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Inoculation with industrialized mycorrhiza powder in a forest plant nursery with standard production methods.

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Forest Sciences

Preface

This thesis marks the end of my journey as a Forest science student at the Faculty of Environmental Sciences and Natural Resource Management (MINA) at the Norwegian University of Life Sciences (NMBU). The thesis has been brewing since the second year of my bachelor's studies and revolves around whether mycorrhizal inoculation, in the forest nursery Sólskógar, could be integrated into the standard production.

First and foremost, I'd like to express my gratitude to my mentors, Professor Line Nybakken (NMBU), Professor Isabella Børja (NIBIO/NMBU), and Senior Research Scientist Inger S. Fløistad (NIBIO). Without your help, vast expertise, and persistence, this project might not have been completed. Furthermore, I would like to thank Skógræktin, the Icelandic Forest Service, for access and assistance in their laboratory in Mógilsá. Additionally, I am grateful for unwavering support from the forest nursery Sólskógar and their aid in providing plant material, use of equipment, and all-around support and advice.

Finally, I would like to thank my fellow students through the studies, both in Ås and in Göttingen. The last five years have been full of exciting adventures and unforgettable memories. This thesis is both the perfect end to that chapter as well as the start of a brand new one.

Yfir dalinn augun líða
aldnir stofnar blasa við,
klæðist skógi foldin fríða
fegrar sund og klettarið.
Ungplantan í örmum hlíða
á hér stærstan sess og grið

Ungplantan from Úr viðjum vitundar.
Author: Sigmundur Benediktsson

Akureyri, 15th of July, 2023

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Abstract

Icelandic afforestation is almost completely limited to planting of seedlings. On a national scale, the overall survival rate for seedlings in the field is below 50%. An understudied possibility to increase survival in the field is inoculation of mycorrhiza in the forest nursery, but inoculation is presently not a part of the standard production. The aim of this study was to investigate if incorporating mycorrhizal inoculation into the standard nursery production was possible by testing how three factors — inoculation, fertilization, and the use of fungicide — affected mycorrhizal colonization, growth, and root morphology of *Larix sibirica* seedlings.

Seedlings of *Larix sibirica* (N = 1608) were inoculated with an industrialized mycorrhizal soluble at two different time points: germination and visible fine root production. Eleven and eight weeks after inoculation treatments, seedling growth was measured and mycorrhization visually estimated. At the same time, the root traits of inoculated vs. uninoculated seedlings were examined with a WinRHIZO scanner. Individual as well as combined effects of the treatments were examined.

Results showed that inoculated seedlings increased their mycorrhization, while fertilization decreased mycorrhization. Inoculated seedlings had a lower height and stem diameter compared to untreated ones. Low fertilization decreased height and stem diameter, while there was little difference between medium and high fertilization. As multicollinearity occurred between fertilization and fungicide in a regression analysis, the effects of fungicide on mycorrhization as well as height could not be interpreted. Combined effects showed that repeated inoculation and medium fertilization gave the highest mycorrhization. Inoculation did not significantly affect root traits. However, root traits, such as the number of root tips, indicated that mycorrhization had been successful.

This study contributes to an increased knowledge about the possibility of incorporating inoculation of mycorrhiza into the standard nursery production of seedlings. Further studies are needed to address remaining questions about the relationship between repeated inoculation and fertilization levels.

Sammendrag

Skogreising på Island står nesten kun av planting av planter fra planteskoler. På landsbasis ligger gjennomsnittlig overlevelse i felt på under 50%. Muligheten for å øke overlevelsen ved å inokulere plantene med mykorrhiza sopp under produksjonen er en lite studert mulighet. I dag er inokulering ikke del av standardisert skogplanteproduksjonen i Island. Formålet med denne oppgaven er å se om inokulering kan innlemmes i standardisert produksjon. Påvirkning av tre behandlinger – inokulering, gjødsling og bruk av fungicider – på mykorrhisering, tilvekst og rot formasjon ble undersøkt. iada

Lerkeplanter (*Larix sibirica*) (*N* = 1608) ble inokulert på to ulike tidspunkter med et industrielt mykorrhiza-pulver. Elleve og åtte uker etter inokulering ble vekst til plantene målt, samt mykorriserings grad vurdert visuelt. Dessuten ble rotegenskaper hos inokulerte og ikke inokulerte planter undersøkt i WinRHIZO rot skanner. Individuelle og kombinerte effekter av behandleringer ble undersøkt.

Inokulering økte mykorrhisering i plantene, mens gjødsling reduserte mykorrhisering. Inokulerte planter hadde lavere høyde og rothalsdiameter sammenlignede med ikke inokulerte planter. Lav gjødsling mengde reduserte også høyde og rothalsdiameter, mens det var lite forskjell i vekst mellom middels og høy gjødslingsmengde. Dersom multikollinearitet oppstå mellom gjødsling og fungicider, ble effekten av fungicide på både mykorrhisering som vekst umulig å tolke. Kombinerte effekter av gjentatt inokulering og middels gjødslingsmengde ga høyest mykorrhiserings-grad hos plantene. Inokulering hadde ikke en signifikant effekt på root-egenskaper, derimot indikerte resultatene fra WinRHIZO røtte-skanneren, e.g., antall rot spisser, at inokulering hadde lykkes.

Denne oppgaven viste å det er mulig å inkorporere mykorrishering til standardisert produksjon av skogplanter i planteskoler. Dersom mykorrhisering var generelt lav trengs videre forskning på ulike kombinasjoner av faktorer som påvirker inokulerings graden.

Ágrip

Nýskógrækt á Íslandi er að meira eða minna leiti háð útplöntun af bakkaplöntum. Á landsvísu er lifun smáplatna í felti að jafnaði undir 50%. Lítið rannsakaður möguleiki til að auka lifun í felti er að smita bakkaplöntur með svepprót í gróðrastöðum. Hingað til hefur slík smitun ekki verið hluti af hefðbundinni skógarplöntuframleiðslu. Markmið þessarar rannsóknar var að kanna hvort mögulegt sé að innleiða smitun með svepprótum í hefðbundna skógarplöntuframleiðslu. Áhrif þriggja breytna: smitunar, áburðargjafar og noktun sveppaeiturs á myndun svepprótar, yfirborðs vöxt og uppbyggingu róta voru rannsökuð.

1608 lerki plöntur (*Larix sibirica*) voru smitaðar með fjöldaframleiddu svepprótadufti á tveimur mismunandi tímasetningum, við spírun og við myndun fínróta. Ellefu og áttu vikum eftir smitun var vöxtur platnanna mældur og myndun sveppróta metin sjónrænt. Að auki var uppbygging róta smitaðara og ósmitaðara plantna borin saman í WinRHIZO rótarskanna. Stakstæð jafnt sem sameinuð áhrif af hverri breytu voru rannsökuð.

Niðurstöður sýndu að smitun með svepprótardufti jók svepprótamyndun á meðan aukin áburðagjöf minnkaði svepprótamyndun. Einnig dró smitun úr hæðar og breiddar vexti platnanna. Lár áburðastyrkur dró einnig úr hæðar og breiddar vexti, en lítill munur var á vexti plantna sem fengu meðal eða háan áburðastyrk. Þar sem fjölfylgni fannst milli áburðargjafar og notkun sveppaeiturs í aðhvarfsgreiningu var ekki hægt að túlka niðurstöður noktunar sveppeiturs með vissu. Sameinuð áhrif breytna sýndu að svepprótasmit var mest þegar smitun var endurtekin og áburðargjöf var há. Áhrif smitunar á uppbyggingu róta reyndust ekki tölfræðilega markæk. Samt sem áður sáust vísbendingar um að smitun hefði heppnast við skoðun róta í WinRHIZO skannanum.

