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Effect of seed treatment with putative defence priming chemicals on defence-related gene expression and pathogen resistance in Norway spruce (*Picea abies*) seedlings

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Master of Science in Ecology

Preface

This thesis marks the end of my master's degree in ecology at the Norwegian University of Life Sciences (NBMU).

I would like to thank Melissa Magerøy for being a great supervisor. I really appreciate all the time and effort you put into helping me design and carry out the experiments and answering all my questions. Thank you for always making time for me. Many thanks also to my other supervisor Paal Krokene for giving me valuable feedback on the thesis drafts.

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And of course, thank you to my parents for always supporting me.

Ås, June 2020

Claire Devos

Abstract

Chemical seed treatment may be a simple method to protect conifer seedlings from pests and pathogens in plant nurseries and forest stands. In this study, I tested the ability of eight putative defence priming chemicals (methyl jasmonate (MeJA), β-amino butyric acid (BABA), hexanoic acid, gibberellic acid, quinic acid, thiamine, riboflavin and chitosan) to enhance the resistance of Norway spruce (*Picea abies*) seedlings by means of seed treatment. I carried out two different tests on young spruce seedlings grown from seeds soaked overnight in priming chemicals: I tested (1) 10-day-old seedlings for resistance to the oomycete *Pythium ultimum*, and (2) 10-week-old seedlings for expression of selected defence-related genes (*ACS, LOX, PAL1, TPS-Car* and *Chi4*) after mechanical wounding. In addition, I measured germination rates of treated seeds and root length of 8-day-old seedlings grown from treated seeds. None of the seed treatments I tested caused significant changes in seedling resistance to *P. ultimum*, gene expression, or the percentage of seeds that germinated. All seed treatments significantly accelerated seed germination, except from BABA (0.1 mM and 0.5 mM), quinic acid (0.1 mM) and riboflavin (0.5 mM). Seed treatment with MeJA (0.05 mM and 0.1 mM) led to a significant decrease in seedling root length, while treatment with BABA (0.5 mM) and gibberellic acid (0.1 mM and 0.5 mM) led to an increase in root length. Overall, I could not demonstrate that any of the tested seed treatments has the potential to enhance the resistance of young spruce plants. My results do illustrate the importance of evaluating fitness costs in studies on seed priming.

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1. Introduction

Conifers dominate temperate and boreal forests and are of great economic and ecological importance. To defend themselves against the wide range of organisms they might encounter during their long life spans, conifers have evolved various defence mechanisms. Constitutive or preformed defences include anatomical structures that prevent tissues from being penetrated or chewed on, as well as chemicals with toxic or inhibitory effects, such as phenolics and terpenoids. When attacked, conifers can activate additional inducible defences, such as the formation of traumatic resin ducts and the activation of polyphenolic parenchyma cells. In addition, exposure to certain stimuli can induce resistance to future attacks. This form of resistance is known as acquired or induced resistance (Franceschi *et al*., 2005, Krokene, 2015).

Acquired resistance in conifers was first described by Christiansen *et al.* (1999), who found that mechanical wounding and sublethal fungal infection made Norway spruce (*Picea abies)* more resistant to subsequent fungal inoculation. Since then, various studies have shown that conditioning with wounding, fungal inoculation and stem application of the phytohormone methyl jasmonate (MeJA) can increase resistance in Norway spruce and other conifer species (e.g. Martin *et al.*, 2002; Erbilgin *et al.*, 2006; Zeneli *et al.*, 2006; Magerøy, Christiansen *et al.*, 2020). Acquired resistance in conifers may be based on one or both of two mechanisms that involve inducible defences: (1) prolonged upregulation of inducible defences and (2) priming of inducible defences. In the case of a prolonged upregulation of defences, the defences induced by a stimulus remain upregulated for a period of time, and this provides resistance against subsequent attacks. In the case of defence priming, inducible defences are not activated but become sensitized, and this allows for a stronger and/or faster upregulation of inducible defences upon future attacks (Wilkinson *et al.*, 2019). Compared with a prolonged upregulation of defences, priming probably causes low fitness costs (Martinez-Medina *et al*., 2016).

Pretreatment with chemical elicitors such as MeJA may be a promising method to protect conifers without introducing pesticides to the environment (Berglund *et al*., 2015). Spraying, painting and fumigation of conifer plants are currently the main application methods. Seed treatment, a well-known practice in the agricultural sector, could be a simple and cost effective alternative for large-scale nursery protection. So far, chemical seed treatment has only been reported to protect conifers by Berglund *et al*. (2015). In their study, 6-month-old Norway spruce seedlings grown from seeds treated with jasmonic acid, 5-azacytidine or nicotinic acid

experienced less pine weevil damage than seedlings grown from untreated seeds, but the difference was not statistically significant.

Much is still unknown about the molecular mechanisms underpinning acquired defence responses in Norway spruce. Studies using MeJA application suggest that endogenous jasmonates play a key role in defence signalling and biosynthesis of defence-related metabolites in conifers (e.g. Franceschi *et al*., 2002; Martin *et al.*, 2002; Hudgins *et al*., 2003; Hudgins & Franceschi, 2004). The biosynthesis of jasmonates in Norway spruce probably starts with the oxygenation of linolenic acids by the enzyme lipoxygenase (LOX) (Turner *et al*., 2002; Arnerup *et al*., 2013). Jasmonates have been found to interact and antagonize or synergistically regulate defence responses with other hormones such as ethylene in various plant species (Yang *et al*., 2019). In conifers, exogenous ethylene treatment elicits defence responses similar to those induced by MeJA application, and ethylene is thought to mediate MeJA-induced defences by acting as a signalling agent (Hudgins & Franceschi, 2004). The first step in ethylene biosynthesis is the conversion of *S*-adenosyl methionine to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) (Wang *et al*., 2002).

Jasmonate and ethylene-mediated signalling pathways seem to be involved in the synthesis of numerous chemical compounds, including many products of the phenylpropanoid and isoprenoid pathways. The phenylpropanoid pathway gives rise to various phenolics involved in conifer defences, such as flavonoids and lignin (e.g. Brignolas *et al.*, 1995; Hudgins & Franceschi, 2004). The phenylpropanoid pathway is initiated by deamination of phenylalanine by the enzyme phenylalanine ammonia-lyase (PAL) (Vogt, 2010). The isoprenoid pathway synthesizes different terpenoid resins. One example is 3-carene, a monoterpene formed by the enzyme 3-carene synthase (TPS-Car) (Fäldt *et al.*, 2003). Induced resistance in Norway spruce also involves pathogenesis-related proteins (PRs). Recently, MeJA treatment of adult Norway spruce has been found to prime 10 different families of PRs, including chitinases (Magerøy, Wilkinson *et al*., 2020). Chitinases can inhibit fungal growth by breaking glycoside bonds of chitin in the cell wall of many fungal species. Chitinolytic breakdown products act as signalling compounds that stimulate further defence responses in plants (Kasprzewska, 2003).

In the current study, several putative defence priming chemicals (MeJA, β-amino butyric acid, hexanoic acid, gibberellic acid, quinic acid, thiamine, riboflavin and chitosan) were tested for their ability to regulate defence-related genes and induce resistance in Norway spruce seedlings by means of seed treatment. Treatment with the non-protein amino acid β-amino butyric acid (BABA) has been reported to protect about 40 non-coniferous species from 80 pathogens and

pests (Cohen *et al*., 2016). Hexanoic acid, a carboxylic acid, has been shown to enhance resistance in *Arabidopsis thaliana*, Fortune mandarin (*Citrus clementina* x *Citrus reticulata*) and tomato (*Solanum lycopersicum*) to fungal and bacterial diseases (Aranega-Bou *et al*., 2014). Gibberellic acid, a pentacyclic diterpene acid known for its role as a plant growth regulator, has been less studied for its involvement in plant defence mechanisms and its potential to enhance resistance by exogenous application. However, studies on *Arabidopsis* and rice (*Oryza sativa*) suggest that gibberellic acid signalling components might play major roles in plant resistance (Bari & Jones, 2009). The cyclitol quinic acid is an intermediate of the shikimate pathway, a pathway that leads to the synthesis of aromatic amino acids such as phenylalanine (Ghosh *et al*., 2012). So far, enhanced resistance following quinic acid treatment has only been reported in tomato plants (Carrasco *et al.*, 1978). Thiamine (vitamin B1), riboflavin (vitamin B2) and chitosan, a derivative of chitin, have all been found to enhance resistance in a wide range of plants (Aranega-Bou *et al*., 2014; Boubakri *et al*., 2016).

