Spring 2023

Impact of Riverbank Lupine (Lupinus rivularis) on Grand Fir (Abies grandis) Ectomycorrhizal Symbioses

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Recommended Citation
Labay, Andrew, "Impact of Riverbank Lupine (Lupinus rivularis) on Grand Fir (Abies grandis) Ectomycorrhizal Symbioses" (2023). *WWU Graduate School Collection*. 1165.
https://cedar.wwu.edu/wwuet/1165

Impact of Riverbank Lupine *(Lupinus rivularis)* on Grand Fir (*Abies grandis*) Ectomycorrhizal Symbioses

By

Andrew Labay

Accepted in Partial Completion
of the Requirements for the Degree
Master of Science

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Master's Thesis

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1/30/2023

Impact of Riverbank Lupine *(Lupinus rivularis)* on Grand Fir (*Abies grandis*) Ectomycorrhizal Symbioses

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

by
Andrew Labay
May 8, 2023

Abstract

Lupinus rivularis, or riverbank lupine, is used in the revegetation of nitrogen-poor soils surrounding the Elwha basin in Washington State due to its ability to improve soil conditions by fixing nitrogen, deterring erosion, and adding organic matter. Previous research illustrated that seeding lupine with conifers increased conifer growth and foliar nitrogen, however, a significant decrease in vital below-ground fungal symbionts, commonly referred to as ectomycorrhizal fungal symbionts (ECM), was observed. ECM fungi are plant symbionts that access and transfer nitrogen and water to conifers in exchange for carbohydrates. Therefore, a decrease in ECM may be due to either lupine increasing soil nitrogen, or neighboring roots retaining nutrients and moisture, thus negating the plant's need for carbohydrate allocation to maintain a fungal symbiont. A field survey and a greenhouse experiment were conducted to investigate the effect of lupine and synthetic nitrogen on ECM and the growth of grand fir (*Abies grandis*). The field study was designed to provide insight into how ECM is correlated to lupine cover and to document any subsequent effects on conifer growth. To accomplish this, grand firs were planted under variable lupine cover in 2017 and then surveyed in 2020. We measured ECM colonization of grand fir roots and identified the colonizing fungal species. We also measured conifer height, basal diameter, and distance to an established forest edge. Our second study was a controlled greenhouse experiment designed to test the hypothesis that the nitrogen added by lupine is the primary factor driving a decrease in ECM symbioses. To answer this question, grand fir trees were grown in the greenhouse under four treatments: 1) synthetic nitrogen, 2) nitrogen-fixing lupine, 3) non-nitrogen fixing plants, Oregon sunshine (*Eriophyllum lanatum*) and Blue Wild Rye (*Elymus glaucus*), and 4) a control. All treatments were grown under identical watering regimes and contained soil inoculum from the field in an effort to ensure similar soil community starting points to the field. At the end of the study, we measured ECM colonization, conifer height, basal diameter, dry biomass, and foliar nitrogen content. In the field, ECM colonization was negatively correlated with lupine cover (r = -0.46, P = 0.02), and conifer height was negatively correlated with distance to the edge of an established forest (r = -0.45, P = 0.01). We did not detect any correlation between lupine cover, conifer height, or basal diameter. In the greenhouse, ECM colonization decreased significantly within the nitrogen treatment (χ^2= 116.2, df = 3, P >0.001) while neither basal diameter nor height differed among treatments. Lupine had no effect on ECM colonization in the greenhouse, however, conifers growing within the lupine and competition treatments had significantly more foliar nitrogen in their needles than in the nitrogen or control treatments ($F_{(3,28)}$ =8.92, P<0.001). Root mass, shoot mass, and root: shoot ratio did not vary among greenhouse treatments. Ectomycorrhizal fungal species richness was greater in the field study than in the greenhouse with the most abundant species in both studies being *Thelephora terrestris* and *Wilcoxina mikolae*. While the field study demonstrated a negative correlation between ECM colonization and lupine, the greenhouse study did not. The greenhouse study illustrated that increased synthetic N can reduce ECM abundance, supporting the hypothesis that nutrient conditions play a role in maintaining ECM symbiosis when water is not a limiting factor, however, this relationship was not observed in the field. Overall, we find that while synthetic nitrogen additions decrease ECM colonization on grand fir root tips, many other biotic and abiotic factors likely contribute to the dynamics of ECM symbioses in the field. Namely, our study highlights that the presence of roots increases N uptake, suggesting that companion planting with native species can increase nutrient acquisition for conifers growing in these coarse sediments, which could help to inform restoration efforts.

