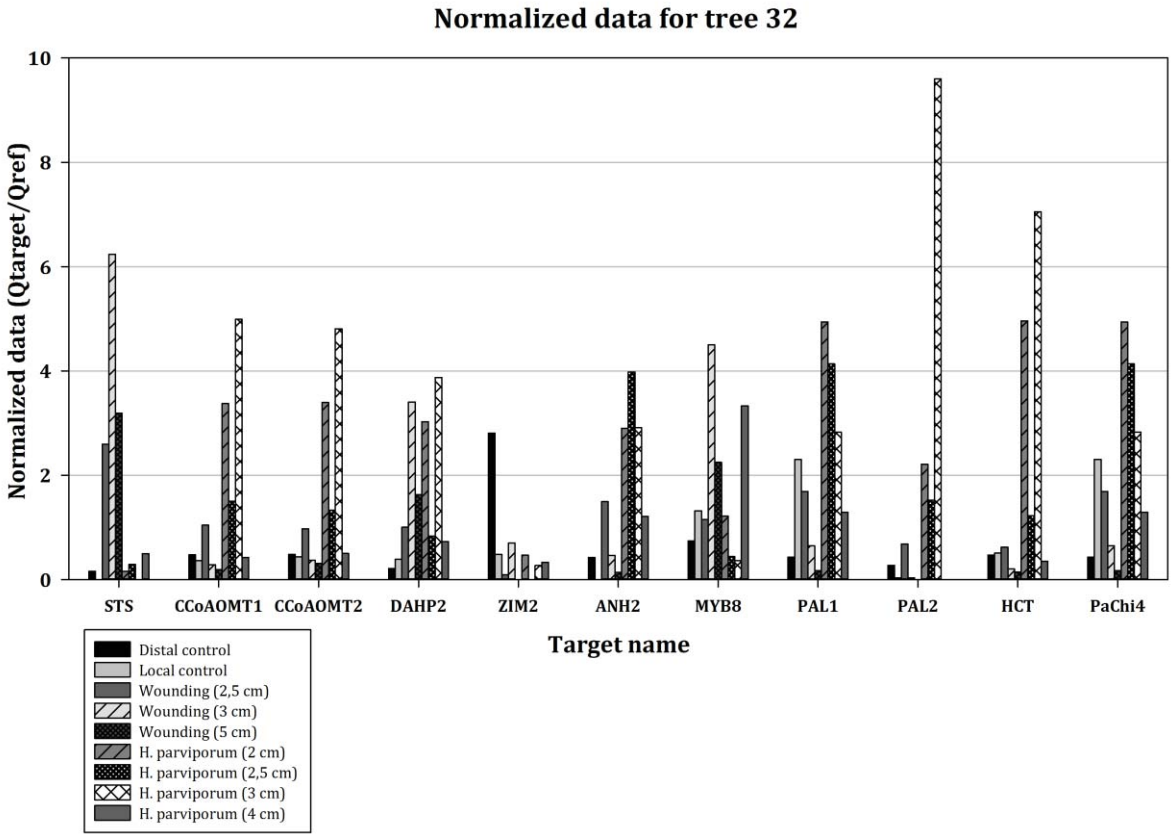

Figure 6: Normalized gene expression for samples at different drilling depths in tree 32, experiment 1. Four treatments were included; Distal control, local control, wounding at different depths and inoculation of *H. parviporum* at different depths.

In experiment 2, clone 275 (figure 7), wounding up regulated PAL2 (p = 0.04). There was also a difference between pathogen inoculations and wounding (p =0.016).