The main objective of this study was to investigate whether seed treatment with MeJA, BABA, hexanoic acid, gibberellic acid, quinic acid, thiamine, riboflavin or chitosan can enhance the defensive capacity of Norway spruce seedlings. In order to do so, I conducted two experiments with seedlings grown from treated seeds. In the first experiment, I subjected seedlings to *Pythium ultimum*, an oomycete known to infect and kill young Norway spruce seedlings (Kozlowski & Métraux, 1998), and assessed seedling resistance through visual observations. In the second experiment, I wounded seedlings and quantified the expression of five defence-related genes (*ACS*, *LOX*, *PAL1*, *TPS-Car* and *Chi4*) by RT-qPCR. In addition, I studied the impact of seed treatment on seed germination and seedling development.

2. Methods

To study the effect of seed treatment on Norway spruce resistance I treated seeds with different chemicals, let the seeds germinate and ran experiments on 10-day-old and 10-week-old seedlings. The youngest seedlings were used to study resistance to *Pythium ultimum*, while the older seedlings were used to study defence-related gene expression after mechanical wounding. In addition, I measured seed germination and root length of 8-day-old seedlings (Figure 1).

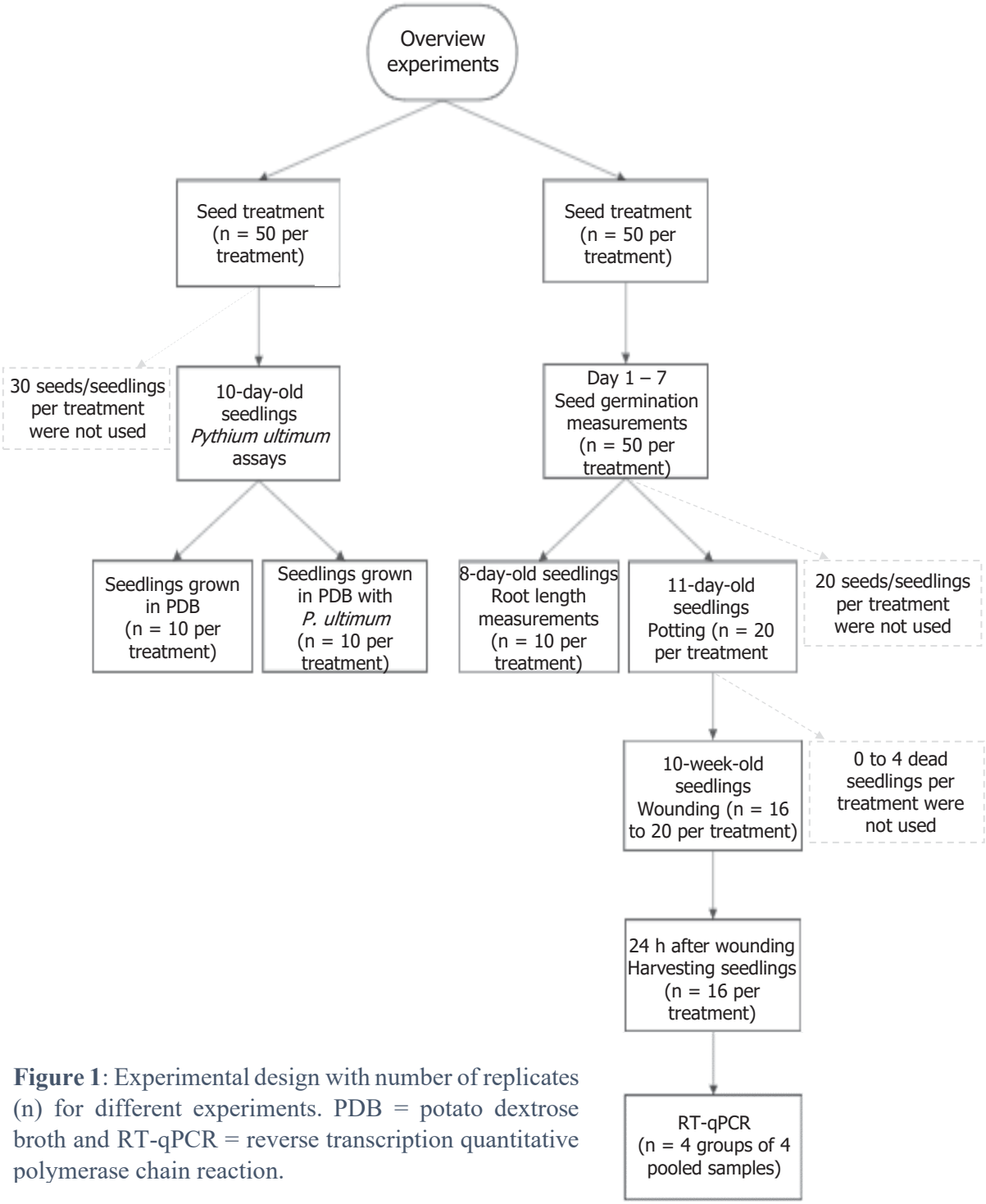


Figure 1: Experimental design with number of replicates (n) for different experiments. PDB = potato dextrose broth and RT-qPCR = reverse transcription quantitative polymerase chain reaction.

2.1 Seed treatment

Norway spruce seeds originating from Kilen (59°18'N, 8°47'E, altitude 89 m.a.s.l.), Norway, were soaked overnight under gentle shaking in jars filled with distilled water solutions of methyl jasmonate (MeJA), β-amino butyric acid (BABA), hexanoic acid, gibberellic acid, quinic acid, thiamine, riboflavin and chitosan. Two solutions with different concentrations were made with each chemical (Table 1). Seeds soaked in distilled water served as control seeds. To make water solutions of riboflavin and chitosan, I first made 10 mg ml^{-1} solutions of riboflavin and chitosan in 0.1 M sodium hydroxide and 1 M ascorbic acid, respectively, before diluting these with water to the desired concentrations. The other chemicals were diluted directly with water. Because of time constraints, seed treatments were divided into two groups (treatment group A and B) and performed on separate days with their own control (Table 1). All seed treatments were carried out twice over the course of the study (n = 2 batches of all 18 seed treatments with 50 seeds per seed treatment = 100 seeds per seed treatment over the course of the study). One batch per seed treatment was used for the *Pythium ultimum* pathogenicity assays, while the other one was used to study germination rates, root lengths and gene expression (Figure 1).

Table 1: Overview of chemical treatment of Norway spruce seeds. For practical reasons seed treatments were divided into two groups (treatment group A and B) and done on two separate days.

Group A		Group B	
Chemicals	**Concentrations**	**Chemicals**	**Concentrations**
Water (control)		Water (control)	
Methyl jasmonate (MeJA)	0.05 mM and 0.1 mM	Quinic acid	0.1 mM and 0.5 mM
β-amino butyric acid (BABA)	0.1 mM and 0.5 mM	Thiamine	0.1 mM and 0.5 mM
Hexanoic acid	0.1 mM and 0.5 mM	Riboflavin	0.1 mM and 0.5 mM
Gibberellic acid	0.1 mM and 0.5 mM	Chitosan	0.01 % and 0.05 %

2.2 Growing conditions for seedlings

After being soaked overnight in the solutions, the seeds were rinsed with distilled water and placed in Petri dishes in between wet filter papers. The Petri dishes were sealed with laboratory film to reduce moisture loss, placed at 4 °C in a dark room for 5 days, before being moved to a growth room with a temperature between 23 and 28 °C and 16 hours of light per day. Seedlings used in the wounding experiment were potted after 11 days in the growth room in 6 cm diameter plastic pots with fertilized soil (Blossom plantejord, Europris). The seedlings were watered approximately every 4 days.