Acknowledgments

Thank you to all who gave me their time as I worked through the process of becoming an academic, an adult, and a scientist. The opportunities afforded me at Western opened my eyes to the great art of scientific inquiry, the ceaseless curiosity of the human spirit, and how truly deep the rabbit hole goes. I would like to express my undying gratitude to all my committee members and their limitless patience as I dealt with balancing real-life responsibilities and those I have as a scientist working towards a common goal. To Rebecca Bunn, I thank you for allowing me to clutter your lab with bags upon bags of tree parts and giving me a huge amount of support for the greenhouse portion of this study. To my chair, Jenise Bauman, I consider you a stout ally and mentor; I look forward to bothering you endlessly as I move forward in my academic career.

This work would not have been possible without the contributions of many volunteers, Austin Kmetovic, Emma Kolden, Olivia Taylor-Manning, and Liz Zimmerman, thank you all for all the hours payed and mountains of sediment I made you all collectively move.

Most of all, I would like to thank Western Washington University and its incredible Environmental Science program for giving me the tools to make a contribution and see the natural world through the eyes of a scientist. This work was funded in part by the Brooks Family Foundation, and without them, the findings presented here may never have been published; I extend to them my deepest gratitude.

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Introduction:

The Pacific Northwest hosts diverse forests and rivers that commonly intersect environments that support a grand array of both aquatic and terrestrial life. Much of the research done in the past century examined how human activities impacted these natural systems, and how we may best balance human progress with the preservation of these dynamic, living communities. Among these were the Glines Canyon and Elwha dams, which while providing hydroelectric power and recreation, were deleterious for local anadromous fish populations (Petts, 1984). Further, these dams dramatically changed the hydrogeology of the region by denying downstream ecosystems of vital sediments while also destroying the food webs and migration paths required to support salmon populations (Shaffer et al., 2017). After decades of advocacy by local tribes and ecologists to get the dams removed, the deconstruction commenced in 2014. Once the dams were decommissioned, 14 million cubic meters of sediment were released and the watershed, nearshore, hydrogeology, and ecology of the Lower Elwha River were transformed (Chenoweth et al., 2011).

Much of the released sediment was quickly transported to the nearshore and the riparian floodplains, and the area of the Elwha delta increased to over 150 hectares. In addition to nearshore impacts, the spatial range of many aquatic species greatly increased following dam removal, with eight anadromous species observed upstream of the Elwha dam 2.5 years after dam deconstruction (Shaffer et al., 2017; Duda et al., 2021). Other sediments that did not make it to the nearshore settled along the riverbank, reshaping the landscape and impacting vegetation (Shafroth et al., 2002). Prior to dam removal, riparian and forested ecosystems typical of the Pacific Northwest could be found on the banks of the Elwha (Duda et al., 2008). In lower elevations, these forested communities were typically dominated by large conifers, predominately *Pseudotsuga menziesii*, *Thuja plicata*, *Tsuga heterophylla,* and *Abies grandis*. Following the massive influx of sediment, these previously established forested riparian communities became novel sediment terraces disconnected from the water table and lacked almost all biological legacy (Prach et al., 2019). It has been demonstrated that these ecologically disturbed forest sites perform significantly fewer ecosystem services such as erosion control, habitat production, and water filtration and contain reduced species diversity (Chazdon, 2008). Therefore, the remediation of these ecologically degraded sites represents a crucial endeavor to revitalize native forest and river systems in an effort to rehabilitate native species diversity, suppress invasives, and stabilize ecosystem processes (Chenoweth, 2011).

