Norges miljø- og biovitenskapelige universitet

Master's Thesis 2018 30 ECTS
Plant Science (IPV)
Halvor Solheim

Mycorrhizal inoculation methods on Lutz spruce seedlings for afforestation of treeless landscape in Iceland

Hlíf Böðvarsdóttir
Plant science

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Abstract

Seedling mortality is a great problem in afforestation in Iceland and is most severe in a treeless landscape. It is not uncommon for new afforestation areas to have 30 – 40 % mortality in seedlings in the first five years. One of the causes to this low survival is the lack of mycorrhizal fungi in the soil. In this study, effects of mycorrhizal inoculation treatments where examined on one summer old Lutz spruce (*Picea x Lutzii)* in two study sites in the South-West of Iceland. Methods were categorized into three treatments 0) Un-inoculated (control), 1) Inoculation in autumn and 2) inoculation in spring. The effect of winter storage on field performance was also examined. The storage methods were, outside winter storage in nursery and plant freezer storage. After one year in the field a subsample of the seedlings was examined. Three morphotypes were found, mycorrhizal root tips were counted and colonization was calculated. The analyses showed that inoculation treatments increased mycorrhizal colonization and generally increased root growth. Winter storage seemed to have negative effect on shoot growth, though not statistically explained seedlings stored in plant freezer seemed to have more dieback after the first winter.

Keywords: Mycorrhiza, inoculation, winter storage, field performance

Ágrip

Mikil afföll hafa verið eftir gróðursetningu á flestum stöðum á landinu og eru afföllin mest þar sem plantað er í trjálaust mólendi. Það er ekki óalgengt að afföll plantna séu um 30 - 40 % fyrstu fimm árin eftir útplöntun. Ein af ástæðum þessara mikilla affalla er vöntun á gagnlegum rórarsveppi í jarðveginum. Í þessari rannsókn voru áhrif svepprótar smitunnar á sumar gamlar Sitka bastarðs plöntur (*Picea x lutzii)* metin á tveimur tilraunastöðum á suð-vestur landi. Smitunnar meðferðum var deil í þrennt 0) Ósmitaðar (kontról), 1) smitaðar að hausti, og 2) smitaðar að vori. Áhrif vetrargeymslu voru einnig könnuð. Yfirvetrunnar aðferðir voru: úti undir plasti í gróðrarstöð og í frysti geymslu. Einu ári eftir útplöntun var hluti af plöntunum rannsakaðar, svepprætur greindar og taldar og þéttleiki reiknaður. Út úr greiningu gagna kom í ljós að það var marktækur munur á þéttleika svepprótar eftir smitunnar meðferðum og að smitun jók rótar vöxt. Yfirvetrunn í frysti geymslu virtist hafa áhrif á vöxt plantnanna þar sem plöntur yfirvetraðar í frysti geymslu kólu meira eftir fyrsta veturinn en þær sem voru úti yfir veturinn, enn þetta var ekki hægt útskýra tölfræðilega.

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Hlíf Böðvarsdóttir
9. of Mai 2018
Iceland

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1 Introduction

1.1 Forest history of Iceland

After the Vikings settled in AD 870 it has been estimated that Iceland has lost more than 50% of its vegetation cover, and over 90% of its forests (Ólafsdóttir et al. 2001). The main cause of vegetation loss is clearcutting of birch woodlands and overgrazing by livestock (Greipsson 2012) in combination with harsh and cool climate and volcanic activity. Eysteinsson (2017) estimates that in the mid. 20th century the birch-woods had reached the absolute minimum of less than 1% land cover. Since the forestry and soil conservation act was established in 1907 continuous work has been done in reintroducing forests into the Icelandic landscape (Eysteinsson 2008).

The Icelandic Forest Service (IFS) was founded in 1908, and during the first half of the 20th century, the prime focus was on protecting birch woodland remnants. By protecting these woodlands from grazing, a practice still necessary today, these woodlands among other more recent afforested areas make up the national forest system (Eysteinsson 2017) that covers 1906 km^2 which is 1,9 % of the total land area (Traustason & Snorrason 2008). Since 1950 to early 2000, there has been an exponential increase in afforestation ranging from 1,5 million to 6 million seedlings per year respectively. The species planted were mainly the native downy birch (*Betula pubescens Ehrh)* and exotic species like Norway spruce (*Picea abies (L.) Karst.,* sitka spruce (*Picea sitchensis Bong. Carr.),* Scots pine (*Pinus sylvestris L.),* lodgepole pine *(Pinus contorta Douglas ex Loudon)* and Siberien larch *(Larix sibirica Ledeb.).* With these experiments came a great deal of knowledge that today's forestry is based on (Eysteinsson 2017). The five most used species in forestry in Iceland today are the native birch, Siberian larch, sitca spruce, lodgepole pine and black cottonwood (*Populus trichoocarpa Torr. & Gray)* (Gunnarsson 2014). Since the financial crisis in 2008 there has been a dramatic decline in planting, in 2015 there were around 3 million seedlings planted. Planting of sitka spruce has increased in the last two decades as older stands have grown very well (Eysteinsson 2017). Sitka spruce is originated from the west coast of North-America and it is now a foundation species in Icelandic forestry. It is a fast-growing tree and it´s best qualities are that it´s both wind and salt tolerant which makes it suitable for the oceanic climate. Sitka spruce hybridizes easily and naturally with white spruce (*Picea glauca* (Moench) Voss) forming Lutz spruce (*Picea x lutzii* Little.*).* The use of lutz spruce has increased in afforestation in Iceland over the last decade. Like sitca spruce, the hybrid is fast growing, tolerates wind and salt well but the hybrid is hardier for autumn frosts (Skúlason et al. 2001).

1.2 Health and vigor of young forests

On average the mortality rate in new plantations is approximately 30-40 % within the first five years (Eggertsson 2004; Snorrason 2007; Þórsson 2008). There has been extensive research on this topic identifying many different potential causes. Frost heaving is a great problem, especially in degraded areas caused by the soil characteristics (Goulet 1995; Orradóttir & Arnaldsson 2006; Óskarsson 1997). Most of the soil in Iceland is classified as Andisols which has a fine, sandy texture and has a large capacity to retain water. These characteristics enhance the processes that lead to frost heaving (Arnalds et al. 1995). Nutrient deficiency, in particular, nitrogen (N) and phosphorus (P) is a problematic factor of plant vigor in many areas (Óskarsson 1997). Rikala et al.(2004) and Jónsdóttir (2011), found that increased autumn fertilization in nurseries increased the size and survival after planting. Others have found that late summer fertilization in the nursery increases frost hardening (DeHayes et al. 1989; Rikala & Repo 1997) all though the opposite findings exist (Nihlgård 1985; Stimart et al. 1985). Others speculate that high mortality in seedlings when planted in a cultivated land where no trees have been present for centuries is due to lack of appropriate soil biota. Experiments have shown that in exposed or degraded sites as is typical in Iceland, there is a lack of mutualistic fungi (mycorrhiza) and by adding it while or prior to planting, growth and fitness increases (Óskarsson 2010) and damages caused by root-herbivores are reduced (Oddsdóttir 2010). According to Guðmundsson (2017), sitka spruce plants have higher success rate when planted within established birch trees/shrubs communities, that could indicate that within a mature woodland the appropriate soil biota is present which boosts the growth. The effect fertilization has on mycorrhiza populations has been studied and there have been different results. Óskarsson & Halldórsson (2008) conducted an experiment with applications of N and P fertilizers on *Betula pubescens* in a nutrient-poor site and nutrient-rich sites. He found that with the highest amount of N there was a decrease in ECM colonization in the first year in the rich site. At the poor site the highest N application had the same effect, however, these seedlings had the best growth response. After three years, the N effect had disappeared and ECM colonization increased. This is in accordance with earlier studies (Allen et al. 2003; Wallander 1995; Wallenda & Kottke 1998) though most studies only look at the short time effect. Ronsheim (2012) studied the effect of different phosphorus concentrations on mycorrhizal colonization on *Allium vineale* and he found that only after 15 months was there a significant difference in total biomass and only at lower P consecrations. This shows the importance of long-term studies cause the benefits aren´t immediate.

Studies have proven that mycorrhiza inoculation are beneficial for seedling growth and survival (Ortega et al. 2004; Óskarsson 2005) however the results are not always so straightforward

(Ronsheim 2012; Stenström & Ek 1990; Stenström et al. 1990) and it has been speculated that co-inoculations are more beneficial than single species inoculations.

1.3 Mycorrhiza

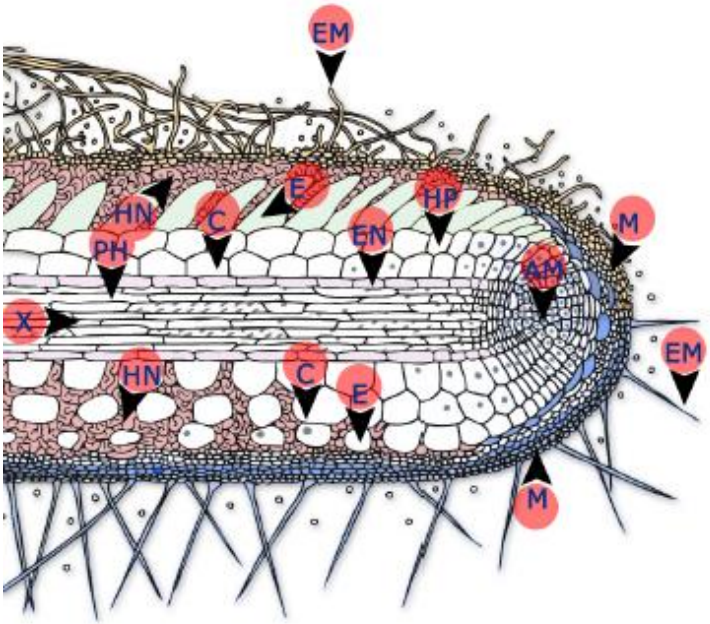
Mycorrhizas are according to Brundrett et al. (1996) „highly evolved, mutualistic associations between soil fungi and plant roots". Almost all ecosystems are dominated by mycorrhizal plants. It is estimated that 95% of natural plants form mycorrhizal association in undisturbed sites, whereas, in highly managed fields, early successional communities and soils with high P values are extremely poor of mycorrhizal plants. There are also 6% of flowering plants that are nonmycorrhizal (NM) or are either NM or AM (8%) (Brundrett 2009; Ortega et al. 2004).
Mycorrhiza fungi increase the root volume with its extensive hyphal network enabling it to reach further to absorb water and mineral nutrients, thereby increasing the plants nutrient uptake and its survival (Bonfante & Genre 2010). In addition to enhanced nutrient acquisition (Landeweert et al. 2001), it also increases drought tolerance (Morte et al. 2000) and pathogen resistance of their hosts (Branzanti et al. 1999). In return, the plant provides the fungus with 1- 25% of its photosynthetically derived carbon compounds (Meyer et al. 2010) which is essential for fungus growth and reproduction (Bonfante & Genre 2010). The fungus forms a net that can connect the roots together (Kropp & Langlois 1990) and thereby create a nutrient pathway between plants.