Þessi rannsókn leggur grunninn að mögulegri innleiðingu svepprótasmitunar í hefðbundninni skógarplöntuframleiðslu. Frekar rannsóknir eru nauðsynlegar til að skoða til hlítar fleiri breytur í framleiðsluferlinu sem hafa áhrif á myndun svepprótar. Meðal annars er mikilvægt að rannsaka áfram áhrif samspils endurtekinnar smitunnar og styrks áburðargjafar á svepprótamyndun.

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1 Introduction

According to the Icelandic definition of a forest, the forest cover in Iceland is 2% (Hausner, 2021). However, according to the FAO forest definition, the forest cover in Iceland is 0.6%, where plantations and natural forests consist of 0.5% and 0.1%, respectively (Umhverfis- og auðlindaráðuneytið, 2022). Forest cover prior to human settlement has been estimated to be between 25 and 40% of the total land area, with downy birch (*Betula pubescens* Ehrh.) being the dominant species (Oddsdottir & Snorrason, 2017). Deforestation (Umhverfis-og auðlindaráðuneytið, 2022) along with overgrazing by livestock (Oddsdottir, 2010) drove and maintained the forest's disappearance. Forest regeneration generally occurs in one of two ways: natural regeneration, by e.g., seed- or retention trees, or planting, where nursery produced seedlings are planted (Holgén & Hånell, 2000). Planting is the dominant regeneration method in Iceland (Elsner, 2022) due to the low forest cover. In Northern- and Eastern-Iceland, the most common species planted is Siberian larch (*Larix sibirica* Ledeb.) followed by lodgepole pine (*Pinus contorta* Bol.). In Southern- and Western-Iceland, lodgepole pine is the most common species planted, followed by Sitka spruce (*Picea sitchensis* Bong.) (Benjamínsdóttir, 2023b). A summary from 2007 showed the average seedling survival rate to be 47% for the whole country (Oddsdottir, 2010), with Eastern- and Northern-Iceland averaging around 70% survival (Halldórsson, 2014; Reynisson, 2007). Seedling mortality is highest during the first three years after planting and is usually low to non-existent five years after planting (Benjamínsdóttir, 2023b). Newly planted areas are open, barren landscapes where protection for seedlings is limited. Abiotic factors have a significant impact on seedlings growing in places with extreme weather, such as drought or frost (Tingstad et al., 2015). In addition to weather, soil conditions have a significant impact on seedling survival (Martini et al., 2020), with survival being low in drained wetlands and soils with high concentrations of sands (Oddsdottir, 2010). To increase survival in the field, methods such as soil scarification (Kristjánsson, 2020) and fertilization (Óskarsson et al., 2006) when planting have shown good result. A little-studied possibility for increased survival is mycorrhizal inoculation in nurseries. However, studies performed in Iceland have shown that seedling inoculation increases survival in the field (Böðvarsdóttir, 2018; Óskarsson & Sigurgeirsson, 2001; Oskarsson, 2010).

Most land-living plants are associated with mycorrhizae in one way or another (Traveset & Richardson, 2014). Mycorrhiza is defined as the symbiotic association between a plant and a fungus, in which the fungus absorbs water and nutrients in exchange for the plant's photosynthesis-derived sugars (Brearley, 2012). The association increases surface area for

water and nutrient uptake, which can reduce stress related to resource deficiency (Perry et al., 2008). Additionally, mycorrhizal association has shown other advantageous properties, such as fine root protection against pathogens (Liu et al., 2020) and resistance against salinity (Onwuchekwa et al., 2014). When mycorrhiza colonizes roots, the root structure can change, with roots becoming shorter and wider (Brundrett, 2002). Based on their growth within the roots, mycorrhizae can be categorized into two basic groups: ectomycorrhiza (ECM), where the fungal hyphae do not penetrate the cells, and endomycorrhiza, where the hyphae do penetrate the cells. The ECM is known for its characteristic Hartig-net that forms when fungal hyphae grow between the root cortex cells (Brundrett, 2002; Smith & Read, 2010). In boreal forests ECM is the dominant type of mycorrhiza, together with ericoid mycorrhiza associated with understory vegetation (Dahlberg, 2002). Around 20,000 ECM species can form symbiosis with trees (Martin et al., 2016). Most ECM species are found in old-growth forests and are believed to be an important inoculation source for saplings (Hagerman et al., 1999).

In 2022, approximately 7 million seedlings were produced in Iceland, in two forest nurseries, Sólskógar and Kvistabær. The Icelandic forest nursery production consists of five tree genera: *Betula, Pinus, Larix, Picea,* and *Populus*, each with several species or subspecies (Ásgrímsdóttir, 2022; Benjamínsdóttir, 2022). In Sólskógar, *P. contorta* and *B. pubescens* account for 40% of the production each. During the standardized production in Sólskógar, seedlings germinate in heated greenhouses, where they grow for three to six weeks, followed by five weeks to a year outside, depending on the species and the multipot container size. Seedlings are produced in three different multipot container sizes, with 24, 40, and 67 cells in each container, with a volume of 150, 93, and 50 cm^3 per cell, respectively. With three sowings each season, species with the longest growing period are sown first, e.g., *Picea*. Thereafter, other species are sown in medium to large-sized multipot containers, e.g., fp-40 *Pinus*. Lastly, species that require a shorter growing period in small multipot containers are produced, e.g., *Betula* and *Larix*. During each sowing, a 2000 m^2 greenhouse is filled, where the seedlings from the former sowing are either moved to smaller greenhouses or to the outside area (Ásgrímsdóttir, 2022). In order to deliver seedlings, requirements for minimum height and stem diameter must be met (Benjamínsdóttir, 2023b). To control seedling growth, factors such as watering, fertilization, and shading are used. As the application of these factors is weather-dependent, they are therefore the driving force behind variation in the production.

Mycorrhizal inoculation is not a part of the standard production practice in Iceland. However, sparse, natural inoculation with ECM fungi has been observed in seedlings during production

in Sólskógar (Ásgrímsdóttir, 2022) as well as in forest nurseries in Norway (Benjamínsdóttir, 2023a). This is often noticeable in nurseries located in or near forests, where spores from the surroundings are dispersed by wind (Selosse et al., 2000). To be able to inoculate whole parties in nurseries, both the production methods as well as the inoculation material must be considered, as the success of inoculation is affected by both (Oskarsson, 2010). Fertilization is perhaps the most important factor in nursery production when it comes to successful inoculation. High fertilization is generally correlated with a low mycorrhization level (Quoreshi & Timmer, 2000). Repetition of inoculation is another factor that could affect inoculation success. However, repeated inoculation seems to be a scarcely studied factor in relation to mycorrhization and production practices.

The purpose of the study was to evaluate if mycorrhiza inoculation could be incorporated into the standard seedling production in a forest nursery, testing the most easily manipulated factors during the production: Inoculation, fertilization, and usage of fungicide, to see their impact on mycorrhization. If inoculation would be successful, it is my hope that this could aid afforestation in Iceland by improving survival in the field.