Upland forests are an important buffer against sediment erosion along river banks, resulting in increased water quality while controlling water flow and providing shade to moderate stream temperatures (Beschta, 1997; Neary et al., 2010). Consequentially, in the absence of preserved forested areas, the diversity of aquatic and terrestrial species is adversely impacted (Corbacho et al., 2003). In particular, salmonid species rely heavily on large woody debris (LWD) sourced from conifers in riparian zones for both refuge and habitat complexity. In Pacific Northwest stream ecosystems that support these salmonids, downed conifers create some of the longest residency time LWD, which also shapes the hydrogeology of the river itself, producing plunge pools, redirecting and slowing flowing water, and exerting influence on localized sediment deposition (Naiman et al., 2000; Balian and Naiman, 2005). As such, in degraded forests such as in the novel dewatered terraces of the Lower Elwha River basin, getting these ecologically valuable trees to quickly establish is an area of active research.

Spontaneous revegetation of the dewatered sediments has not occurred consistently throughout the watershed. Tree recovery has been driven by substrate; areas with fine sediments and organic matter were fast to revegetate with pioneer willows, cottonwoods, and alder, where coarse sediments required active revegetation (Prach et al., 2019; Chenoweth et al., 2021). Low amounts of soil organic matter (OM), low nutrient availability, and low water holding capacity are the greatest hurdles to establishing conifers in the coarse sediments of these novel terraces, as low nitrogen can lead to reductions in photosynthetic rate and growth (Lupi et al., 2013). The intense summer droughts and desiccating winds create conditions that are particularly lethal due to the substrates low water holding capacity (Chenoweth et al. 2011). Additionally, as these are essentially novel sediments following dam removal, they lack any form of biological or mycorrhizal legacy which has been shown to help support establishment (Cortese and Bunn 2017; Kałucka and Jagodziński, 2017; Nara and Hogetsu, 2004; Deyn et al., 2004; Walker and del Moral 2003). If a stable forested community containing conifers is to be established, direct remediation efforts may be necessary to create environmental conditions that are more supportive of their historical populations.

To mitigate nitrogen stress, riverbank lupine (*Lupinus rivularis)* was directly seeded across the coarse sediments. Lupine is considered a pioneer species and is able to grow in nutrient-poor conditions and was one of the first arrivals in the pyroclastic sediments produced in the eruption of Mount St. Helens. Riverbank lupine is a nitrogen-fixing plant sourcing bioavailable nitrogen from its *Rhizobium* symbiont present in root nodules and thus does not rely on soil-derived nitrogen, and upon decomposition, increases levels of OM and bioavailable nitrogen in the soils they occupy (Morris and Wood, 1989). Conifers growing next to neighboring lupine can have increased height, root collar diameter, and foliar nitrogen (Mauer et al., 2013). In addition to ameliorating bioavailable soil nitrogen stress and increasing OM, lupine may benefit conifer seedlings by blunting desiccating winds, stabilizing temperature fluctuations through shading, and increasing moisture availability (Morris and Wood, 1989).

Previous field research conducted along the novel terraces of the Elwha suggests that companion seeding of lupine with conifer saplings increases conifer growth and foliar nitrogen content (Kardouni 2020). Kardouni (2020) demonstrated that conifers growing in plots containing a moderate amount of lupine had the highest height:diameter ratios, while those growing in plots containing sparse amounts of lupine had the lowest. Foliar nitrogen was highly correlated with lupine abundance as well, with conifers growing in plots that were densely populated with lupine having the greatest foliar N content, while conifers growing in plots with sparse lupine had the least. The data collected during their study provided evidence that companion planting with lupine has measurable benefits for establishing conifers.

These increases in growth were accompanied by a corresponding decrease in root tip colonization by ectomycorrhizal fungi. Ectomycorrhizal fungi are a prominent component of the rhizosphere in forests and perform a variety of functions that ultimately contribute to survivability, resilience, and plant establishment (Smith and Read, 2008). Ectomycorrhizal fungi are a broad class of fungi with a high affinity for colonizing the roots of forest trees. The fungi allow for greater access to water and soil nutrients and in return receive carbon in the form of photosynthates from the plant (Anderson and Cairney, 2007). What distinguishes them from other classes of symbiotic fungi is that they form a fungal sheath around the root, produce extensive soil-radiating hyphae, and contain a Hartig Net (Smith and Read, 2008). This partnership between forest trees and ECM fungi allows for increased recruitment rates for establishing conifers and has been demonstrated to correlate with increased conifer growth metrics and alleviate low nutrient stress (Smith and Read 20008). The fungi also help establishing conifers have increased resilience to pathogens through the production of antibiotics, creating a physical barrier to infection through the fungal sheathe, as well as priming plant immunity (Ferlian et al., 2018, Jung et al., 2012).

