Norwegian University
of Life Sciences

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# Impacts of Abiotic Stress on Priming of Defense Responses and Pathogen Resistance in Norway spruce

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# Preface

This master thesis marks the end of my degree in General Ecology at the Faculty of Environmental Sciences and Natural Resource Management (MINA) at the Norwegian University of Life Sciences (NMBU). This is where I have studied for five years, and have met some amazing people, and made memories for life. This master thesis has enabled me to cooperate with another institute outside of NMBU, the Norwegian Institute of Bioeconomy Research (NIBIO) which I am truly grateful for. I would like to thank my supervisors Paal Krokene (NIBIO & NMBU) and Melissa Magerøy (NIBIO) for their patience, guidance and for keeping my spirits up for more than a year. I could not have been more grateful for having such skillful and encouraging supervisors. I would also like to thank the employees at NIBIO for assisting me during this master thesis. Finally, I would like to thank my family and friends for their encouragement and support during the work with this thesis.

# Abstract

Norway spruce (*Picea abies)* is one of the most important tree species in Norway due to its high economic and ecological significance in forests. Pathogens, fungi and insects may have detrimental impacts on Norway spruce trees and cause economic losses for the timber industry. Insects such as the European spruce bark beetle (*Ips typographus*) carry fungi and help them enter the trees as the fungi cannot penetrate the bark alone. *Grosmannia penicillata* is a bluestain fungus that causes discoloration and necrosis in Norway spruce trees. Together this mutualistic pair may overcome the defenses of healthy trees and kill thousands of trees during outbreaks. There have been several studies experimenting with either methyl jasmonate (MeJA) or drought against pathogens, fungi and insects, but few have studied the combined effects of drought and MeJA on Norway spruce. This study will look at the effects of MeJA, a natural defense-inducing compound found in trees, and whether Norway spruce seedlings sprayed with MeJA are more resistant to *G. penicillata* infection than uninfected control seedlings. Drought is also going to be a main part in this study, as drought is known to act as a priming agent in trees which enables them to respond to threats faster but may also stress trees to the point where they become more vulnerable to pathogens, fungi and insects. In this study 144 seedlings, 72 of these were sprayed with MeJA, while the other 72 seedlings were treated with Tween as a control. Half the seedlings in each treatment group were exposed to drought while the other half received ample water. The results of this study showed that the aboveground growth of Norway spruce seedlings, such as height and stem growth, were not significantly different in control and MeJA-treated plants exposed to drought. However, belowground root biomass in Norway spruce was negatively affected by the combination of MeJA application and drought exposure. The necrotic lesions caused by *G. penicillata* infection were significantly longer in control plants, than MeJA-treated plants. Our study shows that the combination of mild drought and minimal amount of MeJA increases Norway spruce resistance to fungi, but further studies should be done to better understand the effects of combinations of stresses on spruce defense.

# Content

# 1. Introduction

Norway spruce (*Picea abies)* is a conifer species that is widespread in Europe due to its ability to adapt well to different environments (Krokene. 2015). Not only does this conifer species have high economic value as a timber species, but it also serves as an important keystone species in several ecosystems for many insects, birds and mammals (Nystedt et al. 2013). Norway spruce trees are affected by both abiotic stress factors such as drought, nutrient deficiencies, climate and others, and by biotic stress such as insects, fungi and pathogens (Desplanque et al. 1999). Drought is one of the main factors which determines the growth of plants as well as their ability to survive in different environments (Lévesque et al. 2013). The effect of the drought depends on the severity of the drought periods, how long they last and how well the seedlings can adapt to this environmental challenge (Moya et al. 2018). Due to the importance of drought on Norway spruce seedlings, it will be a focus of our study as it can either act as a priming agent but may also cause stress to seedlings and expose them to pathogen, insects and fungi.

*Grosmannia penicillata* is a deadly tree fungus that is transported by bark beetles (Hammerbacher et al. 2013). *G. penicillata* is categorized as an ophiostomatoid fungi and belongs to the group bluestained fungi. These fungi are well known to cause discoloration in wood, and some may also be lethal to trees through necrosis, which in turn leads to economic losses for loggers and other interest groups (Zhou et al. 2001; Repe et al. 2013). *G. penicillata* is not well studied in scenarios where the host trees have been previously exposed to potential stressors such as drought in combination with methyl jasmonate (MeJA) treatment.

The vector of *G. penicillata* is the European spruce bark beetle (*Ips typographus*), which is one of the biggest threats to Norway spruce as they attack trees in large numbers periodically and increase tree mortality. The spruce bark beetles can weaken the trees with the help of associated ophiostomatoid fungi, causing the trees to be more vulnerable, have higher stress levels and reduced growth (Krokene 2015; Hlásny et al. 2019). The mutualistic relationship of the fungi and the beetle enable them to overcome the tree defenses and to kill healthy adult trees (Krokene 2015; Zhao et al. 2019). The beetles dig inside the phloem of the trees where the female creates galleries and lays eggs (Wermelinger et al. 1999). The larvae then excavate feeding tunnels towards the outer bark where they pupate and start searching for new host trees (Hlásny et al. 2019). Bluestained fungi also grow in these galleries which enables them to spread in the trees (Zhao et al. 2019). Insecticides and pesticides are known to repel attacks from bark beetles and pathogens, but these are rarely used today since these are not good for the environment (Mahmood et al. 2016). MeJA, a naturally occurring chemical in plants, can induce plant defenses (Cheong et al. 2003; Zas et al. 2014; Moreira et al. 2012 A; Franceschi et al. 2002). Conifers respond to application of MeJA by boosting their defenses which may last from weeks to months (Erbilgin et al. 2006).

Conifer defenses can be split up into two categories: constitutive and induced, with constitutive defenses being a standing defense which is always present in the plants, such as cork bark, needles, and stored chemicals that are released under attacks (Krokene 2015; Franceschi et al. 2005; Mageroy et al. 2019). The constitutive defenses are costly as they must be maintained all the time, while induced defenses only occur during or after attacks from insects and pathogens. The induced defenses are for example monoterpenes, and resin acids that are types of terpenoids (Martin et al. 2002). Traumatic resin ducts (TRD) are also an important antiherbivory strategy since they contain oleoresin which has monoterpenoids and diterpene resin acids in them that protects the trees (Martin et al. 2002). TRDs have an increased cost associated with them, and often cause a tradeoff in conifers, that must choose to either grow or increase their defenses at the cost of growth (Franceschi et al. 2005; Wilkinson et al. 2019). Prolonged induction of defenses, caused by TRDs, help the trees respond faster to similar attacks in the future, and keep these defenses induced for a period (Krokene 2015; Mageroy et al. 2019). Research is therefore needed to understand how these defenses function.

The goals of this study are to better understand the combined effects of MeJA-treatment and drought stress on 1-year-old Norway spruce seedlings. This study will explore whether the combination of MeJA treatment and drought-exposure on Norway spruce seedlings increases or reduces their resistance to *G. penicillata*. The questions that will be the focus of our study are: 1) how does MeJA treatment affect 1-year-old Norway spruce seedlings?; 2) are seedlings that are sprayed with MeJA and exposed to drought more resistant to attacks from *G. penicillata* than control seedlings, and 3) how do these two factors affect the seedlings*?*

# 2. Methods

A total of 144 Norway spruce seedlings were used in our study. First, 72 seedlings were treated with MeJA, while 72 seedlings were treated with Tween as a control. A few weeks later, half the seedlings in each treatment group were exposed to drought while the other half received ample water. This gave four different treatment combinations: seedlings treated with MeJA or Tween and exposed to drought, plus seedlings treated with MeJA or Tween and given ample water. Several analyses were performed on all seedlings, such as root analysis ( n = 40), microscopic analyses of stem growth and measurement of traumatic resin ducts ( n = 40 ) and inoculation of *Grosmannia penicillata* or mock-inoculation with potato-dextrose-agar (PDA) (control) (n = 64 ) (Figure 1).