The following hypotheses were tested:

1. Mycorrhization will be affected by inoculation, fertilization, and fungicide.

    a) Inoculated seedlings will have a higher mycorrhiza colonization degree, compared to uninoculated seedlings.
    b) The degree of mycorrhization will increase with repeated inoculations in the nursery.
    c) Seedlings kept out of fertilization shortly after inoculation will have a higher degree of mycorrhization.

2. Seedling height and stem diameter will be affected by inoculation, fertilization, and fungicide.

    a) Inoculation will increase the height and stem diameter of seedlings.
    b) Increased fertilization will increase the height and stem diameter of seedlings.

3. Mycorrhizal colonization levels will differ between different combinations of treatments.

2 Materials and methods

2.1. Location

The experiments took place from June 4th until September 12th, 2022, in Sólskógar, a forest nursery located in Kjarnaskógur, Northern Iceland (65°38'53.2" N, 18°05'22.8" W45).

2.2. Production of seedlings

On June 4th, *P. contorta* and *L. sibirica* seedlings were sown in multipot containers. Each container consisted of 67 cells, 870 seedlings per square meter, where each cell had a volume of 50 cm^3. The seed provenances were Skagway, seed number 221003, and Lassima, seed number 221008, for *Pinus* and *Larix*, respectively. A pre-fertilized peat, FPM 420 Airboost, from Kekkila was used with a pH of 4.3. Amounts of soluble nitrogen (N), phosphorus (P), and potassium (K) were 1000–3000 mg/kg, 250–750 mg/kg, and 1050–3150 mg/kg, respectively. On June 21st, the seedlings were watered with the product Entonem *(Steinernema feltiae*), commonly known as nematodes, as a standard precaution against dark-winged fungus gnats *(Sciaridae).*

At germination, on June 22nd, every 10th multipot container was picked out until 24 multipot containers had been selected, for each species. Each multipot container was then randomly assigned to one of the six treatment groups and marked. Each group was treated according to Table 1 and 2. Groups one, two, four, and five were inoculated at germination on June 22nd. Inoculation was repeated for groups four and five at fine root development on July 14th. Group three was inoculated once, at fine root development. Group two was kept out of inoculation two weeks after the first inoculation, while group five was kept out of fertilization until two weeks after the second inoculation and joined the standard production on July 28th. The control group followed standard production, see Table 1. On June 29th, the seedlings were transferred outside. Between July 11th and 25th, a fine net was placed over the seedlings due to slow growth and low temperatures. Simultaneously, the root system was visually examined. As fine roots were apparent, a clear plastic tarp was placed over the groups waiting for the second inoculation to shelter them from the rain. Due to continuous low growth, the seedlings were moved back into a greenhouse on July 28th until they were measured between September 5th and 8th.

Table 1: The three treatments – fertilization, inoculation, and fungicide - used for each group. Fertilization and inoculation consisted of three levels each. Fungicide consisted of two levels. Treatment abbreviations are explained in Table 2.

	Fertilization	Inoculation	Fungicide	Treatment level
Group 1	High fertilization: standard nursery fertilization	At germination	Applied	Fz2, I1, F1
Group 2	Medium fertilization	At germination	Applied	Fz1, I1, F1
Group 3	High fertilization: standard nursery fertilization	At fine root development	Applied	Fz2, -, F1
Group 4	Medium fertilization: kept out of fertilization for two weeks after first inoculation, standard nursery fertilization after second inoculation	At germination and fine root development	Applied	Fz1, I2, F1
Group 5	Low fertilization: kept out of fertilization until two weeks after second inoculation	At germination and fine root development	Not applied	Fz0, I2, F0
Group 6 – Control, Standard production	High fertilization: standard nursery fertilization	Uninoculated	Applied	Fz2, I0, F1

Table 2: List of treatment abbreviations.

Treatment level	Explanation of treatments
Fz0	Kept out of fertilization after first and second inoculation (low fertilization)
Fz1	Kept out of fertilization after first inoculation (medium fertilization)
Fz2	Standard fertilization (high fertilization)
F0	With out fungicide
F1	With fungicide
I0	Without inoculation
I1	Inoculated once at germination
I2	Inoculation twice, at germination and fine root development

2.3. Inoculation

A mycorrhiza soluble from Glückspilze® was used as an inoculation source. The soluble contains 11, 9, and 2 species of ECM, AM, and Trichoderma fungi, respectively, as well as numerous soil microorganisms. The ECM consisted of the following species: *Pisolithus tinctorius, Rhizopogon villosulus, Rhizopogon luteolus, Rhizopogon amylopogon, Rhizopogon fulvigleba, Scleroderma citrinum, Scleroderma cepa, Suillus granulatus, Suillus punctatipes, Laccaria bicolor,* and *Laccaria laccata*. The soluble was mixed with water, in accordance with the producer's manual, or approximately 8.4 g per m^2. Therefore, the total amount of soluble used for the first and second inoculations were, 16.8 g diluted in 30 L of water and 8.4 g diluted in 15 L, respectively. During inoculation each multipot container was placed in a wheelbarrow and drenched in the mycorrhizal solution. After inoculation the multipot containers were placed back into normal production until fertilization started on June 30th, Figure 1.

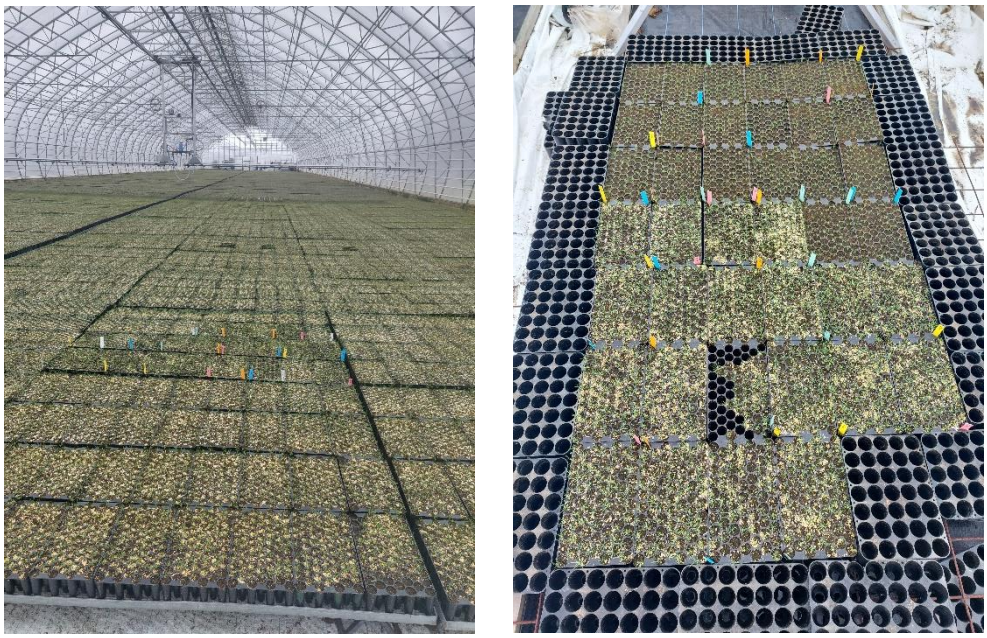

Figure 1: Larix seedlings after first inoculation on June 22nd inside the greenhouse and seedlings on June 30th after being moved outside to the field, Larix and Pinus.