Other studies have directly identified a positive correlation between ECM colonization and the health and survival of outplanted seedlings (Menkis et al., 2011, Nara and Hogetsu, 2004, Ortega et al., 2004, Garbaye and Churin, 1997). Nara and Hogetsu (2004) documented the health of newly outplanted *Salix reinii* seedling on the novel pyroclastic sediments of Mt. Fuji and was able to identify that colonization by ECM increased seedling growth and nitrogen content. In terms of seedling survivability, Menkis (2011) examined the efficacy of a mycorrhizal cultivation system for outplanted *Picea abies* seedlings by comparing a bare root treatment against a mycorrhizal inoculation and found that seedlings that were inoculated with ECM had significantly higher survivability and increased growth following a two-year growing period.

Given all the benefits ECM colonization confers to seedlings discussed above, this increase in growth in conjunction with the decrease in colonization recorded by Kardouni (2020) seems counterintuitive. One proposed framework that can explain this disparity lies in resource limitation and resource allocation influencing the relative benefit of maintaining or forming symbioses. This hypothesis draws from a functional equilibrium model, which posits that to be competitive, an organism must be maximally efficient in its allocation of internal resources such that all nutrients are equally limiting. In other words, a competitive organism will utilize what it has in abundance to acquire what is limiting to its growth and reproduction. In this way, organisms that are most efficient at striking a resource balance in their respective environments are strongly selected for (Bloom et al., 1985). Additionally, it has been proposed that resource limitation may be an important driver among organisms involved in nutritional symbioses and plant-soil feedbacks (Revillini et al. 2016). In terms of nutrient economics, studies have shown that investing carbon into N-fixing symbionts is comparatively more costly than acquiring N from soils (Perkowski et al., 2021; Rastetter et al., 2001). When two possible sources of N are present, plants will allocate carbon to the less costly source (Gutschick, 1981). Further, Hobbie (2006) showed that carbon allocation to ECM symbionts was highest when nutrients in the soil were limiting and plant growth was slowest in culture.

It has been hypothesized that this mutualistic balance exists on an environmental continuum and is maintained when both partners receive benefits within the symbiosis (Johnson et al. 1997). Under this paradigm, observing a decrease in ECM on the root tips of conifers may be due to lupine decreasing nutrient stress by increasing bio-available forms of nitrogen in the early years of dewatering the reservoir, thereby negating the need for a fungal partnership. This would then benefit the host plant by satisfying the N requirement while eliminating the carbon cost expended to maintain that fungal symbiont. Further, in sites like the Elwha where the lack of fungal propagules and organic matter may limit the formation of functional mycorrhizas (Cortese and Bunn 2017), companion seeding with lupine would relax the need for mycorrhizal symbioses in the early years of tree establishment.

In many environmental contexts, increased levels of available nitrogen have been shown to reduce ECM colonization and species diversity in deciduous species (Treseder 2004; Newton and Pigott, 1991; Lilleskov et al. 2008). In a meta-analysis, Treseder (2004) was able to determine that N additions decreased mycorrhizal abundance by approximately 15%, while elevated CO_2 conditions increased abundance by 47%. In agreement with those findings, Newton and Pigott (1991) planted English oak (*Quercus robur*) and birch (*Betula pendula*) on two woodland sites that contained nutrient-poor soils. After one growing season, both species demonstrated reduced ECM colonization following nitrogen additions. In terms of species composition, in a large-scale N deposition study, Lilleskov et al. (2008) determined that as N concentrations in root tips increased, *Piloderma* spp, *Cenococcum geophilum,* and other ECM species declined, while other smooth mantle morphotypes increased resulting in an altered community structure over a broad geospatial scale.