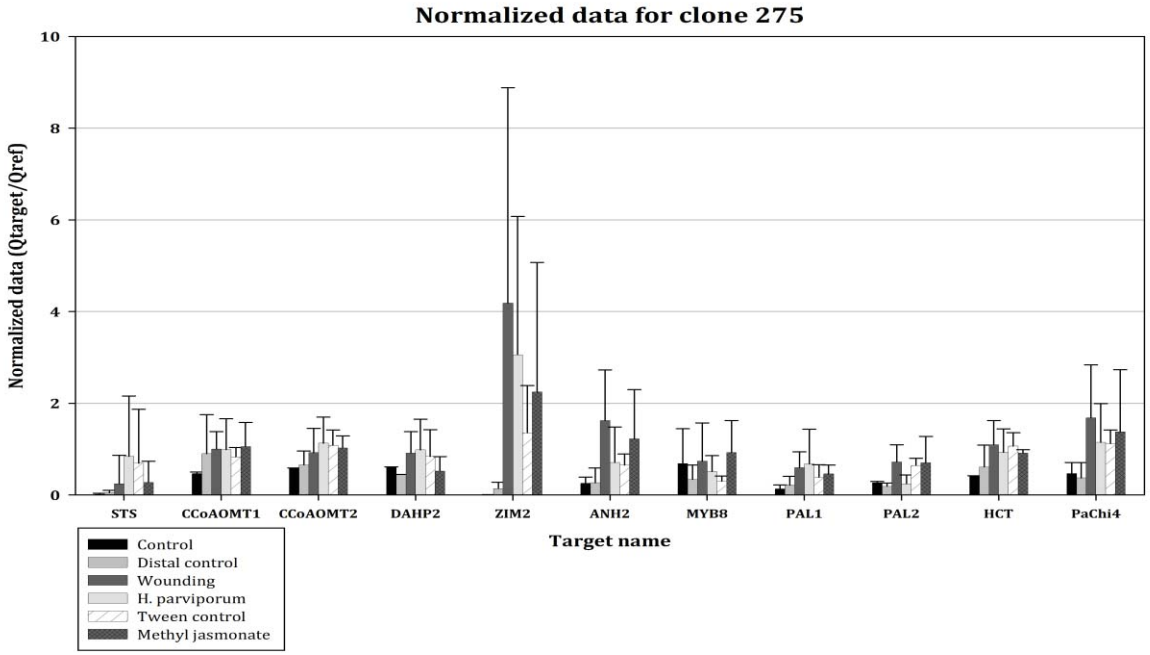

Figure 7: Means of expressed values of genes for each treatment in clone 275 with its respective standard deviation. Data have been normalized to the constitutively expressed genes Actin and α-Tubilin.

In clone 288 (figure 8), wounding up regulated CCoAOMT1. The effect was statistically significant compared to control and distal control (p = 0.025 and 0.018, respectively).