2.4. Fertilization

Fertilization began on June 30th, approximately 4 weeks after sowing, and a week after the first inoculation. Two fertilizer solutions, Kekkila peat suprex (NPK: 11-5-26) and forest superex (NPK: 22-11-19), were dissolved in irrigation water and applied throughout the growing season. The seedlings were watered with a 0–1.2 millisiemens (mS/m) fertilizer solution during the growing period, in accordance with the measured electrical conductivity. However, between July 19th and 26th, the seedlings were watered with a 2.5 mS/m solution, except group five, which was still not receiving fertilization and thus separated from the standard production. After July 28th, once inside again, all groups were watered with a 0-0.8 mS/m solution, in accordance with the measured electrical conductivity. Three levels of fertilization were applied: high fertilization, the amount used in a standard production in the nursery; medium fertilization, where seedlings were kept out of fertilization for two weeks after inoculation; and low fertilization, where seedlings were kept out of fertilization until two weeks after the second inoculation. Hereafter, the different levels will be addressed as high, medium, or low.

2.5. Fungicides

The use of fungicides was not part of the original experimental design. However, on July 25th, when the fungus Needle cast (*Meria laricis)* was discovered, the seedlings were treated with the fungicide Tilt®. All groups were treated with the fungicide except group five, which was separated from the standard production at the time.

2.6. Measurements and evaluation

Due to insufficient growth and development of roots, the *Pinus* seedlings were not measured. Hereafter, seedlings will refer only to the *Larix* seedlings. The seedlings were measured during the period between September 5th and 8th. Seedling height was measured with a tape measure (precision 1 mm). The stem diameter was measured at the base of the seedling with a digital calliper (precision 0.01 mm). Mycorrhization was evaluated visually by examining the root system of each seedling for visible mycelium formations and recorded as either present or non-present, see Figure 2. The percentage of mycorrhizal colonization was calculated from the number of seedlings in each group with mycorrhiza.

Figure 2: Visible mycelium found during the visual evaluation of mycorrhizal colonization on September 6th in inoculated L. sibirica seedlings.

2.7. Root scanning and weighing

On September 12th five seedlings were chosen at random from group four, the group with the highest mycorrhization percentage, along with five seedlings from group six, the control group, to compare the root morphology of inoculated vs. uninoculated seedlings. Each seedling was divided into the above-ground part and roots. From each root sample, roots were thoroughly washed and scanned using a WinRHIZO scanner, Figure 3, where root length, diameter, and number of root tips were calculated. Both tops and roots were then weighed using an AA-160 scale from the Denver Instrument Company (precision 0.0001 g). Each sample was separately packed in a paper bag and dried in a drying cabinet at 50°C for one day. Then the samples were weighed again. Ten paper bags without any samples were dried and weighed, and the average was subtracted from each sample after drying. Lastly, the specific root length (SRL) was calculated by dividing dry root weight by root length.

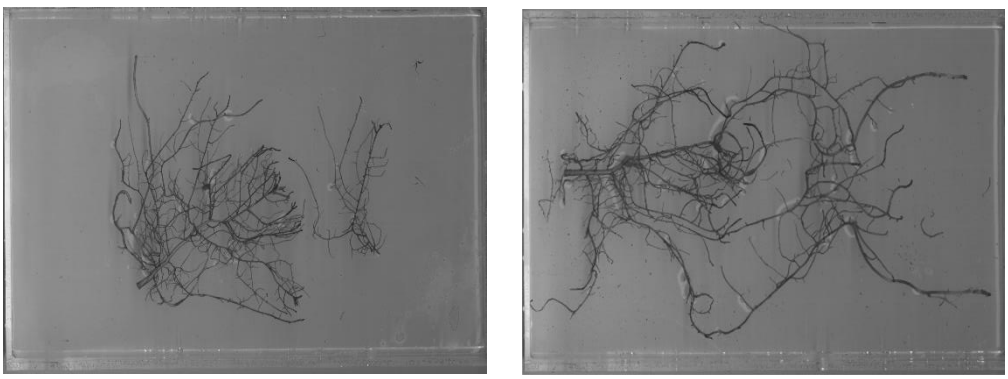

Figure 3: Roots of Larix seedlings from the WinRHIZO scanner from September 12th, showing one sample from group four and group six (control), respectively.

2.8. Statistics

2.8.1. Analyses

For statistical analysis as well as graph production, R.studio version 4.2.3 was used. Before analysis group three was removed from the dataset in order to make the results more compressible. The normality of the two numerical variables; height and stem diameter, was examined via histograms, where both variables were deemed normally distributed. Thereafter, the variables were checked for outliers. As stem diameter had three extreme values, those measurements were removed from the dataset.

Four types of analyses were carried out: binary logistic regressions, ANOVAs, chi-squared analyses, and t-tests. Level of significance was defined as $p < 0.05$. A binary logistic regression was used to examine the effects of the three different treatments on mycorrhization. ANOVAs were used to examine the effect of the treatments on both height and stem diameter. The chi-squared test was performed to determine whether mycorrhization differed between groups. Lastly, the t-tests were used to analyse data from the WinRHIZO root scanner and compare root traits for an inoculated group with an uninoculated group.

2.8.2. Multicollinearity

One of the assumptions in regression analysis is the absence of multicollinearity. This assumption was not met in the case of fertilization and fungicide, as group five was both the group that did not receive fungicide (F0) and the group that received low fertilization (Fz0), Table 2. Therefore, it was impossible to separate the effects and significance of fungicide from fertilization. To see the effects of fungicide as an individual variable, as well as inoculation and fertilization, a simple binary logistic regression and One-Way ANOVA were tested for each treatment level.

3 Results

3.1. Treatment effects on mycorrhization

A binary logistic regression showed that both inoculation treatments had a statistically significant effect on mycorrhization percentage, I1 (p < 0 .001) and I2 (p < 0.001), compared to the uninoculated seedlings. Seedlings inoculated twice (I2, consisting of groups four and five) had the highest mycorrhization percentage of 17.3%, followed by groups with a single inoculation (I1, groups one and two) at 8.5%, and uninoculated seedlings (I0, group six) with a mycorrhization percentage of 1.9%, see Figure 4.

Fertilization did not show a statistically significant effect on mycorrhization. Nevertheless, the mycorrhizal percentage was found to be 16.1% in the groups that received low fertilization (Fz2, group 5), 12.6% in groups that received medium fertilization (Fz1, groups two and four), and 6.3% in the group that received high fertilization (Fz0, groups 1 and 6).

The effect of fungicide could not be tested due to the multicollinearity. However, mycorrhization for groups that did not receive fungicide (F0, group five) and groups that received fungicide (F1, group one, two, four, and five) were 16.1% and 9.5%, respectively.

Three simple logistic regressions were used to test each treatment level on its own. All treatments except Fz1 had significant effect on the percentage mycorrhization; I1 ($p < 0.001$), I2 ($p < 0.001$), Fz1 ($p = 0.210$), Fz2 ($p < 0.001$), F1 ($p < 0.001$).