Given the amount of support for high nitrogen environments resulting in reduced ECM colonization and growth, we propose that functional equilibrium models are especially applicable to early successional sediments like those observed in the dewatered sediments of the Elwha. Under this regime, the reduced ECM colonization observed near lupine may be due to this investment dynamic being shifted away from ECM allocation by either increasing bioavailable soil nitrogen or increasing moisture content by virtue of increased root density. Under this paradigm, we propose that ECM symbioses will be disfavored when soil-derived nitrogen is no longer limiting to growing conifers. We conducted two studies. The first study was a field trial in which we measured the abundance and community composition of ECM fungi in the roots of 29 grand fir seedlings that were planted with different densities of lupine in the Elwha riparian zone in 2017. Our second study was a controlled greenhouse study to determine if the ECM response to lupine was related to elevated levels of nitrogen or increased water-holding capacity due to the additional root mass. We tested this by growing grand firs in a greenhouse study under four treatments: 1) synthetic nitrogen treatment, 2) nitrogen-fixing lupine, 3) non-nitrogen fixing plants, Oregon sunshine (*Eriophyllum lanatum*) and Blue Wild Rye (*Elymus glaucus*), and 4) a control plot where grand firs are planted without fertilizer or neighboring vegetation. We predicted that increasing the available N by companion planting with the nitrogen-fixing lupine and the addition of a nitrogen fertilizer would result in an increase in growth, an increase in foliar N, and a decrease in ECM colonization

Methods:

Field Methods:

The objective of this study was to determine if ECM colonization in grand fir correlated with lupine cover after three field seasons and to document the ECM fungal community on the grand fir roots. The field study is located in the Olympic National Park on the Elwha River at the former Lake Mills, the reservoir contained behind the Glines Canyon Dam. The dam was removed slowly over the course of three years and was completely deconstructed in 2014. The study plot is located on the eastern terrace, approximately 1.6 kilometers from the former dam, and is 0.5 hectares in area (Figure 1a). In 2015, pure-live seed of forbs and graminoids were introduced, all harvested from regional seed sources (Chenoweth et al. 2011). Seeding was applied at 430 pure live seeds per square meter. From live seed, lupine was one of the few species that grew vigorously and spread to dominate many restoration areas. However, at the time of seeding, lupine was not applied in equal amounts, and this resulted in variable lupine densities along the terraces (J. Chenoweth, per. comm). As a biennial, lupine set seed in the second year (2016), and progeny from the initial application germinated in the early spring of 2017.

On March 3, 2017, 90, three-year-old, grand fir (*A. grandis*) were planted using a hand shovel digging to a depth of 18 cm (Figure 1b). All grand firs were measured (height and basal diameter) and tagged upon planting. In July 2020, all surviving seedlings (n=29, 32% survival after three years) were re-measured for height (cm), basal diameter (mm), and distance from the forest edge (m). Lupine, being the most abundant species surrounding the grand fir seedlings was also quantified at this time. To accomplish this, vegetation surveys were conducted using the grand fir as a center point creating a 2 m^2 circular area around each surviving grand fir seedling (n = 29). Lupine was placed into the following cover-class categories based on visual estimation: 1) < 1%, 2) 1 to 10%, 3) 11 to 25%, 4) 26 to 50%, 5) 51 to 75%, 6) 76 to 90%, and 7) > 90%.