## 2.1 Plant material and growing conditions prior to drought stress

One-year-old Norway spruce seedlings (M95 type) were bought from a nursery and planted on May 13, 2019 in a greenhouse in Ås. A total of 144 seedlings that had been stored in a cold-room were potted in 0.8 L pots with a fertilized soil containing nitrogen, phosphate and potassium (Blossom plant soil, Europris). The pots were placed in watering trays with 10 pots in each. The seedlings were given a month to acclimate to the greenhouse conditions before their total height was measured on June 11, 2019. The seedlings were given 0.2 L of water every other day on weekdays and daily during weekends prior to the drought exposure and continued after drought exposure. Plant height was measured again September 26, 2019 to determine plant growth over the course of the experiment (Figure 1).

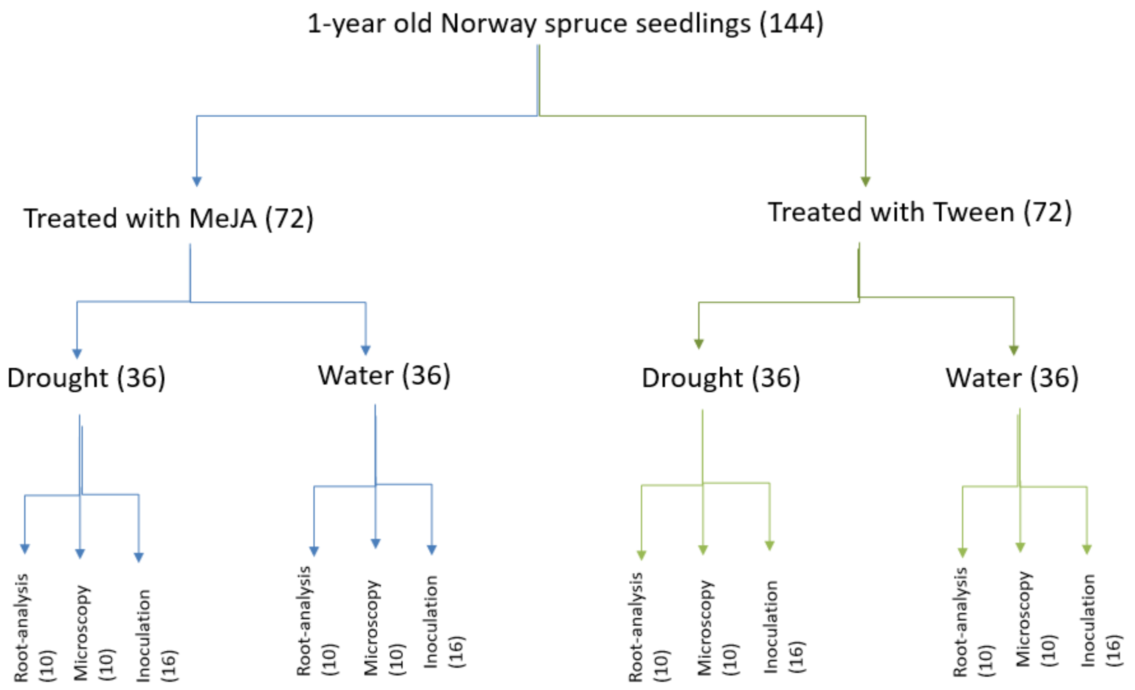

**Figure 1** Overview of the experimental design, treatments and analyses used in this study. Numbers in parentheses refer to the number of seedlings used.

## 2.2 Application of methyl jasmonate and Tween

On June 12, 2019, 72 seedlings were sprayed with 10 mM MeJA and 0.1 % Tween (Sigma-Aldrich® 392707, USA) and 72 were sprayed with 0.1 % Tween (Sigma-Aldrich® P9416, USA) (Figure 1). The seedlings were sprayed outside using a 1.5 L pressurized spray bottle (Bürkle GmbH, 0309-0100). Seedlings were given eight sprays along the length of the stem. This was enough to saturate the stem and droplets could be seen on the needles. After spraying, the seedlings were left for an hour outside to dry. The weather outside was cloudy and windy with no rain. The seedlings were put back in the greenhouse room after MeJA application in a randomized pattern.

On June 15, 2019 the temperature-regulating system in the greenhouse failed and the roof of the greenhouse remained closed for nine hours. This increased the temperature to > 35 °C for several hours and some seedlings became discolored and shed some needles. Because of this technical error we decided to delay the start of the drought experiment to August 13, 2019. This was done to avoid stressing the seedlings further, which might have killed them due to the combination of heat stress and MeJA application.

## 2.3 Drought exposure

The drought experiment started on August 13, 2019 and continued until September 10, 2019. There were in total 72 seedlings which were exposed to drought and received 1 L of water per watering tray once a week, while the other 72 seedlings received 2 L of water per watering tray three times a week (i.e. six times more water per week; Figure 1).

## 2.4 Fungal inoculation with *Grosmannia penicillata*

*Grosmannia penicillata* (Isolate = 1980-91/54, Collected: 1980, Akershus (Ås), Norway) was taken out from the -150 °C freezer on September 5, 2019 to grow a month prior to the inoculation. Eight seedlings per treatment combination (seedlings treated with MeJA or Tween that were either exposed to drought or given ample water) were inoculated with *G. penicillata* on October 02, 2019 or were mock inoculated with PDA Seedlings were inoculated by cutting a small bark flap with a scalpel in last year's internode. Then 5 ml of PDA (control) or 4.5 ml of the *G. penicillata* culture were inserted into the wound using a small syringe. After inoculation, the wound was covered with Parafilm. The difference in the inoculation applied for the two treatments was due to the fungi being slightly waterier than PDA, causing the difference in the amount added.

About seven weeks after inoculation (November 26, 2019) the seedlings were examined and scored for symptoms. The outer bark was removed, and the length of the necrotic lesions in the inner bark was measured using an electronic caliper (Cocraft Vernier Caliper, Clas Ohlson, England).

## 2.5 Root analysis

The root analysis of Norway spruce was on September 27, 2019. Ten seedlings from each treatment combination (in total 40 seedlings) were used. First, the root mass of each seedling was divided in two equal halves. One half was discarded, and the other half was placed in a transparent tray, covered with water and scattered to ensure that none of the roots overlapped. Only half the root mass was used for the root measurements to avoid overcrowding the tray and getting inaccurate measurements. The tray was placed in a flatbed scanner (Epson Expression 11000 XL, Epson Inc., Japan) connected to the root analysis software WinRhizo (Regent Instruments Inc. 1991) that was used to analyze root images. The software sorted the roots into four different root diameter classes (0.0 – 0.5 mm; 0.5 – 1.0 mm; 1.0 – 2.0 mm and ≥ 2.0 mm) and measured the total length of roots in each diameter class.

When the roots had been scanned, they were collected and dried for three days at 70 °C in a Termaks Laboratory drying oven (Termaks AS, Norway). A Mettler-Toledo PB602 was used to weigh the root materials with a precision of 0.01 gram. Specific root length (SRL) was calculated by dividing the total root length (mm) by the total root weight (g) for each seedling. Root tissue density (RTD) was calculated by dividing the total root weight (g) by total root volume ($mm^3$) for each seedling.

## 2.6 Microscopic analyses of stem growth and traumatic resin ducts

To measure the stem growth, total cross-sectional area of bark, and to count resin ducts, stem cross-sections were examined in a binocular microscope (AxioCam MRc 5, Zeiss, Germany). A thin cross-section was cut from each of 10 seedlings per treatment combination with a scalpel and imaged using the microscope software Axiovision Rel. (4.8.2, 2009, Germany) (Figure 2). A second stem section was cut of each seedling and examined in a light microscope (Leica Type 020-525.732, Leica Microsystems GmbH Wetzlar, Germany) to measure TRDs at higher magnification (10×) since it was difficult to measure them in binocular microscope (Figure 3 A & B). The software ImageJ was used to make measurements of the light microscope images together with the TRDs by highlighting them in each seedling. ImageJ was used to measure and count the TRDs, together with stem growth, and to ensure that the previous calculations taken by Axiovision software were correct.