2.3 Seed germination and root length measurements

Germination percentages of treated seeds were recorded over the first 7 days after the seeds were moved to the growth room. On the eighth day, the root length of seedlings (n = 10 per treatment) was measured with a Zeiss SteREO Discovery.V20 stereomicroscope (Carl Zeiss Microscopy, Germany) equipped with AxioVision imaging software (v4.8.0.0, Carl Zeiss Microscopy) (Figure 2).

Figure 2: An 8-day-old Norway spruce seedling with seed capsule and measured root (red line).

2.4 *Pythium ultimum* pathogenicity assays

Pythium ultimum (isolated in 1983 from Norway spruce grown in Telemark Skogplanteskole, Gvarv, Norway) was grown in Petri dishes with potato dextrose agar. After 7 days, scoops of the *P. ultimum* culture were removed from the Petri dishes and placed inside Erlenmeyer flasks with potato dextrose broth (PDB). The flasks were placed on a shaking device in a dark room. After 3 days of gentle shaking, 5 ml black microcentrifuge tubes were filled with either inoculated PDB or non-inoculated PDB. With a Neubauer improved cell counting chamber (Brand GmbH + CO KG, Germany) the concentration of hyphae in the inoculated PDB was estimated to be 7.5×10^4 hyphae ml^{-1} PDB. The roots of five 10-day-old seedlings grown from treated seeds were threaded through small holes in the lid of each microcentrifuge tube and submerged in the PDB inside the tube (Figure 3). For each seed treatment, 10 seedlings were grown in inoculated PDB (n = 10) and 10 seedlings were grown in non-inoculated PDB (n = 10). Eight days later, the seedlings were inspected and categorized into three categories: (1) alive seedlings with expanded needles, (2) alive seedlings with seed capsule attached, and (3) dead seedlings.

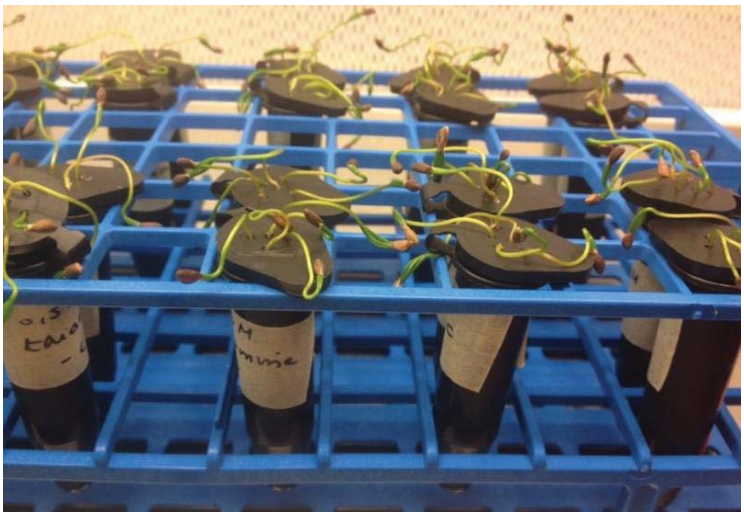


Figure 3: Setup of *Pythium ultimum* pathogenicity assays. Norway spruce seedlings were grown in microcentrifuge tubes with *P. ultimum*-inoculated growth medium (n = 10), or in tubes with non-inoculated growth medium (n = 10). Five seedlings were grown in each tube.

2.5 Wounding experiment

Wounding and harvesting

Ten-week-old seedlings grown from treated seeds were wounded by cutting off one needle (n = 16 to 20 per treatment, depending on the number of seedlings that was still alive). Twenty-four hours after wounding, the above-ground part of 16 seedlings per treatment was harvested and immediately frozen in liquid nitrogen (n = 16). To obtain enough plant material for RNA extraction, four harvested seedlings per treatment were pooled and used as one sample (n = 4 groups of 4 pooled seedlings per treatment). Needle tissue was ground with a Retsch MM300 TissueLyser mill (Retsch GmbH, Germany) and stored at -80 °C until further analysis.

RNA extraction

RNA was extracted using a modified version of the protocol accompanying the MasterPureTM Complete DNA and RNA Purification Kit (Epicentre, USA). For each sample, 10 mg of frozen tissue was suspended in 610 µl master mix, consisting of 1 µl proteinase K, 6 mg polyvinylpyrrolidone, 3 µl β-mercaptoethanol and 600 µl Tissue and Cell Lysis solution. The samples were incubated at 56 °C for 15 minutes and vortexed after 5 and 10 minutes. Next, the tubes were centrifuged at 4 °C and 16500 × g for 10 minutes. The supernatant was transferred to new tubes and placed on ice for 5 minutes. 250 µl of MPC Protein Precipitation Reagent was added to each sample and the tubes were vortexed for 10 seconds. The tubes were then

centrifuged again at 4 °C and 16500 × g for 10 minutes. The supernatant was transferred to new tubes and 400 µl of 7.5 M LiCl precipitation solution was added. The tubes were inverted 40 times to mix the supernatant with the LiCl before being stored overnight at -20 °C. The following day, the tubes were first centrifuged at 4 °C and 16500 × g for 30 minutes. Then the pellets were rinsed twice by adding 0.9 ml 70% ethanol, centrifuging at 4 °C and 16500 × g and removing the ethanol. The pellets were allowed to air dry for 5 minutes before being resuspended in 30 µl 56 °C nuclease-free water. Finally, the tubes were placed on ice for 10 minutes, vortexed for 10 seconds, centrifuged for 10 seconds and stored at -80 °C.

cDNA synthesis and RT-qPCR

cDNA was synthesized using a Thermo Scientific Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Desired RNA concentrations for cDNA synthesis, 25 ng μl^{-1}, were quantified for each sample with the NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA).

Transcript expression levels of three housekeeping genes and five defence-related target genes were quantified by RT-qPCR using a ViiA 7 Real-Time PCR System (Applied Biosystems, USA) and QuantStudio Real-Time PCR Software (v1.3, Applied Biosystems). Amplification reactions were carried out in 10 µl reaction volumes, consisting of 5 µl Fast SYBR Green Master Mix (Applied Biosystems, Latvia), 3 µl distilled water, 1 µl primer (4 µM) and 1 µl cDNA (25 ng/µl). All primer sequences are listed in Table 2. Cycling conditions were as follows: 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.

Relative gene expression was determined based on the obtained threshold cycle (Ct) values, which represent the number of cycles needed for the fluorescence generated within a reaction to cross a threshold line. First, raw fluorescence data were exported to the LinRegPCR software (v2020.0.0.0.3, Ruijter *et al*., 2009) to correct Ct values for variation in PCR efficiency. Since the expression of three different housekeeping genes was quantified, average housekeeping Ct values were calculated for each sample. Then, relative gene expression (ΔΔCt) was calculated as

$$\Delta Ct = Ct\,(\text{average housekeeping genes}) - Ct\,(\text{target gene}), \text{ and}$$

$$\Delta\Delta Ct = \Delta Ct\,(\text{treatment}) - \Delta Ct\,(\text{control})$$

Positive ΔΔCt values represent upregulation of genes compared to the control group, while negative values represent downregulation. One ΔΔCt unit corresponds to a two-fold difference in transcript amount.

Table 2: Overview of primers used in RT-qPCR. *Actin*, *α-tubulin* and *ubiquitin* were used as housekeeping genes.