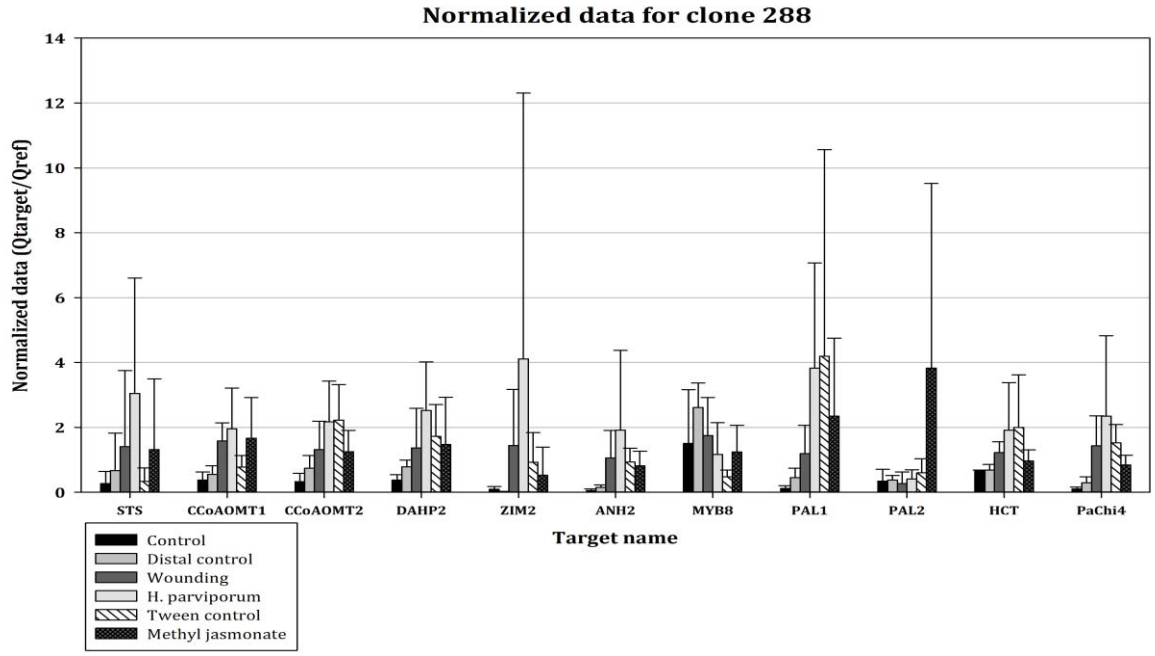

Figure 8: Means of the normalized data for each treatment in clone 288 with its respective standard deviation. Data have been normalized to the constitutively expressed genes Actin and α-Tubilin.

Wounding had a down regulating effect on STS ($p \leq 0.001$) compared with local control in clone 335 (figure 9). PAL2 was also significantly up regulated under methyl jasmonate inoculation in clone 335 ($p = 0.040$).