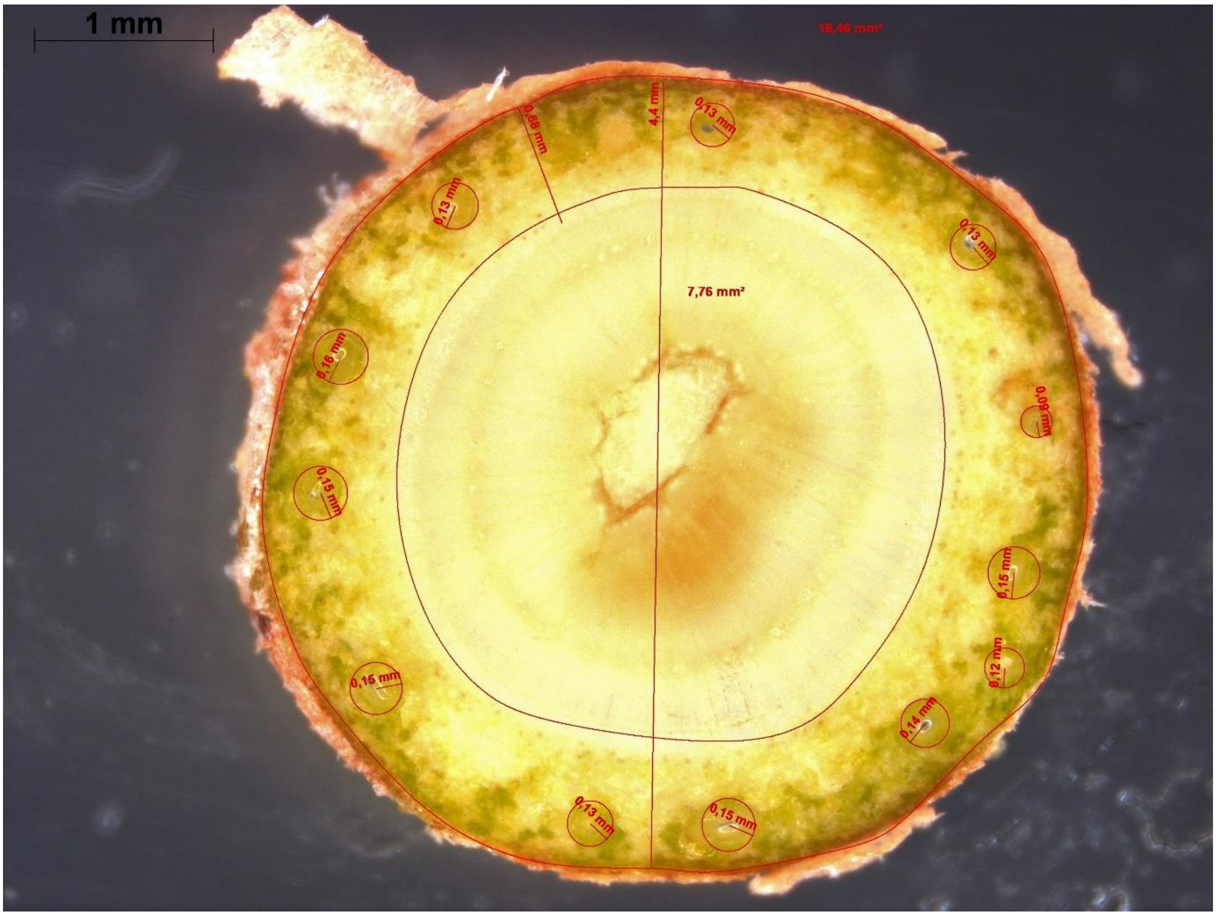

**Figure 2**: Stem cross-section of a 1-year-old Norway spruce seedling showing the different measurements that were made, such as the stem circumference (outer circle), stem diameter (vertical line), and the circumference of cortical resin ducts (red circles). Bark thickness was determined by subtracting the sapwood circumference (inner circle) from the stem circumference.

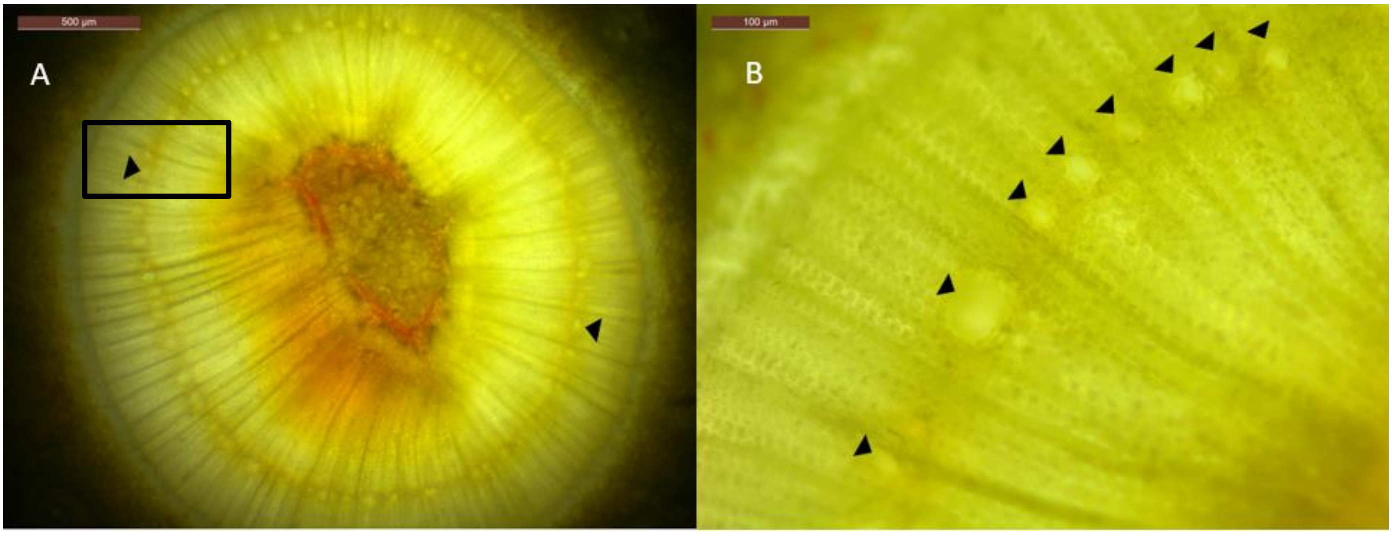

**Figure 3**: A) Cross-section of a stem taken by a light microscope from a Norway spruce seedling. Traumatic resin ducts (TRD) form a circle inside the stem. Black triangles indicate TRDs. B) A close-up of Figure 3 A to measure TRDs more accurately.

## 2.7 Statistics

All statistical tests were done in R Studio (R Studio, USA). First, a Shapiro test was used to test if the residuals were normally distributed. When normal distribution was indicated, a one-way ANOVA test was used. Tukey's post hoc test was used to identify differences between treatments if the ANOVA was significant ($p < 0.05$). For data that was not normally distributed, the non-parametric Kruskal-Wallis test was used to test for significance. If the test was significant ($p < 0.05$), a post-hoc Dunn test was used to identify statistically significant treatment differences.

# 3. Results

## 3.1 Aboveground growth

There were no significant differences in either stem height ($F_{3,9} = 1.846$, $p = 0.16$) or stem growth ($F_{3,9} = 0.383$, $p = 0.766$) between seedlings allocated to the four different treatment combinations (Figure 4).

