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Phenolic defense mechanisms in needles of nursery plants and naturally regenerated Norway spruce (Picea abies)

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Preface

This thesis marks the end of my studies at the Norwegian University of Life Sciences (NMBU).

I would like to thank my supervisors Line Nybakken (NMBU), Melissa Magerøy (NIBIO) and Johan Asplund (NMBU) for their time and support during this process.

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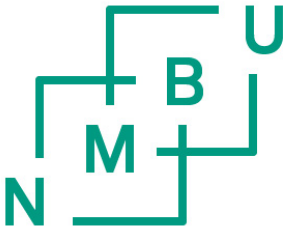
Special thanks to my family and friends for always supporting me.

Oslo, May 2022,

Femke E. de Ruiter



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Abstract

To incorporate sustainable management in increasing forestry practices in Norway, low impact management of Pine weevils *(Hylobius abietis)*, the most common pest on clear cut area in boreal silviculture, is of great importance. This project has looked at differences between inducible and constitutive defense mechanisms, comparing naturally regenerated and planted Norway spruce seedlings on a clear-cut area in Lomsdal, Eastern Norway under treatment with MeJA, Wounding, MeJA+Wounding. Chemical defense, specifically phenolics as well as to carbon, nitrogen, C: N ratio and defense related gene expressions were studied to investigate potential differences in defense mechanism between the two plant types by using HPLC, acid butanol assay, CHNS analyzer, and RT-qPCR. For single phenolic compounds, but not total phenolics, namely stilbene and Acetylphenone picein, as well as single flavonoids (+)catechin, gallocatechin dicoumaroylastragallin2, dicoumaroylastragallin3 and two unknown flavonoids differences for sample type could be observed. Differences in concentrations due to seedling treatment were true for insoluble condensed tannins, Dicoumaroylastragallin1 and dicoumaroylastragallin2. Trade-offs between defense allocation and growth could be one possible explanation for differences in carbon and phenolic concentrations between sample types. Additionally, or alternatively, seedling age, resource allocation to treated areas and UVB light exposure in early development could explain differences in defense composition. Gene expression analysis revealed possible explanation of higher concentrations of stilbenes due to a tradeoff between STS and CHS and CHI precursor use. This work has shown that differences between planted and naturally regenerated seedlings of Norway spruce (*Picea abies*) exist regarding carbon concentrations, C:N ratio, secondary metabolic compounds and gene expression. It can therefore be concluded that defense against external biotic stressors such as the pine weevil are potentially higher in natural regenerated seedlings which should be considered in future forest management to promote tree intrinsic defense mechanism.

Table of Content

1. Introduction

Boreal forest ecosystems make up about 27% of all forest cover and is thus the second largest forest biome (Bright et al., 2014). In addition to environmental, economic, and cultural ecosystem services, boreal forest has the potential to mitigate climate change. Carbon sequestration, nutrient recycling, regulation of water fluxes and preservation of biodiversity are just some examples of possible contributions to cope with human induced environmental changes, which can be mitigated by active forest management (Bright et al., 2014; Huuskonen et al., 2021).

In Norway, forestry is of high importance when it comes to land use change and impacts on environment, as more than half of the area beneath the timberline is covered in boreal forest (Bevanger, 2018; Statistics Norway, 2021). Modern forestry measures have shaped these ecosystems, while natural forests have gradually disappeared in the last 100 years (Bevanger, 2018). Norwegian forestry is currently characterized by even aged monocultures, clear cutting, replanting, and site preparation practices like, thinning, fertilization, and pesticide use are designed to maximize yield (Statistics Norway, 2022). Norway has set a goal to increase products and services related to forestry and is currently increasing spruce production with 12.8 million cubic meters. Additionally, Norway has pledged to manage production in a sustainable way, taking environmental values into account and combining conservation and sustainable use (Ministry of Agriculture and Food, 2018).

To manage boreal forests, it is necessary threatening factors as well as enhance and support its coping mechanisms to ensure sustainable use of forest ecosystems services. Threats to Norway spruce can be abiotic, such as drought and high temperatures, which are expected to increase even more in the upcoming years. Abiotic stressors can also make Norway spruce more vulnerable to biotic stressors such as herbivory and pathogens (Allan et al., 2021, Netherer et al., 2021, Virjamo et al., 2014). We can already observe these changes in Southern and Central Europe where insect abundance ultimately led to loss of big areal of forest, as seen for example in Germany (Ganthaler et al., 2017, Inward et al., 2012, Obladen et al,. 2021, Tan et al., 2010).

The large pine weevil (*Hylobius abietis)* is considered the biggest pest to Norway spruce seedlings, as they feed on stems of young plants leading to damage and death. In addition to increased reproduction, survival rate of pine weevils will also increase with warming temperatures (Inward, et al., 2012, Lalík et al, 2020, Lalík, et al., 2021, Zas et al., 2017). The

pine weevils usually feed on a clear-cut area with new seedling for at least 2 years before moving to a new clear cut where stumps of harvested trees serve as nurseries for pine weevil larvae. At present, up to 63% of seedlings can be killed and 23% of seedlings showed damage by the pine weevil, which is likely to increase in the future (Hanssen, 2011, Lalík et al., 2020, Tan et al., 2010). Measures against pine weevils are currently chemical treatment of seedlings or other labor-intensive physical barriers. However, pesticide use is problematic as it is likely to be prohibited in the future for environmental reasons. The EU, for example, has set the goal to cut down pesticide use by 50% by 2030 (European Comission, 2019, Haukeland, 2007, Lalík et al., 2020, Norwegian Food Safety Authority, 2019). To reduce chemical treatment against pine weevils in young stands, alternative measures as for example increasing trees resistance should be considered. Thus, our knowledge about trees intrinsic defense mechanisms needs to be strengthened (Zas et al., 2017).

Norway spruce trees make use of a combination of physical and chemical defense mechanisms to cope with external biotic stressors. As true for all interspecies interactions, spruce trees have evolved defense mechanisms through the evolutionary arms race, to minimize damage through herbivore or pathogen attack. Physical defense mechanisms are morphological properties such as bark and leaf toughness and other physical barriers (Howe & Jander, 2008, Nemesio-Gomez et al., 2017). Chemical defenses consist of secondary metabolic compounds which in contrast to primary metabolites (growth and storage) are used for defense and protection (Huang et al., 2019). Secondary metabolites can further be divided into constitutive defense metabolites, which are always present and induced defenses which are a reactionary process to external factors, like herbivory grooming through arthropods such as pine weevil (Huang et al., 2019; Huang, Kautz, et al., 2020a).

Norway spruce synthesize secondary metabolic compounds, such as phenolics, terpenes and steroids and alkaloids as their chemical defense (Bourgaud et al., 2001, Metsämuuronen & Sirén, 2019; Huang, Rückner et al., 2020b). These compounds contribute to individual plant fitness through their antifungal, antibiotic, UV protection, antifeeding and antiviral properties and are thereby protecting plants against pathogens (Bourgaud, et al., 2001, Ganthaler et al., 2017, Strack, 1989). The important group of phenylpropanoids or phenols can further be divided in several groups, i.e., stilbenes, flavonoids and tannins and are found in high concentrations in needles of Norway spruce (Ganthaler et al., 2017, Paasala, 2017, Metsämuuronen & Sirén, 2019, Strack, 1989)

After induced defense is triggered by external cues, the signaling pathways through phytohormones like jasmonic acid elicits plants response (Arnerup et al., 2013). This phytohormone, which, in addition to its role in regulating defense mechanisms, is also involved in reproductive and developmental processes, can therefore be considered crucial in endogenous plant communication. External application of methyl jasmonate (MeJA), a jasmonic acid derivative has been proven to generate analogous anatomical changes and inducing defense mechanisms in Norway spruce (Arnerup et al., 2013, Howe & Jander, 2008, Krokene et al., 2008, Li, 2022, Tamogami et al., 2008).

Wounding or methyl jasmonate induce changes in specific defense mechanisms through gene and transcription factor up and downregulation (Ganthaler et al., 2017, Nemesio- Gomez et al., 2017, Wendland & Bawa, 1996). Upregulation of phenylpropanoid biosynthesis related genes has been associated with the accumulation of carbon based secondary metabolic compounds (CBSC).

Phenolic compounds are produced through the phenylpropanoid pathway. These secondary metabolites are generated from aromatic amino acid produced by the shikimate pathway. Phenylalanine ammonia-lyase (PAL) performs the first step in the phenylpropanoid pathway by deaminated L-phenylalanine to cinnamic acid. Cinnamic acid is then further converted to cinnamoyl-CoA or *p*-coumaric acid and then *p*-coumaroyl-CoA. These molecules are then used in the synthesis of flavonoids and stilbenes. Chalcone synthase (CHS) is the first enzyme in the flavonoid pathway, which synthesized chalcone from three malonyl-CoA and one cinnamoyl-CoA or *p*-coumaroyl-CoA. Chalcone is then isomerized by chalcone isomerases (CHI) forming a precursor of flavones and dihydroflavones. Following several other steps, leucoanthocyanidin reductase (LAR) converts leucocyanidin into Catechin. (Hammerbacher et al., 2018, Metsämuuronen, & Sirén, 2019, Appendix B).

Similar to the flavonoid pathways, Stilbene Synthase (STS) uses p-Coumaroyl-CoA to synthesis resveratrol, which is the precursor for all other stilbenes Similar to the flavonoid pathways, Stilbene Synthase (STS) as well condensates p-Coumaroyl-CoA, resulting in the formation of a linear tetraketide intermediate and later formation of stilbenes (Hammerbacher et al., 2011). A deeper understanding of how external stressors influence phenolic composition should include analysis of different steps throughout the phenylpropanoid pathways.

Several factors influence the presence of constitutive and inducible defense metabolites in Norway spruce. CBSCs concentrations are suspected to be influenced by temperature, seasonality and intraspecific differences in concentrations of defense molecules (Bag et al, 2022,

Ganthaler et al., 2017, Virjamo et al., 2014). Additionally, as resources are finite, use of nutrients always comes with trade-offs on investments in either defense or growth. Carbon and nitrogen availability is thus one factor influencing investment in secondary metabolites (Bryant et al, 1983, Herms & Mattson, 1992, Huang et al., 2020b, Koricheva et al., 1998, Nybakken et al., 2018,).

This study is a follow-up project of an experiment previously conducted on the same study site investigating effects of herbivory specifically on bark of Norway spruce seedlings. Disregarding this project, no prior comparisons have been conducted between planted and naturally succeed Norway spruce seedlings (Nybakken, 2020).

In this thesis, Norway spruce seedling constitutive and inducible defense mechanisms regarding external stressors are investigated. More specifically, concentrations of phenolics in planted and naturally regenerated seedlings are measured after wounding. Additionally, carbon and nitrogen concentration were analyzed and changes in relative genome expression of defense related genes were evaluated. My main aim was to test if phenolic compound concentrations and expression of defense related genes (i) differed between naturally regenerated and planted Norway spruce, and if this was related to their nitrogen concentrations, and, furthermore, if (ii) these responses were affected by wounding and MeJA treatment.

2. Material and Methods

2.1 Study System

Study site

The study was conducted in Lomsdal, Søndre Land, Innland county (Figure 1). The vegetation cover is conifer woods, with Norway spruce (*Picea abies*) as dominating vegetation cover (Lomsdal, 2020). In this area, Norway spruce was harvested in 2017 and no further site preparation was conducted. The mean temperature in the last 5 years for June in this area is 16,8℃ and corresponding precipitation is 260,68 mm (Norwegian Center for Climate Services, 2022).

Plant material

Seedlings used for this experiment were both naturally regenerated, and nursery grown seedlings. The naturally regenerated seedlings were approximately three years old at the time

of the experiment, i.e., regenerated before the clear-cutting, but had about the same height as the nursery plants. Healthy naturally grown seedlings were selected and nursery grown seedlings were placed in the same area as the naturally regenerated seedlings (Figure 1). One year old M95 Norway spruce nursery grown seedlings were acquired at Skogplanter Østnorge AS in Biri, originating from seeds from Huse seed plantation in Lillehammer. The M95 Norway spruce nursery grown seedlings had no prior chemical or wax treatment (Lomsdal, 2020).