The mycorrhizas are commonly divided into two broad categories based on their anatomical features, there are ectomycorrhizas (ECM) and endomycorrhizas (Bonfante & Genre 2010; Brundrett et al. 1996). These categories are based on whether the fungus colonizes between the root cells (ecto-) or penetrates the cells (endo-). Endomycorrhizas are further divided into three groups, vesicular-arbuscular mycorrhizas (VAM), Ericoid-, Orcidmycorrhiza. Then there is ectendo-, arbutoid- and monotropid mycorrhiza which have many similarities with ectomycorrhizal associations but have some unique characteristics (Brundrett et al. 1996).
AM associations are found in all ecosystems and are usual in grasslands though less common in boreal and temperate forest ecosystem (Meyer et al. 2010). It is estimated that 74% of all plant species form AM associations with fungi of the Glomeromycota. Ericoid form associations with plants from the Ericaceae family which constitutes 1% of all plant species. Orchid mycorrhiza, as the name implies associates with plants from the orchid family and constitutes 9% of the total plant species (Brundrett 2009). Ecto- (ECM) and ectendomycorrhizas (EECM) are associated with gymnosperms and a few angiosperms mainly trees and shrubs (Brundrett 2008; Smith & Read 2008) and are typical

in the boreal and temperate forest (Meyer et al. 2010). According to Brundrett (2009), 6 % of Angiosperms are non-mycorrhizal but have other means of foraging nutrients like parasitism, carnivore or cluster roots. The final 8 % of Angiosperms are families that consist of both AM and NM species. Lutzi spruce, which is the subject of this thesis, is predominately ectomycorrhizal and therefore I will subsequently focus on ECM fungi associations.

1.4 Ectomycorrhiza

Ectomycorrhizas (ECM) only associate with about 2% of the total number of plant species but because of their effect on forest trees in the Northern hemisphere, it is considered very important for wood production (Smith & Read 2008). In the coniferous forest in the cold boreal zone, ECM associations rule. The fungi play a vital role in the forest growth and survival. There are thousands of fungi species that form ECM associations, most are Basidiomycetes, few are Ascomycetes and a single Zygomycete (Miller Jr 1982). ECM is primarily found on the short lateral roots of the host and has three main characteristics (*figure1*). After the hyphae have made contact with the root 1) it forms a sheath or a mantle of fungal tissue around the root tip. The sheath can differ in appearance depending on which plant- and fungi species are involved. On genus level, the structure of the mantle is invariable and is used for fungal recognition (Nedelin 2014). 2) Hyphae from the mantle penetrate between the root cells, branch and form a coat called the Hartig net which is different within every host and fungal species. 3) From the mantle and into the soil, grow strands of mycelium, often called the extraradical mycelium, which is the primary connection to the soil and increase the root ability to exploit the nutrients and water in the soil. These networking elements (Hartig net and Seath) have their unique function. The Hartig net is the boundary where the exchange of carbohydrates from the plant to the fungus happen. The fungal Seath is a storage unit for 1) nutrients transferred from extraradical mycelium, intended for the Hartig net and 2) carbohydrates harvested by the Hartig net and tended for the soil growing extraradical mycelium (Jordy et al. 1998).
Fruit bodies (sporocarps) grow from the extraradical mycelium, commonly called mushrooms. Around 80% of the fungi that form ECM grow above ground fruit bodies (epigeous species) the rest only grows underground (hypogeous species) (Smith & Read 2008). Many fruit bodies of ECM are edible and very important for the food industry, f.exc. truffles (*Tuber spp.)*, Chanterelle (*Cantharellus spp.),* and Porcini (*Boletus edulis) (Horton & Bruns 2001).* Others are used for their medical potentials. In many countries, these fungi are a large portion of the diet and a source of income (Hall et al. 1994).



Note: M - Mantle (blue and Yellow), HN – Hartig net, EM – extraradical mycelium, E – epidermis cells, HP – hypodermis, C – cortex cells, EN – endodermis, PH – phloem in the main cylinder, X – xylem in the main cylinder, AM – apical meristem.

Figure 1 ECM properties in gymnosperms (lower half) and angiosperms (upper half) (Nedelin, 2014)

1.5 The function of ectomycorrhizal fungi

Environmental factors and soil conditions determine the colonization of the mycorrhiza fungi, and it is assumed that mycorrhizas are an adaptation to nutrient-poor sites (Brundrett 1991; Meyer et al. 2010). This correlates with the findings in Óskarsson and Halldósson (2008) experiment where they found that the treatment with the lowest fertilization at a nutrient-poor site gave the highest mycorrhizal colonization. Ronsheim (2012) mentions studies that have shown that in connection with mycorrhiza the plant biomass has decreased under high P conditions. In such conditions, the plant itself can take up P from the soil but the fungi continue to harvest C from the plant, in these cases, the association is no longer mutualistic but parasitotic. In mycorrhizal plants the photosynthesis rate increases with levels of colonization for some fungal species making the fungal biomass a big carbon sink (Treseder et al. 2007). The fungi increase the C demand for the root to maintain a net flux of C in their favor (Nehls 2008). Other nutrients, NO_3^- , P, K, Ca, SO_4 , Cu, Fe, and Zn have been reported for fungus transport to the plant, providing it with the micro- and macro nutrients it needs (Ames et al. 1983; Ek 1997). In the nitrogen-poor boreal forests ECM´s play the key role in cycling of carbon (C), nitrogen (N) and phosphorus (P), in fact it has been measured that the uptake of inorganic N by the ectomycorrhizae is ten times the root uptake rates (Plassard et al. 1991). The extraradical mycelium forms a hyphal network in the soil which connects the coexisting plants together and moves C along these connections. This hyphal network has appropriately been called the “Wood-Wide Web” by Helgason et al (1998). Phosphorus is known to move along these extraradical paths to the host plant

but doesn´t seem to move to other plants (Kropp & Langlois 1990). The movement of carbon seems to be controlled by the source-sink relationship, Finlay et al. (1986) noticed that shaded plants got more C than none-shaded plants. Thus the availability of light in the forest controls the movement of C, which can increase the survival of young seedlings (Kropp & Langlois 1990).

1.6 Winter storage

The fitness of seedlings in spring is often associated with how they are stored over winter in the nursery (Malmqvist et al. 2017). When plants are stored outside on open land they can get exposed to adverse temperatures due to lack of snow cover which increases the possibility of frost damages of the roots caused by the fluctuations in the weather and even more so if stored above ground. Containerized plants are more vulnerable than bare-root plants because the soil isolates the roots from the cold. Roots are less frost tolerant than the top growth and young roots even more than mature ones. Young roots of container plants are usually found on the outer side of the root plug and are therefore more exposed to cold temperatures. The growing season is longer for roots than it is for shoots and roots lose their frost toleration sooner in the spring so the danger of damages by spring frost are substantial (Bigras & Dumais 2005). Because shoots don't show immediate signs of root damages, they often go unnoticed (Landis & Luna 2009). Plants with damaged root system grow less or die, mostly because of lower water and nutrient uptake, depending on how severe the damages are (Bigras & Dumais 2005). For plants to overwinter successfully it is crucial for roots to develop frost hardiness. It has become a common practice in Scandinavia and N- America to overwinter plants in frozen storage (-3°C to -5°C) but it is relatively new in Iceland. In Scandinavia, the plants are usually kept in storage from October-November until planting at the beginning of April (Malmqvist et al. 2017). Keeping containerized plants in a freezer during the long, unstable winter is said to ensure the quality of the plants especially the roots (Jónsdóttir & Jóhannesdóttir 2009). Before plants can be placed in winter storage they must be cold acclimated which according to Bigras et al. (2001) "is the transition from a non-hardy state to a hardy one". This transition is influenced by thermo- and photoperiod though the interaction is different for species, provenances, roots, and shoots (Bigras et al. 2001; Malmqvist et al. 2017). For the plants to acclimate they have to be exposed to cold temperature and/or shorter photoperiod. It is common in plant nurseries to move plants outdoors in the autumn, exposing them to gradually lower temperatures, and at higher latitudes, to shorter day length until they develop freezing tolerance (Stattin & Lindström 1999). The most important parameter for roots to become fully hardy is the soil temperature. Roots stop growing at temperatures between 2-5 °C and get to a full hardy state (Bigras et al. 2001). Measuring freezing

tolerance provides an indication of field performance potential. There are many methods to measure root freezing tolerance the most common are *root growth capacity* (RGC) and shoot/root electrolyte leakage (REL/SEL) (for a detailed description see (Stattin & Lindström 1999).

1.7 The goal of this study

A high mortality rate on young forest seedlings is extremely expensive. In disturbed- and cultivated areas with no trees present for centuries, the mortality rate is highest. Since most of the tree species used in afforestation in Iceland are often the first generation and not native it is likely that the microbial soil community is lacking the species needed for healthy growth. In this study, I want to investigate if inoculation of mycorrhizal fungi will increase growth rate and survival in lutz spruce (*Picea x lutzii*) in treeless land. Three methods of mycorrhizal inoculation of forest nursery seedlings were tested using soil inoculum from a mature sitka spruce stand, along with non-inoculated control plants. The methods were evaluated depending on subsequent root colonization and plant performance in the field. The inoculation was carried out before and after winter storage and the influence of the two storage techniques employed, storage outside or in a plant freezer is also evaluated based on plant responses.