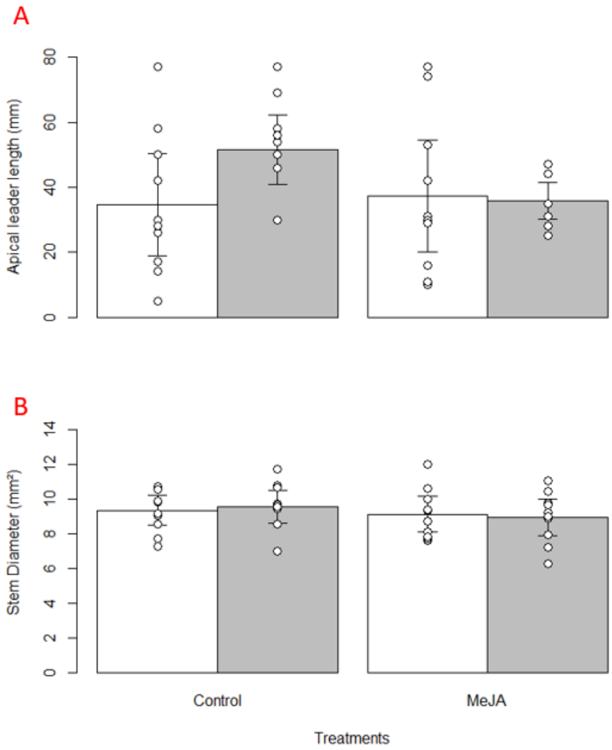

**Figure 4** Height and stem growth of 1-year-old Norway spruce seedlings after treatment with methyl jasmonate (MeJA) and subsequent exposure to drought. Seedlings were first sprayed with Tween (control) or methyl jasmonate (MeJA). Seven weeks later, seedlings were either subjected to drought (white bars) or received ample water (grey bars) over a 3-week period. A) Height growth was measured as the difference in tree height at the time of MeJA treatment and at the end of drought exposure. B) Stem growth was determined from stem cross-sections examined in a microscope. White circles show values for individual seedlings (n = 10). Error bars show 95% confidence intervals.

## 3.2 Belowground root biomass

For total root weight and total root length, seedlings treated with MeJA had significantly lower values than seedlings treated with Tween, but only for seedlings that received ample water. For seedlings that had been subjected to drought there was no significant difference between MeJA-treated and control seedlings (Figure 5 A-B). For root diameter, there was no significant effect of MeJA treatment, but drought-exposed seedlings had significantly thinner roots than fully watered seedlings (Figure 5 C).  Root volume was significantly smaller in MeJA-treated seedlings than in control seedlings. This was true for both fully watered seedlings and seedlings subjected to drought (Figure 5 D). For drought-exposed seedlings there was a significant negative effect of MeJA treatment on root volume, but for fully watered seedlings there was no significant effect.

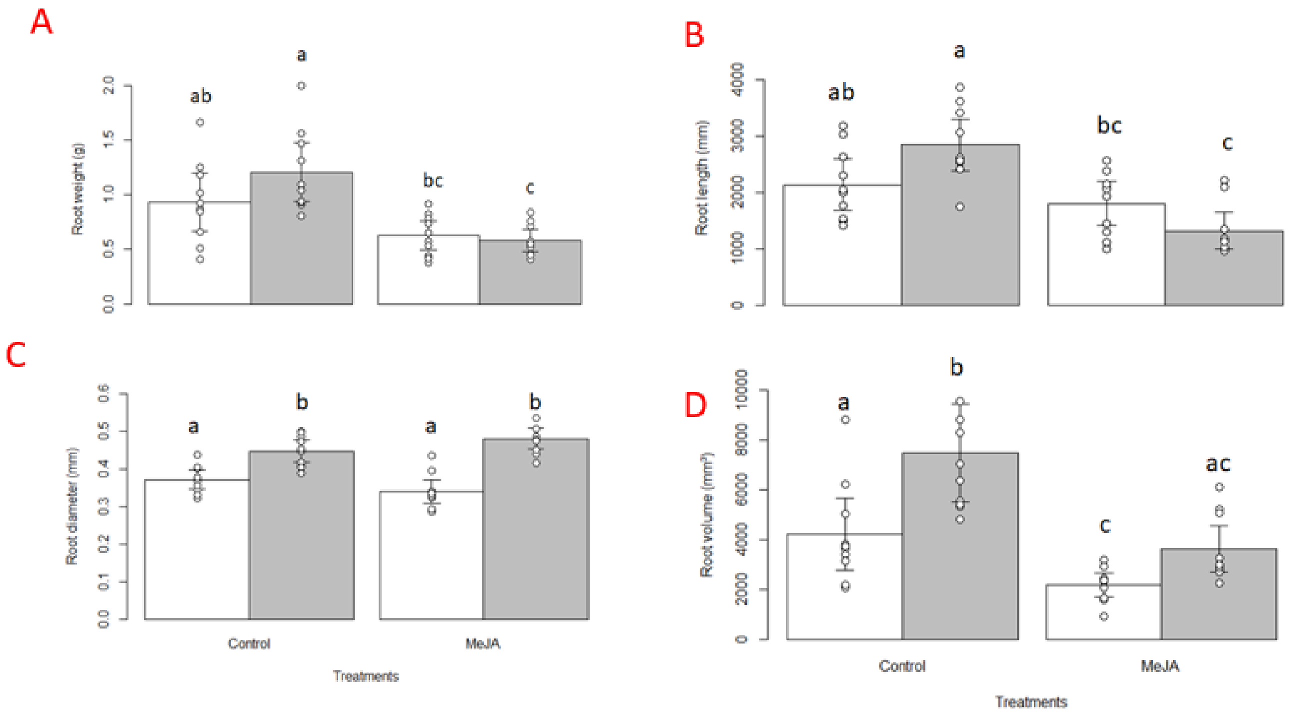

**Figure 5** Root weight, root length, root diameter and root volume of 1-year-old Norway spruce seedlings. Seedlings were first sprayed with Tween (Control) or methyl jasmonate (MeJA). Seven weeks later, seedlings were subjected to drought (white bars) or received ample water (grey bars) over a 3-week period. White circles show values for individual seedlings (n = 10). Error bars show 95% confidence intervals. A Kruskal-Wallis was used to test for differences between treatments in root weight ($\chi^2_3 = 19.80$, $p < 0.001$) (Figure 5 A) and root volume ($\chi^2_3 = 23.80$, $p < 0.0001$) (Figure 5 D). A 1-way ANOVA test was used to test for differences in root lengths ($F_3 = 12.30$, $p < 0.0001$) (Figure 5 B) and root diameters ($F_3 = 26.00$, $p < 0.0001$) (Figure 5 C). Treatments with the same letters are not significantly different.

Fully watered seedlings that had been sprayed with Tween had more roots in all diameter classes than seedlings subjected to all other treatment combinations, but for many comparisons the differences were not significant (Table 1).  Seedlings sprayed with Tween tended to have higher values than MeJA-treated seedlings, but the difference was significantly only for roots < 1 mm in diameter for fully watered seedlings and for roots > 2 mm in diameter for drought exposed seedlings (Table 1). The coarse roots (≥ 2.0 mm) seemed to be most negatively affected by MeJA treatments (Table 1 & Figure 6).

**Table 1:** Average root length for different diameter classes of 1-year-old Norway spruce seedlings. Seedlings were first sprayed with Tween (Control) or methyl jasmonate (MeJA). Seven weeks later, seedlings were subjected to drought or received ample water over a 3-week period. A Kruskal-Wallis test ($\chi^2$) or a 1-way ANOVA (F) was used to test for treatment differences. For each root diameter class treatments with the same letter are not significantly different.

| | **Root classes (mm)** | | | |
|---|---|---|---|---|
| **Treatment** | ≤ 0.5 | 0.5 - 1.0 | 1.0 - 2.0 | ≥ 2.0 |
| Control_watered | 2136.0 ± 119.7 a | 509.8 ± 14.3 b | 160.8 ± 11.2 c | 33.9 ± 5 a |
| Control_drought | 1765.3 ± 114.1 a | 278.3 ± 17 a | 73.0 ± 7.2 ab | 21.5 ± 3.6 a |
| MeJA_watered | 934.1 ± 78.1 b | 274.8 ± 16.9 a | 93.1 ± 6.5 bc | 13.9 ± 2 a |
| MeJA_drought | 1491.0 ± 106.0 ab | 274.6 ± 19.4 a | 36.0 ± 3.9 a | 1.4 ± 0.2 b |
| Statistics | $F_3 = 10.43$,<br>**$p < 0.00001$** | $\chi^2_3 = 20.24$,<br>**$p < 0.0001$** | $\chi^2_3 = 28.18$,<br>**$p < 0.00001$** | $\chi^2_3 = 25.95$,<br>**$p < 0.00001$** |