Seedlings originating form Biri nursery were fertilized during the vegetative phase (Mai-July), with the nursery's self-designed designed fertilizer "*Biri 2 spesial*" (NPK 10-7-18 + 4 Mg + micronutrients; Ammonium sulfate) as well as with commercially available fertilizer ("*Kristalon Blue"*: NPK; 19-3-17 + Mg, S, mikro). In the regenerative phase, the treatment regime was: NPK 7-7-28 + 3 Mg + micro and monopotassium phosphate.
Germination of seeds took place in a greenhouse and seedlings were moved outside or kept in greenhouse depending on the weather conditions. During winter period, the seedlings were stored at freezing temperatures or under a protective snow layer. Through selective breeding processes, seedlings originating from Biri nursery are expected to have a 10-20% yield when compared to naturally regenerated Norway spruce seedlings (Jansson et al., 2017).

2.1.1 Experimental Design

Two clearcut areas were selected at 175–275 m a.s.l (60◦36′ N, 10◦15′ E) and five experimental sites established (3 blocks in area A and two Blocks in area B) (Figure 1). A total of 400 plants, 200 planted and 200 naturally regenerated, were included in the study. Planted seedlings were placed as follows:
For each block, four 3x3 m treatment squares were established containing 10 naturally regenerated seedlings and 10 planted seedlings per treatment (Figure 2). Treatments conducted for both type of plants were application of 50 mM MeJA (in water with one drop of Tween20® per liter), wounding of bark to simulate herbivory, a combination of treatments (MeJA + Wounding) and a control group each for one seedling per block. On May 28, 2019, the above-mentioned treatments were executed. The MeJA solution was applied by brushing on the lower ¾ of seedling stems. For the wounding treatment, two squares of approximately 0.5 × 0.5 cm were scraped off the bark at different heights and opposing sides to avoid ringbark effect. For wounding and control treatments, a solution of Tween20® and water was brushed on the stems like MeJA treatment to eliminate potential effects of Tween20®.

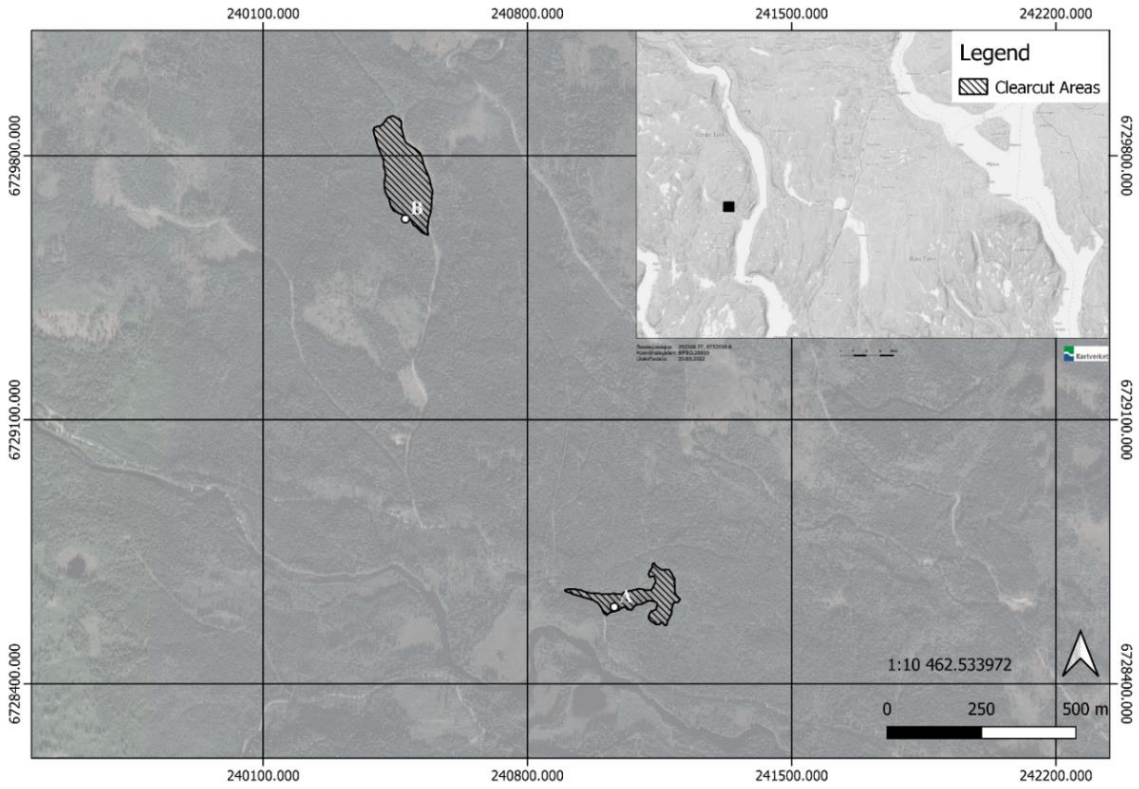

Figure 1: Map of study site. Clear-cut areas A and B, where the experiment was performed are marked.

2.2 Seedling Collection and Sample Preparation

From September 1st to 4th, 2019, five individual seedlings from each block and treatment (in total 40 seedlings) were harvested. The apical 10 cm of each seedling was cut from the stem and the upper 2 cm of shoot tips removed. In the field, the sampled shoots were divided into two halves, one of which was packed in aluminum foil and frozen in liquid nitrogen in a dry shipper, while the other half was put into a paper bag with silica to ensure dryness. The frozen samples were stored in a freezer at -80°C. The samples in the paper bags were dehydrated for 48 hours at 30°C in a drying cabinet, and stored in a cool, dark environment until further analysis.

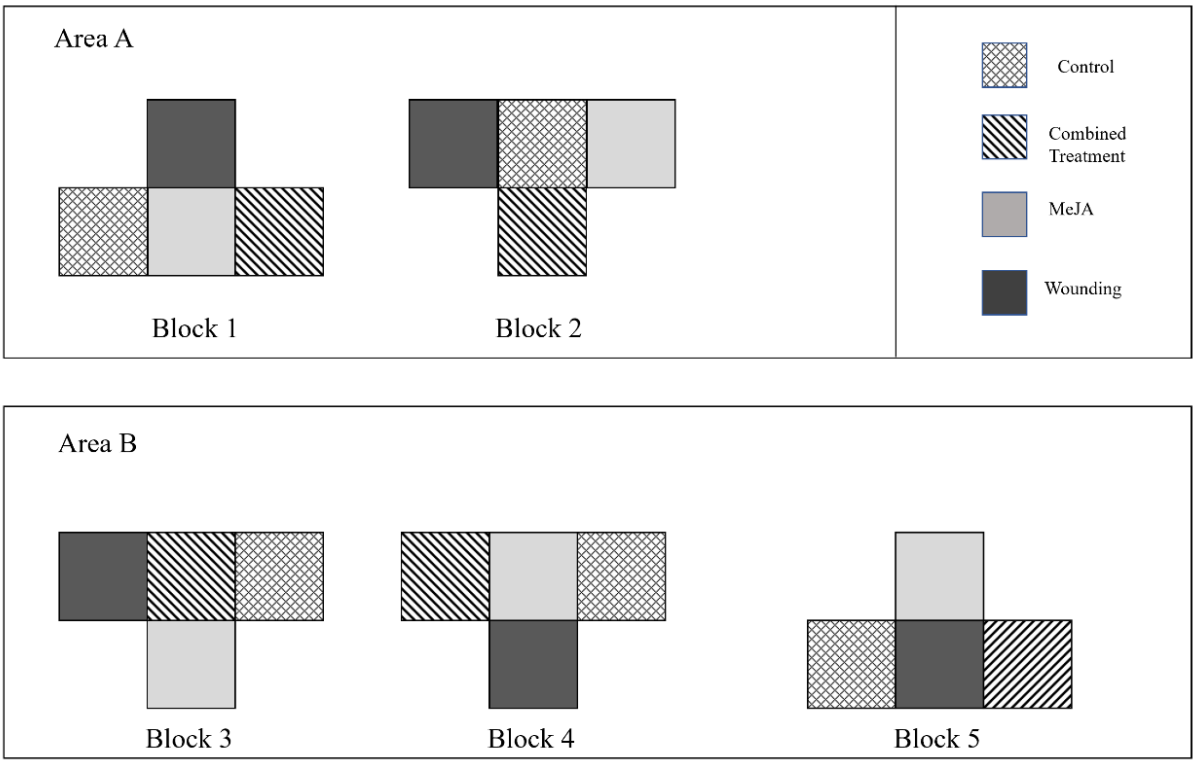

Figure 2: Experimental design of study conducted in Lomsdal, 2019 using naturally regenerated and planted seedlings of Norway spruce. Four treatment types (MeJA, Wounding; MeJA + Wounding and Control) in five blocks (1-5).

2.3 Chemical Analysis

2.3.1 Sample Preparation

All 200 collected samples were included in the phenolic analysis, except for one sample with too little material. Dried samples were then sorted, transferred to 2mL Eppendorf tubes and stored in a dark dry place to prevent degradation through UV light (Figure 3).

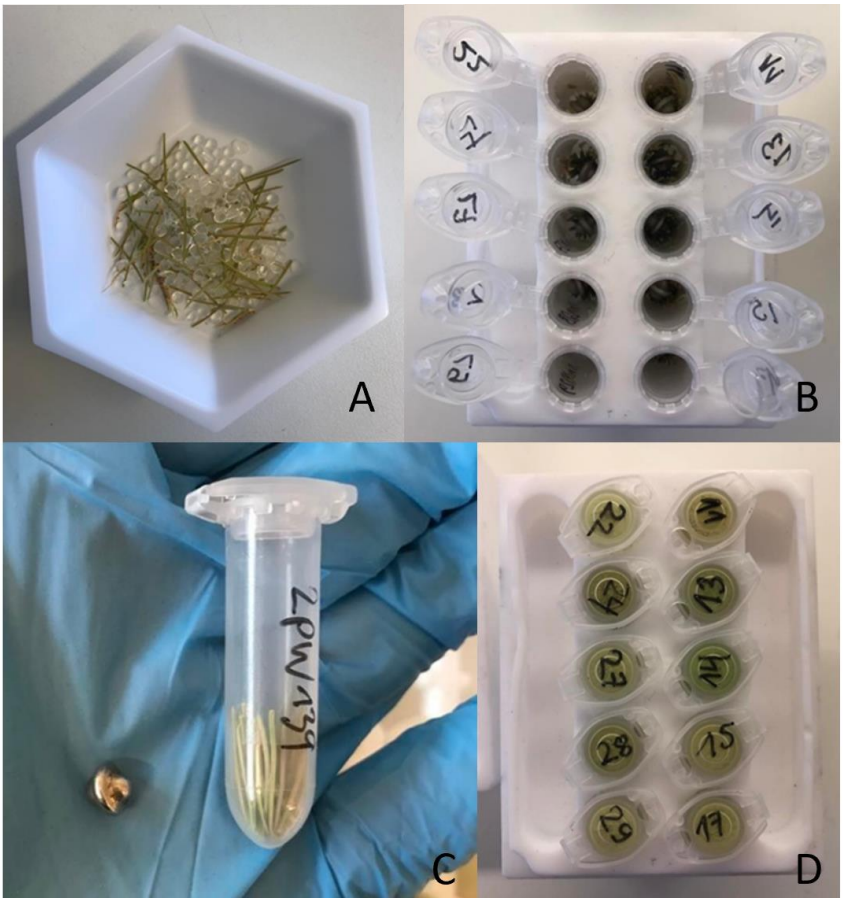

Figure 3: Overview over sample preparation documented through pictures. **A**: Norway spruce needles stored with silica to ensure dryness ready to be sorted. **B**: 2 mL Eppendorf tubes filled with Norway spruce needle samples prior to grinding. **C**: 2 mL Eppendorf tube and stainless-steel bead before grinding. **D**: Norway spruce needs samples after being ground to fine powder stored in 2 mL Eppendorf tubes.

The samples where then ground using a Retsch MM300 TissueLyser mill (Retsch GmbH, Germany) with a stainless-steel bead at a frequency of 30 /s for 2 min per sample. More fibrous samples were run for longer to ensure same sample consistency, which was controlled visually. Ground powder was then stored in a 2mL Eppendorf tubes at room temperature until further analysis (Figure 4).