The questions I want to answer are:
Does timing and method of inoculation have any impact on mycorrhizal colonization.
Does mycorrhizal inoculation increase plant survival and growth in the field.
Do techniques of winter storage have an impact on mycorrhizal colonization and plant field performance.

2 Method and materials:

2.1 Seedlings and soil inoculum

The plants used for the experiment are a summer old lutz spruce, (seed source Þjórsárdalur F09-008). The seeds were gathered in Selhöfði in Þjórsárdalur oktober 2008 and then treated and stored at Frøsentralen in Hamar, Norway. The mother trees are from Homer, Alaska.

The seedlings were produced and stores over winter in Kvistar, a plant nursery in Reykholt (64.1766° N, 20.4719° W) in southern Iceland. Seeds were sown one seed per hole in multipots into a mixture of *Sphagnum-peat* and 10% perlite in 2014. The seedlings were cultivated in a 40 cell (100 cm^3 root volume) plastic conical multipots (BCC HIKO V93, Sweden), according to standard nursery procedures; seedlings were fed repeatedly since germination with a mineral nutrient solution EC 0.5 mS (Brøste, NPK 14 - 3 – 23, Azelis Co.). During the growth phase in the greenhouse, seedlings were watered with EC 0.8-1.2 mS, and once outside they are watered with fertilizer EC 2.0 mS once to twice a week. The plants for this study were treated once with the fungicide Topsin (Nippon Soda Company,Ltd) in August and with Amistar (Syngenta, Australia) in September. Seedlings that were to go into freezer storage were kept in the greenhouse. In the end of the summer seedlings were short day treated and stored at 3-4 C° until placed in freezer on December 1st 2014.

The soil providing the mycorrhizal inoculum for the study was taken from a healthy, mature (60-year-old) stand of sitka spruce located in Snæfokstaðir in South-West Iceland. The litter layer was removed and soil from the top 20 cm was excavated and stored for further use. New soil sample was excavated for autumn inoculation and spring inoculation. The specific composition of the inoculum was not identified but was the same for all treatments.

2.2 Winter storage

The plants were stored over winter in either a plant freezer or outside in frames under white plastic tunnel. Before placing the seedlings in the freezer the plants were wrapped in plastic, 10-20 seedlings together, with the top shoot sticking out. These rolls are then placed in a wooden box with open sides (*Photo1*).

The plants were placed in the freezer on 1.12.2015. The temperature was set to gradually decrease from 1 to -5 °C,

Photo 1 Plants prepared for freezing. @Hallur Björgvinsson

and maintain the latter value for the duration of the storage. Plants were removed on 11.5.2016 and left for 24 hours in water (8 C°) for thawing.

2.3 Experimental treatments

A combination of two methods of seedlings overwintering storage and two methods of inoculation application, along with two non-inoculated controls, gave a total of six experimental units (*Table 1*).

Table 1 Experimental treatments/units

Inoculation Method	**Time of inoculation**	**Plant overwintering**	**Method no in text**
None applied (control)		Outside	M2
Soil onto root plugs	Autumn 2015	Outside	M1
Soil under plant trays	Spring 2016	Outside	M3
None applied (control)		Freezer storage	M6
Soil onto root plugs	Autumn 2015	Freezer storage	M5
Soil in thawing water	Spring 2016	Freezer storage	M4

2.3.1 Method details

Method 1 (M1, *Table 1*). One summer old plants of Lutz spruce were inoculated by dispersing soil on top of the root plugs. In all, 300 plants overwintered in trays placed outside under white plastic at the nursery Kvistar the winter 2015-2016

Method 2 (M2, *Table1*). Non-inoculated and stored outside. Used as control plants for M1 and M3,

Method3 (M3, *Table 1*). Plants inoculated in spring 2016 but otherwise identical to control plants (M2). In spring, multipots were placed on a bed of forest-soil in the pine forest at Snæfokstaðir, for spring mycorrhizal inoculation (*Photo 2*).

Photo 2 Plants standing on forest soil (2016), Outdoor winter storage. @Böðvar Guðmundsson

Method4 (M4 *Table 1*). Plants inoculated in spring 2016. The plants were stored over winter in plant freezer and retrieved on 13.05.2016. In spring, rolls were opened, and the roots exposed, and water mixed with the soil inoculum pored over the root systems.

Method5 (M5, *Table1*). 300 plants were inoculated at the same time and with the same method as in M1, and then packed (see *Figure 2*) and overwintered at the freezer storage at Kvistar.

Photo 3 Seedlings from freezer, inoculation by pouring soil mixed with water over the seedlings. @Böðvar Guðmundsson

Method6 (M6, table1). Un-inoculated control plants that were stored in the same way as M4 and

In the spring of 2016 (12.05.) all plants, both inoculated and un-inoculated were moved from the nursery at Kvistar to Snæfokstaðir. The plants, except groups M4 and M5, were placed on plastic sheet in a pine forest, 7 m from the workstation in Snæfokstaðir to avoid contact with soil. Contacts between plant groups was also avoided. After resting for 14 days, during which time M3 and M4 were inoculated (see above), 720 seedlings were planted on June 6th and 8th on two locations, Skálmholt and Kluftir, both in south-west Iceland.

2.4 Study area and field experiment

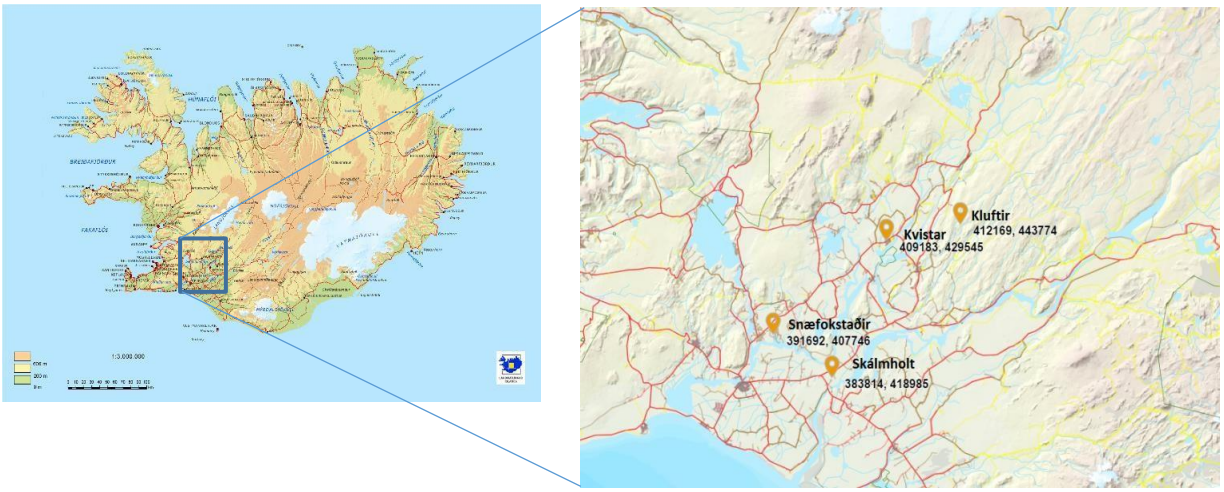


Figure2 Map showing all locations involved in the experiment. Study plots at Skálmholt and Kluftir. Nursery at Kvistar and Snæfokstaðir where spring inoculation took place.

2.4.1 Kluftir

Kluftir is a farm located far inland in south-west Iceland (64° 13' N, 20° 9' W). The area is heathland and been used for grazing for centuries, and was until recently tree- less. The vegetation in the study site is dominated by grasses, low-growing herbs, mosses and lichens (see *Table 2*) The site receives precipitation on average 10 days per month. August has the most precipitation, 13 days and May the fewest, 7 days. The average temperature ranges from -6.5 in January to 7.4 in July (yr.no 2007-2018). The study plot is located on a small hill to avoid water- and frost accumulation.

Photo 4 The study plot at Kluftir, poles mark each row. In total 36 rows. Taken in October 2017

2.4.2 Skálmholt

The second study area is located at Skálmholt, a farm located in south-west of Iceland (63° 56' N, 20° 39' W). The area has, like Kluftir, been used for grazing for centuries. It´s a tree less heathland dominated by grasses, low growing herbs, mosses and lichens (see *Table2*). The mean annual precipitation is 12.5 days per month/year, most days of rain in March (15) and the fewest in June/July (10) and the normal temperature rages from -0.6°C in January to 10.7 ° C in July (yr.no 2007-2018). The study plot is located in a small hill facing South-West, to avoid water- and frost accumulation.

Photo 5 Study plot at Skálmholt located in a SW facing hill, poles mark each row. Taken in April 2018.

Table 2 Vegetation in the study area at Skálmholt and Kluftir. Star market are the most common species.

Latin names	Icelandic names	English names	Skálmh	Kluftir
Grasses	**Grös**			
*Agrostis capillaris**	Hálíngresi	Common Bent	x	X
*Festuca richardsonii**	Túnvingull	Artic fescue	x	X
*Festuca vivipara**	Blávingull	Mountain bunchgrass	x	X
*Luzula multiflora**	Vallhæra	Common woodrush	x	X
Deschampsia cespitosa	Snarrótarpuntur	Tussock		x
Forbs	**Jurtir**			
Alchenilla filicaulis	Maríustakkur	Hairy Lady´s-mantle	X	
*Bistorta vivipara**	Kornsúra	Gray alpine bistort	X	X
Cardamine nymanii	Hrafnaklukka	Lady´s smock	X	
*Galium boreale**	Krossmaðra	Northern bedstraw	x	X
Galium normanii	Hvítmaðra	Bedstraw	x	X
*Galium verum**	Gulmaðra	Lady´s bedstraw	x	X
Ranunculus acris	Brennisóley	Meadow buttercup	X	
Thymus praecox ssp. arcticus	Blóðberg	Creeping thyme	x	X
Mosses	**Mosar**			
*Hylocominum splendens**	Tildurmosi	splendid feather moss	x	X
*Racomitricum squarrosus**	Engjaskraut		x	X
Racomitrium canescens	Hærugambri	Hoary fringe-moss	x	x
Licheins	**Fléttur**			
Cladonia arbuscula	Hreindýrakróki/mosi	Reindeer lichens	x	X
Ferns	**Byrkningar**			
Equisetum pratense	Vallelfting	Meadow Horsetail	x	x

2.4.3 Climate

The climate in Iceland is characterized as oceanic in the lowland but with higher elevation it changes to low-artic. The summers are cool and the winters are mild. Iceland is located where warm and cold ocean currents meet, and warm and cold air often collides near the island. This causes frequent changes in the weather and more rain in the Southern and the Western part of the island (Einarsson 1984).