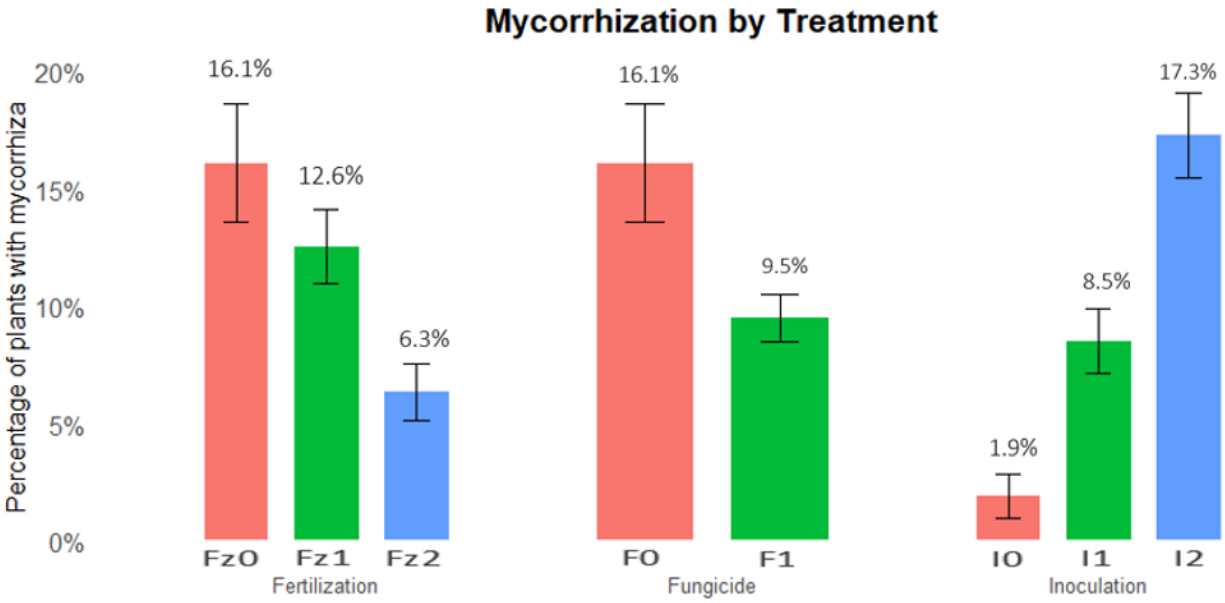

Figure 4: Mycorrhizal percentage for each level of the treatments – fertilization, fungicide, and inoculation -.
The treatment variables from left to right: Kept out of fertilization until two weeks after second inoculation (Fz0), Kept out of inoculation two weeks after first inoculation (Fz1), Standard fertilization (Fz0), Without fungicide (F0), With Fungicide (F1), Uninoculated (I0), Inoculated once at germination (I1), Inoculation twice, at germination and fine root development (I2). It should be noted that Fz0 and F0 both consist of only group 5.

3.2. Treatment effects on growth parameters

3.2.1. Height

A Two-way ANOVA analysis revealed that both inoculation (p = 0.045) and fertilization (p = 0.031) had a significant effect on height. Non-inoculated seedlings (I0) were the tallest, followed by seedlings that were inoculated once (I1), and seedlings that were inoculated twice (12) were the shortest. Seedlings receiving the least amount of fertilization (Fz0) were the shortest. Seedlings receiving high (Fz2), and medium (Fz1) fertilization were higher, with little difference between them, see Figure 5. The effects of fungicide could not be tested due to the multicollinearity.

Additionally, three One-Way ANOVA analyses were performed to test each treatment separately. Individually fungicide (p = 0.034) significantly increased height, while both inoculation (p = 0.100) and fertilization (p = 0.344) did not significantly affect height.

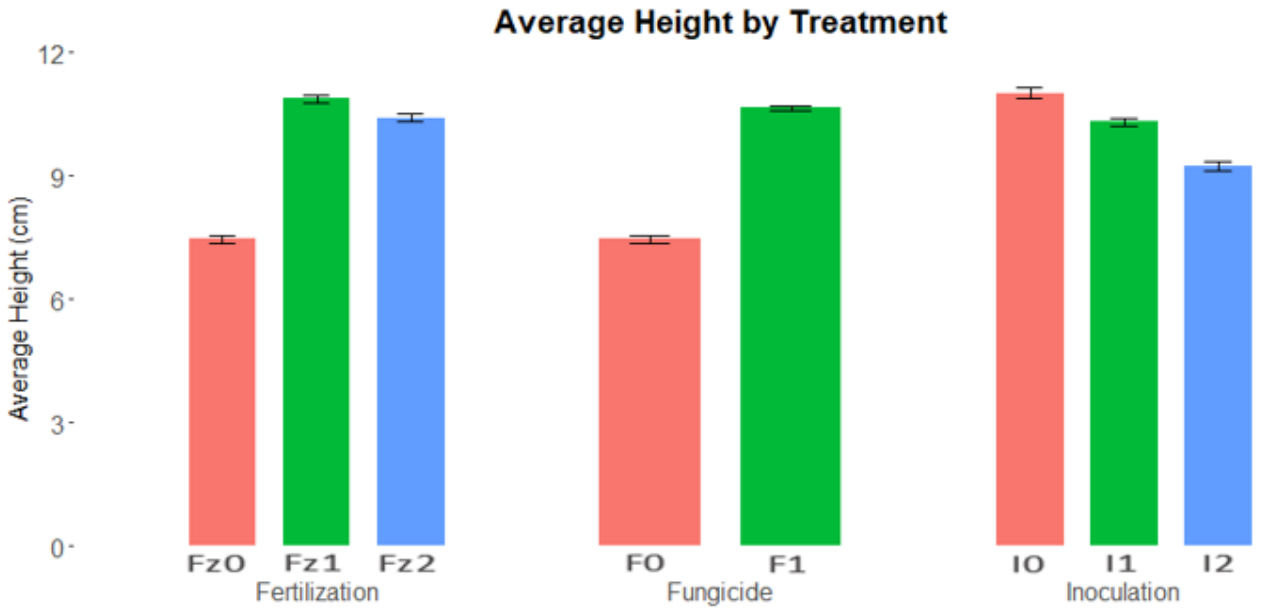

Figure 5: Average height in centimetres (cm) of seedlings for each level of the treatments– fertilization, fungicide, and inoculation - The treatment variables from left to right: Kept out of fertilization until two weeks after second inoculation (Fz0), Kept out of inoculation two weeks after first inoculation (Fz1), Standard fertilization (Fz0), Without fungicide (F0), With Fungicide (F1), Uninoculated (I0), Inoculated once at germination (I1), Inoculation twice, at germination and fine root development (I2). It should be noted that Fz0 and F0 both consist of only group 5.

3.2.1. Stem diameter

A Two-Way ANOVA was performed, which revealed that both inoculation (p = 0.017) and fertilization (p = 0.090) had a significant effect on stem diameter. Non-inoculated seedlings (I0) had the largest stem diameter, followed by seedlings that were inoculated once (I1), and seedlings that were inoculated twice (12) had the lowest average diameter. Seedlings receiving low fertilization (Fz0) had the lowest diameter. Seedlings receiving high (Fz2), and medium (Fz1) fertilization had a similar diameter, see Figure 6. The effects of fungicide could not be tested due to the multicollinearity.

Additionally, three One-Way ANOVA analyses were performed to test each treatment separately. Individually, fertilization (p = 0.118) and fungicide (p = 0.117) did not significantly affect stem diameter, while increased degree of inoculation (p = 0.047) significantly decreased stem diameter.