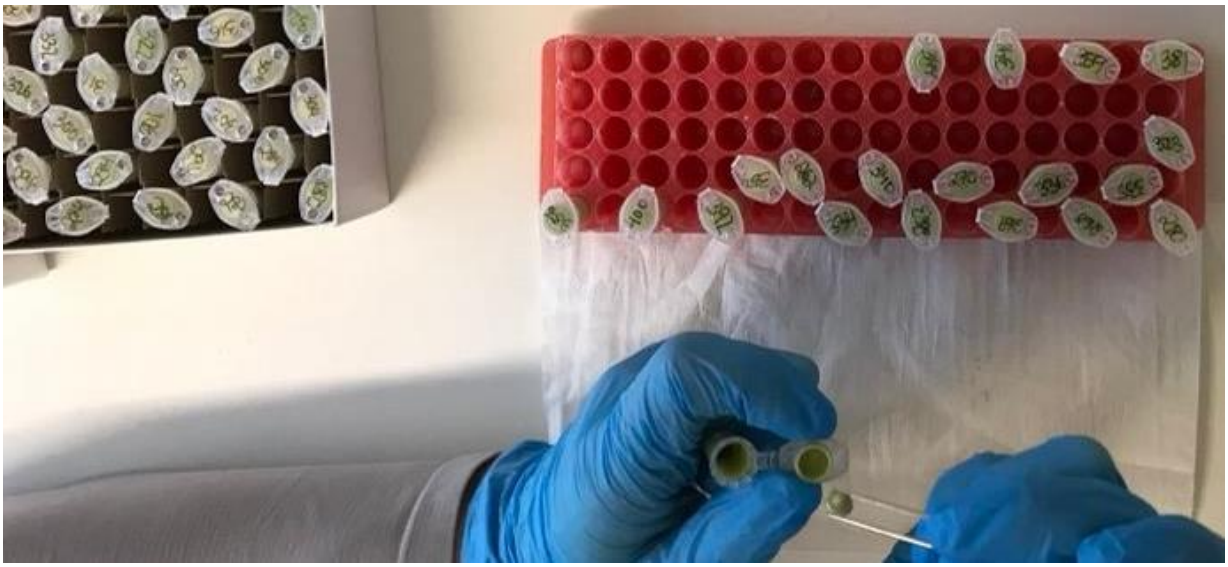

Figure 4: Ground tissue of Norway spruce needles.

2.3.2 Phenol Extractions

Soluble Phenol Extractions

6 mg (+/- 0.5mg) of the stored samples was transferred into 2 mL-Reinforced Preccellys tubes. 500 µL 70% MeOH and 1-3 zirconium oxide beads were added to the tubes for extractions.

Tubes were then mixed thoroughly with FastPrep-24TM 5G (MP biomedicals, USA) for 30 seconds at 5000 rpm under vacuum. After homogenization, the samples were placed on ice for 15 minutes and then centrifuged at maximum speed for 3 minutes. The supernatant was pipetted into 5 mL plastic tubes (Sarstedt AG& Co, Germany). The pellet was washed three more times by adding 500uL MeOH, vortexing and centrifuging, until the supernatant was clear. The supernatant in the marked 5 mL tube was evaporated in a vacuum centrifuge (Eppendorf concentrator plus, Eppendorf, Hamburg, Germany) for three hours. The dried extracts were stored at -20°C until further (HPLC) analysis (Figure 5). After extractions, tissue from which soluble phenols were extracted remained in the 2mL-Preccellys tubes. This residue pellet was stored at -20 °C and later used for MeOH-insoluble condensed tannin analysis.

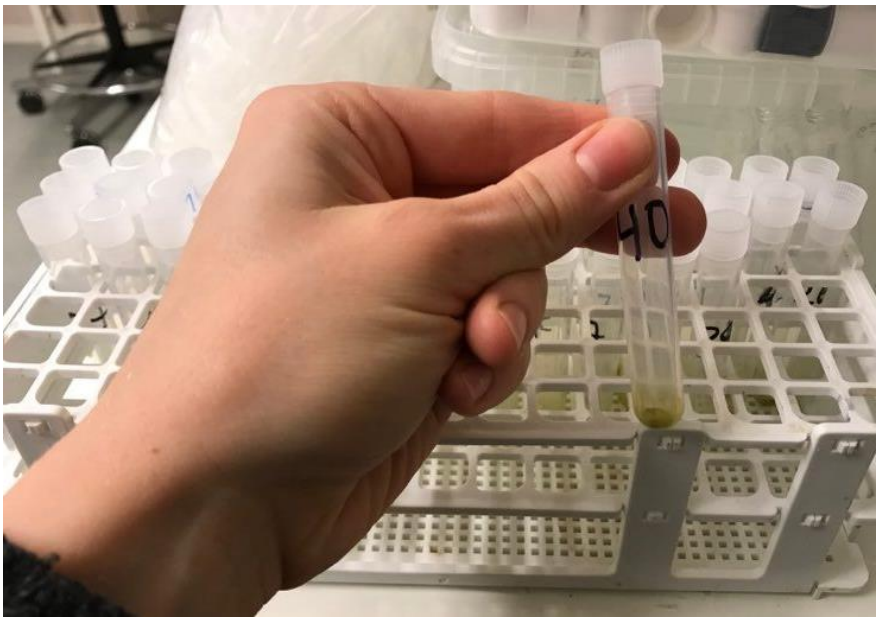

Figure 5: 5mL tubes containing soluble phenolic compounds after extraction and evaporation of MeOH.

HPLC Analysis

The samples were taken out of the freezer and left in the lab sealed for 20-30 minutes until they reached room temperature. The extracts were then dissolved in 200 µL MeOH and 200 µL ultrapure H_2O (USF ELGA Maxima HPLC; Veolia Water Technologies, Saint- Maurice, France) by using an ultrasound bath. The liquid was then transferred into a 2 mL Eppendorf tube and centrifuged at maximum speed for three minutes. After this, the samples were transferred into a HPLC vial and sealed.

Samples were then analyzed an HPLC system (Agilent Series 1200, Agilent Technologies, Waldbronn, Germany) with G1312, a binary pump, a G1329 autosampler, a G1316A column , an oven with thermostat and G1315D diode-matrix detector. The column used to separate the phenols (50 × 4,6 mM (length, inner diamer) contained ODS Hypersil (3 µM) (Thermo Fisher Scientific Inc., Waltham, USA). Two mobile phases, A (30 mL tetrahydrofuran; 5 mL orthophosphoric acid; 1965 mL ultrapure water) and B (MeOH, 70%) were used. Temperature under analysis was 30°C, with an injection volume of 20 µL. To identify low molecular phenolic acids, retention times at absorption spectra at 270 and 320 nm were used.

For low molecular weight phenolics, values used for statistical analysis derived from values received from HPLC analysis were converted as follows:

$$Concentrations\ (mG\ g-1\ dryweight) = \frac{Area\ HPLC \times responsefactor \times\ Volume\ dissolvant}{weight\ of\ sample \times Volume\ injectionweight}$$

Non detectable phenolic acids (area HPLC <25) where recorded as 0.

Condensed Tannins

Concentrations of both MeOH-soluble and MeOH-insoluble CTs were quantified using the acid butanol assay for proanthocyanidins (Hagerman, 2002).

MeOH-Soluble Condensed Tannin Analysis

MeOH-soluble condensed tannins were analyzed from HPLC-extract within 48 hours after HPLC analysis. For analysis, 100 µL sample extract of dissolved sample was transferred to a 10 mL glass tube, and 400 µL MeOH, 100 µL ferric reagent (2 M HCI; 2 % Ammonium iron (III) sulfate) and 3 mL acid butanol (950 mL butanol; 50 mL HCI) were added. Samples were mixed by inversion. All glass tubes were sealed and incubated in a water bath at 99° for 50 minutes. As a reference, 100 µL MeOH was placed in a glass tube as described above, replacing the sample. A spectrophotometer (UV-1800, Shimadzu Corp., Kyoto, Japan) was used for absorbance measure with 550 nm wavelength. Duplicate analyses were run for all samples (thus A and B samples for n=197). The average absorption measure was then used for further statistical analysis. Soluble condensed tannins, analyzed from residual liquid from the HPLC analysis, and values received from the photo spectrometer of 550 wavelength were calculated according to standard curves made from analyses of purified condensed tannins from spruce.

MeOH-Insoluble Tannin Analysis

Of the extraction residue, 1.5 mg (± 0.5 mg) were weighed with a microscale (Mettler-Toledo XP6, GmbH, Switzerland). The powder was then transferred to a 50mL glass tube and stored at -20°C until further analysis. Similar to MeOH- soluble tannin analysis, the powdered sample was dissolved by adding 500µL MeOH, 100 µL ferric reagent (2 M HCI with 2 % Ammonium iron(III) sulfate) and 3 mL acid butanol (950 mL butanol; 50 mL concentrated HCI). Due to little sample material, duplicating the analysis was not possible and thus only one subsample per sample boiled at 99°C for 50 minutes in a water bath. Results from absorbance measure with 550 nm wavelength, spectrophotometer (UV-1800, Shimadzu Corp., Kyoto, Japan) of n=199 samples were included for further statistical analysis. Transformation of raw

data photo spectrometric analysis from insoluble condensed tannins for statistical analysis has taken place according to standard curves made from analyses of purified condensed tannins from spruce.

2.3.4 Carbon and Nitrogen Concentrations

Ground samples were weighed (5 mg (± 0.5 mg)) with a microscale (Mettler-Toledo XP6, GmbH, Switzerland), placed in tin foil and then analyzed using Vario Micro cube Elementar CHNS analyzer (Elementar Analysen systeme GmbH, Hanau, Germany). Three samples had insufficient material and thus could not be analyzed. Therefore, the total amount of analyzed samples used for further statistical analysis is n=197.

2.3.5 Gene Expression Analysis

Primer Selection

Genes for gene expression analyses were selected after literature research and prior experience from phenolic quantification of bark from the same seedlings (Lomsdal, 2020).

Analysis was run on several genes, however not all primers were selected for statistical analysis due to their poor melting curves and/or threshold values

The final genes selected are genes involved in the biosynthesis of stilbenes (STS) and flavonoids (PAL; CHI1; LAR2; LAR3; CHS) or related to defense (Chi4). In addition, the transcription factor MYB35 was included, as it is suspected to be involved in the regulation of phenolic biosynthesis (Table 1) (Nemesio-Gorrez et al, 2017).

Sample Preparation

For gene analysis, samples were pooled by combining all five needle samples of the same type, treatment, and block. These samples will further be referred to as “pooled sample” in this thesis (n= 40 for pooled samples).

Pooled samples were ground in liquid nitrogen using a mortar and a pestle to create a fine powder and checked for consistency visually. Pooled samples with the same treatment and type where ground in the same mortar, which was cleaned thoroughly with 70% etOH between pooled samples (Figure 6).

The pooled samples were transferred with a spatula into a marked 2mL Eppendorf tube. Pooled Samples were then stored at -80 °C until further RNA extraction.

Fig 6: Pooled sample chilled with liquid nitrogen with mortar and a pestle before grinding.

RNA Extraction

Prepared pooled samples were used for RNA extraction which was performed by using MasterPureTM Complete DNA and RNA Purification Kit (Epicentre, USA) and adapting the provided protocol.

Approximately 40 mg of frozen ground tissue of the pooled samples were placed in a 2 mL Eppendorf tube together with 610 µL Mastermix 1. The Mastermix 1, consisting of 1µL proteinase K, 6 mg PVP and 3 µL of βME, 600 µl Tissue and Cell Lysis. The Mastermix was mixed thoroughly by pipetting

Pooled samples were then incubated at 56°C for 15 minutes with vortexing after 5- and 10-minutes incubation time. After, samples where centrifuged at 4°C/ max speed(10,000 × g) for 10 minutes in prechilled centrifuge. Supernatant is then transferred into a new 2 mL Eppendorf tube and 250 µL MPC Protein Precipitation Reagent was added. Pooled samples are then vortexed and centrifuged at 4°C/ max speed (10,000 × g) for 10 minutes. This step was repeated until supernatant looked clear.

Depending on corresponding MPC treatment of each sample, 370 µL or 500 µL of 7.5 M ammonium acetate was added to 610 µL of supernatant and mixed carefully through inversion. Samples were then left at -20°C for at least 24 hours until further analysis.

Afterwards, pooled samples were centrifuged at 4°C at max speed (10,000 × g) for 30 minutes to pellet the debris. The extracted pellet was rinsed with 75% ethanol carefully and centrifuged between rinsing.

The ethanol was removed, and the dry pellet was dissolved in 30 µL of 56°C nuclease-free water. Resuspension was assisted by pipetting water against the pellet. The samples were placed on ice for 10 minutes and vortexed for 10-15 seconds before placed in a -80°C freezer.