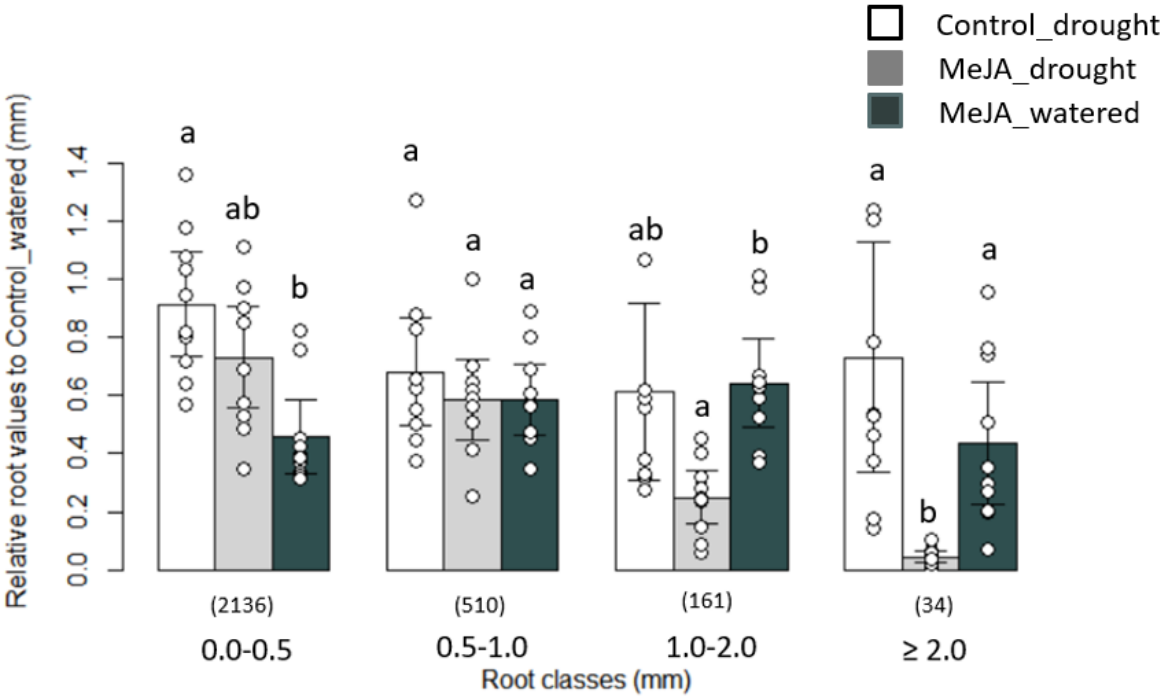

**Figure 6** Root diameter classes of 1-year-old Norway spruce seedlings. Seedlings were first sprayed with tween (Control) or methyl jasmonate (MeJA). Seven weeks later, seedlings were subjected to drought or received ample water over a 3-week period. For each diameter class the mean for fully watered control seedlings are set to 1.0 and the other treatments are expressed relative to this. The mean value of Control_watered is shown for each class in parentheses. A Kruskal-Wallis test ($\chi^2$) or a 1-way ANOVA (F) was used to test for treatment differences. White circles show values for individual seedlings (n = 10). Error bars show 95% confidence intervals. Treatments with the same letter are not significantly different for each diameter class.

There were no significant differences between treatments for specific root length (SRL) (Kruskal-Wallis test, $\chi^2_3 = 5.50$, p = 0.14) (Figure 7 A). However, for root tissue density (RTD) fully watered seedlings had significantly lower values than drought-exposed seedlings in both MeJA-treated and control seedlings, but there were no significant differences between spray treatments (Kruskal-Wallis test, $\chi^2_3 = 30.40$, $p < 0.000001$) (Figure 7 B).

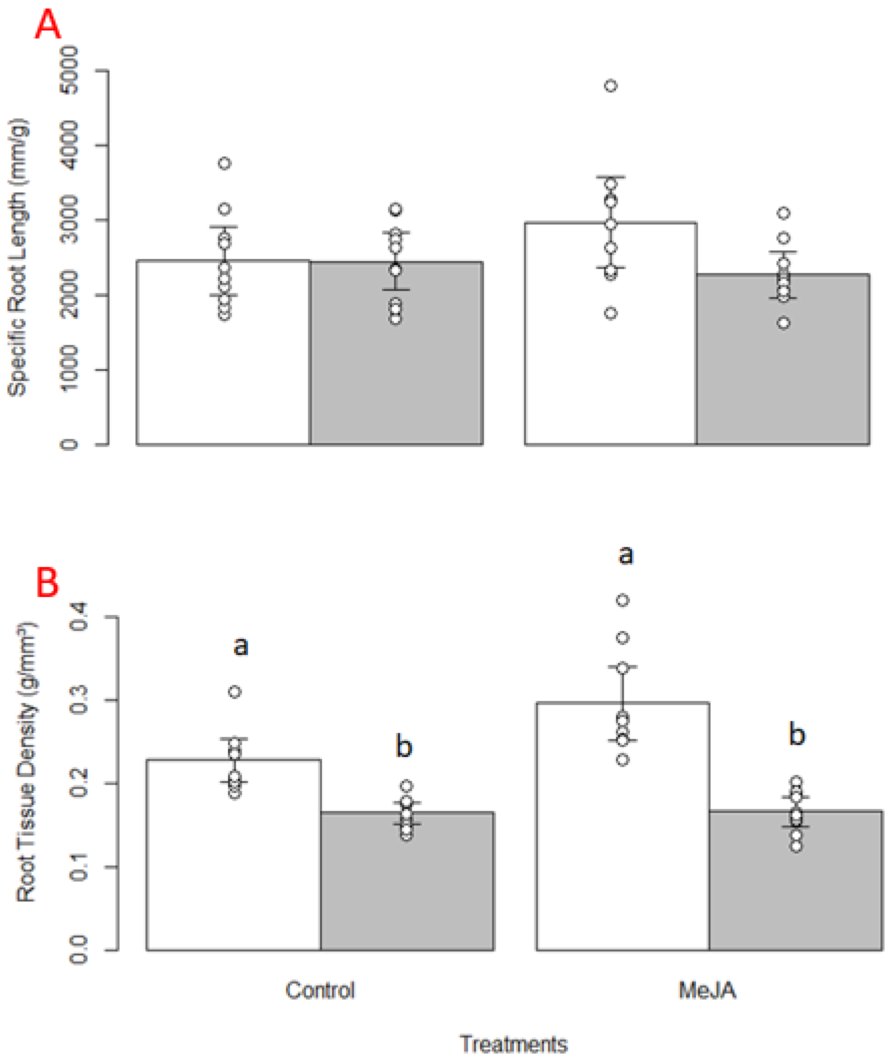

**Figure 7:** Specific root length (SRL) (A) and root tissue density (RTD) (B) of 1-year-old Norway spruce seedlings. Seedlings were first sprayed with Tween (Control) or MeJA. Seven weeks later, seedlings were subjected to drought (white bars) or received ample water (grey bars) over a 3-week period. SRL was calculated as total root length (in mm) divided by total root weight (in gram) for each seedling. RTD was calculated as total root weight (in gram) divided by total root volume (in mm$^3$) for each seedling. White circles show values for individual seedlings for (n = 10) and error bars show 95% confidence intervals. Treatments with the same letter are not significantly different (Kruskal-Wallis test followed by Dunns post-hoc test; SRL: χ23 = 5.50, p = 0.14, RTD: χ23 = 30.40, p < 0.000001.