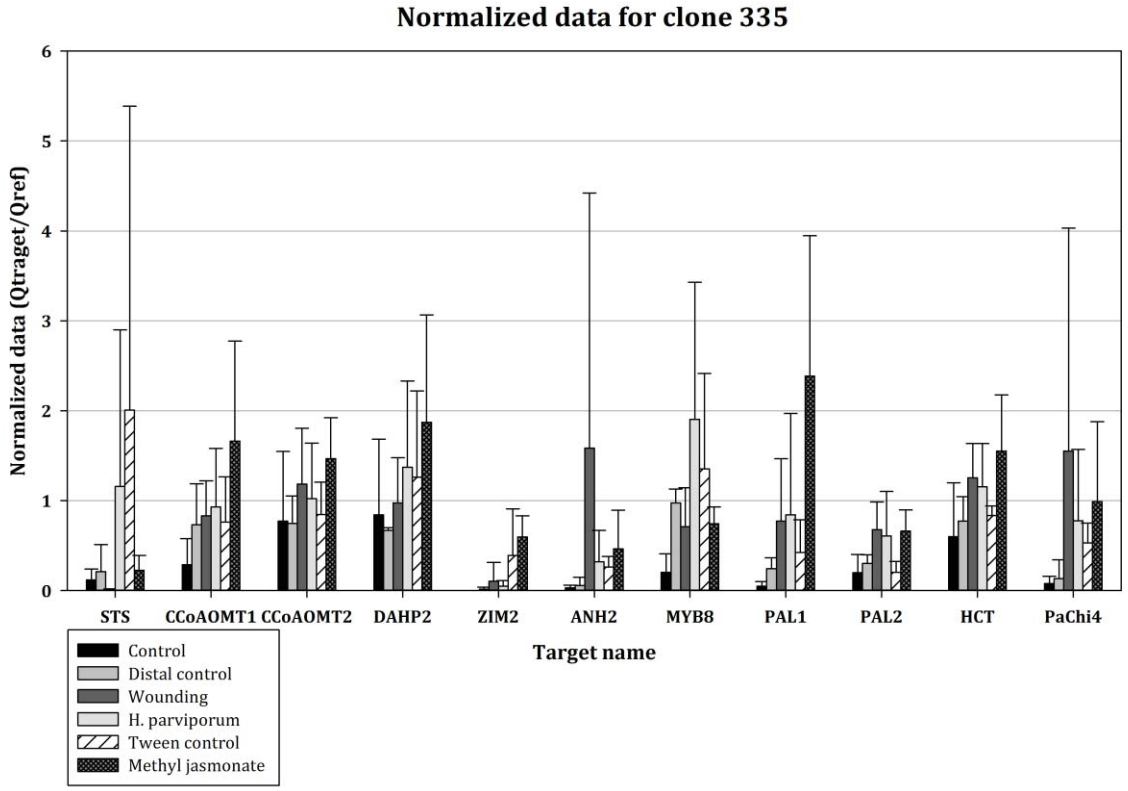

Figure 9: Means of expressed values of genes for each treatment in clone 335 with its respective standard deviation. Data have been normalized to the constitutively expressed genes Actin and α-Tubilin.

4 Discussion

This work is among few that involve both the expression of genes as well as the chemical changes in the reaction zone of Norway spruce when sapwood is infected with the white-rot fungus *Heterobasidion parivporum*. The reaction zone is an important part of the induced defense in Norway spruce. However, there has been little research on the connection between chemical compounds and the change in gene expression within the reaction zone. This leads to the opportunity of increasing our understanding of the behavior of Norway spruce when faced with fungal attack.

4.1 Differentially expressed genes and chemical changes

4.1.1 Stilbene glycosides and stilbene synthase (STS)

Stilbenes have in previous studies shown defense related properties in sapwood (Hart and Shrimpton, 1979). Iso-rhaphontin and E-Astringin are among these stilbenes, and have shown constitutive antifungal functions (Toscano Underwood and Pearce, 1991, Hammerbacher et al., 2011, Rommel, 2012). The connection between STS and the stilbene synthesis (Rommel, 2012) predicted an increase of STS expression and concentration of stilbenes as part of the line of defense in infected or wounded samples in Norway spruce.

Our results revealed that wounding had a down regulating effect on STS, contrary to expectations. According to Hammerbacher et al. (2011), an up regulation of STS will lead to increased levels of stilbenes. This statement is in accordance with our findings, as we did indeed identify lower concentrations of in wounded samples with down regulated STS. However, the concentration of these compounds was decreased for fungal infected samples as well, where STS was not significantly different.

Contradictory studies did not find significant STS transcript level changes in sapwood, whether samples were wounded or inoculated with fungus (Oliva et al., unpublished, Rommel, 2012). In bark however, the transcript level increased in both treatments (Oliva et al., unpublished). Hammerbacher et al. (2011) also found increased levels of STS when white spruce saplings and bark (*Picea glauca*) where inoculated with fungus (*C. polonica*). Effects of wounding alone were not performed. Thus there are differences

in STS response to wounding alone in Oliva et al. (unpublished) and Rommel (2012) and our work. The method of the experiment by Oliva et al. (unpublished) and Rommel (2012) was based on the one we have developed here in Norway, and the inoculation period in Oliva et al. (unpublished) was based on our setup, and differences in results may be caused by other factors.

In regards to phenolic compounds, Lindberg et al. (1992) found a decrease of E-Astringin and iso-rhaphontin in response to in vivo infection of *H. parviporum* in Norway spruce bark, which corresponds with our results. However, these were algycones while our samples were glycosides. The same compounds were also reduced in Sitka spruce bark (*Picea sitchensis*) after inoculation of *H. annosum* in a study by Woodward and Pearce (1988), and the same author finds evidence of aglycones having derived from the glycosides. They also found that these compounds accumulated around wounds (Woodward and Pearce, 1988) and inoculation (Danielsson et al., 2011), which may explain the decrease in the bark. The decrease seen in our samples could perhaps be that they were re-synthesized to form other compounds with defense related properties, but this is hard to believe, as there were no bigger peaks that we did not include in our analysis.