RNA purity and quantification was determined using a NANODROP 200 (Thermo Scientific). Additionally, RNA quality was tested by gel electrophoresis using a 1% E-Gel Precast Agarose Gels with SYBR Safe (https://www.thermofisher.com/). If visualization showed sign of RNA degradation, a new extraction was performed.

Dilution and cDNA Synthesis

Extracted RNA from the pooled samples was diluted with RNA free H2O to achieve a concentration of 25 ng / µM following formular:

$$\frac{(25\text{nG}/\mu\text{M})}{(\text{total DNA nG}/\ \mu\text{M})} \times 20\ \mu\text{M H20} = \text{amound of sample per solution } (\mu\text{M})$$

RNA nG/mL data from spectrometric analysis was used in this calculation. The diluted samples were transferred to an Eppendorf Fast PCR Tube Strips (Eppendorf, Germany) and stored at -20 °C until further synthesis of cDNA.

Using Thermo Scientific Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase by Thermo Scientific, cDNA synthesis was executed according to the provided protocol and using 200 nG total nucleotides. First, 10x dsDNase Buffer (1 µL), dsDNase (1 µL) and template DNA (8 µL) were added in Eppendorf Fast PCR Tube Strips and mixed gently and vortexed, then incubated at 37°C for 5 minutes. Afterwards, 10 µL reaction mix (5x Reaction mix (4 µL, Maxima Enzyme Mix (2 µL) and nuclease-free H2O (4 µL) was added to each tube. Pooled samples were then incubated in a qPCR machine for 10 min at 20°C, 30 minutes at 15°C and 5 minutes at 85°C. The cDNA was stored at -20 °C until RT-qPCR analysis.

RT-qPCR Analysis

Quantification of selected genes of interest as well as Actin, chosen as housekeeping gene, was executed by using a ViiA 7 Real-Time PCR System (Applied Biosystems, USA) and QuantStudio Real-Time PCR Software (v1.3, Applied Biosystems) for real time quantitative polymerase chain reaction (RTqPCR) (Table 1).

A primer mix containing forward and reverse primer sequence with a concentration of 4 µM was made according to the following formula:

$$100\mu L = (\,Yield\ (nmol) \times 10) + X\ H2O\ \mu L$$

A 10 µL amplification reaction containing Fast SYBR^TM (5 µL), above mentioned primer dilution for selected genes (1 µL) and nuclease-free H2O (3 µL) and 1 µL cDNA was used for RT-qPCR analysis. Analyses were run using the comparative Ct program for fast SYBR: 20 seconds at 95 °C, 40 cycles of 1 second at 95 °C, 20 seconds at 60 °C, 15 seconds at 95 °C, 60 seconds at 60 °C and 15 seconds at 95 °C. A no-template control was run for each primer.

For genes that were low in expression (MYB 35; STS; CHS; CHI 1; LAR2, LAR3) the amount of cDNA was increased to 2µL and reaction amplification was adjusted: Fast SYBR^TM (5 µL), primer dilution for selected genes (2 µL) and nuclease-free H2O (2 µL). This concentration was then also increased for Actin.

Threshold cycle (Ct) values were used to determine relative gene expression. Ct values represent the number of reaction cycles specific target nucleic acids need to pass a fluorescent threshold. Ct values are inversely proportional to the amount of target nucleic acids and are thus lower if the amount of target nucleic acids is higher in the sample. A Ct of 40 was used as a maximum threshold and all values above were disregarded and noted as 40.

Further, Ct values were organized in EXEL and Delta Ct (ΔCt) and Delta Delta Ct (ΔΔCt) were calculated, which explain relative gene expression in comparison to the housekeeping gene (Actin 1uL; Actin 2uL) and then used in statistical analysis.

Relative gene expression was calculated by using Delta Threshold Cycle (Ct) (ΔCt) and Delta Delta Ct (ΔΔCt). These values were calculated from the threshold cycles received from the real time qPCR machine and calculated relative to the housekeeping gene Actin (ΔCt). Thereafter, naturally regenerated control samples were used as a common base for further comparison in ΔΔCt. (i.e Rao et al., 2013, Zhang et al., 2016):

$$\Delta Ct = Ct\ (target\ gene) - Ct\ (Actin)$$

And

$$\Delta\Delta Ct = \Delta Ct\ (target\ sample) - \Delta Ct\ (average\ naturally\ regenerated\ control)$$

Table 1: Primers used for Overview of primers used in RT-qPCR were Phenylalanine Ammonia-lyase (PAL2); (MYB35); Leucoanthocyanidin Reductase (LAR2, LAR3); Chalcone Synthase (CHS); Chalcone Isomerases (CHI1, CHI4); Stilbene Synthase (STS). Actin was used as a housekeeping gene

Gene	**Forward/ reverse primer**	**Reference**
Actin	GGCATACCGGCAGCTCTTC / AAGTTGTTGGCGGCGTCTT	Hietala et al., 2003
PAL2	TTGCTCGTAGGCACCAATAGC/ GCCTTGCCTTCGTTGATAGC	Yaqoob et al., 2012
MYB35	AAAGGAATCGACCCCAAAAC/ CTGTCATACCGTGCTCGAAA	Nemesio-Gorriz, et al. 2017
LAR2	ACAAGAACTTTTGCATTAGCCG/ GAAATCTCTGGATATAGTTGTGAC	Nemesio-Gorriz et al., 2017
LAR3	CGGACATTGTGACACGAAAC/ CGGAGTTTATACCCGTTCCA	Nemesio-Gorriz et al., 2017
CHS	CCGCCTCTCAAATAAATCGTATTAGT/ ATCAATTATTTGGGTTTCAGTTCTG	Nemesio-Gorriz et al., 2017
CHI1	TCGAGGAGGAGGAAGAGGA/ TCCGCCCAAGTACCATTTCT	Wilkinson et al., 2022
CHI4	GCGAGGGCAAGGGATTCTAC/ GTGGTGCCAAATCCAGAAA	Yaqoob et al., 2012
STS	ACAAGTTCAAGCGAATATGTGAA / ATGTTTCCGTACTCGCTCATAAC	Paasela et al., 2017

2.4 Statistical Analysis

For C:N ratio, Nitrogen Concentration and Carbon concentration, raw data from microcube was used without further transformation.

Data summary has been carried out using summary function in "psych" (R package "psych", Revelle, 2021) for carbon, nitrogen and C:N ratio as well as condensed tannins. Soluble condensed tannins and non-soluble condensed tannins concentration in mg g^{-1} as well as low molecular weight phenolics, carbon, nitrogen and C:N ratio and relative gene expression ($\Delta\Delta$Ct) were analyzed using a linear mixed effects model "lme4" (R package lme4; Bates et al., 2015). Sample Type and Treatment were used as fixed effects while *Block* nested in *Area* were used as random effects for analysis as follows:

*(lmer(X~(SampleType*SampleTreatment) + (1|Area/Block), data).*

Post hoc testing was conducted using "emmeans" for multiple comparison using Tukey adjustment (Lenth, 2022). Normal distribution of residuals was confirmed visually through QQplotting. Type III ANOVA Analysis of Variance Table was used for printing results, applying Satterthwaite´s method to estimate degrees of freedom as well as to generate p-values. All statistical analyses were conducted with R version 4.0.3 and all visualized displayed results were plotted for in R, using "ggplot" (R Core Team, 2020; R package "ggplot2", Wickham, 2016).

3. Results

3.1 Phenolics

3.1.1 Low Molecular Phenolic Compounds

HPLC analysis revealed differences in concentration of individual low molecular weight phenolics of Norway spruce seedling needles when comparing both seedling types and seedling treatment treatments. Phenolics were analyzed in groups of flavonoids (n=11), stilbenes (n=5) and acetophenones (n=1) as well as individual compounds to observe naturally regenerated and planted seedling response to MeJA, Wounding and MeJA + Wounding treatments.

The differences in the acetophenone picein concentrations were based on type of seedling (Table 2).

The flavonoids gallocatechin, (+)catechin, dicoumaroylastragallin2, dicoumaroylastragallin3 as well as two unknown flavonoids showed differences in concentrations based on type of seedling. Dicoumaroylastragallin2 and one unknown flavonoid showed differences depending on sample treatment. For two unknown flavonoids, there was an interaction effect (Type × Treatment) (Table 2).

In sum, concentration of flavonoids did not differ with type, treatment of sample or interaction effects.

The concentration of all stilbenes, namely gallic acid, piceatannol glucoside, resveratrol, isorhapontin glucoside and piceatannol aglycon was found to be differing between naturally regenerated and planted seedlings dependent on seedling types (Table 2). For gallic acid, additionally, interaction effects seemed to impact differences in concentrations. In sum, stilbene concentration differed due to seedling type and were higher in naturally regenerated samples, differing slightly between treatments (Figure 7). Post hoc testing confirmed statistically significant differences between naturally regenerated and planted seedlings of all treatments (Control = P-Value: 0.002; MeJA, Wounded and MeJA+Wounding = P-Value: <0.001)

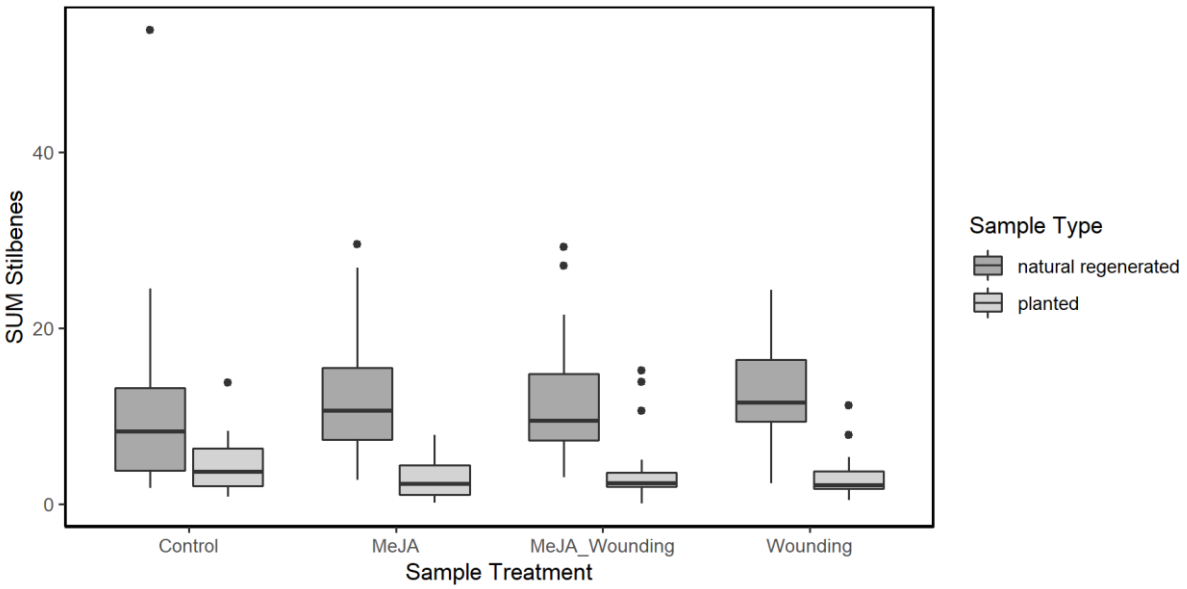

Figure 7: Sum of stilbenes from average concentration (mG g^{-1}) in needles of Norway spruce displayed as bar plot differentiating sample types and treatments.

Table 2: Average concentration (mg g^{-1} (± sd)) of low molecular weight phenols in needles Norway spruce seedlings (n=198) and statistical analysis (ANOVA) of linear mixed effects model, taking sample type (naturally regenerated/ planted) and sample treatment (MeJA, Wounding, MeJA + Wounding and Control) into account. Phenolics are grouped into acetophenones (Picein), flavonoids (Gallocatechin, (+)catechin, Monocoumaroylastragallin, Dicoumaroylastragallin1-3; Unknown flavonoids (n=5) and the sum of all flavonoids), stilbenes (Gallic acid, Piceatannol glucoside, Resveratrol, Isorhapontin glucoside, Piceatannol aglycon and sum of all stilbenes). Statistical results are displayed through F-values and P-values where P <0.005 is considered statistically significant and marked in bold. All numbers excluding (Sum of flavonoids, Gallic acid, Dicoumaroylastragallin3) were square transformed after inspecting the normality of residuals.