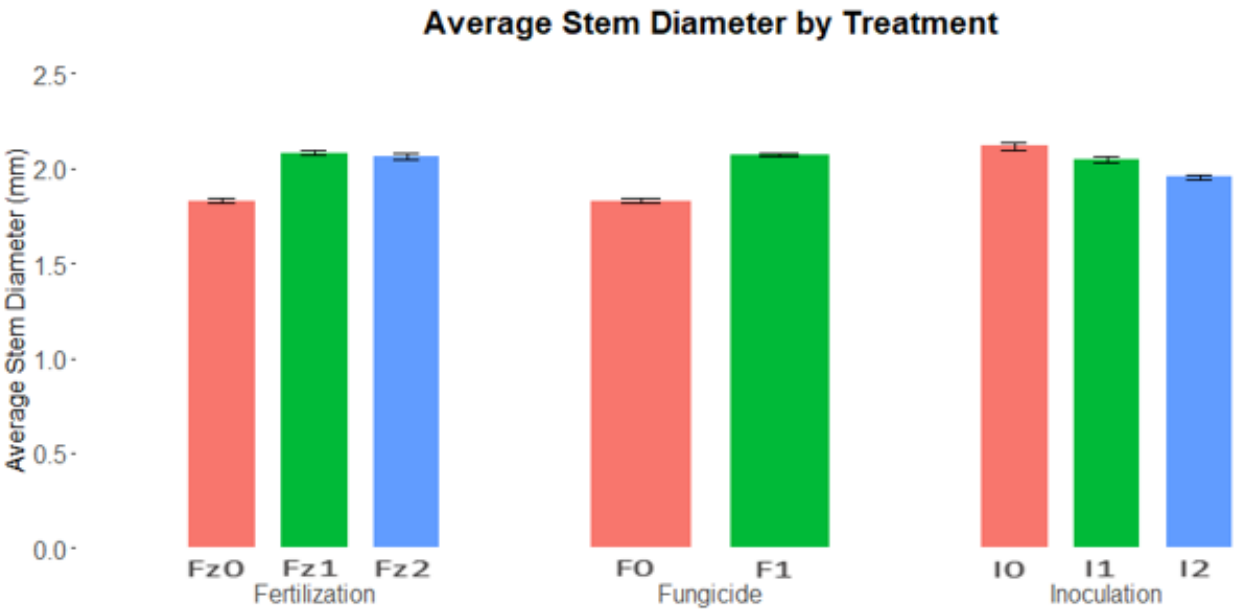

Figure 6: Average diameter in millimetres (mm) of seedlings for each level of the treatments – fertilization, fungicide, and inoculation -.
The treatment variables from left to right: Kept out of fertilization until two weeks after second inoculation (Fz0), Kept out of inoculation two weeks after first inoculation (Fz1), Standard fertilization (Fz0), Without fungicide (F0), With Fungicide (F1), Uninoculated (I0), Inoculated once at germination (I1), Inoculation twice, at germination and fine root development (I2). It should be noted that Fz0 and F0 both consist of only group 5.

3.3. Combined effects of treatments on mycorrhization

Chi-squared analyses showed that mycorrhization varied significantly between different combination of treatments ($p < 0.001$), see Figure 7. Additional chi-squared analyses showed that both inoculation ($p < 0.001$) and repeated inoculation ($p < 0.001$) significantly increased mycorrhization.

Groups inoculated twice, groups four and five, had the highest mycorrhizal percentages at 18.5% and 16.1%, respectively. Groups inoculated once, groups one and two, followed with a mycorrhizal percentage of 10.9% and 6.2%, respectively. Group six, the non-inoculated control group, had the lowest mycorrhization at 1.9%. When looking at fertilization, groups that received more fertilization, groups four and one, had a higher mycorrhizal percentage compared to their inoculation counterparts, groups five and two.

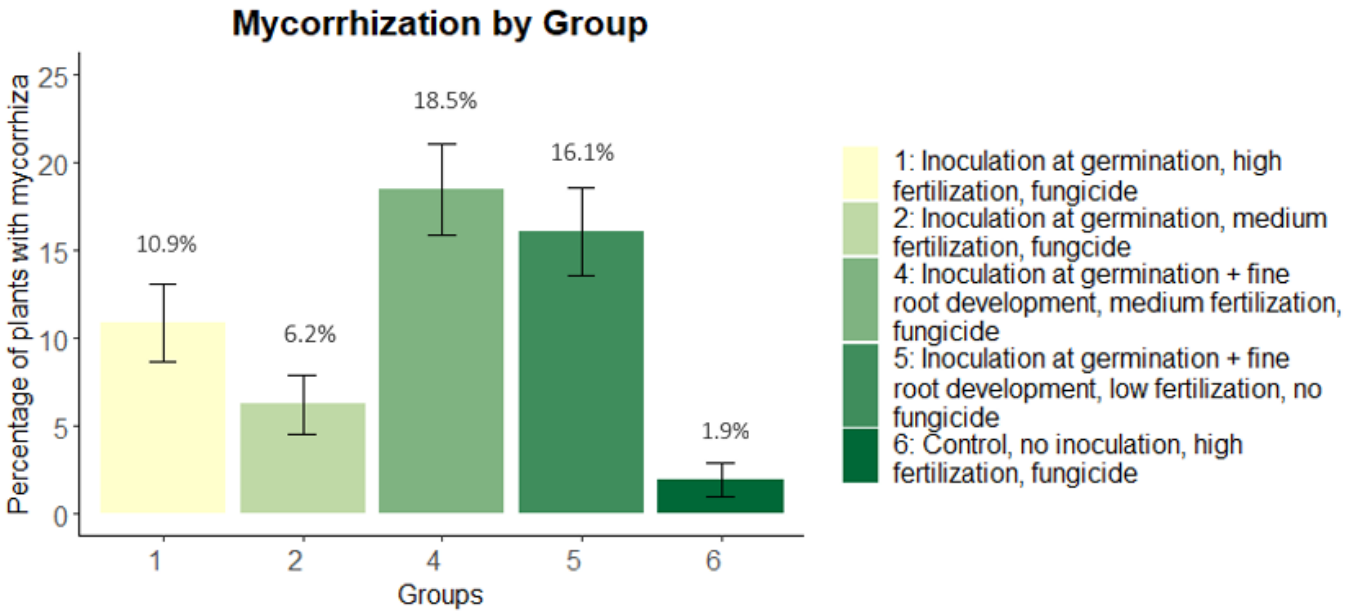

Figure 7: Percentage of plants with mycorrhiza with combined effects of all three treatments -inoculation, fertilization, and fungicide -. The groups consisted of the following treatments. Group 1 was inoculated at germination, received standard fertilization and fungicide. Group 2 was inoculated at germination, kept out of fertilization two weeks after inoculation and received fungicide. Group 4 was inoculated at both germination and fine root development, kept out of fertilization after the first inoculation and received fungicide. Group 5 was inoculated at germination and fine root development, kept out of fertilization until two week after the second inoculation and did not receive fungicide. Group 6 was the control group that followed standard production, was not inoculated, received standard fertilization and fungicide.

3.4. Root morphology

T-tests for root morphology traits showed no statistical significance between an inoculated group, group four, and an uninoculated group, group six, the control group, with average root length ($p = 0.434$), average root diameter ($p = 0.717$), Average root tips ($p = 0.267$), and average specific root length ($p = 0.122$). Although non-significant, the inoculated group had more root tips, a larger root diameter, and a higher SRL, but a shorter root length compared to the uninoculated group, see Figure 8.