Gene [a]	**Forward/reverse primer**	**Reference**
Actin	GGCATACCGGCAGCTCTTC / AAGTTGTTGGCGGCGTCTT	Hietala *et al.*, 2003
α-Tubulin	GGCATACCGGCAGCTCTTC / AAGTTGTTGGCGG CGTCTT	Hietala *et al.*, 2004
Ubiquitin	GTTGATTTTTGCTGGCAAGC / CACCTCTCAGACGAAGTAC	Schmidt and Gershenzon, 2007
ACS	CAAGCAGAATCCCTATGATGCCGAAA / TCTGGATGAGACTTGAGCCAACCTTC	Yaqoob *et al.*, 2012
LOX	ACCCTTGGTATAGCCCTCATA / ATCGTCACTCCATTCTCTCGT	Arnerup *et al.* 2011
PAL1	GGCAGATCATTTGGGTGATC / TAAAGTTCCATTTTCAACTATAGGACTAAT	Koutaniemi *et al.*, 2007
TPS-Car	GGTGGTACCAGGCAGACAGG / CAGTGTAGCCATCTCGATAATTGT	Zulak *et al.*, 2009
Chi4	GCGAGGGCAAGGGATTCTAC / GGTGGTGCCAAATCCAGAAA	Hietala *et al.*, 2003

[a] *ACS: aminocyclopropane-1-carboxylic acid synthase, LOX: lipoxygenase, PAL1: phenylalanine ammonia-lyase 1, TPS-Car: 3-carene synthase, Chi4: chitinase 4.*

2.6 Statistical analysis

Statistical analyses were performed using R (v3.6.1, R Core Team, 2019) in RStudio (v1.2.1335, RStudio Team, 2018). Differences in root length and relative gene expression (ΔΔCt) between seed treatments were assessed using one-way ANOVA followed by Tukey's post hoc test after having confirmed normality of the data with Shapiro-Wilk tests. An independent samples *t*-test was used to compare root length in control treatment A and B. Differences in germination rate and health class distributions were analysed with Fisher's exact tests. The package ggplot2 (Wickham, 2016) was used to make graphs.

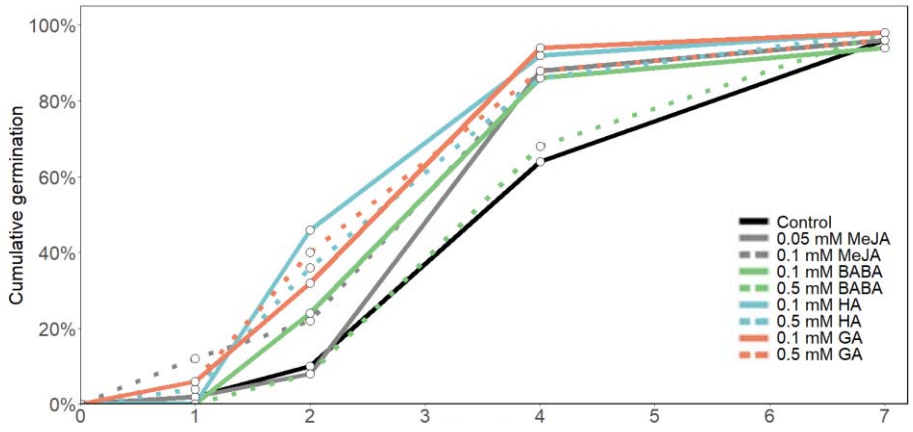
3. Results

3.1 Impact of seed treatment on seed germination and seedling development

Seed germination

To assess treatment differences in germination speed, I compared the cumulative germination percentage reached each observation day. In treatment group A, seeds treated with hexanoic acid (0.1 mM and 0.5 mM) and gibberellic acid (0.1 mM and 0.5 mM) had a significantly higher germination percentage than the control treatment on day 2. Seed treatment with methyl jasmonate (0.05 mM and 0.1 mM), hexanoic acid (0.1 mM) and gibberellic acid (0.1 mM and 0.5 mM) led to a significantly higher germination percentage than the control on day 4 (Fig. 4A, Table 3A). In treatment group B, seed treatment with quinic acid (0.5 mM), thiamine (0.1 mM and 0.5 mM), riboflavin (0.1 mM) and chitosan (0.01% and 0.05%) gave a significantly higher germination percentage on day 3. All of these treatments except 0.1 mM riboflavin still had a significantly higher germination percentage than the control on day 5 (Fig. 4B, Table 3B). By day 7, all treatments had reached a uniformly high germination percentage of 94 to 100%.

A



B

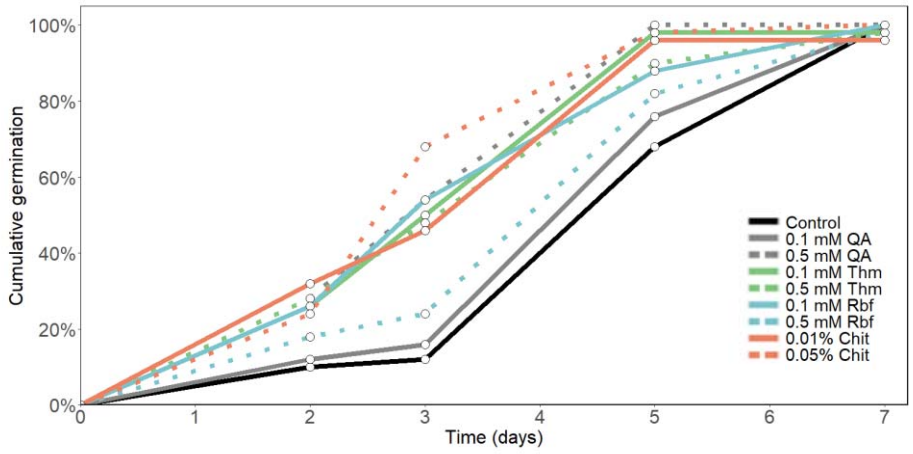


Figure 4: Cumulative germination percentage of treated Norway spruce seeds. For practical reasons seed treatments were divided into two groups and carried out on different days (treatment group A and B). Seeds were soaked overnight in water (control), methyl jasmonate (MeJA), β-amino butyric acid (BABA), hexanoic acid (HA), gibberellic acid (GA), quinic acid (QA), thiamine (Thm), riboflavin (Rbf) or chitosan (Cht) (n = 50 seeds per treatment).

Table 3: Statistical comparison and overview of cumulative germination percentage of treated Norway spruce seeds per observation day. Values in brackets are *P*-values (treatment vs. control) determined by Fisher's exact tests. Values in bold are statistically significant ($P < 0.05$).

A

Treatment [a]	**Day 1**	**Day 2**	**Day 4**	**Day 7**
Control	2 %	10 %	64 %	96 %
0.05 mM MeJA	2 % (1.00)	8 % (1.00)	88 % (**0.047**)	96 % (1.00)
0.1 mM MeJA	12 % (0.792)	22 % (0.28)	88 % (**0.047**)	96 % (1.00)
0.1 mM BABA	0 % (1.00)	24 % (0.194)	86 % (0.079)	94 % (1.00)
0.5 mM BABA	0 % (1.00)	8 % (1.00)	68 % (1.00)	98 % (1.00)
0.1 mM HA	0 % (1.00)	46 % (**0.001**)	92 % (**0.02**)	98 % (1.00)
0.5 mM HA	4 % (1.00)	36 % (**0.015**)	86 % (0.079)	98 % (1.00)
0.1 mM GA	6 % (1.00)	32 % (**0.038**)	94 % (**0.014**)	98 % (1.00)
0.5 mM GA	6 % (1.00)	40 % (**0.006**)	88 % (**0.047**)	96 % (1.00)

B

Treatment [a]	**Day 2**	**Day 3**	**Day 5**	**Day 7**
Control	10 %	12 %	68 %	100 %
0.1 mM QA	12 % (1.00)	16 % (0.917)	76 % (0.648)	100 % (1.00)
0.5 mM QA	28 % (0.353)	54 % (**<0.001**)	100 % (**<0.001**)	100 % (1.00)
0.1 mM Thm	26 % (0.353)	50 % (**<0.001**)	98 % (**<0.001**)	98 % (1.00)
0.5 mM Thm	28 % (0.353)	48 % (**<0.001**)	90 % (**0.046**)	98 % (1.00)
0.1 mM Rbf	26 % (0.353)	54 % (**<0.001**)	88 % (0.073)	100 % (1.00)
0.5 mM Rbf	18 % (0.873)	24 % (0.314)	82 % (0.297)	98 % (1.00)
0.01 % Cht	32 % (0.353)	46 % (**0.001**)	96 % (**0.003**)	96 % (1.00)
0.05 % Cht	24 % (0.409)	68 % (**<0.001**)	98 % (**<0.001**)	100 % (1.00)

[a] MeJA: methyl jasmonate; BABA: β-amino butyric acid, HA: hexanoic acid, GA: gibberellic acid, QA: quinic acid, Thm: thiamine, Rbf: riboflavin, Cht: chitosan.