	Planted mean±sd				**Naturally regenerated** mean±sd				**ANOVA linear mixed effects model** F-Value(P)		
	Control	MeJA	MeJA+Wounding	Wounding	Control	MeJA	MeJA+Wounding	Wounding	Type	Treatment	Type ×Treatment
Acetonphenols											
Picein	15.36±14.22	8.30±9.95	13.25±17.52	12.43±10.05	13.74±11.86	22.01±19.98	24.15±21.62	21.90±18.30	**10.47(0.001)**	0.39 (0.726)	1.95 (0.123)
Flavonoids											
Gallocatechin	0.22±0.31	0.32±0.43	0.20±0.43	0.15±0.11	0.35±0.44	0.40±0.65	0.24±0.34	0.40±0.69	**4.84 (0.029)**	0.96 (0.409)	0.38 (0.764)
(+)Catechin	0.27±0.16	0.31±0.18	0.25±0.13	0.28±0.13	0.30±0.12	0.36±0.16	0.35±0.23	0.38±0.19	**10.29 (0.002)**	1.46 (0.226)	0.28 (0.843)
Monocoumaroylastragallin	0.21±0.15	0.15±0.11	0.22±0.13	0.26±0.17	0.32±0.18	0.25±0.28	0.26±0.25	0.23±0.16	3.27 (0.072)	1.57 (0.198)	1.42 (0.237)
Dicoumaroylastragallin1	0.40±0.18	0.50±0.22	0.38±0.24	0.50±0.24	0.41±0.24	0.51±0.29	0.43±0.23	0.49±0.21	0.00 (0.969)	**3.138 (0.027)**	0.17 (0.918)
Dicoumaroylastragallin2	0.09±0.20	0.16±0.24	0.21±0.21	0.17±0.21	0.11±0.19	0.33±0.30	0.29±0.28	0.18±0.26	**4.63 (0.033)**	**8.27 (<0.001)**	2.28 (0.081)
Dicoumaroylastragallin3	7.25±1.93	6.59±1.49	7.03±2.11	7.11±2.18	6.89±2.21	6.65±1.96	5.80±2.12	5.29±1.60	**9.03 (0.003)**	1.830 (0.143)	2.32 (0.077)
Unknown flavonoids											
Flavonoid 1	0.22±0.23	0.13±0.16	0.16±0.23	0.18±0.50	0.41±0.42	0.59±0.62	0.56±0.42	0.54±0.32	**78.97 (<0.001)**	0.04 (0.988)	2.29 (0.080)
Flavonoid 2	0.21±0.08	0.11±0.09	0.18±0.07	0.17±0.07	0.21±0.15	0.27±0.17	0.25±0.18	0.31±0.15	**28.37 (<0.001)**	1.34 (0.263)	**4.67 (0.00361)**
Flavonoid 3	0.66±0.36	0.56±0.35	0.46±0.37	0.80±0.52	0.52±0.38	0.64±0.41	0.57±0.41	0.59±0.36	0.36 (0.546)	1.29 (0.280)	1.93 (0.124)
Flavonoid 4	0.23±0.32	0.17±0.27	0.38±0.39	0.39±0.41	0.29±0.32	0.22±0.26	0.12±0.17	0.32±0.37	0.26 (0.608)	2.63 (0.051)	**4.23 (0.006)**
Flavonoid 5	0.30±0.22	0.19±0.21	0.23±0.26	0.22±0.21	0.25±0.24	0.17±0.22	0.30±0.29	0.26±0.29	0.15 (0.695)	2.488 (0.062)	1.21 (0.309)
Sum of flavonoids	10.07±2.20	9.19±1.83	9.67±2.67	10.23±2.56	10.04±2.49	10.38±2.52	9.17±1.69	8.98±1.90	0.21 (0.647)	0.71 (0.546)	2.57 (0.055)
Stilbenes											
Gallic acid	3.64±3.36	5.69±2.867	5.15±4.41	3.01±2.38	7.58±4.53	5.31±4.80	5.07±4.68	4.29±4.31	**4.36 (0.038)**	2.55 (0.057)	**3.02 (0.031)**
Piceatannol glucoside	2.24±1.92	1.40±1.57	1.93±2.64	1.20±1.59	6.15±7.08	6.56±4.52	5.86±3.45	5.79±4.09	**108.77(<0.001)**	0.48 (0.69)	0.32 (0.814)
Resveratrol	1.88±1.24	1.39±1.10	1.48±1.13	1.54±0.87	4.40±4.43	5.36±4.67	5.98±4.26	6.12±3.73	**115.14 (<0.001)**	0.78 (0.509)	1.98 (0.118)
Isorhapontin glucoside	0.12±0.26	0.03±0.05	0.11±0.28	0.04±0.10	0.239±0.461	0.420±0.915	0.216±0.370	0.305±0.819	**14.42 (<0.001)**	0.24 (0.869)	0.78 (0.503)
Piceatannol aglycon	0.12±0.10	0.10±0.08	0.14±0.12	0.17±0.12	0.11±0.10	0.10±0.08	0.09±0.10	0.09±0.08	**8.21 (0.005)**	0.99 (0.401)	1.23 (0.299)
Sum of stilbenes	4.36±2.96	2.91±2.32	3.65±3.80	2.95±2.37	10.90±11.05	12.44±7.67	12.14±7.04	12.30±5.80	**147.38 (<0.001)**	0.06 (0.981)	1.95 (0.123)

3.1.2 Condensed Tannins

Insoluble condensed tannins differed depending on treatment. The concentrations were generally higher in control groups than samples with any other treatments (Table 3, Figure 8). However, the post hoc testing did not reveal which treatments differed significantly from each other.

Table 3: Mean concentration (mg g^{-1}) (± sd)) of insoluble (n=199) and soluble tannins for samples grouped after sample type (naturally regenerated and planted) and treatment (MeJA, Wounding, MeJA + Wounding and Control). Statistical results after analysis (ANOVA) of linear mixed effects model of log transformed concentrations of insoluble and soluble condensed tannins for samples grouped after sample type (naturally regenerated and planted) and sample treatment (MeJA, Wounding, MeJA + Wounding and Control) are displayed through F-values and P-values were P< 0.005 is marked in bold. Area and block were included as random effects.

	Insoluble Condensed Tannins	**Soluble Condensed Tannins**
Planted	Mean ± sd	Mean ± sd
Control	43.26±19.58	51.69±20.23
MeJA	37.50±18.83703	59.72±19.91
MeJA+Wounding	34.34±19.61	59.80±22.77
Wounding	35.03±18.21	52.78±25.53
Mean	37.53±19.06	55.99±22.11
Naturally regenerated		
Control	41.99±20.96	55.79±18.82
MeJA.	36.33±17.18	65.81±16.87
MeJA+Wounding	40.41±16.45	57.65±29.57
Wounding	33.84±15.11	65.84±24.90
Mean	38.14±17.42	61.27±22.54
	F-value (P-value)	
Type	0.46 (0.501)	3.19 (0.076.)
Treatment	**2.67 (0.049 *)**	1.67 (0.175)
Type×Treatment	1.24 (0.296)	1.62 (0.187)

There were no differences in soluble condensed tannins of Norway spruce needles between plant types or treatments.

Statistical analysis for condensed tannins was conducted for both non soluble and soluble tannins separately.

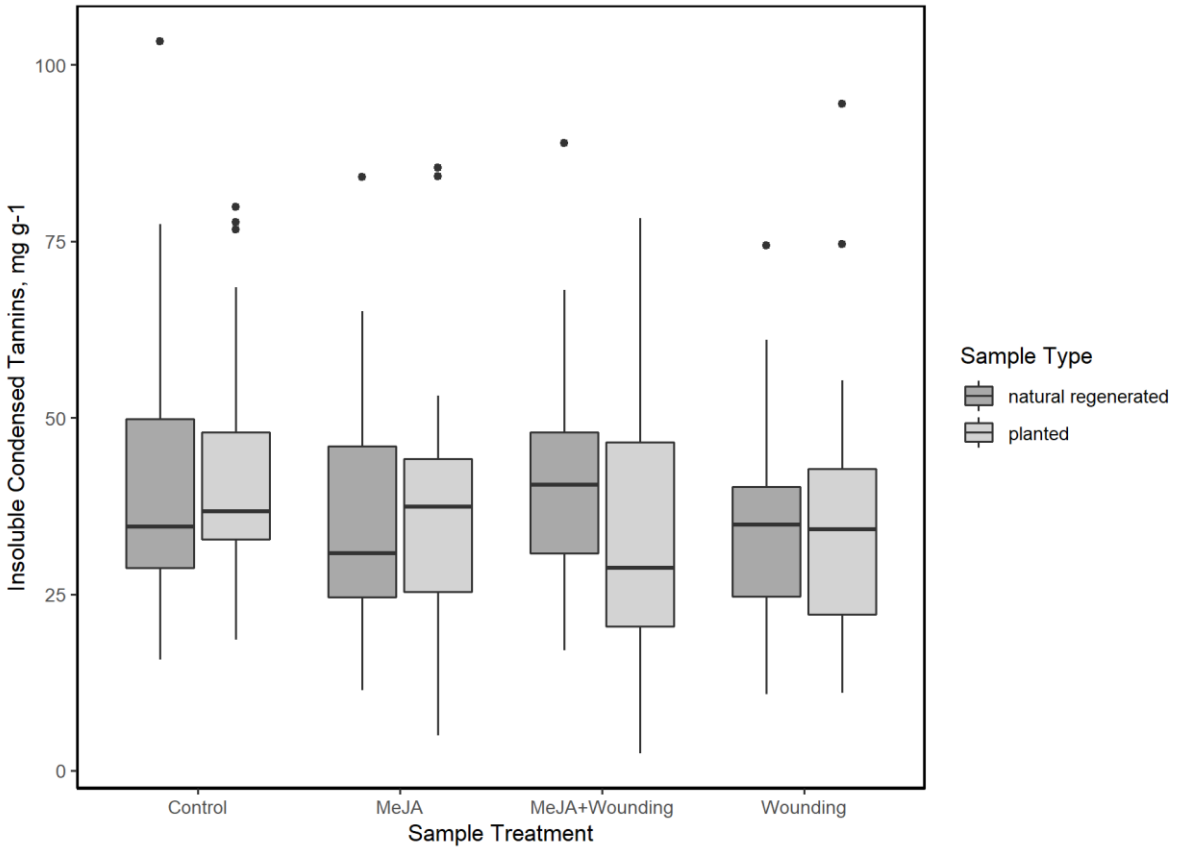

Figure 8: Insoluble condensed tannin concentrations for Norway spruce seedling needles. Differences are displayed between seedling type (naturally regenerated and planted) as well as treatment (MeJA, MeJA+Wounding, Wounding and Control).

3.2 Carbon and Nitrogen Analysis

Carbon-Nitrogen ratio was significantly different due to seedling treatment, seedling type and interaction effects (Type×Treatment). Concentrations can be observed to be higher in planted seedlings compared to naturally regenerated seedlings (Table 4 and Figure 9). This is true for treatments but not for the controls.