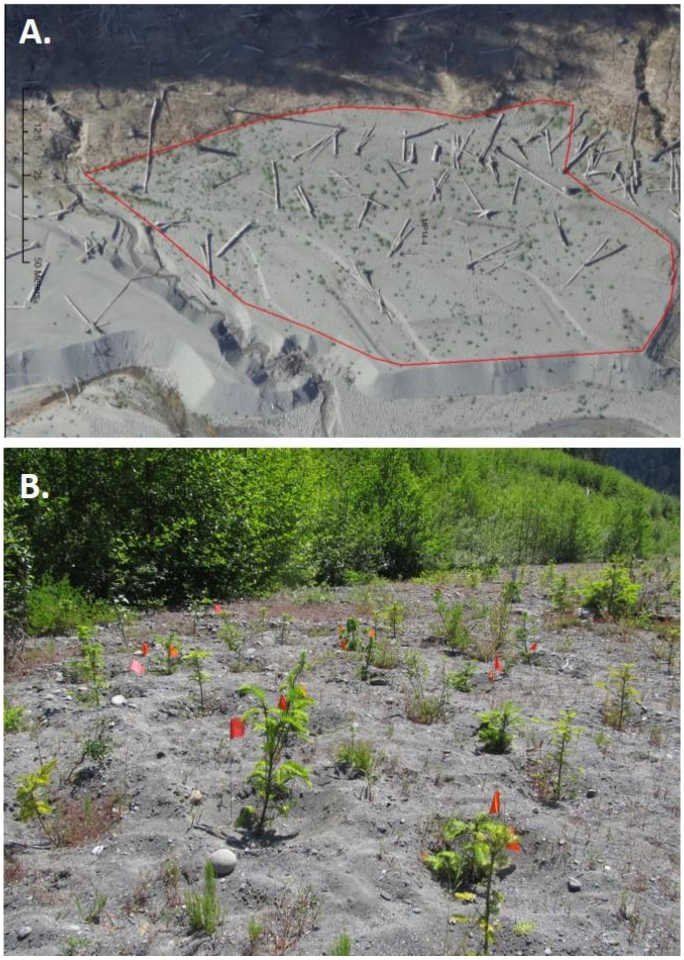

Figure 1. Panel A) Aerial view of the study plot which is approximately 0.5 hectares taken in 2014. Panel B) Photo from June 2017 as an example of grand firs planted in coarse soils along the eastern terrace.

ECM Field Sampling:

In July 2020, all surviving grand fir seedlings were sampled non-destructively for ECM. Soils were trenched to a depth of 18 cm and exposed roots were collected and returned to the laboratory. In the laboratory, roots were washed, quantified for ECM colonization, and sorted into morphotypes based on their appearance, and photographed. Three root tips of each morphotype were stored in dH20 at -20°C prior to being selected for DNA extracting and sequencing using a 5-mm section of the root tip.

ECM Abundance Methods:

Lateral root segments that were designated to be quantified for their ECM abundance were taken out of their 4°C storage and aseptically placed into a petri dish using sterilized forceps and rinsed with DI water to remove any remaining soil particulates. The rinsed root segments were then transferred aseptically to a new petri dish containing approximately 2-3 milliliters of DI water and placed under a dissecting microscope. Individual root tips were then assigned a category of positive or negative for the presence or absence of ECM morphotypes. Root tips exhibiting a monopodial morphology, a fungal sheathe, and hyphae were identified as positive and those not exhibiting that morphology were identified as negative for ECM colonization (Massicotte et al., 1999, Matsuda & Hijii, 1999).

The presence and type of ECM species on the root tip were confirmed by DNA extraction followed by PCR and DNA sequencing. Briefly, a 3 mm segment of root tip was homogenized using a mortar and pestle and DNA was extracted using appropriate buffers and filter columns provided by QIAGEN® DNeasy Plant Pro Kit per manufacturers protocol (QIAGEN, Germantown MD).

Approximately 10 ng of this DNA was used for PCR amplification using primers ITS1-F (5’ cttggtcatttagaggaagtaa 3’) and ITS4 (5’ tcctccgcttattgatatgc 3’), which will target the highly variable internal transcribed spacer (ITS) region of ECM fungal ribosomal DNA (Gardes and Bruns 1993). PCR reactions were based on the following concentrations for a 25 µl reaction: 12.5 µl of GoTaq® Green Master Mix (Promega, Madison WI), 0.25 µl of 25µM of each primer, 11 µl of molecular grade water, and 1 µl of DNA template. Temperature cycling was accomplished using GeneAmp PCR systems 9700, which allowed for a programmable Thermal Cycler Heating regime as described by Gardes and Bruns (1993): The initial denaturation step is 94 ºC for 85 s followed by 35 amplification cycles of denaturation, annealing, and extension. The temperature and times for the first 13 cycles are 95 ºC for 35 s, 55 ºC for 55 s, and 72 ºC for 45 s. Cycles 14-26 and 27-35 repeated the above parameters with lengthened extension steps 120 and 180 s, respectively. When the 35 cycles are completed, the samples will be programmed to incubate for 10 min at 72 ºC for 45 s.