Root length

After 8 days in the growth room, root lengths of seedlings in treatment group A varied significantly between treatments (ANOVA: $F_{8,80} = 28.45$, $P < 0.0001$. Tukey's test revealed that seedlings from seeds treated with 0.05 mM methyl jasmonate ($P = 0.002$) and 0.1 mM methyl jasmonate ($P < 0.0001$) had significantly shorter roots than the control, while seedlings from seeds treated with 0.5 mM BABA ($P = 0.003$), 0.1 mM gibberellic acid ($P < 0.0001$) and 0.5 mM gibberellic acid ($P = 0.039$) had significantly longer roots (Fig. 5A). Root lengths in treatment group B did not differ significantly from the control group (Fig. 5B). An independent samples *t*-test indicated that the root length of control seedlings in treatment group B was significantly longer than those in treatment group A ($t_{18} = 6.28$, $P < 0.0001$).

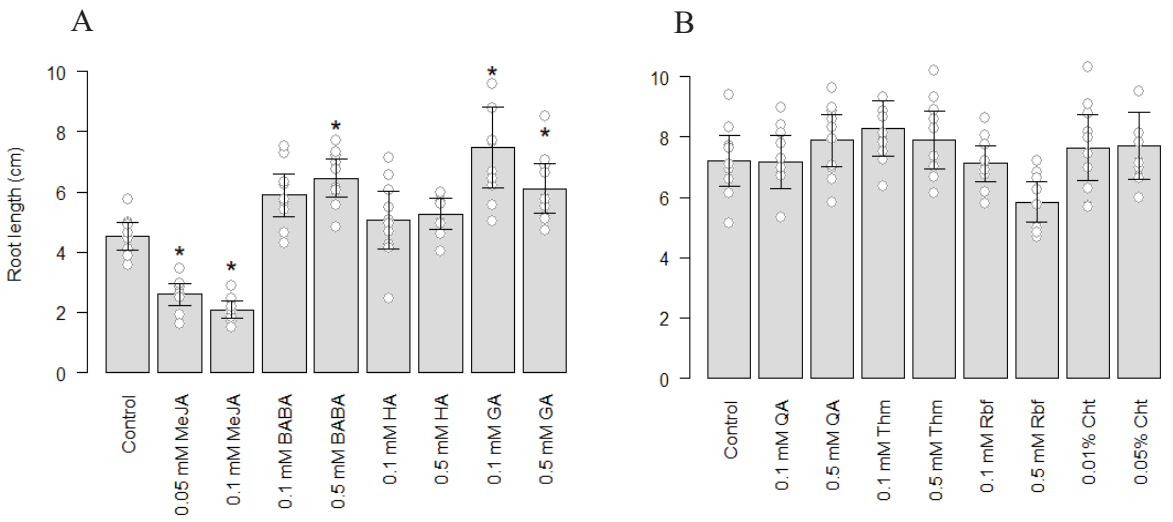


Figure 5: Root length of 8-day-old Norway spruce seedlings grown from seeds treated with water (control), methyl jasmonate (MeJA), β-amino butyric acid (BABA), hexanoic acid (HA), gibberellic acid (GA), quinic acid (QA), thiamine (Thm), riboflavin (Rbf) or chitosan (Cht) (n = 10 per treatment). Seed treatments were divided into two groups and carried out on different days (treatment group A and B). Error bars represent 95% confidence intervals, circles represent individual data points and asterisks indicate significant differences from the control at the $P < 0.05$ level, as determined by Tukey's tests following ANOVA.

3.2 Impact of seed treatment on seedling resistance and gene expression

Resistance to Pythium ultimum

No significant differences in resistance to *Pythium ultimum*, measured as seedling health status, were observed between seedlings grown from chemically treated seeds and seedlings grown from control seeds. This was true for all chemical treatments. In addition, seedling health within seed treatment group A and B did not differ significantly between seedlings grown in non-inoculated PDB and seedlings grown in inoculated PDB (Fisher's exact tests: $P > 0.05$ for all comparisons) (Fig. 6).