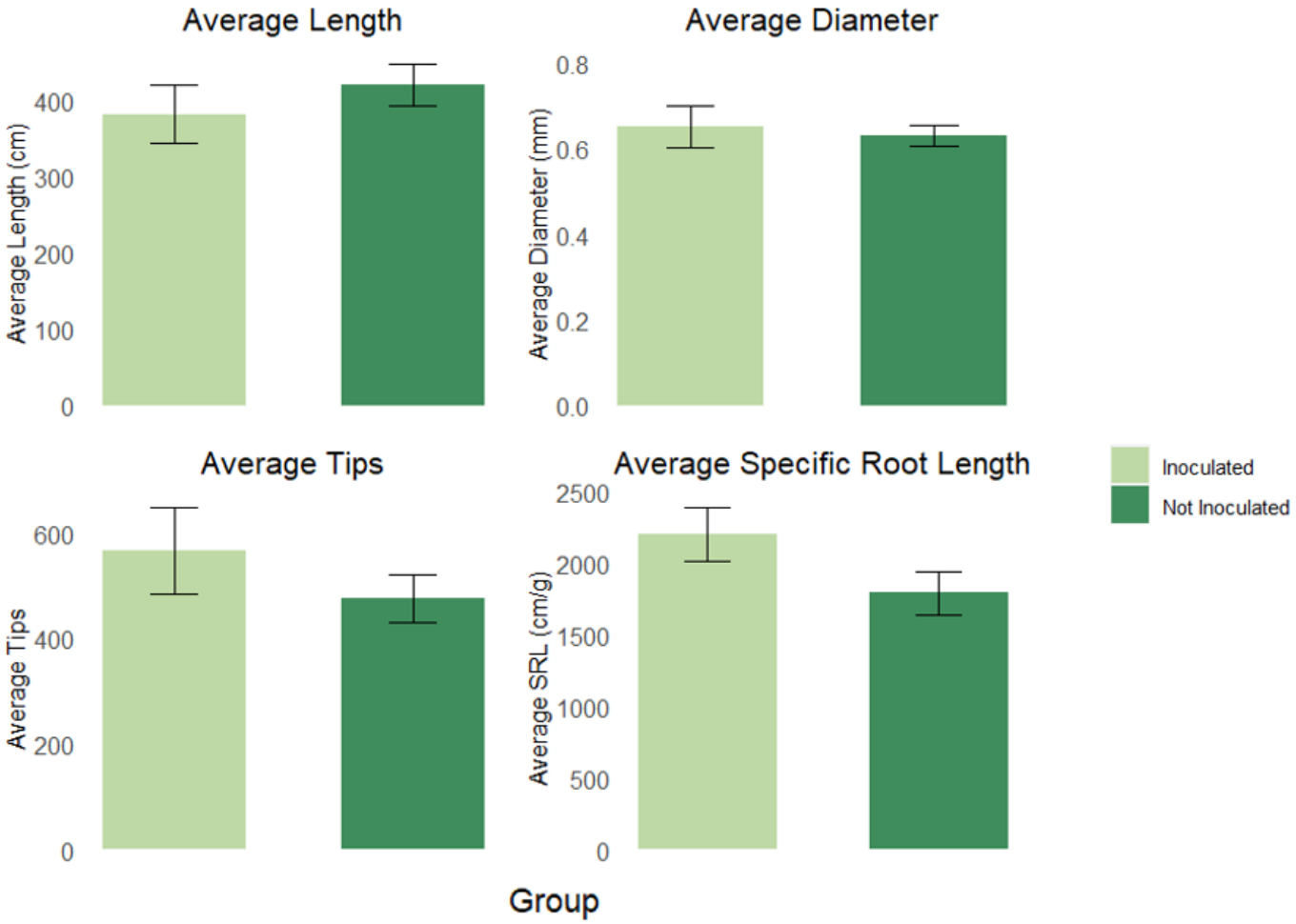

Figure 8: Average length, diameter, specific root length (SRL) and number of root tips for inoculated (group four) vs. uninoculated group (group six, control) measured in the WinRHIZO root scanner.

4 Discussion

Inoculation is not a part of the standard nursery production in Iceland, and to my knowledge, the incorporation of inoculation into standard production has not been examined before. Globally, inoculation as a part of the standard nursery production is an understudied field, even though multiple studies have shown the benefits of inoculated seedlings, such as a higher survival rate in the field (Oskarsson, 2010; Steinfeld et al., 2003) and a higher tolerance for environmental stress (Fini et al., 2011; Kozdrój et al., 2007). The aim of this study was therefore to examine the possibility of incorporating inoculation into the standard production in the forest nursery Sólskógar in Iceland.

In agreement with the first hypotheses, mycorrhization increased with inoculation, 1a, and with repeated inoculation, 1c, which is consistent with previous studies (Oskarsson, 2010; Zhang, Z. et al., 2019). However, the subject of repeated inoculation is a scarcely studied one. To my knowledge, no studies have been done with examining if mycorrhization increased with repeated inoculation of the same seedlings with the same inoculum. Nevertheless, mycorrhization has been shown to change root morphology (Zhang, X. et al., 2019) and increase fine root production (Cairney, 2011). Therefore, a repeated inoculation should, in theory, increase mycorrhization, with the first inoculation boosting fine root production and therefore increasing opportunities for colonization during the second inoculation.

The first hypothesis, 1b, stated that increased fertilization would decrease mycorrhization. Even though this trend was observed, the results were not statistically significant. Generally, fertilization is thought to decrease mycorrhizal colonization (Diaz et al., 2010; Singh, 1999). However, some studies have suggested that fertilization has no effect on mycorrhization (Khasa et al., 2001; Salto et al., 2020). The varying results can partly be attributed to other factors that affect mycorrhization success, such as pH and mycorrhizae type. The pH of soil affects mycorrhization (Gerz et al., 2016). The optimum pH for ectomycorrhizal species lies between pH 4.5 and 5.5 (Hung & Trappe, 1983). In low-pH soils the use of granular calcite, which increases soil pH, has been shown to increase mycorrhization (Lamhamedi et al., 2020). In this experiment the soil pH was 4.3, which is quite close to the optimum pH and is therefore unlikely to affect the mycorrhization success greatly. The fertilization effect on successful mycorrhization can also be attributed to the type of mycorrhizae used, where some strains of mycorrhiza can tolerate higher amounts of fertilization (Diaz et al., 2010) such as the *Laccaria* species (Ortega et al., 2004). As the mycorrhiza inoculum consisted of two *Laccaria* strains, that could partly explain why mycorrhization was not significantly affected by fertilization.

Furthermore, the type of fertilization may also play a part. A study showed that the type of fertilization had little to no effect on mycorrhization of *L. laccata*, while *P. tinctorius* showed a much lower mycorrhization percentage when a soluble fertilizer was used compared to a slow-release fertilizer (Rincón et al., 2007). The fertilizer used in the experiment was a soluble type, which could therefore hinder the colonization of some species, such as *P. tinctorius,* from the mycorrhiza soluble.