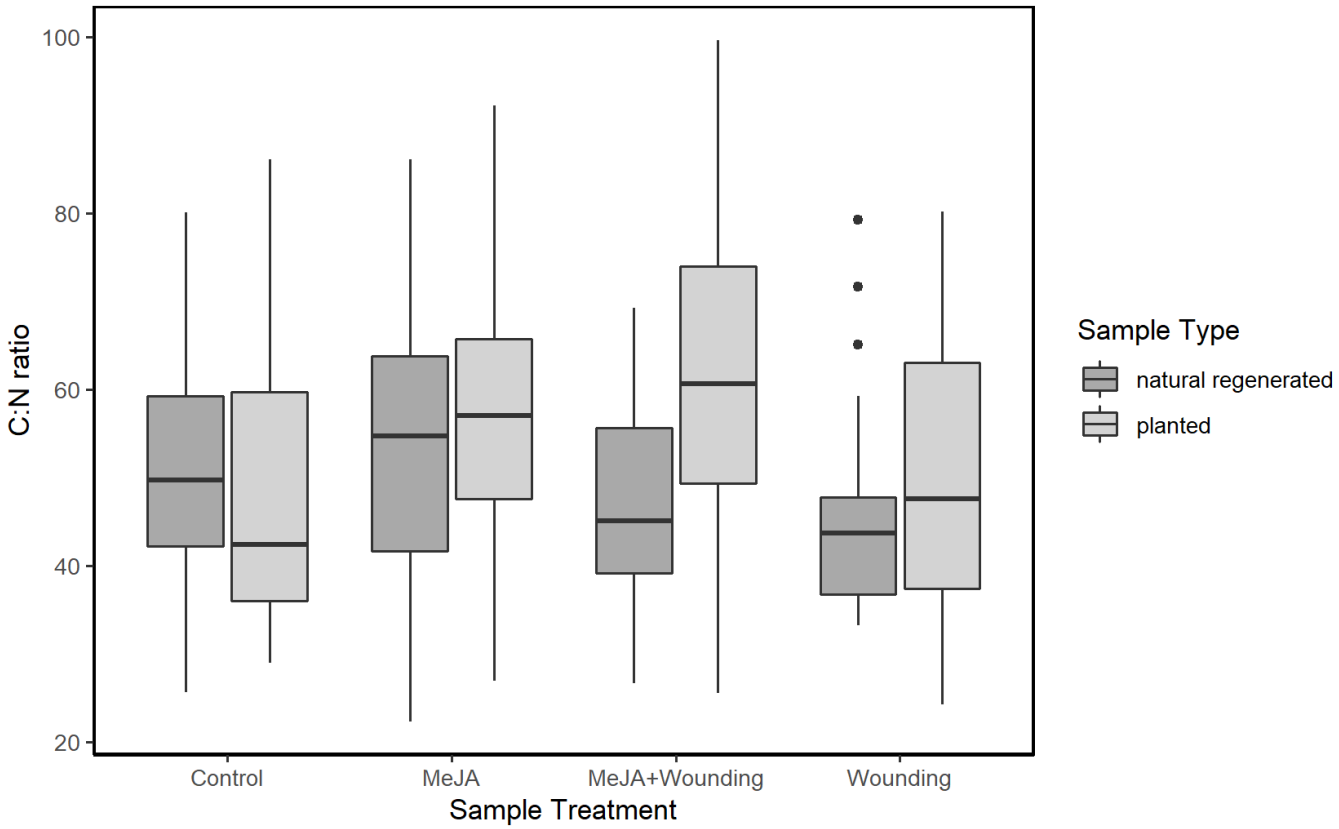

Figure 9: Differences in C:N ratio between types of samples (naturally regenerated and planted Norway spruce seedlings) treated with MeJA, Wounding, MeJA + Wounding or Control (n=197).

Table 4: Carbon (C%), Nitrogen (N%) and Carbon:Nitrogen ratio (C:N ratio) displayed (mean±sd) by type (naturally regenerated and planted Norway spruce seedling) and treatment (MeJA, MEJA+ Wounding, Wounding, Control) (n=197). Statistical analysis was performed with linear mixed effects modeling. Statistical results are displayed through F-values and P values were P < 0.005. Area and block were included as random effects.

	C:N ratio mean±sd		**N% mean±sd**		**C% mean±sd**	
	planted	naturally regenerated	planted	naturally regenerated	planted	naturally regenerated
Control	48.435±16.103	50.788±13.890	1.076±0.318	1.029±0.334	47.361±0.924	48.080±1.127
MeJA	57.641±16.257	53.552±15.477	0.898±0.296	0.993±0.370	47.491±0.955	48.134±1.406
Wounding	49.342±16.884	45.826±12.179	1.079±0.411	1.109±0.244	47.052±1.065	47.450±1.743
MeJA+Wounding	62.772±20.566	46.953±10.950	0.858±0.350	1.065±0.258	47.567±1.857	48.076±0.964
	F-Value (P)					
Type	**6.21 (0.014)**		2.49 (0.116)		**9.75 (0.002)**	
Treatment	**3.27 (0.023)**		2.49 (0.062)		0.60 (0.596)	
Type×Treatment	**3.19 (0.025)**		1.40 (0.245)		0.13 (0.129)	

Concentrations of carbon differed between sample types, and are observably higher in naturally regenerated samples, while no significant differences in nitrogen concentrations between samples were found (Figure 10, Table 4).

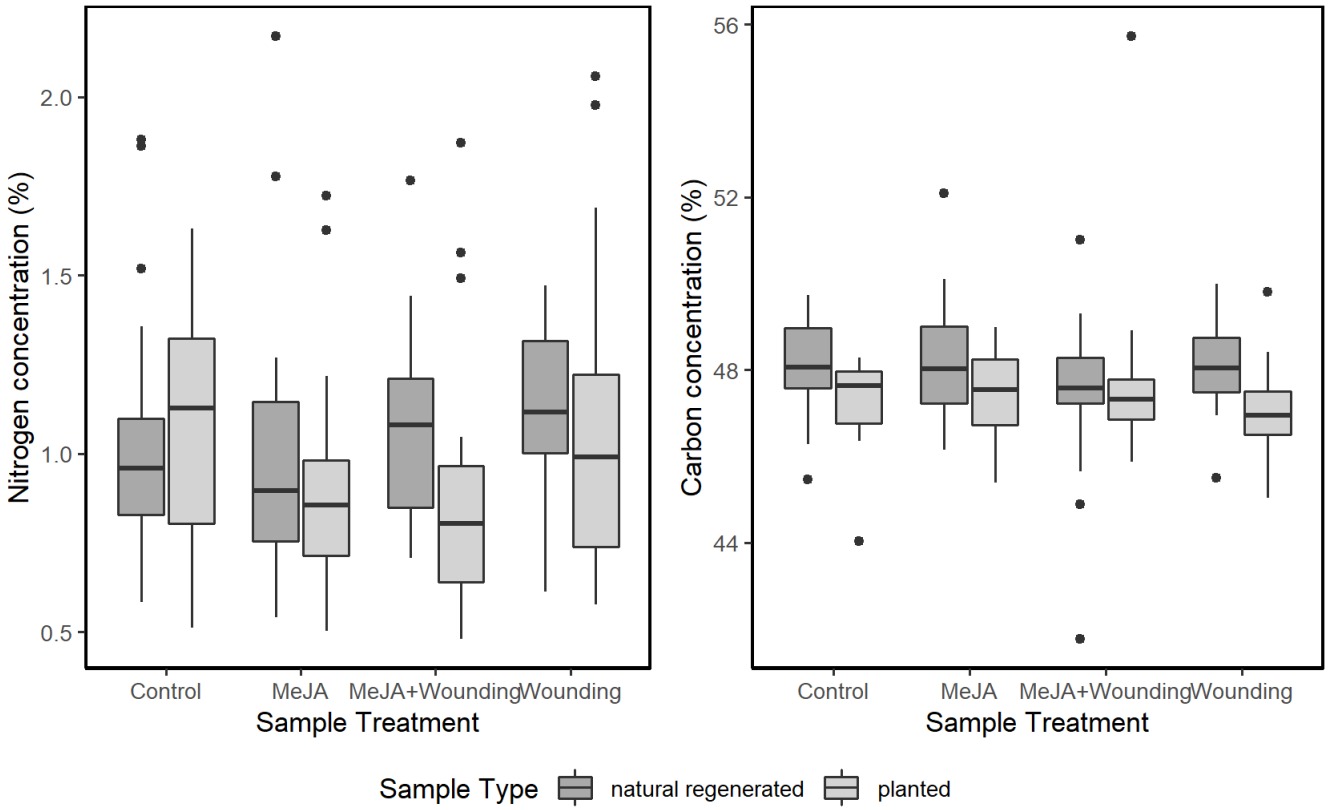

Figure 10: Differences in nitrogen and carbon concentration (%) between types of seedling (naturally succeeded and planted Norway spruce) treated with MeJA, Wounding or MeJA + Wounding. Random effects included in the analysis were Block nested in Area, n=197.

3.3 Gene Expression

Relative gene expression for Norway spruce needle samples with varying types and treatments were accessed for several defense related genes using RT-qPCR. Genes selected included PAL2, LAR2, LAR3, CHS, CHI1, CHI4, STS and the transcription factor MYB35.

ANOVA analysis for linear mixed effects model of ΔΔCt values revealed no statistical significance in gene expression between samples for PAL2 and CHI4.

In general, naturally regenerated seedlings that were wounded showed downregulation of LAR3, STS, CHS, CHI1 and LAR2, while planted control and MeJA treated seedlings showed upregulations for all genes compared to naturally regenerated control seedlings.

Transcription factor MYB35 was generally higher expressed in treated naturally regenerated samples than planted treated seedlings (Figure 11). MYB35 showed statistically significant differences for treatment and type of seedling and interaction effects (P-Value= 0.010 ;0.022 ;0.048, respectively) (Appendix A). Post hoc testing showed significant differences for planted wounded (pW) and naturally regenerated MeJA+Wounding (nMW; P- Value= 0.0117); naturally regenerated MeJA treated (nM; P-Value= 0.0019) and planted control seedlings (pC; P-Value= 0.013)

LAR3 was upregulated in planted control seedlings as well as all types of seedlings treated with MeJA. Linear mixed effects model showed statistical significance for seedling treatment (P-Value= 0.001) (Appendix A). Tukey post hoc revealed statistical significance (P-Value < 0.05) for planted wounded seedlings (pW) compared with all other treatments conducted on planted seedlings (pM: P-Value= 0.048; pMW P-Value= 0.028; pC P-Value= 0.049) (Figure 11).

CHS was generally higher expressed in planted compared to naturally regenerated seedlings. Statistical testing showed significance for type and treatment (P-value < 0.001 and 0.034 respectively) (Appendix A). Post hoc testing revealed significant downregulation for naturally regenerated wounded (nW) compared to planted seedlings, with the exception of planted wounded seedlings (*MeJa* (pM; P-value<0.001); *MeJA + Wounding* (pMW; P-Value= 0.016) and controls (pC; P-Value= 0.014)). In addition, naturally regenerated seedlings treated with MeJA (nM) differ significantly from planted, MeJA treated seedlings (pM; P-Value= 0.027) (Figure 11).

CHI1 was upregulated in all planted seedlings compared to the naturally regenerated controls (Appendix A). Statistical testing showed significance in differences between type of seedlings (P-value <0.001). More detailed, lower expressions of naturally regenerated wounded seedlings (nW) compared to planted seedlings were observed regardless of treatment (*Control* (pC; P-Value= 0.003), *MeJA* (pM; P-value <0.001), *Wounding* (pW; P-Value=0.009) and MeJA + Woundings (pMW; P-Value=0.0217)). In addition, planted MeJA (pM) treated seedlings were upregulated for CHI1compared to naturally regenerated seedlings with all

treatments (*Control* (nC; P-Value=0.034), *MeJA* (nM; P-Value=0.008), *Wounding* (nW; P-value <0.001) and MeJA + Woundings (nMW; P Value= 0.013) (Figure 11).

Planted seedlings were upregulated for LAR2 in comparison with naturally regenerated controls (nC), except from planted wounded seedlings (pW). Both naturally regenerated and planted seedlings downregulate expression of LAR2 after wounding compared to the controls (nC). Differences between seedlings comparing ΔΔCt values showed significant differences in both seedling type (P-Value= 0.020) and treatment (P-Value= 0.002) (Appendix A). Post hoc testing revealed significant differences comparing planted controls (pC) with naturally regenerated wounded (nW; P-Value=0.018) and planted wounded (pW; 0.006). Additionally, planted seedlings treated with MeJA (pM) differed from planted seedlings that were wounded (pW; P-Value=0.046) (Figure 11).

Treatments involving wounding showed downregulation of STS in both naturally regenerated and planted seedlings of Norway spruce. STS expression showed statistical significance difference for sample treatment (P-Value= 0.047) (Appendix A). No statistical significance was found when conducting post hoc testing (Figure 11).