## 3.3 Total traumatic resin ducts

The total area of traumatic resin ducts differed significantly between seedlings sprayed with MeJA and Tween, but there were no significant differences between seedlings exposed to drought and fully watered seedlings (Kruskal-Wallis test $\chi^2{}_3 = 31.3,$ p < 0.0001) (Figure 8)

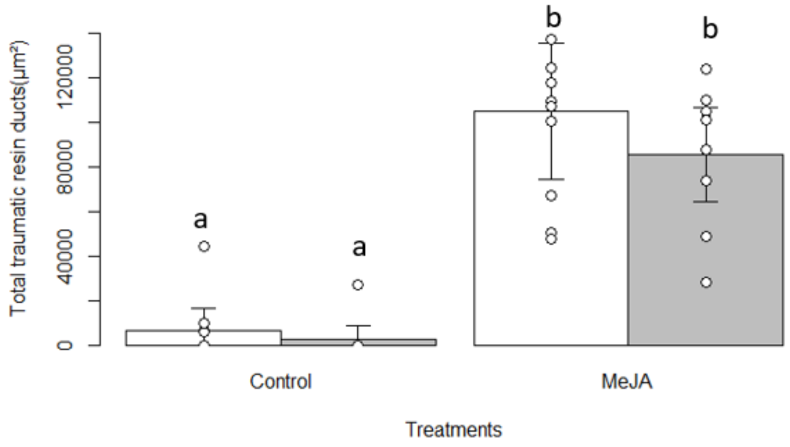

**Figure 8** Traumatic resin ducts in the sapwood of 1-year-old Norway spruce seedlings after treatment with methyl jasmonate (MeJA) and exposure to drought. Seedlings were first sprayed with Tween (Control) or MeJA. Seven weeks later, seedlings were subjected to drought (white bars) or received ample water (grey bars) over a 3-week period. Traumatic resin ducts in the current annual ring were measured on stem cross-sections using a microscope. White circles show values for individual seedlings (n = 10). Error bars show 95% confidence intervals. Treatments with the same letter are not significantly different (Kruskal-Wallis test followed by Dunns post-hoc test, $\chi^2_3 = 31.3$, $p < 0.0001$).

## 3.4 Inoculation with *Grosmannia penicillata*

In mock-inoculated seedlings there were no significant differences between any treatment combinations (Figure 9). Fully watered seedlings that had been treated with Tween had significantly longer lesions than mock-inoculated seedlings. MeJA-treated seedlings had significantly shorter lesions than fully watered control seedlings but did not differ significantly from mock-inoculated seedlings. This was true for both fully watered seedlings and seedlings subjected to drought (Figure 9). There was no significant effect of drought on necrosis length within control or MeJA-treated seedlings.

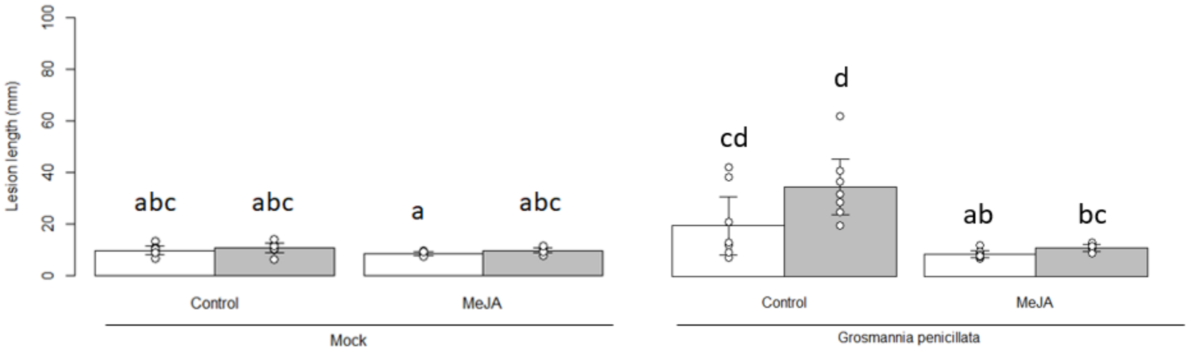

**Figure 9** Necrotic lesion lengths in the bark of 1-year-old Norway spruce seedlings. Seedlings were first sprayed with Tween (Control) or methyl jasmonate (MeJA). Seven weeks later, seedlings were subjected to drought (white bars) or received ample water (grey bars) over a 3-week period. After a 7-week recovery period, seedlings were either mock inoculated with sterile media as a control or inoculated with the bluestain fungus *Grosmannia penicillata*. White circles show values for individual seedlings for (n = 8). Error bars show 95% confidence intervals. A Kruskal-Wallis test was used to test for differences between treatments ($\chi^2_7 = 37.70$, $p < 0.001$). Treatments with the same letter are not significantly different.

# 4. Discussion

In our study, Norway spruce seedlings were subjected to four different treatment combinations: treatment with MeJA or Tween and then exposure to drought, or treatment with MeJA or Tween and then ample watering. We found that MeJA treatment affected negatively belowground root biomass by reducing the total root volume and the total length of roots in different diameter classes. Drought-exposed seedlings treated with MeJA or Tween had lower root biomass than fully watered seedlings receiving the same treatments. However, we found no significant treatment differences in aboveground biomass such as stem growth and height. Prior to this study, it was known that MeJA caused some negative effects on Norway spruce, such as reduction of aboveground and belowground biomass. Also, it was known that MeJA treatment effectively reduced necrotic lesions caused by the bluestain fungus *Grosmannia penicillata*. Drought has the potential to either stress seedlings to the point where they become more vulnerable to attacks from pathogens and fungi, or to enhance their resistance. The combined effects of defense priming with MeJA-treatment and drought stress have not been addressed in previous studies. As it is important to understand the effects of defense priming in stressful environmental conditions, we chose to investigate this further.

## 4.1 Effects of MeJA and drought on above-and belowground growth

We found no significant differences between any four of the treatment combinations on the height or stem growth in Norway spruce, although the seedlings treated with MeJA in our study had a 15% reduction in height compared to Tween-treated seedlings. This differs from other studies that have shown a negative effect of MeJA on aboveground growth in Norway spruce (Fedderwitz et al. 2016; Zas et al. 2014). These authors found that MeJA-treated spruce seedlings grew significantly less than control seedlings. The reason for the differences between these previous studies and our results may be that Fedderwitz et al. (2016) used 3-year-old rooted cuttings from six spruce clones while we used seedlings from a plant nursery with diverse genotypes. Additionally, they treated their plants with a concentration of MeJA that was five times higher than our study (50 mM vs 10 mM). Previous studies observed that higher dosages of MeJA leads to several negative impacts such as reduced growth in both aboveground and belowground biomass for Norway spruce seedlings (Fedderwitz et al. 2016; Zas et al. 2014; Moreira et al. 2012 B).

Zas et al. (2014) did not find any significant differences in plant growth between MeJA and control treated Norway spruce seedlings for the lowest MeJA concentrations they tested (5 and 10 mM). These results agree with the results of our study. However, Zas et al. (2014) observed that a higher MeJA concentration of 25 mM had negative impacts on the seedlings, such as a decreasing plant height and stem growth. Other conifer species such as Maritime pine (*Pinus pinaster*), Monterrey pine (*Pinus radiata*), and Scots pine (*Pinus sylvestris*) included in their experiment showed a negative effect of MeJA treatment on height growth across all concentrations. This indicates that Norway spruce is more tolerant to high levels of MeJA than other tree species. In their study, Norway spruce seedlings experienced a 10% reduction in height growth when 25 mM of MeJA was applied, but growth recovered the next year. Untreated seedlings, however, faced a higher growth loss because they were more susceptible to attacks from insects and pathogens. When the seedlings were placed in the field 17 months after MeJA treatment, there were no significant differences in stem growth and stem height between control seedlings and seedlings treated with MeJA. This suggests that with time, seedlings can make a full recovery from the negative effects of MeJA.