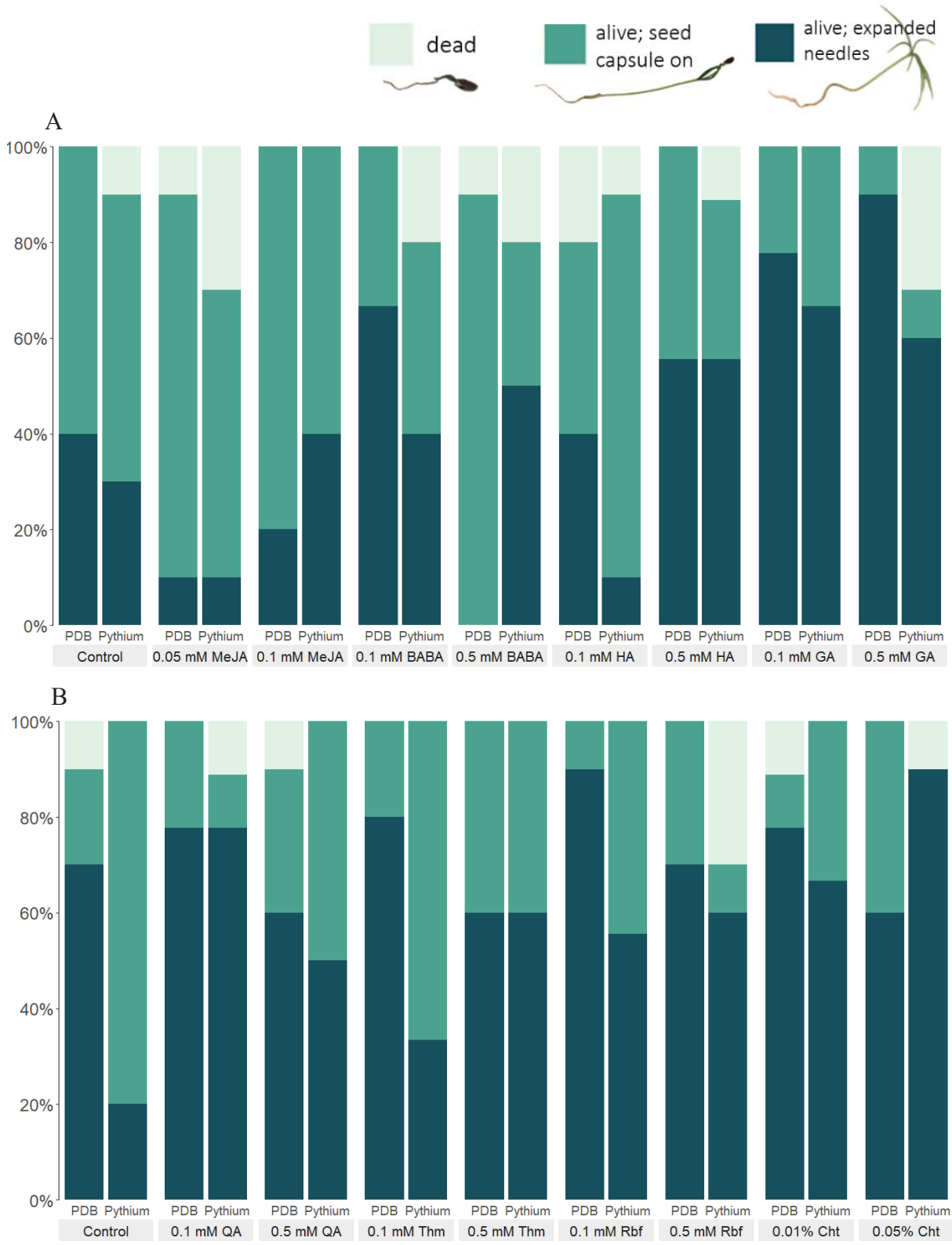
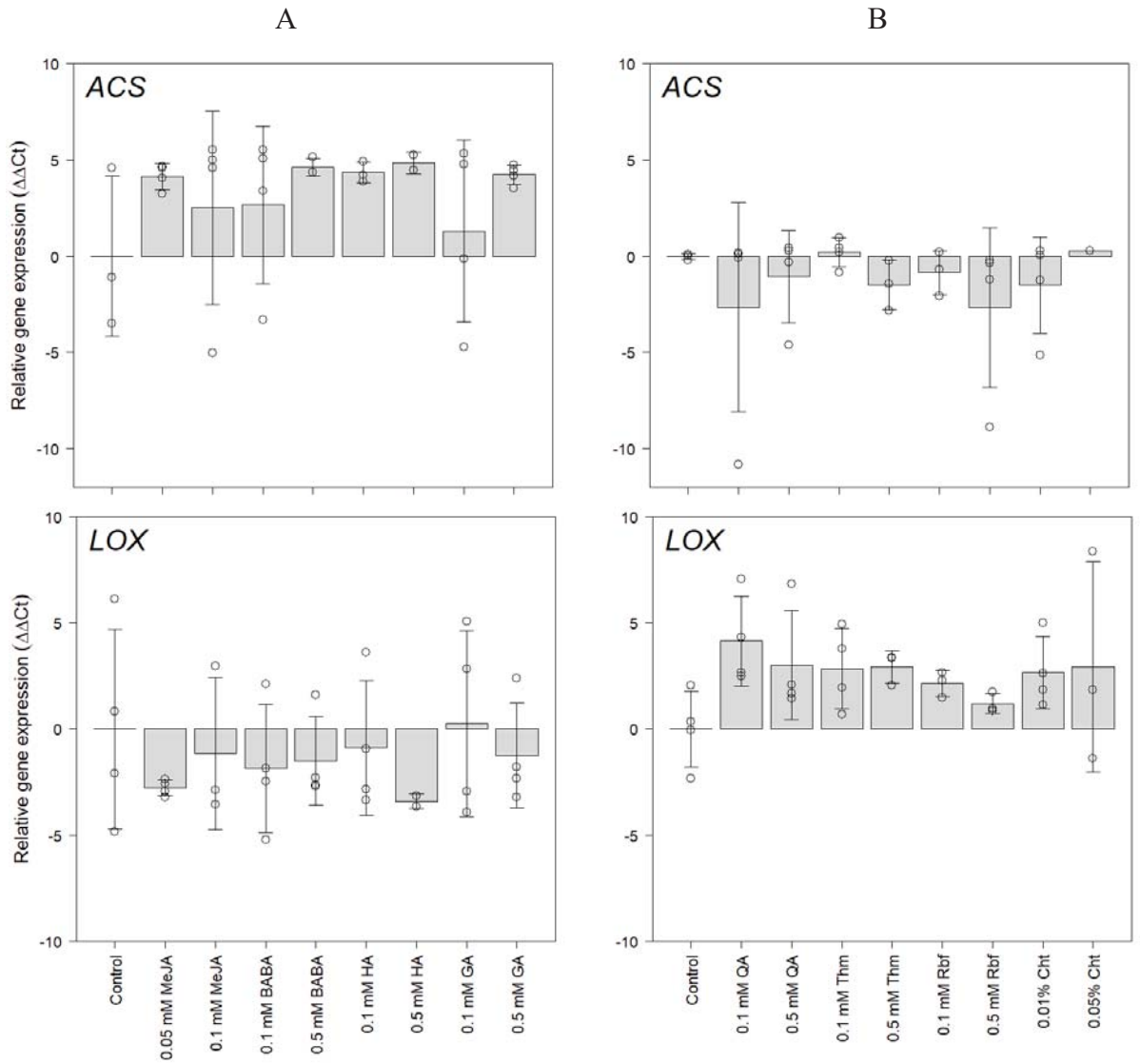


Figure 6: Percentages of Norway spruce seedlings in different health classes after 8 days of infection with *Pythium ultimum*. Seeds were treated with water (control), methyl jasmonate (MeJA), β-amino butyric acid (BABA), hexanoic acid (HA), gibberellic acid (GA), quinic acid (QA), thiamine (Thm), riboflavin (Rbf) or chitosan (Cht). Seed treatments were divided into two groups and carried out on different days (treatment group A and B). Ten-day-old seedlings were grown in non-inoculated PDB (PDB) (n = 10 per seed treatment) or in PDB colonized by *P. ultimum* (Pythium) (n = 10 per seed treatment) for 8 days. There were no significant differences with the control seedlings (Fisher's exact tests: $P > 0.05$).

Regulation of defence-related genes

Using RT-qPCR, I examined the transcript level of one ethylene biosynthesis gene (*ACS*), one jasmonate biosynthesis gene (*LOX*), one phenylpropanoid biosynthesis gene (*PAL1*), one terpene biosynthesis gene (*TPS-Car*) and one pathogenesis-related gene (*Chi4*) in wounded 10-week-old trees. There was no significant up- or downregulation of genes in wounded seedlings grown from chemically treated seeds compared to wounded seedlings grown from control seeds (Figure 7). Overall, Ct values tended to vary greatly between biological replicates, leading to high variability in relative gene expression (ΔΔCt) within seed treatments.