The long photoperiod during summer reduces the risk of night frost, but towards the end of the growing season the risk rises. According to Einarsson (1984) the mean annual temperature ranges from 2.0°C to 5.7°C in the lowlands. In the southern part of the country the average summer and

winter temperature are lower further away from the coast. (Einarsson 1984). The study area Kluftir might be more exposed to late summer frosts than Skálmholt, due to higher elevation and greater distance from the cost.

Precipitation is closely controlled by the topography and is strongly related to winds that come from the east and south. In southwestern and western parts of the country the precipitation measures 1,000 – 1,600 mm in the lowlands, but 700 – 1,000 mm further inland. Most rain falls in early winter and in autumn with the maximum value in October(Einarsson 1984). At Kluftir and Skálmholt the maximum precipitation falls in August and March respectively.
In the year of

2.5 Field experiment design and cultivation technique

Two identical study plots were laid out at Kluftir and Skálmholt in the spring of 2016. The plots were divided into six blocks, and each block contained 6 rows, representing 6 randomly placed treatment combinations. 10 seedlings of the same experimental unit were placed into each row. Plant spacing between rows was 1 m and within rows was 0.5 m. A total of 360 plants were planted in each study plot.

Planting took place on 6 th and 8th of June 2016 using a planting tube. No site preparation was made and no fertilizer was applied during planting.

2.6 Data collection

2.6.1 Temperature recordings during winter storage

For both winter storage methods, temperature sensors (1-Wire/iButton) were placed among the plants to register temperatures every two hours in the freezer storage and every four hours in the outside storage. In the plant freezer storage, the first sensor was in the middle of the container and the other was at the far end of the container. The sensors that registered temperature at the outdoor plant storage facility at the nursery Kvistar were placed under a multi pot (one sensor), into a plant root plug (one sensor), 1 cm above plant root collars (three sensors) and 8 cm above plant root collars (three sensors).

2.6.2 Data collection in the field

The first field observations were made in September 2016, where total height was measured and the survival of the plants was assessed. Vitality was recorded by classifying seedlings, 0 = dead and 1= alive. Seedling not found were presumed dead. At Skálmholt damages by the Broom mouth (*Melanchra pisi*) were detected and recorded. A second observation was conducted in September 2017 at Kluftir and Skálmholt, where the same observations were made as the year before. The total height of living plants was measured, and if the leading shoot was missing/dead the height was measured to the highest living side shoot. Shoot damages were recorded and additional color assessment was made, where the plants were categorized by needle coloration, 1) Blue-green (normal), 2) Green, and 3) Yellow. Each category represented the health/nutrient status of the seedling, 1 being healthy, 2 showing a little health decline and 3 not healthy (*Photo 4)*.

Photo 4. Color assessment, with a shoot of a healthy sitka spruce for comparison. 1) Blue-green (left) 2) Green (Middle), 3) Yellow (right)

Due to extreme difficulties in finding surviving seedlings at Kluftir, only the first three blocks were assessed for survival and other observations and samplings were omitted.
On the 9 th of October randomly chosen plants were excavated, one from each row (36 in total) in Skálmholt, placed in a plastic bag and moved to the laboratory for further analyses. The roots were gently washed and the root collar diameter was measured. For each plant, the shoot- and the root were detached and weight separately. Root samples were taken (ca. 0.2 g wet weight) and viewed under a dissecting microscope, where mycorrhizal colonization and morphotypes were assessed. After examination, the root subsamples were combined with the corresponding sample. Root and shoot samples were placed in a paper bags and dried at 70°C for 48 hours, to a constant weight, allowed to cool and the dry weight recorded. Roots, shoots, and needles were weighted separately. This data allowed for calculations of plant shoot: root ratio and plant relative needle amount, used for the statistical analysis.

2.7 EM colonization

Each root sample was examined under a dissecting microscope (Olympus S2x9) at around 50x magnification, where EM colonization was assessed by a subsample of approximately 100 randomly chosen root tips. Mycorrhizal root tips were grouped visually into three main morphotypes, each containing some variability. These were, (1) an Asco-type, showing weak root thickening and weak external hyphal growth (Cavender-Bares et al. 2009; Rudawska et al. 2006), (2) a Dark type, showing clear thickening of root short ends and displaying a dark brown – brownish-black coloring. Similar to type ITE.5 described by Rudawska et al. (2006). (3) White-type; showing clear thickening of root short ends and whitish abundant hyphae. Similar to *Hebeloma*-like morphotypes (Rudawska et al. 2006). Non-mycorrhizal root tips were also counted.
For each root sample, the data allowed for calculation of the percentage of total EM colonization and individual mycorrhizal types.

3 Statistical analyses

The program JMP 13.1.0. (SAS Institute Inc.,2016) was used for statistical analysis. A data sheet with statistics collected from Skálmholt with mean-, percent values or relative numbers was prepared. While the seedling height and growth data were values for each plant, means for plant survival, the frequency of shoot dieback and insect damage were calculated from each row of 10 plants, repeated six times for each experimental unit. Data collected from plants excavated in the field, one from each

row, were also values repeated six times (six blocks) for each experimental unit. Standard Least Square analysis was used for the analysis. The independent variables for inoculation were classified into three categories: un-inoculated control plants, plants inoculated in autumn (2015), and plants inoculated in spring (2016). Independent variables also included overwintering methods, i.e. plants stored outside and plants from the freezer storage. Furthermore, the block was also included as an independent variable. The main effects of inoculation, winter storage and block were analyzed, along with the interaction between inoculation and winter storage. In cases where the model effects were significant ($P<0.05$), a Student´s t-test was performed, where each pair of group levels and tests only individual comparisons.

4 Results

4.1 Winter storage

4.1.1 Plant freezer

Means from two temperature sensors recordings from the plant freezer storage are shown in (*Figure 3*). Although the temperature was set for -5 °C, one of the sensors registered temperature down to -5.5 °C frequently during the period 14.01 – 12.02.

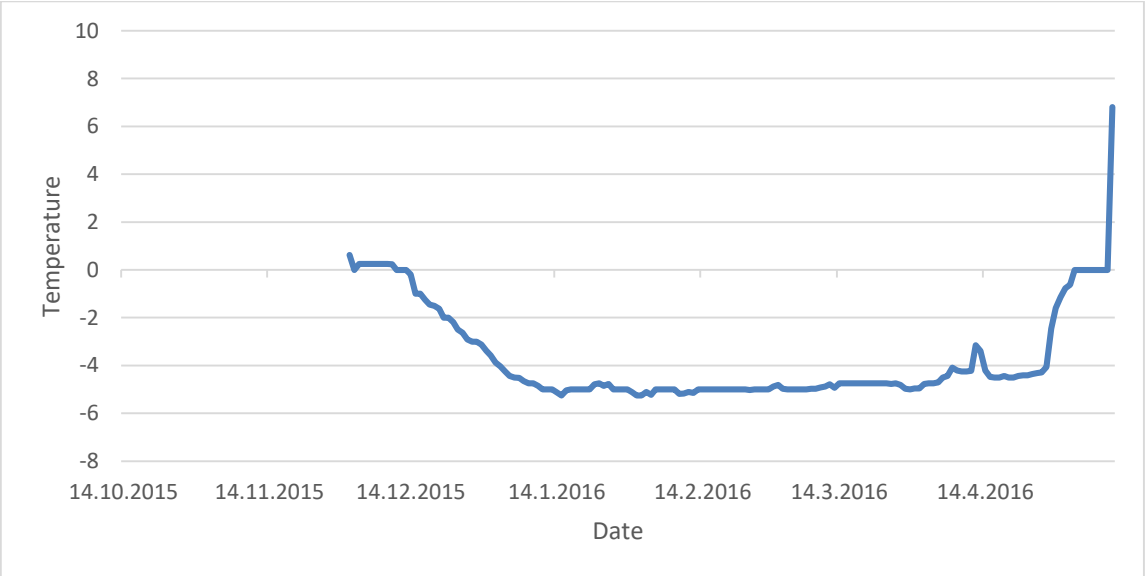


Figure 3 Daily mean temperature registrations from the plant freezer. Registrations of two sensors once every 120 min.