To properly address the effects of fungicides on mycorrhization, the use of fungicides would have needed to be part of the original experimental design to ensure no multicollinearity between the treatments. Therefore, interpreting the results for the effects of fungicide on mycorrhization should be done with great caution. On its own, fungicide was found to significantly decrease mycorrhization. Field experiments have shown that propiconazole, the active ingredient in Tilt®, does reduce ectomycorrhizal growth (Marin, 2011). However, fungicides have been reported to affect mycorrhizal species to varying degrees (Yen et al., 2009). Additionally, propiconazole was found to decrease mycorrhization by only 20% when applied consistently over a 2-year period (Teste et al., 2006). As the fungicide was applied once in the experiment, it is therefore likely that the fungicide had little effect on the mycorrhization. Furthermore, it is plausible that the observed effects could be attributed to fertilization rather than fungicide, given that the group that did not receive fungicide also exhibited the greatest disparity in the amount of fertilization given compared to the other groups. However, a research design that incorporates the use of fungicide would be needed to further assess impact of fungicide on mycorrhization.

Contradictory to the second hypothesis, 2a, inoculation decreased height and stem diameter. Previous studies have shown varied effects of ECM on growth parameters. One study found that ECM inoculation had a negative impact on growth which resulted in lower seedling height, biomass, as well as root length (Agathokleous et al., 2022). However, inoculated seedlings subjected to drought stress showed a higher growth rate (Zhang, Z et al., 2019). This was also the case when seedlings were attacked with disease (Sapsford et al., 2017). Plant condition seems to be of importance when discussing the effects of inoculation on growth, where inoculation under optimal conditions seems to decrease growth in seedlings, whereas stress can result in increased growth. Seedlings in the nursery are generally under low stress, as nutrients and water are supplied generously throughout the growing season, which supports the theory that inoculation decreases growth in a low stress environment.

In agreement with the second hypothesis, 2b, fertilization increased height and stem diameter. However, when individual effects of fertilization on seedling growth were examined, no significant effects were found. Contrary to assumptions, seedlings receiving high fertilization did not result in the largest seedlings. Several studies have shown Larix to have a low growth response to fertilization (Park et al., 2012; Wan et al., 2019; Wei et al., 2014). However, fertilization has been shown to increase height of unfertilized seedlings, compared to fertilized ones (Duan et al., 2013). This supports the results from the study, as there was little difference between high and medium fertilization but a significant difference in growth for the seedlings receiving low fertilization, where the seedlings that were kept out of fertilization until late June.

In agreement with the third hypothesis, mycorrhization differed between combinations of treatments. However, the combined effects of the treatments were larger than expected. While mycorrhization generally decreased with increased fertilization, when repeated inoculation was combined with increased fertilization, mycorrhization increased. A few factors could explain these findings. Firstly, as mentioned, some of the ectomycorrhizal species in the mycorrhiza soluble tolerate high levels of fertilization, often called nursery-adapted species (Ortega et al., 2004). Additionally, other nutrients highly affect the success of mycorrhizal colonization. Phosphorous (P) is a key regulator of mycorrhizal colonization (Walker, 2001). The peat the seedlings grew in has a limited amount of nutrients and a low amount of P compared to both nitrogen (N) and potassium (K). As the mycorrhiza species used could tolerate fertilization, when combining inoculation with increased fertilization, a higher mycorrhiza colonization could be possible due to an increase in the P available to seedlings. The combinations of treatments in the experiment were not fully factorial, and the results indicate that the highest mycorrhization could possibly be achieved with high fertilization combined with a repeated inoculation. More studies are needed to further examine the relationship between fertilization and repeated inoculation to find the balance between the two factors that will yield the highest amount of mycorrhization.

Root morphology was not significantly affected by inoculation. This could possibly be due to the fact that the sample size was only five individual observations. Nevertheless, the root scanning revealed interesting trends. Inoculated seedlings had shorter, wider roots, a higher SRL, and more root tips compared to the uninoculated seedlings. A previous study showed that ECM inoculation on *Larix* seedlings significantly altered the root morphology, with shorter (Cabrera-Ariza et al., 2023) and wider roots (Sun et al., 2010a). As mycorrhiza increases water and nutrient uptake efficiency (Smith & Read, 2010), the longer roots of uninoculated seedlings

may indicate the compensation required for adequate nutrient and water uptake. Additionally, when mycorrhiza is present, roots tend to swell slightly, resulting in an increase in root diameter (Brundrett, 2002). Furthermore, as mycorrhiza generally establishes on root tips, an increased number of root tips can indicate a successful mycorrhization. SRL is generally lowered with inoculation (Sun et al., 2010b). However, in such young plants, when the length is decreased and diameter growth is limited, a higher SRL can be expected. The morphological factors studied all showed indications of mycorrhization in the inoculated group compared to the control group. These results therefore indicate that mycorrhization was somewhat more successful than indicated by visual mycelium examination despite the results not being statistically significant.

Visible mycelium was low at the time of evaluation. Several factors can cause both low visible mycelium as well as a general low mycorrhization colonization. As ECM structures take time to develop (Brundrett, 2002), low visible mycorrhization percentage could have resulted from the short time window from inoculation to inspection. The first six months after inoculation have been regarded as the installation phase (Dib & Fortas, 2019). A study that inspected seedlings three months after inoculation concluded that ectomycorrhizal formation may be incomplete during that timeframe (Cabrera-Ariza et al., 2023). In this study the mycorrhization was evaluated approximately 11 and 8 weeks after inoculation, which is in all likelihood too little time for mycorrhiza to be able to establish enough for visible evaluation.

Another factor contributing to the low visible inoculation could be the use of peat as a substrate. Oskarsson found that peat reduced mycorrhization colonization in seedlings (Oskarsson, 2010). The peat is both sanitized, therefore losing its beneficial bacteria flora, as well as being pre-fertilized. This creates an unhospitable environment for the mycorrhiza fungi which results in a lowered mycorrhization. Presumably, a combination of these factors contributes to the low mycorrhization percentage visible in the seedlings at the time of evaluation.

The results from the study suggest that inoculation with standard nursery production is a possibility feasible option. As the mycorrhiza soluble is designed for greenhouse irrigation systems, a transition from hand inoculation to large-scale production should not cause any difficulties in the nursery. As mycorrhization was overall low, more research is needed to maximize the colonization of seedlings, where more factors affecting mycorrhization in the nursery should be examined. Indeed, it would be interesting to further study how different mycorrhizal strains combined with diverse fertilization levels affect mycorrhization.

Furthermore, the possible benefits of inoculation in the field with the given mycorrhiza soluble need to be examined as well to determine if inoculation as a part of the standard nursery production should be a part of the future.

5 Conclusion

This study suggests that incorporating inoculation into the standard nursery production is possible without drastic changes to the production. Mycorrhiza colonization in the seedlings was increased by inoculation as well as repeated inoculation. Increased fertilization reduced mycorrhiza colonization. Plant growth, both height and stem diameter were both positively affected by inoculation and fertilization. Root morphology traits indicated that mycorrhization had been successful, however, the results were not statistically significant.

As the highest mycorrhiza colonization was found in group four, where inoculation was repeated, and fertilization was medium, this combination would be recommended to incorporate into the standard production. Further studies are needed as the groups were not fully factorial and the results indicate that a higher mycorrhiza colonization could be gained when combining high fertilization with a repeated inoculation.

Further studies should also include a repetition of this study, with tweaks to prevent a multicollinearity and a longer incubation period for the mycorrhiza. Furthermore, a follow up study will be performed in autumn 2023 where inoculated seedlings will be planted in the field and long-term benefits of inoculation, such as survival and growth, will be researched.

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