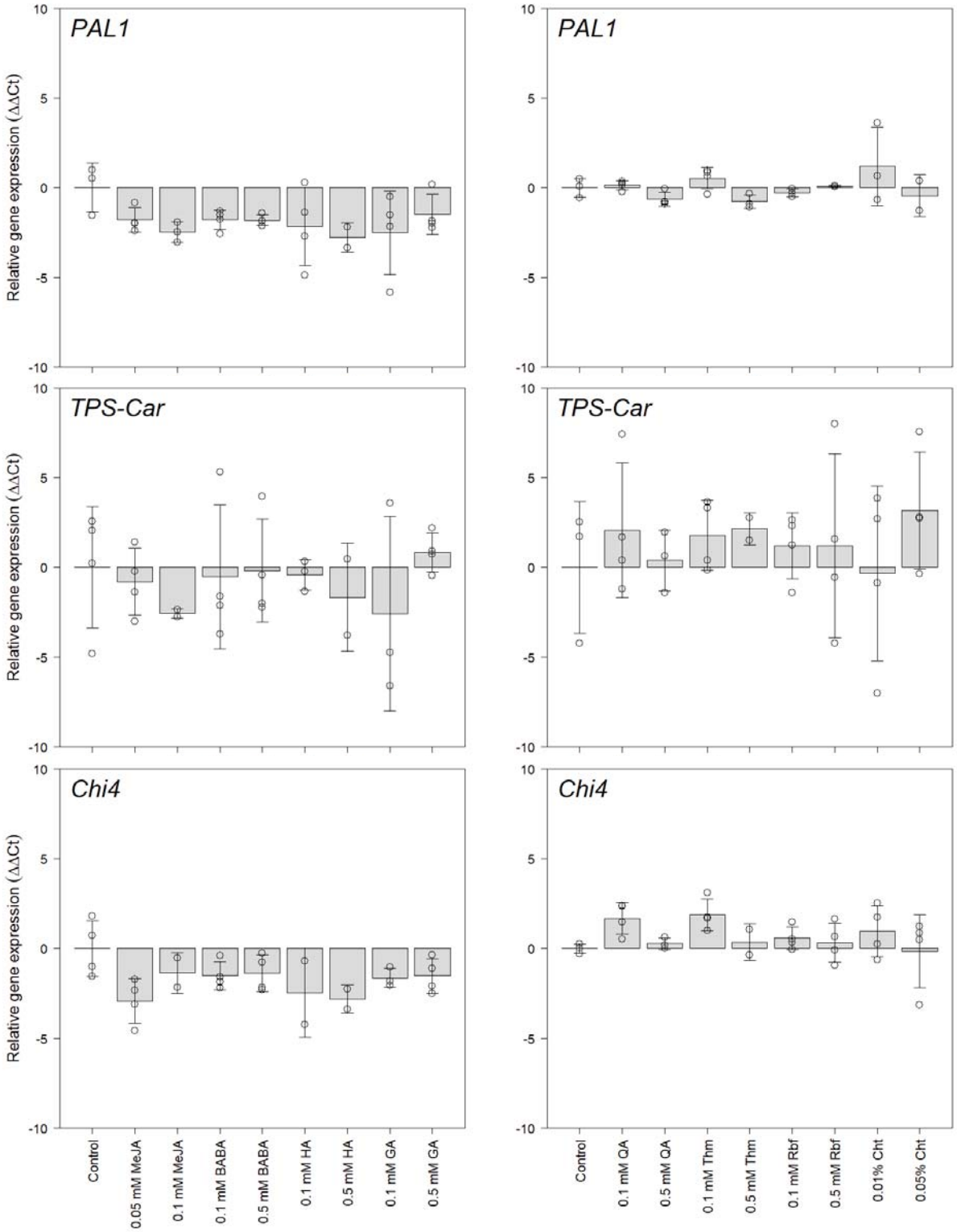


Figure 7: Relative gene expression (ΔΔCt) in 10-week-old Norway spruce seedlings 24 hours after wounding (n = 4 groups of 4 pooled seedlings per treatment). Seedlings were grown from seeds that had been treated with water (control), methyl jasmonate (MeJA), β-amino butyric acid (BABA), hexanoic acid (HA), gibberellic acid (GA), quinic acid (QA), thiamine (Thm), riboflavin (Rbf) or chitosan (Cht). Seed treatments were divided into two groups and carried out on separate days (treatment group A and B). Error bars represent standard deviations and circles represent individual data points. There were no significant differences between treatments for any of the genes (ANOVA: $P > 0.05$).

4. Discussion

4.1 Impact of seed treatment on seedling resistance and gene expression

Stem treatment with the phytohormone methyl jasmonate has been known to induce defences in Norway spruce and other conifers for almost two decades (e.g. Franceschi *et al*., 2002; Heijari *et al.*, 2005; Erbilgin *et al.*, 2006; Zeneli *et al.*, 2006; Mageroy, Christiansen *et al.*, 2020). Little is known about the impact of other chemical compounds or the use of seed treatment as an alternative method to increase conifer resistance. In this study, I tested the ability of methyl jasmonate (MeJA), β-amino butyric acid (BABA), hexanoic acid, gibberellic acid, quinic acid, thiamine, riboflavin and chitosan to regulate defence-related genes and induce resistance to *Pythium ultimum* in Norway spruce seedlings by means of seed treatment.

Resistance to Pythium ultimum

None of the seed treatments I tested seemed to protect seedlings against *Pythium ultimum*. However, since control seedlings grown in potato dextrose broth (PDB) inoculated with *P. ultimum* were not significantly unhealthier than control seedlings grown in non-inoculated PDB, the results could have been impacted by an inability of *P. ultimum* to infect the seedlings. *Pythium ultimum* is a pathogen that may cause serious damage to young Norway spruce seedlings (Kozlowski & Métraux, 1998; Kozlowski *et al*., 1999). However, the particular *P. ultimum* culture used in this experiment might have been non- or low-virulent, perhaps due to repeated subculturing in the laboratory. Subcultering on artificial media has been reported to reduce the virulence of various fungi and oomycetes (e.g. Krokene & Solheim, 2001; Shah *et al.*, 2007; Songe *et al.*, 2014). Reduced virulence is often associated with phenotypic changes, such as slower growth and a reduced ability to secrete enzymes that facilitate host penetration, but little is known about the underlying genetic mechanisms (Butt *et al*., 2007). It is also possible that issues not related to *P. ultimum* impacted my results, since control seedlings grown in non-inoculated PDB also displayed relatively poor health after the assays. For example, only 40% of control seedlings in treatment group A had fully expanded needles. After the pathogenicity assays were completed, I noticed that some of the tubes were not entirely filled with PDB. This might have caused seedlings with shorter roots to wilt and die regardless of their resistance to *P. ultimum*. Overall, due to these uncertainties it is not possible to draw firm conclusions regarding the impact of seed treatment on seedling resistance to *P. ultimum*.

However, the pathogenicity assay used in this study is an important step in the development of a relatively simple and fast method of screening resistance of conifer seedlings to fungal root pathogens. During the course of this project I tried out two different pathogenicity assays. In a preliminary phase, scoops of *P. ultimum* grown on potato dextrose agar (PDA) were placed in the centre of Petri dishes filled with PDA. After the pathogen had colonized the PDA for 4 days, 8-day-old Norway spruce seedlings grown from treated seeds were placed in the dishes with the tip of their roots touching the *P. ultimum* culture. Seedlings were taken out of the Petri dishes and scored for symptoms after one week. This assay was less time-consuming than the final setup, but had the disadvantage that the *P. ultimum* culture grew unevenly across the Petri dishes, covering some seedlings more than others. By placing the seedlings in microcentrifuge tubes with *P. ultimum*-inoculated PDB as described in the methods, the seedlings had a more uniform chance of getting infected. It would have been interesting to repeat the final assay setup with another culture of *P. ultimum* verified to be pathogenic and making sure that the tubes were completely filled with PDB. In future studies, an alternative way to evaluate seedling resistance after seed treatment could be to pot the seedlings and infect them by pouring a suspension of fungal or oocymetal hyphae around the seedling base, as described by Kozlowski & Métraux (1998) and Kozlowski *et al*. (1999).

Regulation of defence-related genes

Seed treatment did not cause significant changes in the transcript levels of the ethylene biosynthesis gene *ACS*, jasmonate biosynthesis gene *LOX*, phenylpropanoid biosynthesis gene *PAL1*, pathogenesis-related gene *Chi4* and terpene biosynthesis gene *TPS-Car* in wounded 10-week-old seedlings. The high variability I observed in gene expression between samples from the same seed treatment could result from issues during the RT-qPCR process. However, since I tried to reduce inaccuracy caused by technical errors as much as possible (e.g. by verifying RNA purity, using master mixes, running no-template controls, correcting Ct values for variation in PCR efficiency etc.), the outcome is most likely a result of true biological variation in gene expression between replicates. This would indicate that none of the seed treatments I tested strongly upregulated the target genes for a prolonged period of time and/or caused seedlings to have a stronger transcriptional response to wounding.