4.1.2 Outdoor winter storage

Figures 4 -7 show temperature readings from sensors at the outdoor storage facility at Kvistar. The sensor placed under a multipot shows that winter temperatures rapidly cooled around the new year and mostly remained around 0°C from January to April, and dropped to a minimum of -2.5°C at 14.1.2016 (*Figure 4*). The temperature readings inside a root plug also show similar trends, but greater fluctuation below 0°C the lowest reaching -3°C at 23.2.2016 (*Figure 5*). Registrations from three sensors 1 cm above the root collar also showed the same trends, though more extreme fluctuations below 0 C° with the lowest registration of -6.5 on 11.1.2016 (*Figure 6)*. The sensors placed 8 cm above root collar show air temperature with the lowest registered temperature of – 14 C° in the evening of 23.2. 2016 and a maximum temperature of 40.5 C° on 9.5.2016 (*Figure 7).*

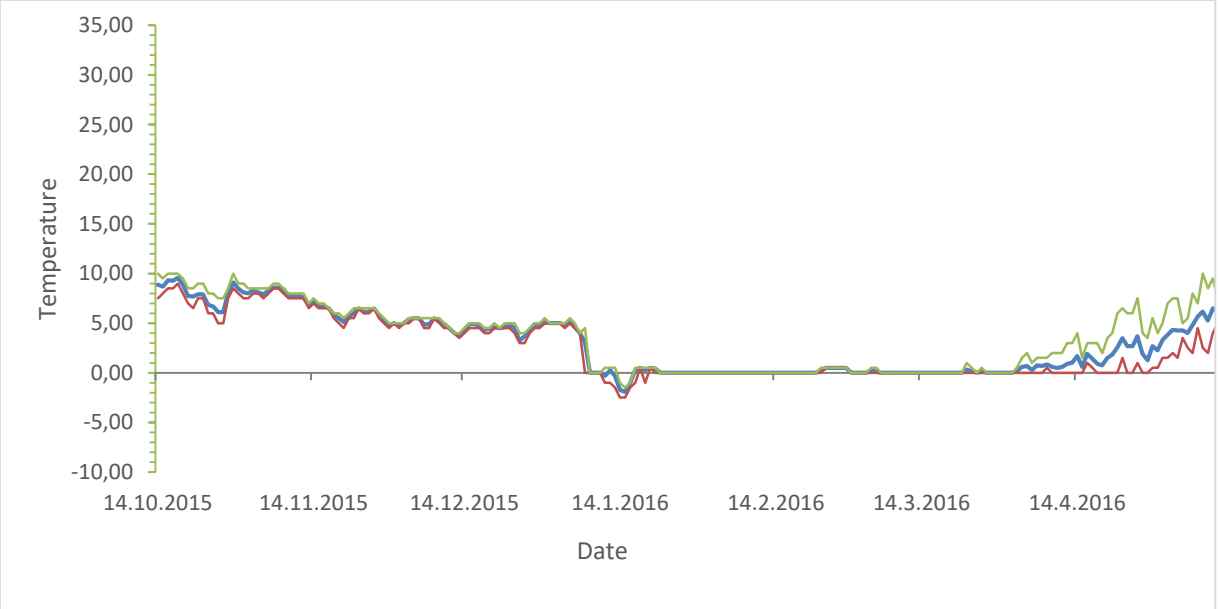


Figure 4 Temperature registrations from out-door winter storage. Sensor placed under the multipot. Blue line represents daily mean temperature, red line represents minimum values and green line represents maximum values.

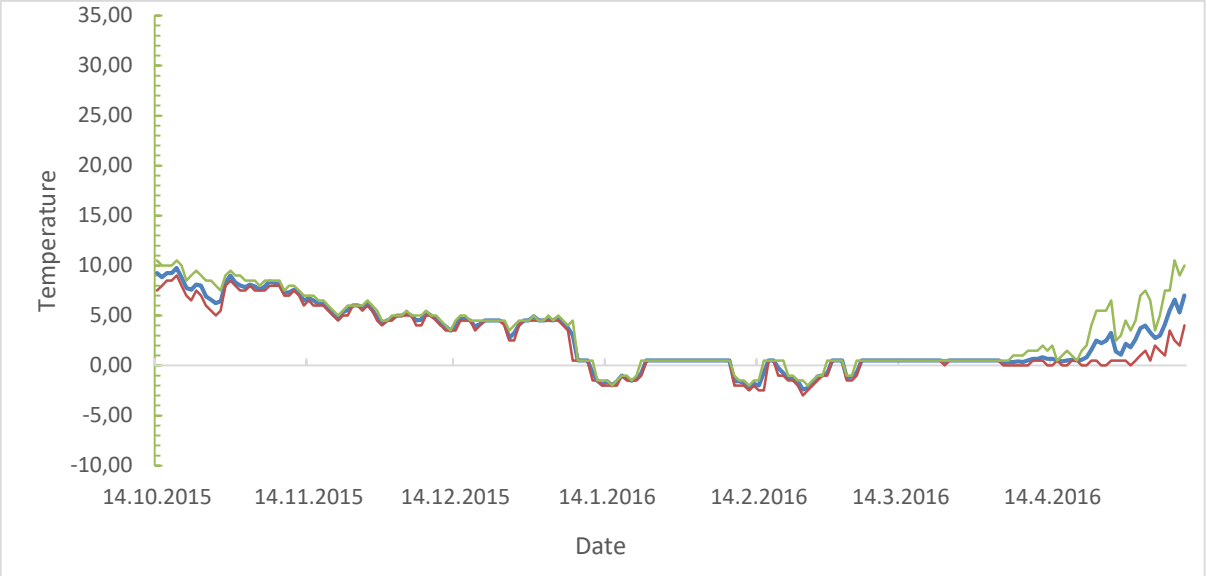


Figure 5 Temperature registrations from out-door winter storage. Sensor placed within the root plug. Blue line represents daily mean temperature, red line represents minimum values and green line represents maximum values.

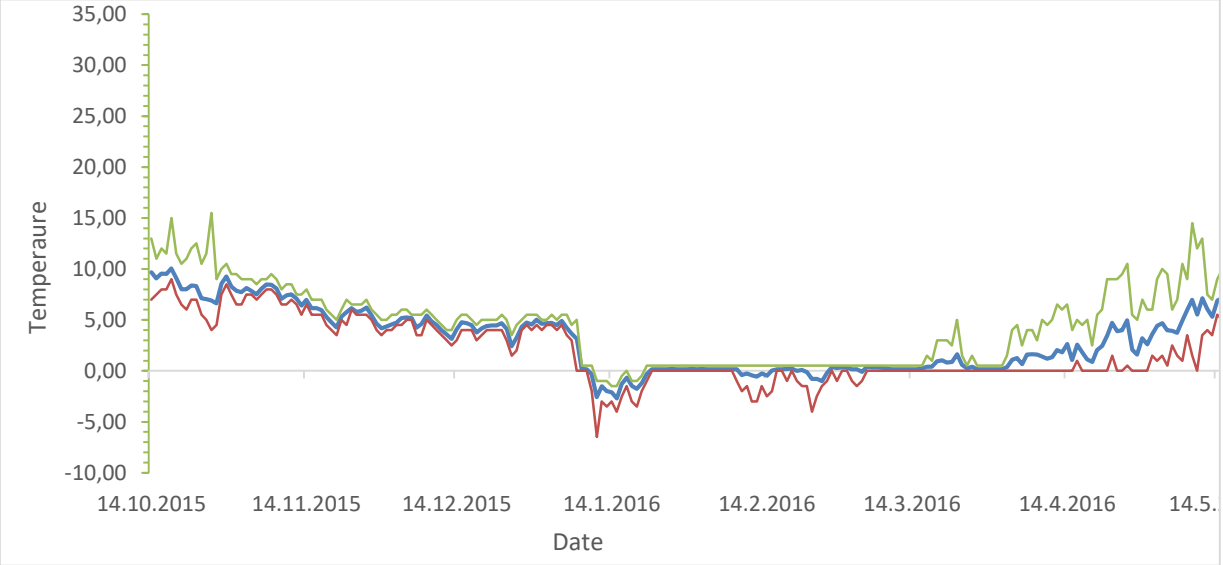


Figure 6 Temperature registrations from three sensors placed 1cm above root collar in out-door winter storage. Blue line represents daily mean temperatures from three sensors, red line represents minimum values and green line is the maximum values.

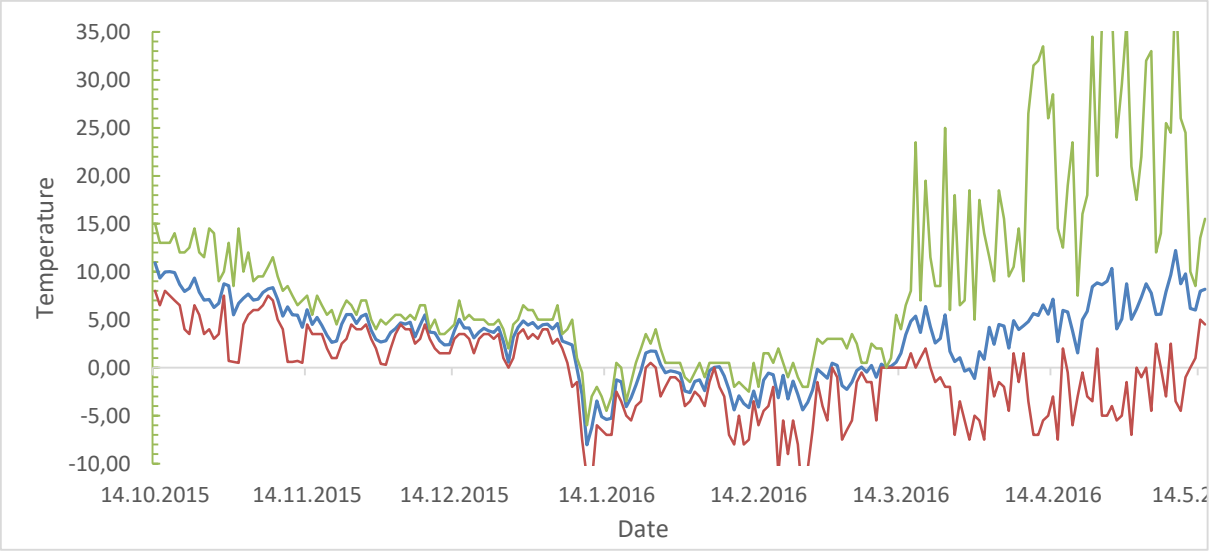


Figure 7 Temperature registrations from 3 sensors, 8 cm above root collar in out-door winter storage. Blue line represents daily temperature means, red line represents minimum values and green line is the maximum values.

4.2 Results from the statistic analysis

4.3 Survival & Growth

At Kluftir only the first three blocks were assessed due to difficulties in finding the seedlings. In those three blocks with a total of 180 seedlings, 102 were considered dead (dead or not found). This gives a survival of 40 %.

The overall seedling survival at Skálmholt was 86% in autumn 2017 and this was not affected by treatment variables. During the first winter in the field around 26 % of plants were affected by shoot dieback. Although the damage had a tendency to be most common among plant from freezer storage this could not be significantly explained by treatment variables.

Seedling growth (i.e. the change in seedling height between 2016 and 2017) was, however, significantly affected by the method of winter storage (P=0,0015). Reduction in seedling height was greater for plants from freezer storage than those stored outside (Figure 8).