The PCR products were confirmed using gel electrophoresis and purified using Wizard® SV 96 Genomic DNA Purification System (Promega, Madison WI). DNA concentration was quantified using a Thermo Scientific 2000 1-position Spectrophotometer (Thermo Fisher Scientific, Pittsburg PA) prior to sequencing. Sanger sequencing was performed using The Applied Biosystem ABI Prism 3730 DNA Analyzer (Retrogen Inc., San Diego, CA). The DNA sequences were analyzed and edited using Retrogen Inc. software. To identify the fungus found on roots, ITS sequences from samples were compared to those in the GenBank using the BLAST search (Altschul et al. 1997). The genera of the fungi reported in this study were based on the best matches of those in the GenBank with a > 97% ITS sequence similarity as a threshold.

Greenhouse Methods:

The objective of this study was to understand the role that soil nitrogen and N-fixing plants such as riverbank lupine have on tree-ectomycorrhizal symbiosis. To understand this relationship we potted 1-year-old grand fir seedlings (*Abies grandis*) and grew them in greenhouse conditions under the following treatments: 1) firs planted next to the nitrogen-fixing lupine, 2) firs planted with the non-nitrogen fixing plants, Oregon sunshine (*E. lanatum*) and Blue Wild Rye (*E. glaucus*), 3) a control plot where firs were planted without neighboring vegetation, and 4) firs planting without neighboring vegetation but given supplemental nitrogen in the form of ammonium nitrate. The grand firs were grown for 5 months under natural light with controlled greenhouse conditions.

The grand fir seedlings, lupine seeds, Oregon Sunshine, and Blue Wild Rye seeds used in this study were provided by Matt Albright Native Plant Nursery, Sequim, Washington. The grand firs obtained were approximately two years old and, while at the plant nursery, were grown in soil that contained osmocoat additives to provide supplemental nutrients. Due to the Oregon Sunshine having a low growth rate, Blue Wild Rye was added in the form of spread seed approximately 2 months into the 5-month growing season. The lupine seeds were placed into 10 Petri dishes with 30-40 seeds per dish. Prior to germination, all seeds were surface sterilized in 3% hydrogen peroxide. The seeds were germinated on filter paper on top of a paper towel and then covered with an additional section of filter paper before being saturated with deionized water (DI) water and left at ambient temperature for 10 days. After the 10-day germination period, the lupine seedlings were transferred to store-bought Jiffy brand peat moss starter plugs (Jiffy 7, manufactured in Brunswick, Canada) and were left to establish for approximately a month before potted in the peat plugs.

To control for the water-holding capacity of the plugs themselves, identical Jiffy plugs that did not have any growing plants in them were also included in both the nitrogen and control treatments and arranged in an equidistant triangle around the main growing conifer, just as the lupine and Oregon Sunshine was planted.

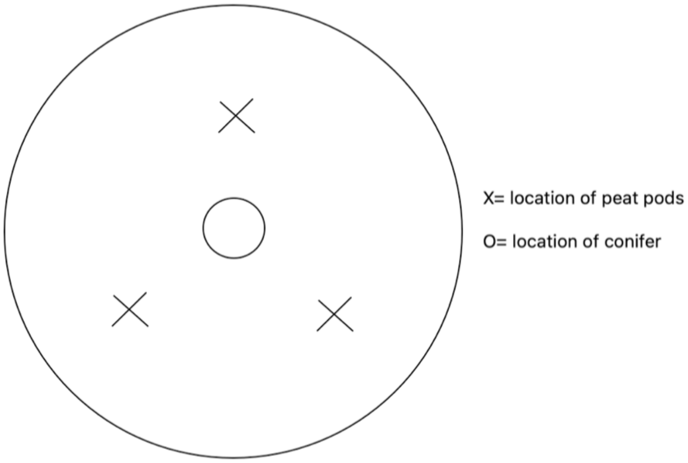

Figure 2: Diagram depicting the top-down view of planting arrangements for treatments. X's represent the location of peat plugs, the O represents the location of the conifers

Figure 3: Image of the first in each treatment at the end of the growing season. Left to right they are as follows: Lupine, Competition, Ammonium Nitrate, and Control.