As far as I know, BABA, hexanoic acid, gibberellic acid, quinic acid, thiamine, riboflavin and chitosan have never before been tested for their ability to regulate *ACS*, *LOX*, *PAL1*, *Chi4* or *TPS-Car* in Norway spruce. MeJA has been studied more thoroughly in this context and stem treatment with MeJA has been reported to upregulate *PAL1*, *Chi4* and *TPS-Car*. However, since these previous studies have a different experimental design than my study, it is difficult to compare my results with their findings. *ACS* has previously been found to be non-responsive to MeJA treatment in 2-year-old Norway spruce saplings (Yaqoob *et al*., 2012). Arnerup *et al.* (2013), who treated 3- to 4-week-old spruce seedlings with MeJA by fumigation, did not find significant differences in *PAL1* expression in cotyledon and hypocotyl tissues between treated and untreated seedlings 48 hours after treatment. Other studies did find *PAL1* to be significantly upregulated in the bark of 2- and 48-year-old Norway spruce in response to MeJA spraying (Yaqoob *et al*., 2012; Magerøy, Christiansen *et al.*, 2020). *TPS-Car* upregulation following MeJA spraying has been observed in the bark of 2-year-old saplings during the first 32 days after treatment (Zulak *et al*., 2009) and in the bark of 48-year-old spruce 14 days after treatment (Magerøy, Christiansen *et al.*, 2020). MeJA treatment has also been found to upregulate C*hi4* in the bark and sapwood of 2-year-old saplings (Yaqoob *et al*., 2012) and in the bark of 48-year-old trees (Magerøy, Christiansen *et al.*, 2020) for at least two weeks. There are no studies involving MeJA treatment of Norway spruce that have quantified transcript levels of the same *LOX* gene I studied. Besides the different methods used to apply MeJA, these previous studies are quite different from my study in the sense that they quantified defence-related transcripts shortly after treatment (< 1 month) without prior exposure to stress. Except from Arnerup *et al.* (2013), the studies also involved much older trees. Thus, little is known about the transcriptional response of very young Norway spruce seedlings to a trigger stress that occurs several weeks after MeJA treatment.

The lack of transcriptional differences between seedlings from treated seeds and control seeds indicates that methyl jasmonate and the other chemicals I tested probably were unable to regulate the studied genes over a prolonged period of time. However, the results could have been different if the trees had been older at the time of wounding and harvesting, as spruce defences and defence-related gene expression probably differ between developmental stages. Ontogenetic variation in defence traits has been documented in many plant species (Barton & Koricheva, 2010), including conifers. For instance, Erbilgin & Colgan (2012) observed significant differences in the magnitude of MeJA-induced monoterpene production between young and mature jack pine (*Pinus banksiana*). They suggested that older trees might be able

to afford higher levels of inducible defences because they have a higher production and storage capacity of photosynthesis products. It is likely that defence-related gene expression in spruce plants varies during development and gives rise to similar variation in defence traits between seedlings and older trees. It would be interesting to repeat the experiment and quantify the expression of genes known to be upregulated in young spruce seedlings in response to exogenous MeJA, such as *PR1* (pathogenesis-related protein 1) and *LURP1* (late upregulated in response to *Hyaloperonospora parasitica* 1) (Arnerup *et al.*, 2013), or to carry out a study involving older Norway spruce plants.

4.2 Impact of seed treatment on seed germination and seedling development

Ideally, a chemical defence priming agent would enhance plant resistance with no or positive effects on important processes such as seedling emergence, root development, shoot growth and seed production. In practice, however, the induction of defences through the application of chemical elicitors often comes with drawbacks. Two commonly discussed costs are allocation costs, which occur when plants allocate resources to defences instead of growth and reproduction, and ecological costs, which result from altered interactions between plants and their environment. Chemical elicitors might also directly reduce plant fitness by having toxic effects (Walters & Heil, 2007; Vos *et al*., 2013). In this study, I measured seed germination and root length to identify potential costs and benefits associated with seed treatment.

Seed germination

None of the seed treatments I tested impacted the germination capacity of the spruce seeds. After 7 days in the growth room, seeds from all treatments reached a germination percentage of 94 to 100%. With the exception of BABA (0.1 mM and 0.5 mM), quinic acid (0.1 mM) and riboflavin (0.5 mM), all chemical seed treatments significantly accelerated seed germination. For spruce nurseries, this high germination capacity and germination speed following seed treatment may be financially interesting. For instance, a high germination capacity means that less seeds have to be sown per unit area to meet seedling production goals. Also, seedlots with a high germination speed require less greenhouse heating since the germination period is shorter (Stoehr & El-Kassaby, 2011).

Root length

My measurements of 8-day-old seedlings showed that seed treatment with MeJA (0.05 mM and 0.1 mM) decreased root length significantly, while seed treatment with BABA (0.5 mM) and gibberellic acid (0.1 mM and 0.5 mM) increased root length. MeJA has so far only been studied for its impact on conifer root development in studies involving stem treatment of older pine saplings. These studies have shown both positive and negative effects of MeJA on root growth. Moreira *et al*. (2012) found that MeJA caused a significant increase in fine roots and a significant reduction in coarse roots in 6-month-old cluster pine (*Pinus pinaster*). Heijari *et al*. (2005), who studied 2-year-old Scots pine (*Pinus sylvestris*), reported significant differences in root biomass between MeJA-treated trees and control trees. The same study did not find significant differences in main root length. However, the negative effect I found for MeJA agrees with studies on various agricultural crops, where seed treatment with MeJA has been found to reduce root growth in young seedlings, especially at higher concentrations (e.g. Tsai *et al*., 1997; Norastehnia *et al*., 2007; Tzorzakis, 2009). In maize (*Zea mays*), root growth inhibition caused by MeJA seed treatment has been associated with decreased α-amylase activity and ethylene production (Norastehnia *et al.,* 2007*)*.

Seed treatment with BABA has previously been reported to enhance seedling vigour (calculated by combining measurements of root length, shoot length and germination percentage) in pearl millet (*Pennisetum glaucum*) and sunflower (*Helianthus annuus*) at concentrations up to 50 mM (Nandeeshkumar *et al*., 2009; Shailasree *et al*., 2001). Similarly, seed treatment with the growth regulator gibberellic acid has been reported to promote root growth in seedlings of several non-conifer species, such as sugar beet (*Beta vulgaris*) and sesame (*Sesamum indicum*), especially at lower concentrations (Kyauk *et al*., 1995; Jamil & Rha, 2007). These positive effects of BABA and gibberellic acid in agricultural crop plants are similar to what I found in Norway spruce seedlings.

The difference I found in root growth between control plants in treatment group A and B could indicate that there were unintentional differences in growing conditions (e.g. humidity and temperature) between the two treatment groups. The early alterations in root length caused by seed treatment with MeJA, BABA and gibberellic acid may directly impact further seedling development by effecting the seedlings' capacity to take up water and nutrients. Additional changes might be caused by interactions with other organisms, such as root-associated microbes, which might impact plant development by for instance facilitating nutrient uptake

and increasing pathogen resistance. Little is known about the precise impact of root system architecture, but plants with greater root length, density and biomass are generally thought to be better at altering soil microbial communities in their favour (Saleem *et al*., 2018).

5. Conclusion

Chemical seed treatment could be a simple method to increase the resistance of young Norway spruce plants to pest and pathogen attacks. By visually assessing seedling resistance to *Pythium ultimum* and quantifying defence-related gene transcripts after mechanical wounding, I could not demonstrate that seed treatment with methyl jasmonate, BABA, hexanoic acid, gibberellic acid, quinic acid, thiamine, riboflavin or chitosan increased the defensive capacity of Norway spruce seedlings. Most of the seed treatments had a positive effect on seed germination speed and seedling root development, demonstrating the importance of evaluating other costs and benefits in addition to resistance in seed treatment studies. With some modifications, the *Pythium ultimum* pathogenicity assays and wounding experiments developed here could be useful in further studies on seed priming. In future studies, it would be interesting to evaluate the defense-inducing capacity of more chemicals at several concentrations, to quantify various allocation and ecological costs associated with defence induction, and to investigate the involvement of DNA methylation and other epigenetic mechanisms thought to underpin defence priming in Norway spruce.

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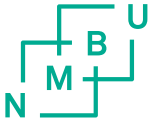

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