Seedlings exposed to drought may have limited photosynthesis activity and growth as discussed by Turtola et al. (2003). It is possible that the drought stress in our study was not severe enough to see clear effects on height and stem growth. In future studies, the drought exposure should perhaps be more intense, up to the point where the foliage becomes brown, and needles start to fall off for drought to act as a stressor. Often severe drought is not realistic under field conditions, mild drought is more likely to occur and may increase defense compounds (Christiansen and Glosli (1996)). The combination of MeJA-treatment and mild drought-exposure in this experiment may have resulted in the induction of higher chemical defenses than treatment of seedlings with only one of these two.

Some of the seedlings may have been harmed as a result of the overheating that happened in the greenhouse a few days after MeJA application. Because of this technical failure we postponed the drought experiment to avoid overstressing the seedlings. We predicted that the seedlings that were treated with MeJA would become more discolored and have higher mortality than control seedlings. based on a previous study on Norway spruce trees treated with MeJA by Mageroy et al. (2019). However, nine control seedlings died after the incident, but only three MeJA-treated seedlings died. The outcome of our experiment might have been different if this incident did not happen since there would have been less time between MeJA application and drought exposure in the seedlings. This might then have affected aboveground growth more, because the seedlings would not have had as much time to recover from the MeJA treatment before they were subjected to drought.

Root weight was not significantly higher in drought-exposed seedlings that had been treated with Tween than in seedlings treated with MeJA (Figure 5 A). Moreira et al. (2012 B) studied the effects of MeJA on roots in young conifers and found that MeJA reduced the overall root biomass of treated seedlings. However, in our study only root volume which indicates the thickness of the roots was significantly reduced. A possible reason why we did not see a reduction in root weight was that we used a lower concentration than Moreira et al. (2012 B) (10 mM vs. 22 mM). Heijari et al. (2005) had similar results to our study with Scots pine (*Pinus sylvestris*). In their study root weight was not affected by application of 10 mM MeJA but was significantly reduced by 100 mM. These results suggest that root growth is negatively correlated to MeJA concentration. We found no significant difference in root weight for drought-exposed seedlings in the two application treatments. These results coincide with the study done by Möttönen et al. (2001) who dried Norway spruce root materials in a forced-air oven at 40 °C and found no significant differences in root weight in fully watered compared to drought-exposed seedlings. However, in this study, watered seedlings treated with Tween had significantly higher root weight than water seedlings treated with MeJA, suggesting that MeJA affects root biomass more than drought does.

Root length was only significantly higher in watered control seedlings against MeJA-treated seedlings (Figure 5 B), while there was not any difference between drought-exposed control seedlings and drought-exposed MeJA-treated seedlings. These results differ from the results in the study conducted by Heijari et al. (2005) who found no differences in root lengths for Scots pine in both 10 mM MeJA and 100 mM MeJA. Gaul et al. (2008) found no difference in fine root biomass (< 2.0 mm root diameter) and root length in Norway spruce that was subjected to drought. No studies were found that showed the combined effects of MeJA-treatment and drought-exposure on Norway spruce seedling root length.

There were no significant differences in root diameters between drought-exposed seedlings sprayed with MeJA or Tween. Seedlings that received sufficient water had significantly larger root diameters than drought exposed seedlings (Figure 5 C). Rötzer et al. (2017) and Wasaya et al. (2018) showed that root diameters were reduced in drought-exposed Norway spruce trees. However, in their experiment, the drought lasted over several periods, and several years of drought was sometimes required to see a significant difference. In our experiment, the drought experiment only lasted a few weeks and therefore the drought was probably not severe enough to lead to significant differences in root diameters.

Root volume was significantly higher in drought-exposed seedlings sprayed with Tween than in corresponding MeJA-treated seedlings. This is also supported in the study by Moreira et al. (2012 B). Overall, seedlings sprayed with Tween had significantly larger root volumes in both drought-exposed and fully watered seedlings. Wasaya et al. (2018) showed that root diameters were positively correlated with root volumes, with higher diameters resulting in larger total volumes. This was also the case in our seedlings (Figure 5 C & D). Blödner et al. (2007) found significant changes in the roots of Norway spruce only after around four weeks of drought as the trees in their study received no water at all for those four weeks, while in our study, the drought-exposed seedlings received water once a week to avoid overstressing the seedlings.  Rötzer et al. (2017) suggested that root volume would be affected negatively by combined drought and MeJA treatment, since roots are negatively affected by MeJA. This results to the reduced roots having less uptake of nutrients and water in the long term. In our study it seems like the combined effects of MeJA treatment and drought-exposure on Norway spruce seedlings reduced the total root volume.

There were several significant differences in the abundance of both fine roots (< 2 mm) and coarse roots (≥ 2) between MeJA-treated and control seedlings. The trend was that MeJA-treated seedlings overall had fewer fine roots than Tween-treated ones in the categories (0 – 0.5, 0.5 – 1.0 and 1.0 – 2.0 mm) (Figure 6 and Table 1). Moreira et al. (2012 B) showed that fine roots increased, while the number of coarse roots decreased when pine trees were exposed to MeJA. The increase in fine roots in their study increased water and nutrient acquisition. This in turn, affected the ability of above-ground regrowth. In this study, the aboveground biomass was not significantly reduced by MeJA-treatment and drought-exposure. However, the belowground biomass was significantly reduced in drought-exposed seedlings treated with MeJA.

A possible explanation to the different results was that in the study conducted by Moreira et al. (2012 B), cloned trees from rooted cuttings were used. Their trees would respond similarly to MeJA treatments while the seedlings used in our study were bought from a nursery with diverse genotypes, where each seedling may respond differently to the different treatment and drought exposure. Another explanation as to why the results were more significant in the study conducted by Moreira et al. (2012 B), was that the concentration of MeJA was twice as much as in our study (22 mM vs. 10 mM). Gaul et al. (2008) experimented with drought in Norway spruce, and saw a 61% decline in fine roots after subjection to drought, but that during this period the production of fine roots increased to compensate for the incoming loss due to drought exposure. However, since the drought exposure in our study was not severe enough, the fine roots that were treated with MeJA and exposed to drought were not significantly different from Tween-treated seedlings.

We found a significant difference for drought exposed MeJA-treated seedlings against control seedlings for the root diameter class ≥ 2.0 mm. This resulted in the MeJA-treated seedlings having significantly less root biomass (Table 1) and correlated with the study conducted by Moreira et al. (2012 B), who found out that MeJA significantly reduced the number of coarse roots in pine trees, however, the trees compensated by boosting the numbers of fine roots as previously mentioned. Rötzer et al. (2017) also found out that heavy drought might be positively correlated with fine roots in Norway spruce, as the trees would allocate more resources to these roots over coarse roots. This means that seedlings that are exposed to drought would have a significantly higher number of fine roots than coarse roots for watered seedlings to compensate for the shortage of water.

Specific root length (SRL) (mm/g) is linked to fine roots (< 2.0 mm) and their uptake of resources and is negatively affected by stress and fertilization (Rose 2017; Ostonen et al. 2007). Since MeJA reduced the root biomass in our study, we predicted that the combination of MeJA application and drought-exposure would increase and lead to lower SRL values (Figure 7 A). However, there were no significant differences in SRL between seedlings receiving a single treatment and those receiving combined treatments. An explanation to this might be that our seedlings were only exposed to mild drought, which did not cause a high enough stress to impact SRL.

Root tissue density (g/mm$^3$) (RTD) is positively correlated with root life span and negatively correlated with root length (Wasaya et al. 2018). Higher RTD values indicate thicker roots, which in turn may increase root pathogen resistance and boost the growth of the seedlings (Kramer et al. 2016; Wasaya et al. 2018). RTD was predicted to increase in seedlings that were exposed to drought, which coincides with the results of this study. Drought exposed MeJA-treated seedlings and drought exposed Tween-treated seedlings had significantly higher RTD-values than watered seedlings but were not significantly different from each other

(Figure 7 B). This result indicates that MeJA application does not influence RTD, which is the opposite of what was predicted.