Contradictory, Deflorio et al. (2011) showed increased concentration of both iso-rhaphotin and E-Astringin in wounded and inoculated samples for Sitka spruce bark, however not in sapwood. They had a shorter inoculation period than ours, as well as a different species, which can explain their lack of response in sapwood. Toscano Underwood and Pearce (1991) also suggests that Sitka spruce does not induce high stilbene levels in the reaction zone, which may indicate a slower or different response to attacks in sapwood compared with bark. E-Astringin increased after wounding and decreased after fungal inoculation (*Ceratocystis polonica*) in Norway spruce, in findings by Evensen et al. (2000).

Other explanations for the decrease in phenolic concentration may be differences between tissues. Lindberg et al. (1992) found that there are differences between bark from different places on the tree, and it is therefore not unlikely that we will find differences between bark and sapwood. This conversion of stilbenes may also be

different between species, and that this happens more rapidly in Norway spruce than in Sitka spruce.

Our findings not only confirms the previous found connection of this gene and the phenolic compounds, but also leads to the hypothesis that wounding causes a reaction that reduces the concentration of these glycosides, and that this reaction may be similar in sapwood as in bark.

4.1.2 Constricting levels of chlorogenic acid derivatives and central genes

Chlorogenic acids are antioxidant in plants and protects against degradation of oxidative lipids (Kojima and Uritani, 1973, Niggeweg et al., 2004). An increase of this soluble phenolic can reduce oxidative stress and possibly increase the resistance of pathogenic fungus like *H. parviporum* (Lepelley et al., 2007). This phenolic compound has been linked to genes in the phenylpropanoid pathway, where they are proposed as important enzymes for the synthesis of chlorogenic acid (Lepelley et al., 2007). These genes are the hydroxycinnamoyl-coenzyme (HCT), and caffeoyl-CoA 3-O-methyltransferase (CCoAOMT). HCT is part of the lignin biosynthesis pathway, and is referred to as a defense related gene (Deflorio et al., 2011) and is thought to be part of the pathway, which reacts with the target genes CCoAOMT (Lepelley et al., 2007). Porth et al. (2012) identified CCoAOMT1 and CCoAOMT2 as resistance markers. These defense properties led us to expect increased transcription levels of HCT and CCoAOMT, and as a result, increased concentrations of chlorogenic acid derivatives upon treatments.

According to the expectations, we found an up regulation of HCT in response to inoculation of *H. parviporum* in experiment 1. Koutaniemi et al. (2007) also showed a significant up regulated expression of HCT in inoculated bark samples. These results were based on bark samples after an inoculation period of 14 days. Yaqoob et al. (2012) and Deflorio et al. (2011), however dispute these findings. Yaqoob et al. (2012) found no significant effect on HCT of inoculation of *H. parviporum*, methyl jasmonate or wounding. Here, the inoculation was applied to bark in 2-year-old saplings. Deflorio et al. (2011) found a down regulation of HCT in sapwood, but the species and inoculation method differed from our experiments.

For CCoAOMT1, its expression was up regulated upon fungal inoculation in experiment 1 in tree 31, and of both genes in tree 32. In experiment 2 the only significant result was found in clone 288, where wounding treatment up regulated CCoAOMT1. Koutaniemi et al. (2007) found an effect of *H. annosum* inoculation and wounding on both target genes, which agrees with our results. Fossdal et al. (2012) found transcript levels for CCoAOMT1 that were up regulated as response to wounding, and these levels were higher than in pathogen-inoculated samples.

The concentration of chlorogenic acid derivative decreased upon wounding in both experiments, in tree 32 and clone 335, which is contrary to our expectations. Unfortunately, there is little research on this phenolic compound in Norway spruce, and the studies regarding chlorogenic acid desagrees with our results. Gajera et al. (2014) identified induced concentration of chlorogenic acid in a tolerant variety of groundnut (*Arachis hypogaea L.*) when infected with a rot pathogen (*Aspergillus niger Van Tieghem*). The tolerance level in our samples was unknown; therefore we cannot say if the behavior of our samples were in accordance with this study. Niggeweg et al. (2004) showed an inhibiting effect of elevated chlorogenic acid concentration in tomato plants when plants were infected with pathogen (*P. syringae*).