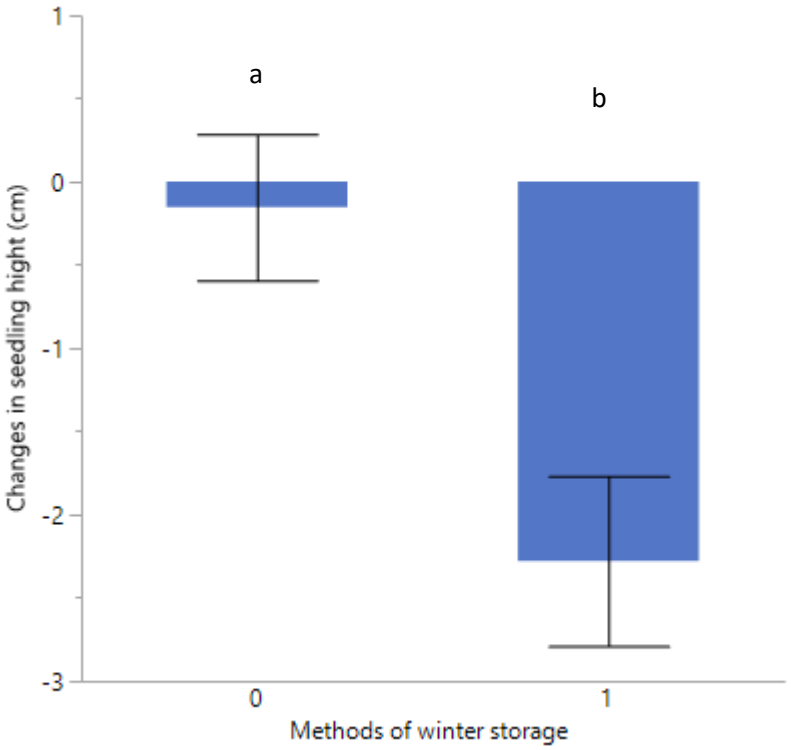


Figure 8 Changes in seedling height between years 2016 and 2017 by methods of winter storage. Methods of winter storage are 0= stored outside and 1= stored in a plant freezer. The columns represent treatments means and horizontal lines indicate Standard Error. Different column letters illustrate significant differences (P<0.05).

4.3.1 Biomass

There was a significant difference in root dry weight by treatment variables. After one year the weight of roots that were stored in plant freezer prior to planting in the field was greater than for those stored outside (P=0.0027) (Figure 9). Inoculation also significantly influenced root dry weight (P=0.0209), plants receiving inoculum in autumn 2015 generally had more root-mass than plants inoculated later (figure 10). The significant interaction of inoculation treatments and storage methods for root dry weight (P= 0.0302), however, revealed that seedlings that were stored in the freezer and inoculated in spring had the greatest root mass (figure 11). Experimental factors did not explain variation in the shoot: root ratio, shoot dry weight, needle weight, plant relative needle amount or root collar diameter.

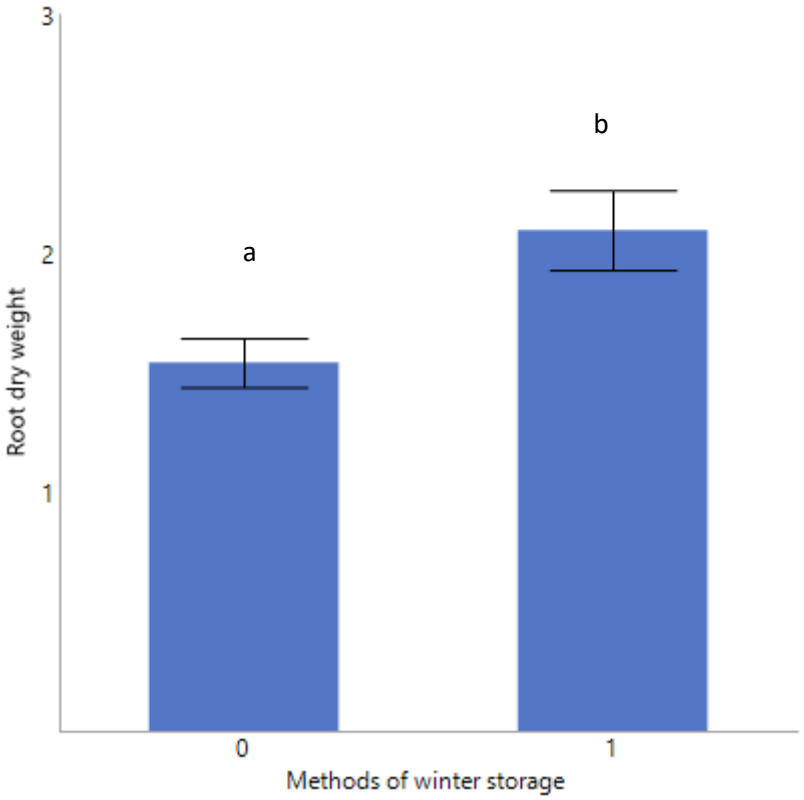


Figure 9 Differences in root dry weight by methods of winter storage. Methods of winter storage are 0= stored outside and 1= stored in a plant freezer. The columns represent treatments means and horizontal lines indicate Standard Error. Different column letters illustrate significant differences (P<0.05).

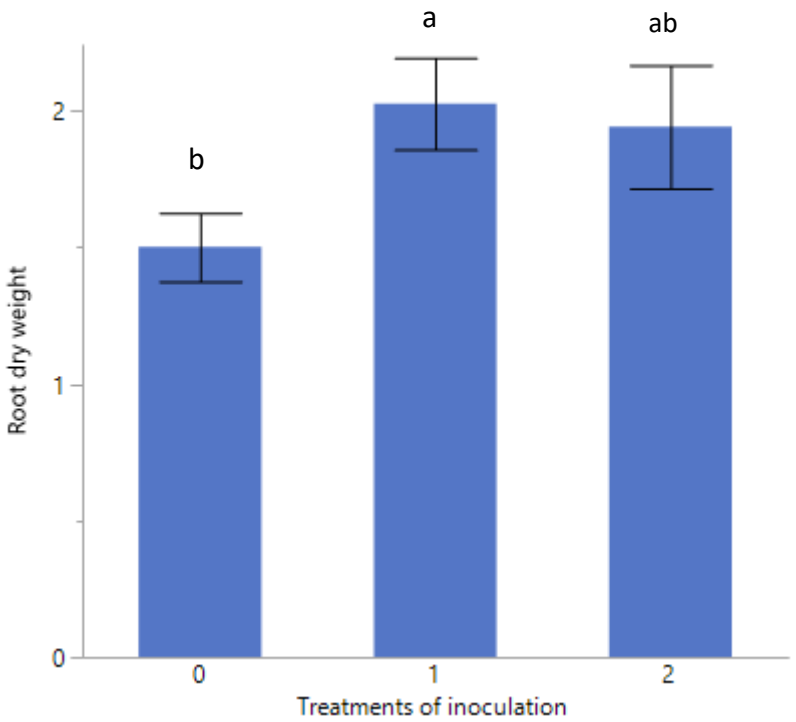


Figure 10 Difference in root dry weight by inoculation treatments. Treatments of inoculation are: 0= no inoculation 1=autumn, 2=spring. The columns represent treatments means and horizontal lines show Standard Error. Different letters above the bars indicate significant differences between inoculation time (P<0.05).

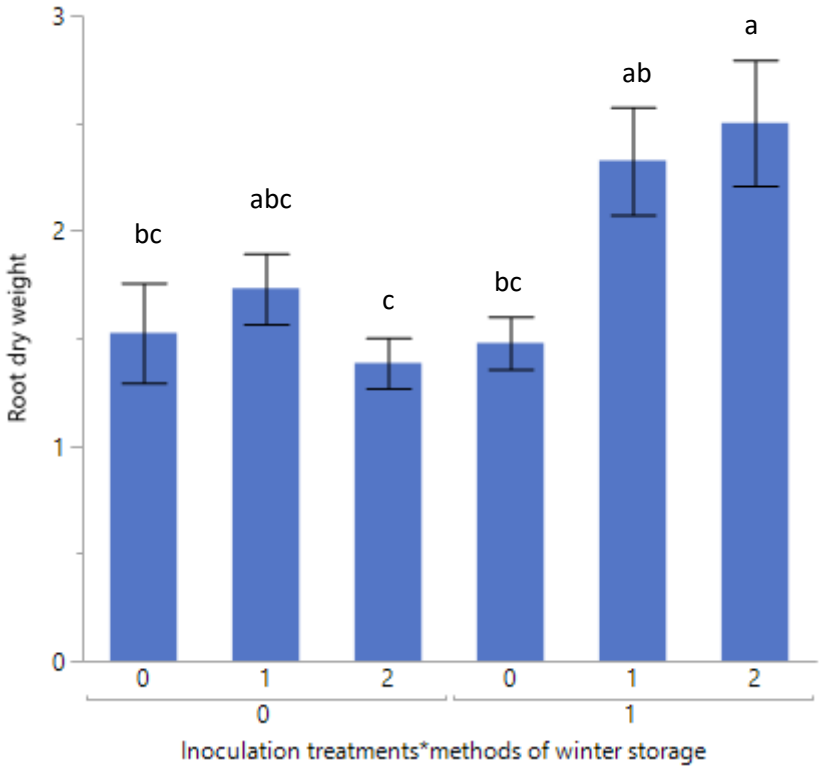


Figure 11 Difference of root dray weight by interaction of methods of winter storage and treatments of inoculation. Methods of winter storage are 0= stored outside and 1= stored in a plant freezer. Treatments of inoculation are: 0= no inoculation 1=autumn, 2=spring. The columns represent treatments means and horizontal lines show Standard Error. Different letters above the bars indicate significant differences (P<0.05).

4.4 Mycorrhiza colonization

There was a significant difference in total mycorrhizal colonization by the interaction of inoculation treatments and methods of winter storage (P = 0.0378). Plants stored outside and inoculated in fall had significantly higher colonization than those inoculated in spring and over wintered the same way. The total mean colonization of Asco-type was 29 % with the minimum of 2 % and a maximum of 70 %. The Asco-type showed a significant difference by winter storage (P = 0.0337) where plants stored in plant freezer had grader relative colonization than those stored outside (*figure 11*). Inoculation also had a significant difference on colonization of Asco-type (P = 0.0079), inoculation in autumn gave lower root colonization than inoculated in spring or no inoculation (*figure 12)*.