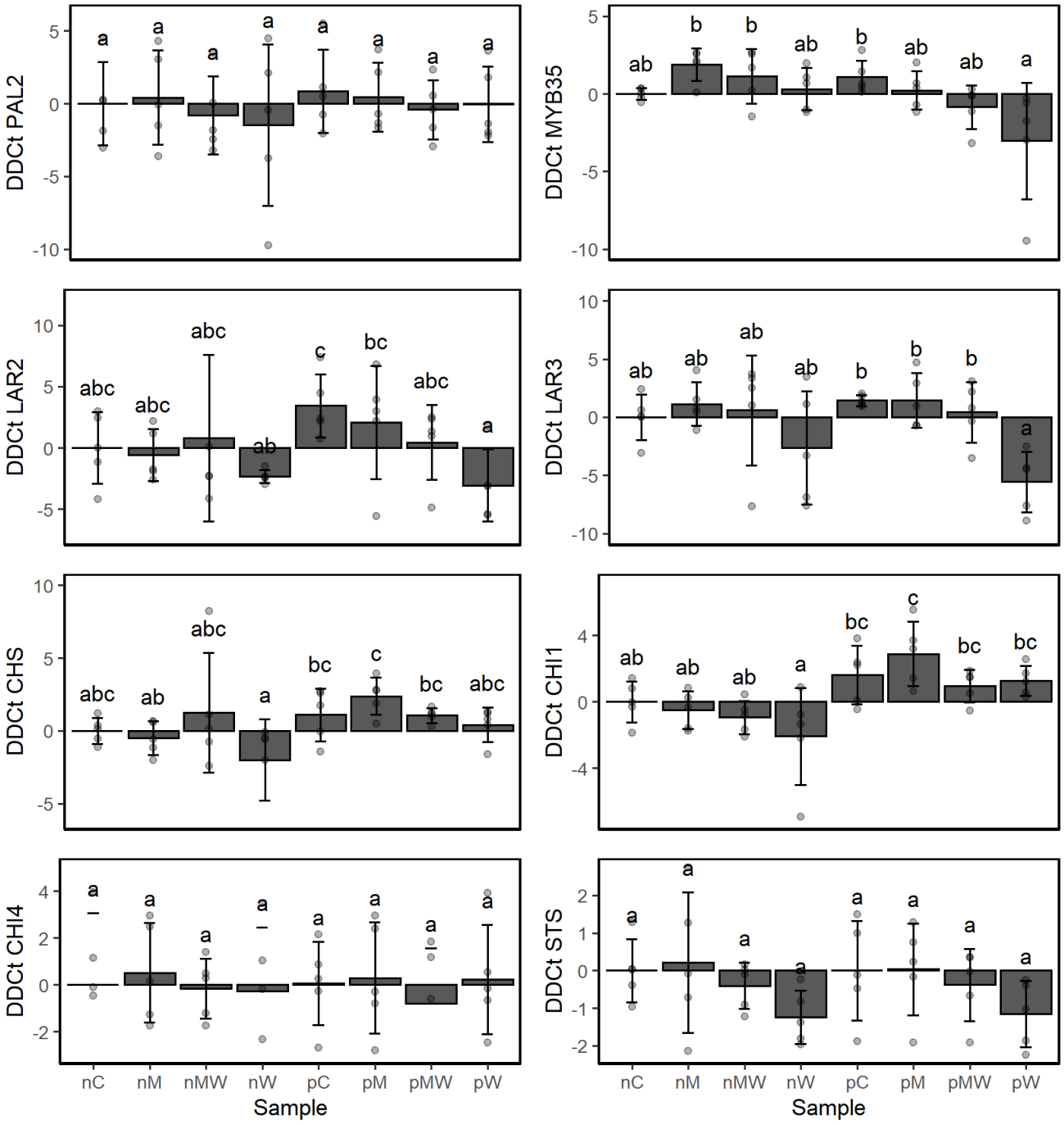

Figure 11: Relative gene expression (ΔΔCt) of phenylpropanoid biosynthesis and defense related genes (PAL2, MYB35, LAR2, LAR3, CHS, CHI1, CHI4 and STS) for samples of different type (naturally regenerated (n); planted (p) Norway spruce) and treatment (Control (C); MeJA(M), Wounding (W) and MeJA+Wounding (MW))(n = 8 groups of 5 pooled Seedling samples per treatment). Error bars represent standard deviations (sd) and data plots represent individual ΔΔCt values. Bars with different letters are significantly different, using Tukey post hoc, where p-value > 0.05.

4. Discussion

Intensified forestry and common forestry measures, such as clear-cutting, replanting, fertilizing and pesticide use, alter boreal ecosystems and can impact ecosystem resilience. Understanding and enabling natural defense mechanisms is important to reduce possible negative anthropogenic impacts. This thesis looked at differences in phenolic defense responses between naturally regenerated and planted Norway spruce seedling needles in response to MeJA, Wounding and MeJA + Wounding.

Concentrations of CBSCs were expected to differ between naturally regenerated and planted Norway spruce seedlings as nutrient availability was impacted by fertilization of planted seedlings, affecting seedling investment in growth and defense. Results showing higher carbon concentration in needles of naturally regenerated spruce seedlings than planted seedlings correspond to their higher bark carbon concentrations (as reported by Lomsdal 2020). These findings are in accordance with higher density of carbon-based tissue due to slower growth through nitrogen limitation which might also explain elevated carbon in naturally regenerated seedlings (Wainhouse et al. 1998). Differences in carbon concentration could additionally be explained by the age of seedlings, as naturally regenerated seedlings have possibly been growing for one more season than planted seedlings. This hypothesis is supported by previous findings showing lower concentration of carbon in first year needles than second year needles (Einig & Hampp, 1990).

Differences in C:N ratio depended on seedling type, treatment, and interaction effects (Type×Treatment). It can be observed that C:N ratio is higher in planted seedlings for all but control treatments (Table 4; Figure 9). Higher C:N ratio in naturally regenerated seedlings as true for controls is cohesive with observations from in previous studies (i.e. Laitinen et al., 2000). As planted seedlings were fertilized, one could assume that previously available nitrogen was not accumulated within the needles but rather invested in seedling growth, which could be an explanation for why no elevated levels of nitrogen were detectable (Huber et al., 2004, Laitinen et al., 2000, Wainhouse et al., 1998, Wingler et al., 1994). Supporting this hypothesis, previous studies have shown that C:N ratio in needles was unaffected by access of nitrogen for Norway spruce (Germany, Bavaria) (Huber et al., 2004; Wingler et al., 1994). The discrepancy of expected findings and actual results in C:N ratio could also be impacted by additional stress through replanting and transportation as well as necessary root establishment in planted seedlings, increasing demand for reallocation of nitrogen to different

plant organs (i.e. roots). As other plant organs were not tested in this study, allocation in other plant organs remains a hypothesis.

Constitutive and inducible defense mechanisms in the form of phenols in Norway spruce seedlings were different for planted and naturally regenerated seedlings. Based on ecological theory, one would expect naturally regenerated seedlings to have higher concentration in CBSCs as growth took place under nitrogen limitation (Coley et al, 1985). However, this hypothesis could only be supported partially as only individual compounds, namely catechin gallocatechin, dicourmaroylasstragallin and two unknown flavonoids as well as stilbenes differed with type of seedling. As elaborated above, carbon concentration also differed significantly with type. As no significant results for nitrogen concentrations were found, no observation of correlation between nitrogen availability and phenolics could be made.

Induced defense altering concentrations of phenolics after treatment was only observed for insoluble tannins, dicoumaroylastragallin1 and dicourmaroylasstragallin 2 while interaction effects were true for two unknown flavonoids and gallic acid.

Acetophenone (further referred to as picein) concentrations were found to be dependent on seedling type. Elevated concentrations in treated naturally regenerated leading to the assumption of genetic or environmental differences between seedlings (Figure 10) (Parent et al., 2018).

Concentrations of insoluble condensed tannins were dependent on seedling treatment. Higher concentration in control seedlings could possibly indicate allocation of defense compounds in proximity of treated areas (stem), increasing inducible defense near wounding or MeJA treatment directly (Table 4). Defense allocation in the stem is supported by several studies, showing increase in defense related molecules near treated areas specifically (i.e. Brignolas et al., 1995, Oliva et al., 2015). However, as this can only be observed in mean concentrations and not be confirmed through statistical testing, it remains an assumption.

Elevated concentration of stilbenes in naturally regenerated seedlings could potentially be explained by trade-offs between growth and defense investments (Herms & Mattson, 1992). Lomsdal (2020) found significantly higher apical growth in planted seedlings relative to naturally regenerated seedlings which can be compared to current needle analysis and showed significantly higher amounts of stilbenes in needles of naturally regenerated seedlings. If true, stilbene allocation in needles could potentially be a trade-off to apical growth of planted seedlings (Brignolas, 1995). To completely adapt this theory, it would be necessary to compare

carbon concentration with both apical shoot and root growth as well as minimizing variation between seedlings age and stressors seedlings were exposed to in future studies.

Alternatively, UVB light exposure during early developmental stages could explain elevated concentrations in naturally regenerated seedlings (stilbenes, picein and individual flavonoids) as growth in UVB light filtering greenhouse is practiced in seedling nurseries. It has also been shown in studies on Norway spruce needles that UVB radiation, temperature and fertilization impacted CBSCs significantly and that fertilization changed reaction to the other two factors. Additionally, resistance to herbivores and other stressor can be reduced by filtering out UVB light through glass for in greenhouse grown seedlings, as UVB exposure elevates levels of terpenes and phenols (Kivimäenpää et al., 2020, Løkke, 1990, Ohlson et al., 2013, Zinser et al., 2000). However, since detailed procedures of growth conditions and light measurements were not documented for either plant type, it is difficult to make conclusions about the effects of UVB on phenolic concentrations in this study.

Differences within CBSCs should be explained through specific evolutionary response, and upregulation of specific genes would thus be expected in seedlings showing higher concentrations of certain CBSCs (Koricheva et al., 1998).

As condensed tannins derive via the flavonoid pathway, elevated concentration should reflect in relative expression levels of PAL2, MYB35, CHI, CHS and LAR. However, since post hoc testing did not reveal which treatments differed, assumptions on regulation through expression levels cannot be made. While LAR genes as well as CHS differed between treatments, CHI1 did only differ between seedling type. Thus, no correlations were found between concentration in condensed tannins and expression of phenolic biosynthesis genes.

Moreover, genes involved in stilbene syntheses STS and PAL2 showed no correlation with concentration of stilbenes. STS showed significant differences in gene expression depending on treatment while stilbene concentration was significantly higher in naturally regenerated seedlings for all treatments. Effects of treatment on STS seems logical as STS is known to be a defense related gene, however timing of sampling as well as the fact that treatment was applied on the bark of seedlings might have influenced absence of correlation. CHI was significantly upregulated in planted seedlings, which could possibly explain elevated stilbene levels. CHI genes are generally involved in chalcone synthesis. Upregulation of CHI in planted seedlings might be related to higher concentration of stilbenes as chalcone and stilbene synthesis as would be a trade of, considering CHI and STS share a common precursor (Appendix B). However, as chalcone was not one of the identified flavonoids in this analysis, this correlation

should be subject of future studies. No correlation between concentrations of stilbenes could be made for the other genes.

Additionally, CHS and CHI transform p-Coumaroyl-CoA into Chalcone and Flavanone respectively, followed up by transformation to catechin by LAR genes and finally condensed tannins (Figure X Appendix). STS also uses p-Coumaroyl-CoA to synthesis resveratrol, the precursor for all other stilbenes (Hammerbacher et al., 2011). Thus, there could be a tradeoff between flavonoids and stilbenes, as upregulation of either CHS and CHI or STS would result in lesser availability of p-Coumaroyl-CoA.

MYB35is a transcription factor has been shown to regulate phenylpropanoid related genes, namely PAL2 and other genes involved in the flavonoid synthesis. As MYB35 is suggested to perform a cell type or organ-specific regulating role, it might be of higher importance in needle-specific defense related secondary metabolic compounds (Nemesio-Gomez, 2017). Additionally, MYB family seemed to be specifically upregulated during the summer month, when herbivory and pathogenic stressor are the highest, which could also explain observable differences in MYB in comparison to other selected genes response (Bag et al., 2021). It was observed that the transcription factor MYB35 was especially upregulated after MeJA treatment, specifically in needles of Norway spruce. Additionally, upregulation of PAL2 and CHS, genes involved in early processes of phenylpropanoid pathway was observed. MYB35 also increased expression of members of the LAR family, involved in downstream flavonoid expression (Chong et al., 2009, Brignolas et al., 1995, Nemesio-Gorrez et al., 2017, Metsämuuronen & Sirén, 2019). A direct effect of MYB35 up and down regulation and gene expression of other genes cannot be observed.

Conclusions based on gene expression analysis are limited, as sampling only took place 12 weeks after treatment. Previous studies observed changes in PAL2 expression 5-6 days after MeJA treatment (Yacoob et al, 2012, Likar & Regvar, 2008). Thus, the design of this study might be insufficient to capture changes in gene expression for PAL2, as well as other selected genes. Considering that treatments on the seedling were conducted in early spring and samples were taken in late summer, defense mechanism might have stayed unnoticed due to active metabolism and modification of defense compounds. If this is the case, gene regulation, too, cannot be linked to actual concentrations of certain defense molecules (Bag et al., 2021, Strack, 1989). Further, to eliminate the factor of differences in seedling age, closer observation is needed in this regard in future studies.