Apart from this, there is little research on both gene expression and chlorogenic acid and its derivatives in Norway spruce. However, these results indicate that our expectations were legitimate in some degree, and that the link between this compound and target genes are of interest.

4.1.3 Genes connected to the lignin synthesis

Lignin increases as the reaction zone is formed, and genes connected to the synthesis of lignin is of relevance for this thesis. The lignin synthesis is part of the phenylpropanoid pathway, and because of this pathways importance to the line of defense in Norway spruce, we expected an up regulation of these target genes.

PAL is the basal enzyme in the phenylpropanoid pathway (Nagy et al., 2014). Our results showed an up regulation of PAL2 in response to wounding and inoculation of pathogen in experiment 1, and to wounding and inoculation of methyl jasmonate in experiment 2.

Koutaniemi et al. (2007), Yaqoob et al. (2012) and Arnerup et al. (2013) found the most comparable results. Koutaniemi et al. (2007) showed an up regulation of PAL2 in response to *H. annosum* inoculation. Although our findings agree with Koutaniemi et al. (2007), their sample material was bark, not sapwood. Their study did not include responses to wounding.

Findings by Yaqoob et al. (2012) and Arnerup et al. (2013) were contradictory to our results. Yaqoob et al. (2012) found no significant increase of PAL2 in sapwood for *H. parviporum* inoculation, methyl jasmonate inoculation or wounding treatment, and Arnerup et al. (2013) found no significant differences in PAL2 after inoculation of *H. parviporum*. However, the experiment by Arnerup et al. (2013) was performed on bark samples from 4-year-old Norway spruce, which were much younger than our sample material, in addition to being a different tissue. Surprisingly, they did not find significant changes in PAL2 after methyl jasmonate inoculation.

Other studies of PAL mainly focus on bark, and on the target gene PAL1. These studies found higher expression of PAL1 in tolerant trees when inoculated with *H. parviporum* (Karlsson et al., 2007). In Nagy et al. (2014) a blue-stain fungus up regulated PAL. Deflorio et al. (2011) found a down regulation of PAL1 in Sitka spruce sapwood (*Picea sitchensis*) after inoculation of *H. annosum*. PAL1 was also highly expressed close to the reaction zone in response to inoculation in Oliva et al. (unpublished) as well as wounding in Rommel (2012). Finally, Danielsson et al. (2011) found differences in regulation of PAL1 and PAL2 in roots of Norway spruce inoculated with *H. annosum*, depending on genotype.

The regulation of lignin may also be of importance when Norway spruce experiences fungal infection. According to Kranz et al. (1998) R2R3-MYB transcription factor (MYB8) is activated during plant defense reactions, and is part of this regulation. The two groups of inoculation depths had significantly different expression of MYB8. However, the inoculation was not significantly different from the controls. This transcription factor has not been explored in Norway spruce before, and as a result of this we do not have enough data to speculate further on its possible role. Nonetheless we can interpret from Kranz et al. (1998) and Bedon et al. (2007), as well as our results, that sequences

from Norway spruce is needed in order to reveal the responses towards pathogen attack. Our selected gene sequence were based on white spruce (*Picea glauca*), and since this provided only significant difference between to inoculation depths, we suggest further investigations within the transcription family in order to obtain the true orthologous sequence in Norway spruce.