The white type was the most commonly identified morphotypes with a total of 45 % colonization of all root samples. Minimum and maximum values varied greatly, from 4 % and 90 % respectively. There was a significant difference in Whit-type colonization by inoculum treatments (*figure 13*), where plants inoculated in autumn had greater colonization than those inoculated later or not inoculated

(P = < .0001). The interaction of winter storage and inoculation also had a significant influence on colonization of White-type (P = 0.0023) where non inoculated plants stored in the freezer had the lowest colonization (*figure 14*).

The least common morphotype was the Black-type with mean colonization of 10 %, with a minimum of 0 % and a maximum of 66 %. Inoculation was the only variable that had a significant difference on Black-type (P = 0,0178), where non treated plants had the greatest colonization (*figure 15*).

Table 5 Mean colonization degree of each morphotype. Minimum and Maximum values show the wide range of colonization.

	Mean Colonization (%)	Min Value (%)	Max value (%)
Asco-type	29	2	70
White-type	46	4	90
Black-type	10	0	66

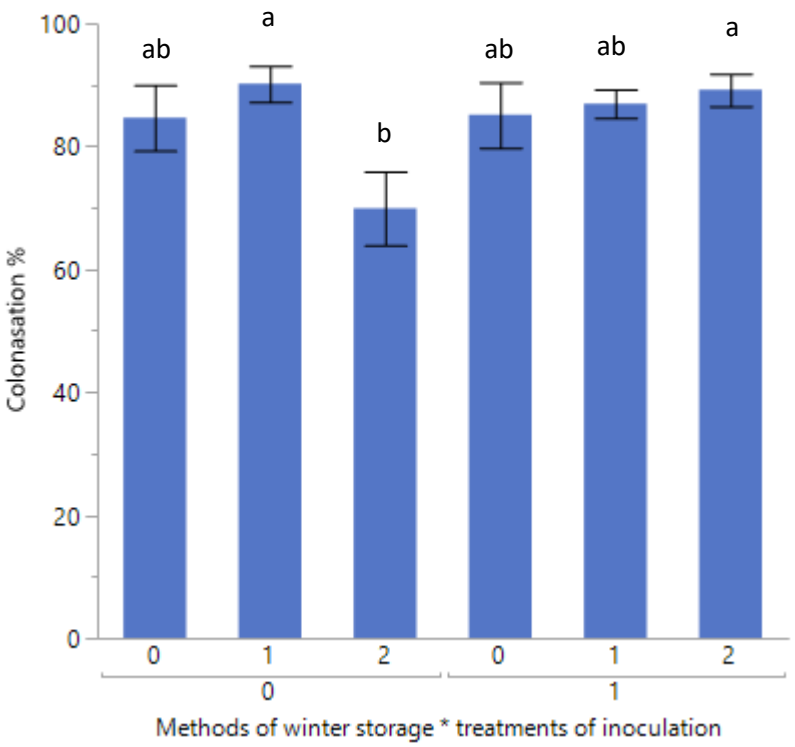


Figure 12 Difference in total root colonization by the interaction of winter storage and treatments of inoculation. Methods of winter storage are 0= stored outside and 1= stored in a plant freezer. The columns represent treatments means and horizontal lines indicate Standard Error. Different column letters illustrate significant differences (P<0.05).

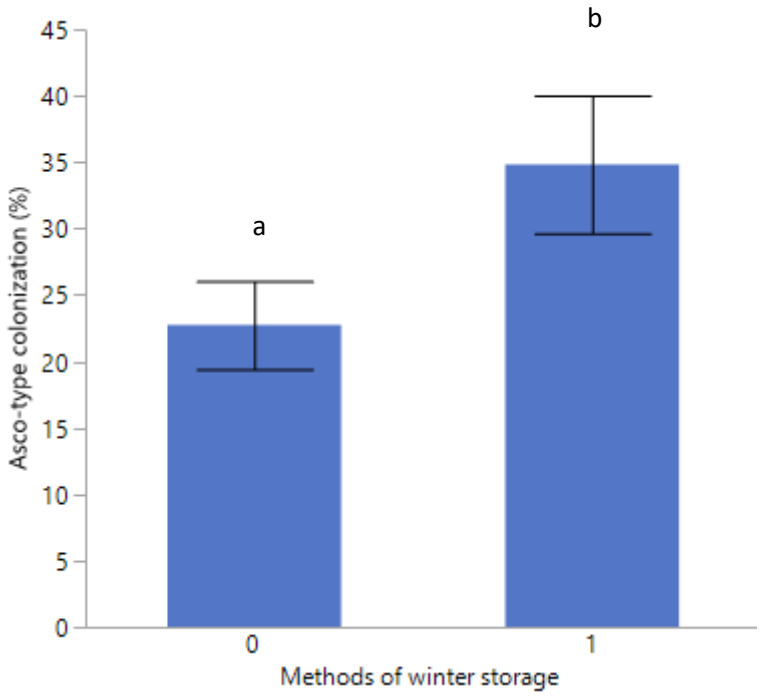


Figure 13 Difference in colonization of Asco-type by winter storage. Methods of winter storage are 0= stored outside and 1= stored in a plant freezer. The columns represent treatments means and horizontal lines indicate Standard Error. Different column letters illustrate significant differences (P<0.05).

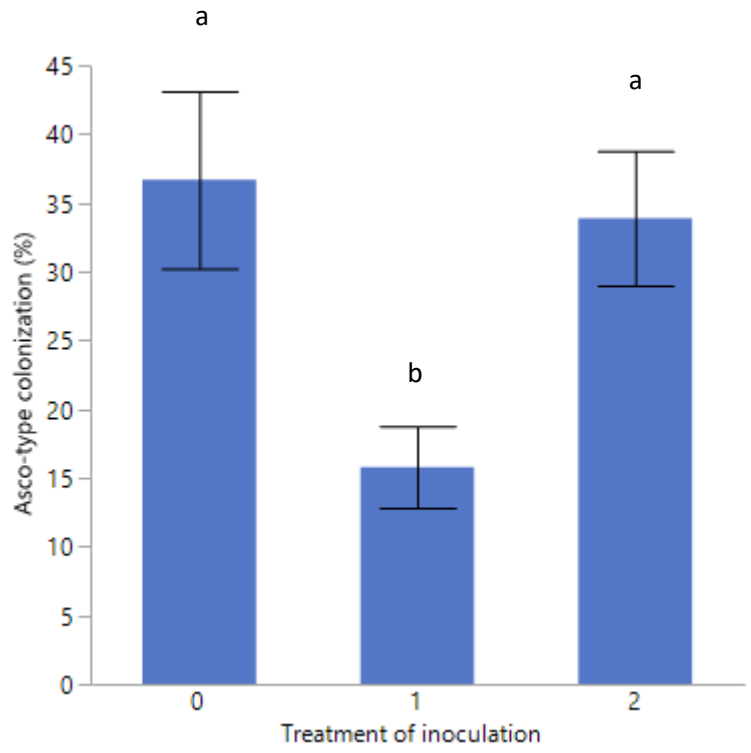


Figure 14 Difference in colonization of Asco-type by treatments of inoculations. Treatments of inoculation are: 0= no inoculation 1=autumn, 2=spring. The columns represent treatments means and horizontal lines show Standard Error. Different letters above the bars indicate significant differences (P<0.05)

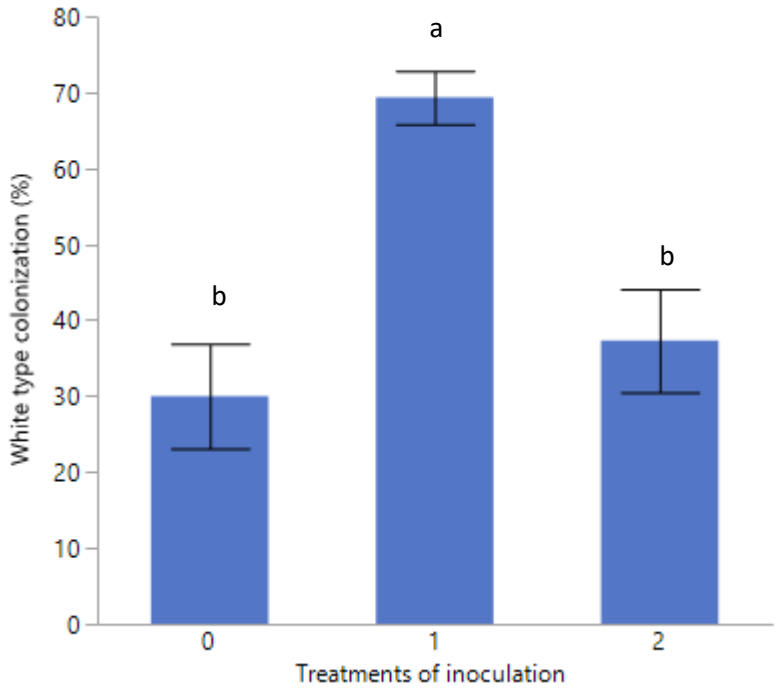


Figure 15 Difference in White-type colonization by treatment of inoculum. Treatments of inoculation are: 0= no inoculation 1=autumn, 2=spring. The columns represent treatments means and horizontal lines show Standard Error. Different letters above the bars indicate significant differences (P<0.05).