## 4.2 Traumatic resin ducts in Norway spruce seedlings

TRDs are positively correlated with tree resistance and defense as they provide secondary resin to areas of the trees that are exposed to pathogens (Christiansen et al. 1999). The amount of TRDs were significantly higher for MeJA-treated seedlings than Tween-treated seedlings in our study. Also, the necrotic lesions caused by *G. penicillata* were significantly less at higher TRD amounts (Figure 8 & Figure 9). Zeneli et al. (2006) and Franceschi et al. (2002) also observed that TRDs helped their seedlings by boosting their defenses against pathogens and insects. TRDs were found to be the main terpenes in Norway spruce trees that were treated with MeJA, and they increased after MeJA application (Schmidt et al. 2011).

There was no significant difference in the amount of TRDs between watered seedlings and seedlings exposed to drought in our study. However, there was a trend towards drought-treated seedlings having more TRDs than seedlings that received ample water. These results were unexpected, as previous studies have shown that conifers that were exposed to limiting factors such as water shortage would produce less TRDs (Ferrenberg et al. 2015; Gaylord et al. 2013). However, other studies suggest that drought may prime seedlings in the same way as pathogens and insects, and thus actually increase the total number of traumatic resin ducts (Turtola et al. 2003; Mauch et al. 2017). The increase in TRDs would increase the defense in the seedlings as they contain antiherbivory compounds as previously mentioned. In the future, a more severe drought should be experimented with, in order to study the response of seedlings and whether the amount of TRDs change or not based on drought-exposure and MeJA application.

## 4.3 Inoculation with *Grosmannia penicillata*

MeJA-treated seedlings had significantly shorter necrotic lesions by *G. penicillata* than Tween-treated seedlings (Figure 9). This result corresponds with previous studies regarding MeJA application and the reduction of fungi infection and insect attacks (Franceschi et al. 2002; Krokene et al. 2008; Zas et al. 2014). There were no significant differences in necrotic lesions between drought-exposed control seedlings and control seedlings that received ample water, although there was a trend in watered control seedlings slightly longer necrotic lesions than drought-exposed control seedlings. We hypothesized that drought-exposed, control seedlings would be more susceptible to infection since drought is a known stressor that can expose plants to pathogens, fungi and insects (Turtola et al. 2003). However, Madmony et al. (2018) showed that cloned Norway spruce trees that were drought-exposed and infected with the pathogen *Heterobasidion parviporum*, did not have less TRDs and were not more susceptible to attacks from the fungus. Barradas et al. (2018) studied the effects of drought on fungal infection in Eucalyptus species. Similarly, their results showed that seedlings exposed to a mild, abiotic stress, such as drought, had a boost in resistance against fungi infection. Christiansen & Glosli (1996) studied mild drought in Norway spruce trees against bluestain fungi and found out that mild drought enhanced the resistance of their trees, while the control trees used in their study became more susceptible to attacks from bark beetles than the mildly drought-exposed trees. Bruce et al. (2007) suggested that plants such as *Arabidopsis thaliana* that are exposed to a stressor, form "stress memory" which primes plant defenses for several days after exposure. The term stress memory applies to Norway spruce trees as well, although the stress memory in Norway spruce trees may last from weeks to several months (Mageroy et al. 2019; Mageroy et al. 2020). It is likely that drought may have acted as a priming agent in our study, boosting the defenses of the seedlings, rather than exposing them further to attacks from *G. penicillata*.

The necrotic lesions caused by *G. penicillata* in control plants were slightly longer in control-watered seedlings than drought-stressed control seedlings as mentioned previously, while the number of TRDs in the seedlings from these two treatments were the same. This indicates that other induced defenses than terpenes, such as defensive enzymes or stilbenes and polyphenolic polymers increase tree resistance to fungi and insects (Hammerbacher et al. 2013; Hammerbacher et al. 2014) and have a significant role in resistance to *G. penicillata* infection. However, this study did not quantify phenolics or analyze the expression of defense proteins in response to *G. penicillata* infection and will therefore not be focused on.

The effects of *G. penicillata* are often detrimental to Norway spruce trees as they cause discoloration and necrotic lesions in the seedlings (Repe et al. 2013). In our study, MeJA application on Norway spruce seedlings reduced the necrotic lesions significantly against Tween-treated seedlings. MeJA treatment did prevent growth of *G. penicillata*, because the lesions were due to the wounding of the seedlings, and not necrotic lesions caused by *G. penicillata*. The lesion lengths in MeJA-treated seedlings were not different from mock-inoculated seedlings, which indicates that the effects of MeJA application on *G. penicillata* are quite efficient.

Spruce bark beetles often carry more than only one type of fungi as discussed by Jankowiak et al. (2009) and Solheim (1992), who found several associates of fungi in the ophiostomatoid group. These may have different resistances to MeJA application for example, as some of the fungi attack trees in later stages of fungal succession. However, this study only conducted research on *G. penicillata* as it is one of the most common fungal species, which lives in a mutualistic relationship with spruce bark beetles. Although this study is quite artificial, as *G. penicillata* infects Norway spruce via being transported by bark beetles that attack adult trees, the results still show that MeJA application is a valuable resource against fungi infestation.

Against *G. penicillata,* based on previous studies and our study, we would recommend MeJA application in small dosages around 10 mM mixed with 0.1% Tween, as higher dosages of MeJA application may cause severe harm in both aboveground and belowground biomass in Norway spruce seedlings. We also recommended that the Norway spruce seedlings should not be exposed to heavy drought as they are not a very drought tolerant species. In mild drought, which was the case in this study, the Norway spruce seedlings were not negatively affected by the drought, on the contrary, the mild drought may have contributed to boost their defenses as there was a trend towards the drought-exposed, MeJA-treated seedlings having smaller necrotic lesions caused by *G. penicillata* than the seedlings that received sufficient water. Based on previous studies, severe drought or high concentrations of MeJA on Norway spruce trees may be detrimental as they might reduce growth, water uptake and may lead to nutrient deficiency, as well as increased susceptibility to pathogens, fungi and insects. These factors may lead to economic losses for the timber industry. However, in our study, we did not find any reduced resistance to fungal infection in Norway spruce seedlings when they are exposed to mild drought and 10 mM of MeJA.

# 5. Conclusion

Norway spruce is a dominant species in Europe with a high level of ecological and economic importance. Due to Norway spruce being affected by both abiotic factors such as drought, and biotic factors for instance insects and fungi, it is vital for us to understand how Norway spruce can cope and adapt to these environmental and biotic challenges. This study looked at the short-term effects of MeJA application and drought in 1-year-old Norway spruce seedlings. MeJA treatment reduced the belowground biomass measured by root length, root volume and root weight. We predicted that the aboveground biomass measured by stem growth and height would also be negatively impacted, however, this was not shown in the results.

The application of MeJA increases the total number of traumatic resin ducts in seedlings, indicating the activation of induced defenses. The MeJA-dosage of 10 mM did not affect as many indicators of biomass as we predicted. There was an overall reduction in both fine roots (< 2.0 mm) and coarse roots (≥ 2.0 mm) when seedlings were treated with MeJA, however, only the coarse roots were significantly reduced. MeJA-treated seedlings had reduced necrotic lesions caused by *Grosmannia penicillata.* However, contrary to our predictions, exposure to drought did not weaken the seedlings defenses and did not increase the necrotic lesions by *G. penicillata*.

MeJA application may be an important asset in forestry, but further studies should be conducted on Norway spruce seedlings to ensure as few negative responses as possible. Further research on the effects of MeJA application and drought exposure in Norway spruce seedlings against *G. penicillata* and its vector, the European spruce bark beetle (*Ips typographus*) is needed as we did not study the combined effects of these biotic factors. A problem that could arise as a result of the reduced root growth caused by MeJA application is less uptake of important nutrients and water, which might in turn harm the seedlings depending on the MeJA-application and how long it lasts. The mortality of seedlings may increase without MeJA-application for Norway spruce trees, so we recommend to loggers that would like to minimize their losses to spray lower concentrations of MeJA, around 10 mM, as the seedlings are more able to withstand these concentrations. Future studies should use a similar research design as ours but should compare seedlings that are exposed to excessive water (flooding) against control seedlings to see if the flooding may prime the seedlings, or they would be stressed and more vulnerable to pathogens and insects.

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