4.1.4 Carbonates and their relation with the reaction zone

The accumulation of carbonates in the reaction zone during formation has been proposed in previous studies (Fossdal, 2014, Shain, 1970, Rommel, 2012, Danielsson et al., 2011, Shain, 1979). Two groups of genes were selected to investigate their influence on the reaction zone as Norway spruce was infected. Deoxy-D-arabinoheptulosonate 7-phosphate synthases (DAHP2) has been thought to synthesize secondary metabolites (Arnerup, 2011), while the carbonic anhydrase gene (ANH2) has a possible defense role against insects, which was identified by Ralph et al. (2006) and Porth et al. (2012). This led us to anticipate an up regulated transcription level for both genes.

Results showed that both genes were significantly up regulated in samples inoculated with *H. parviporum.* However, the responses in Norway spruce towards pathogens have not been tested for carbonic anhydrase genes until now, providing no additional research to compare our results with. Nonetheless our results correspond with our hypothesis, since the up regulation of ANH2 was significant at the inoculation closest to the bark. This may be an indication that the ANH2 gene is affected by this infection, which in turn leads to change in carbon content in the reaction zone hypothesized by Louis Shain back in the early 1970s. The up regulation of DAHP2 is supported by Oliva et al. (unpublished), Arnerup (2011) and (Rommel, 2012), which also found significant up regulation of DAHP2 upon wounding or fungal inoculation. This validates our hypothesis, and recognizes the importance of these genes.

4.1.5 Influences beyond treatments

Since we were not able to identify the compounds UPC 4, UPC 9 and UPC 27, we are unable to compare these with other studies. However, the trend of reduced concentration upon wounding and fungal inoculation appears to be similar in these

unidentified compounds as in the identified ones. The identification was limited by available equipment, standards and little information on the chemistry of Norway spruce sapwood in literature. In addition to the unidentified phenolic compounds, we could not find significant effects on ZIM2 and PaChi4. Particularly we are surprised that PaChi4 was not up regulated in sapwood as it is used as a marker for induced resistance in the bark.

Our results showed that the experiments would have benefitted of additional clones, and more importantly, with a higher number of ramets. This would likely have increased the quality of our findings and strengthened the statistical power. We also saw evidence of unregularly shaped reaction zones globally in the ramets in experiment 2 based on pH measurements (figure 10 and 11, Appendix), which might indicate ongoing damage or stress. We did not see any signs of infection at the time of treatments. However, if the ramets were already highly stressed no additional up regulation of defense genes were warranted. Alternatively, the trade-off between growth and defense must also be balanced (Franceschi et al., 2005, Oliva et al., 2012). In experiment 1, the trees were estimated 60 years old and well established, while in experiment 2 the trees were only 23 years old and in a denser stand. If the young trees have more resources diverted towards growth to outcompete their neighbors, it might have made them less responsive than the trees in experiment 1 (Shain, 1979).

4.1.6 Concluding remarks

We identified changes in extractable phenolic compounds as well as transcriptional changes in defense related genes close to the reaction zone in the sapwood after treatments in Norway spruce. Experiment 1 had several significantly up regulated genes connected to the phenylpropanoid pathway, defense signaling mechanisms and reallocation of carbonates to the reaction zone, which showed effects of inoculation of *H. parviporum*, methyl jasmonate or wounding. The treatments did not affect experiment 2 in the same degree, which might be an indication of an already up-regulated defense caused by ongoing damage or stress.

Several identified and unidentified secondary metabolites decreased in response to treatments. However, we recommend further studies especially on the stilbene

glycosides iso-rhaphontin and E-Astringin, as they appear to have a close connection to stilbene synthase gene STS. In addition, the response of chlorogenic acid to fungal inoculation should be further explored, as there are minimal studies of this phenolic compound in sapwood of Norway spruce.

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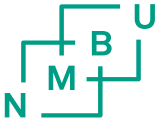
6 Appendix

Pictures of the stems, where a pH-indicator has been sprayed on to illustrate the reaction zone.

Figure 10: Ramet infected with *H. parviporum* in clone 275.

Figure 11: Control tree in clone 275.



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