Temporal scale in two dimensions should be observed, short term during seasonal changes as well as long term ontogenetic changes, as timing of sampling is crucial and should be included in any further study to completely inducible analyze defense mechanisms in Norway spruce (Bag et al., 2021, Day & Greenwood, 2011)

Model plants like Arabidopsis or tobacco suggest that plants do have the ability to differentiate between mechanical wounding as conducted in this study and actual herbivory attack, as cues in saliva indicate herbivore specificity. Thus, mechanical wounding to simulate pine weevil might have been identified as tissue damage but not weevil attack. This could explain for why gene upregulation and plant induced defense mechanisms were not universal across treatments (Howe & Jander, 2008; Li, 2022). In future studies, this factor could be corrected for by a more realistic in situ experimental design including observation of actual weevil attack or including chemical cues form weevil saliva.

Concluding, differences between naturally regenerated and planted Norway spruce seedlings were found for concentrations of individual phenolics, namely catechin gallocatechin, Dicourmaroylasstragallin and two unknown flavonoids as well as stilbenes. Differences after treatment, thus MeJA and Wounding was found in insoluble tannins, Dicoumaroylastragallin1 Dicourmaroylasstragallin 2 while interaction effects were true for two unknown flavonoids and gallic acid. Type of seedling was explanatory for differences in expression of MYB35, LAR2, CHS and CHI while treatment of seedlings did alter gene expression levels for some genes (MYB35, LAR, CHS, STS) however, the up and down regulation did not necessarily reflect the measured concentrations of phenolic compounds that would have been synthesized. Carbon and nitrogen content cannot be linked directly to defense mechanisms in Norway spruce; however, it can be observed that higher carbon content correlates with higher concentrations of stilbenes, which reflects the general assumption that higher carbon concentrations support formation of CBSCs. Additional explanatory factors for difference in concentration of phenols could be age of seedling, UVB exposure in early stages of seedling development and general stress response after planting (Fossedal et al., 2007). Also, a trade-offs between STS expression in planted seedling and upregulation of CHI and CHS could explain for lower concentrations of stilbenes in planted seedlings. However, multiple variants which were not included in this study, such as controlled age of seedlings, resource reallocation due to replanting and transport stress as well as UVB exposure in the field should be included in future research to gasp the complexity of environmental impacts on seedlings. Additionally, sampling at various timescales would be beneficial for gene expression analysis and observation over

phenylpropanoid pathways. Tree defense is likely to become even more important for forest management in the future, as external stressors will become more pressuring. At the same time, artificial measures against pests such as pesticide use will be restricted in the future. Forestry management should not neglect the difference in tree defense between naturally regenerated and planted seedlings and integrate this knowledge fo sustainable forest management in the future.

5. References

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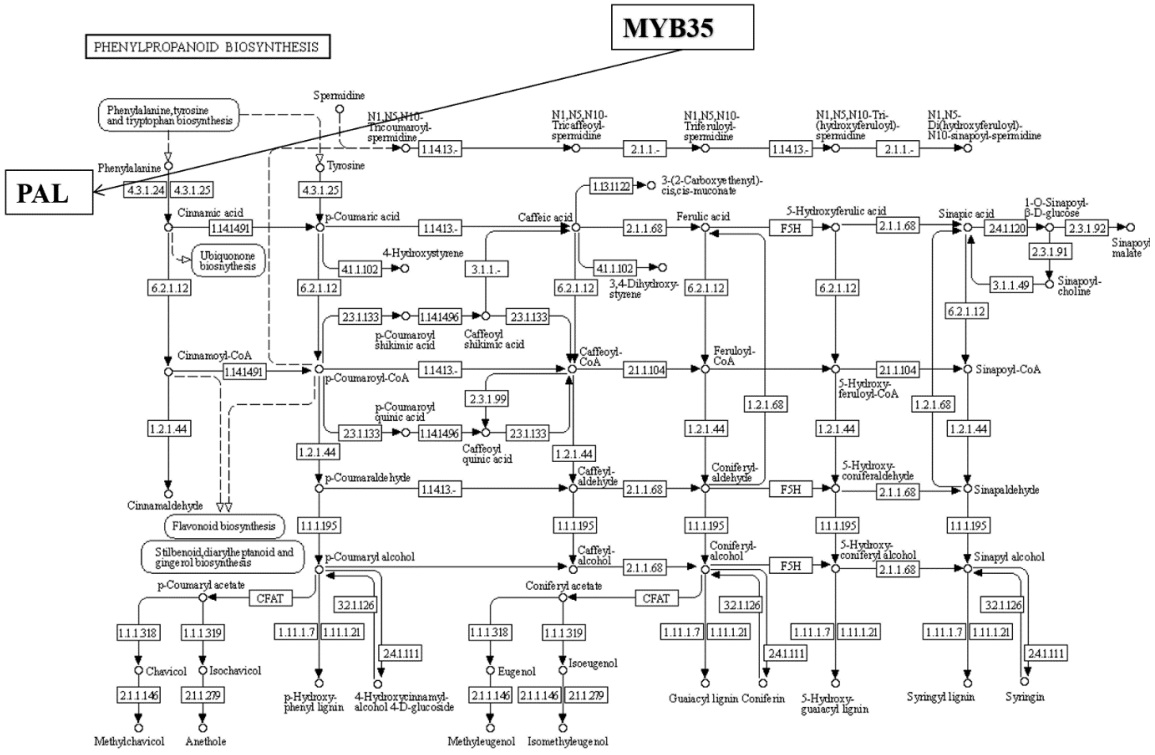
6. Appendix

Appendix A: Table displaying ANOVA analysis following mixed effects modeling for statistical differences for all genes of interest (MYB35; LAR2; LAR3; CHS; CHI1; STS excluded due to NS: PAL2 and CHI4), using relative gene expression (ΔΔCt) for Sample Type (naturally regenerated and planted Norway spruce seedlings); Sample Treatment (MeJA, Wounding, MeJA + Wounding and Control) as well as interaction effects, thus sample Type X Sample Treatment.

Pr(>F)	ΔΔCt **MYB35**	ΔΔCt **LAR2**	ΔΔCt **LAR3**	ΔΔCt **CHS**	ΔΔCt **CHI1**	ΔΔCt **STS**
Sample Type	**0.018**	**0.020**	0.964	**<0.001**	**<0.001**	0.961
Sample Treatment	**0.025**	**0.002**	**0.002**	**0.034**	0.060	**0.047**
Interaction effects	**0.061**	0.263	0.444	0.402	0.230	0.988

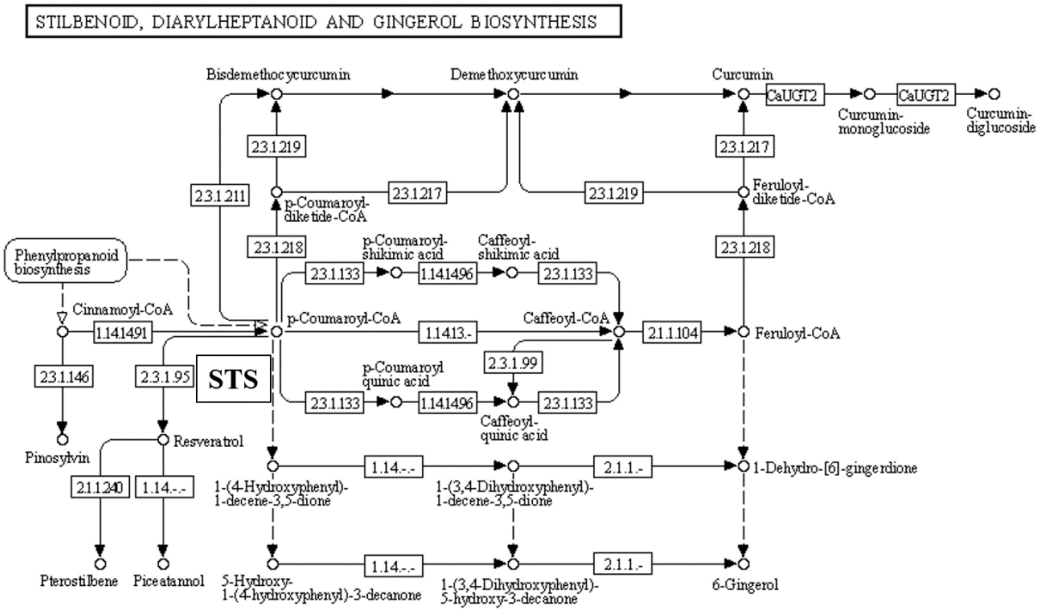
Appendix B: Mappping of (i) Phenylpropanoid Biothiynthesis; (ii) Stilbenoid, diarylheptanoid and gingerol biosynthesis ; (iii) Flavonoid biosynthesis.

(i) Phenylpropanoid Biothiynthesis



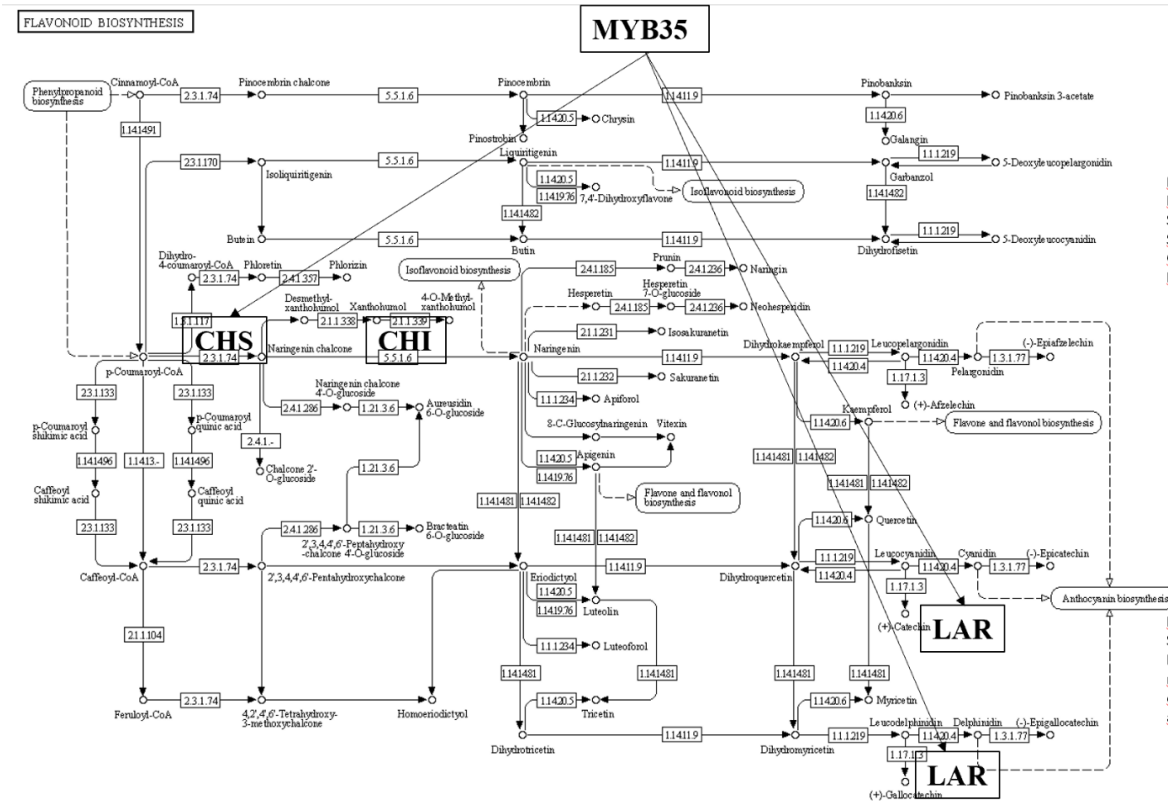
(Kyoto Encyclopedia for Genes and Genome, KEGG (12.10.2021)

(ii) Stilbenoid, diarylheptanoid and gingerol biosynthesis

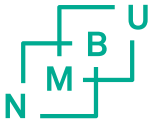


Kyoto Encyclopedia for Genes and Genome, KEGG, (2018)

(iii) Flavonoid biosynthesis



(Kyoto Encyclopedia for Genes and Genome, KEGG, 2019)



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