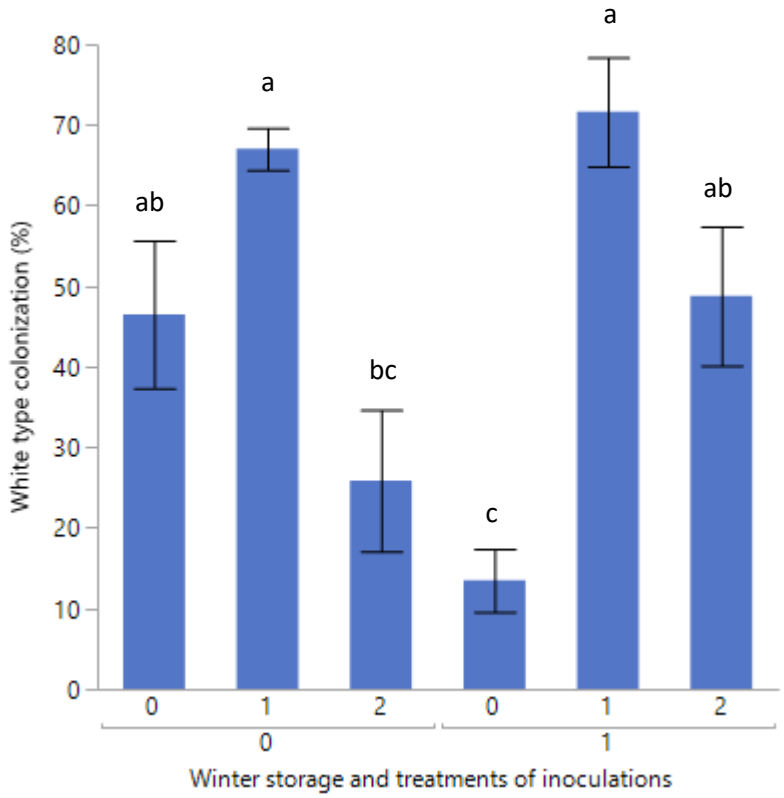


Figure 16 Difference of colonization of Whit-type by interaction of methods of winter storage and treatments of inoculation. Methods of winter storage are 0= stored outside and 1= stored in a plant freezer. Treatments of inoculation are: 0= no inoculation 1=autumn, 2=spring. The columns represent treatments means and horizontal lines show Standard Error. Different letters above the bars indicate significant differences (P<0.05).

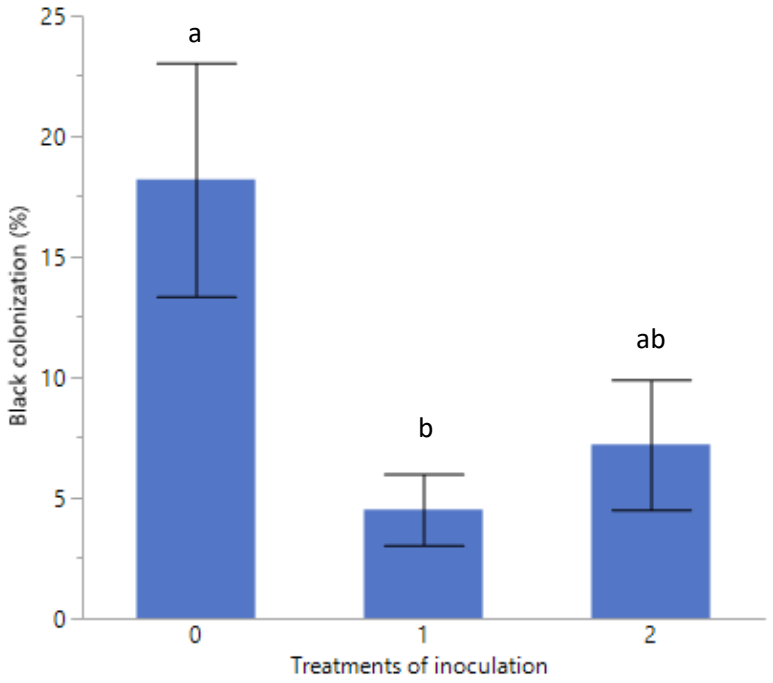


Figure 17 Difference in Black-type colonization by treatments of inoculation. Treatments of inoculation are: 0= no inoculation 1=autumn, 2=spring. The columns represent treatments means and horizontal lines show Standard Error. Different letters above the bars indicate significant differences (P<0.05).

5 Discussion

5.1 Methods of winter storage

The poor survival (40%) at Kluftir in 2017 can´t be explained with certainty however this is a common survival percentage in new afforestation areas of (Eggertsson 2004; Snorrason 2007; Þórsson 2008). No site preparations could also be the cause of low survival as was the results of the experiment by Pennanen at al.(2005).

The overall survival of seedlings at Skálmholt was 85% after the first winter which can be considered very good results when comparing to Kluftir and national survival rates. Even though survival vas very good at Skálmholt, the growth difference between years was negative. This negative growth was influenced by shoot dieback where 26 % of the plants were affected. One factor influencing the dieback could be the Broom mouth larva (*Melanchra pisi*) which was recorded in autumn of 2016. About 50 % of the seedlings were affected. There was also a tendency, though it could not be statistically explained, for plants stored in plant freezer to show more dieback than those stored outside. There are several reasons that can cause this, 1) Desiccation in the freezer storage or in the field. Fine roots suffer most when plants are deprived of water and can inhibit root growth

significantly. In this experiment, seedlings were packed for freezer storage with the shoots exposed which can lead to loss of water. Low water content in shoots can damage the needles which can influence performance after planting (Colombo 1990). 2) Nutrient deficiencies. For plants to resist the stress freezer storage inflicts they need to have enough nutrient reserves. These reserves can be depleted during storage by respiration and other metabolic processes (Camm et al. 1994). According to Wang and Zwiazek (1999), starch content of roots of White spruce decreased with storage duration. Reduction of root non-structural carbohydrates (TNC) is also related to storage duration (Martens et al. 2007; Wang & Zwiazek 1999). In this experiment, no fertilizer was given in the field which could have made the plants vulnerable for stresses the flowing winter. 3) The temperature in freezer storage. Wang and Zwiazek (1999), showed also that White spruce seedlings stored for seven months at -6 C° had much higher electrolyte leakage than seedlings stored at -4 C° and -2 C°. The root growth potential (RGP) was also lower for seedlings stores at -6 C° for seven months. In my study, the seedlings were stored for five months at -5 C° $\pm$ 0.5 which could be too cold for too long for this hybrid/provenance even though Spruces are considered to be very frost tolerant. 4) Storage duration. Although five months of storage is common in Scandinavia there are studies that show that the longer the freezing period the more risk of damages. In the study by Martens et al. (2007) containerized aspen dieback and low RGP was related to storage duration (150 days). They suggest 75 days of cold storage is the optimum for aspen. As said before nutrient reserves can get used up if storage duration is long and the seedlings can´t resist stresses after planting as well as plants packet with nutrients. It is possible that the 26% dieback in this study indicates that five months of storage is too long for this provenance of Lutz spruce. More studies are needed to fully understand the effect duration and temperature in freezer storage have on seedlings of Lutz spruce.

Photo 6 (Left) Kluftir: Seedling dieback, more than half of the shoot shows signs of damage.(Right) Skálmholt: Lower part of the shoot is naked possibly caused by Broom mouth larva.

5.2 Inoculation treatments

To realistically explain the observed treatment effects on plant root growth, it is necessary to take notice of the interaction between inoculation and winter storage methods. This interaction reveals that the winter storage methods were not at all responsible for the differences observed. However, it is one of the methods of inoculation that obviously was influencing the root growth. While the spring inoculations revealed contrasting effects for plant storage methods, this effect seems to have nothing to do with plant storage. It is, however, the different inoculation methods that are likely responsible. In one case the plant´s multi pots are placed on a layer of soil inoculum and in the other case the plant roots are soaked in a water soil inoculum suspension. It is the latter inoculation method that is clearly responsible for the observed treatment effect.

It has been estimated, for ECM to have an effect on survival and growth there is a minimum of 50 % colonization degree (Marx et al. 2002). In this experiment, the mean colonization was 84 %, which is well above the estimate.

According to a study by Palfner et al. (2005), where they studied mycorrhizal community in sitca spruce stands of different ages, the young seedlings had the highest number of non-mycorrhizal root

tips. The Asco-type was most frequent on the non-inoculated seedlings which tells me that most likely this type is formed by fungi already spreading among nursery seedlings. According to Dahlber (1990) and Pennanen et al (2005) it is not uncommon that nursery fungi dominate seedling roots in the first growing season. The black-type was also mostly found on non-inoculated species like the Asco-type indicating that this is also a nursery fungus. The white-type was most frequent on seedling inoculated in autumn independent of winter storage methods. For plants stored outdoors over winter, the white type also was predominant, indicating that the fungi responsible could be present in the substrate of the outdoor facility.

The effect of inoculation experiments has not been straightforward. Some show immediate survival and growth improvement (Halldórsson et al. 2000; Jonsson et al. 2001; Óskarsson 2010) in others it is not so clear (Stenström et al. 1990). The effect is clearly dependent on soil conditions, fungal species, tree species and time (Smith & Read 2008). Mycorrhiza is found on actively growing lateral roots and as the tree matures the proportion of active root tips declines and thereby species richness also reduces with tree age.

Like in the plant kingdom there are successional states of mycorrhizal fungi. There is a change in species composition over the trees life stages. Young trees with active juvenile roots have greater species richness of mycorrhizal fungi than older trees with more senescent root tips (Palfner et al. 2005). Although morphotypes were only classified into three groups in this study, a more variety could have been discovered through a more detailed examination. Seedling growth responses due to mycorrhizal inoculation were not very pronounced in the study, although indications were found mycorrhizal benefits. It is plausible that the soil from the 60-year-old stand used as an inoculant didn´t have the early successional fungal species. In Hrafkelsdóttir master thesis (2009), she found that there were significantly less mycorrhizal root tips on roots grown in soil from a tree-less land, and more mycorrhizal root tips on roots grown in forest soil form a young stand than from a more mature one. There is also a question of time, Óskarsson (2005) found after the first growing season there was no significant growth or survival effect after inoculation of *Pinus cordata and Larix sibirica,* and there was low mycorrhizal colonization. However, after 12 years the difference was significant between inoculated and non-inoculated trees where soil inoculated trees were much taller than those that didn´t receive inoculation or were treated with commercial inoculum.

6 Conclusion

There are clear indications in the present study of effects on Lutz spruce by mycorrhizal inoculation although the early benefits were not decisive. Also, methods of applying the inoculum are clearly important. Some of the results obtained are encouraging for future development if inoculation techniques and studies on inoculum selection.

Freezer storage has proven to be helpful in reducing root damages if the temperature isn´t too low and the duration isn´t too long, and this varies with species. In the present study, indications are that five months at -5 C° might be too low for too long for Lutz spruce. Here is a need for further experiments and evaluation of plant winter storage methods.

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