The pots themselves contained a mix of sand, turface (MVP brand, Buffalo Grove, IL), and sediments collected from the dewatered sediments of the Lower Elwha River. The pots have been prepared where there were three layers of soil. The top and bottom layers were composed of a 1:1 mixture of sand and turface while the middle section is a 2:1:1 mix of dewatered source sediment, sand, and turface respectively.

Plants were initially watered by hand daily and then switched to greenhouse irrigation. Ammonium nitrate was given in the form of an aqueous solution once every other week as 3.78 mg N dissolved in 27 mL of deionized H20. At the time of N fertilization, all plants that were not given ammonium nitrate were given 27 mL of deionized H20 to ensure all plants were receiving equal water. To ensure nitrogen was the only limiting nutrient, all plants also received 27mL of a nitrogen-deficient Hoagland's solution which contained Magnesium Chloride, Potassium phosphate, iron chelated onto ETDA, and other micronutrients every other week.

During the growing season, the basal diameter and height were recorded monthly. The basal diameter was measured using measuring forceps with a resolution to the nearest millimeter, and this measurement was taken one inch above where the shoot of the conifer met the soil. Height was measured using a standard ruler starting where the basal diameter measurement was taken and followed along the main shoot until it reached the terminal bud at the top of the seedling.

After 5 months, the greenhouse plants were harvested, and different parts were partitioned for data collection. At the time of harvest, basal diameter and plant height were recorded and their values at the time of planting were subtracted to obtain a total for growth during the five-month month growing period. Approximately 0.5 grams of terminal foliage was collected from the side branches of each conifer for foliar nitrogen analysis and placed into paper bags and set aside to be dried. Laterally branching root segments were trimmed off the main root structure using scissors and placed in plastic bags over ice, then stored at 4°C for DNA analysis and ECM abundance assays. Following the lateral root tip removal, the remaining root mass was washed to remove sediment and placed into a labeled paper bag, then set aside for dry biomass measurements, this biomass was not accounted for in subsequent calculations. The above-ground biomass consisting of the remaining shoots was also placed into a paper bag set aside for drying. ECM was quantified and identified molecularly as described above.

Biomass Methods:

The collected shoots, roots, and foliar nitrogen trimmings were all placed into an incubator set at 60°C and left to dry for 72 hours before being removed and weighed. Shoot weights were calculated by adding the foliar nitrogen trimming weights to the shoot weight. Root weights were calculated using only the remaining root bulb dry weight obtained after drying the below-ground biomass.

Foliar Nitrogen methods:

Foliar nitrogen was determined using a CE Elantech EA 1112 flash spectrophotometer at Shannon Point Marine Labs. After drying for 72 hours at 60°C, samples to be analyzed were first ground into a fine powder using a mortar and pestle and transferred in plastic bags to the lab. Ground foliar nitrogen samples were then carefully loaded into tin capsules and analyzed according to manufacturer instructions using atropine as a control for nitrogen content.

Statistical Analysis Methods

To determine if ECM abundance was related to lupine cover in the field, a Spearman's rank correlation test was employed. Field measurements for height, basal diameter, and ECM abundance (as a proportion of colonized root tips) failed to meet assumptions of normality when tested using a Shapiro-Wilk normality test. Since these data were continuous, we elected to use the non-parametric Spearman's rank correlation test, as a normal distribution is not a requirement. Height and basal diameter were treated as the Y (response), variables while ECM abundance and distance to edge were used as the X (predictor) variables. The resulting coefficient, rho (r), ranges between -1 and +1 providing a direction to the relationship is also reported.

Variables recorded and analyzed from the greenhouse included the proportion of positive root tip counts, conifer height, basal diameter, and biomass measurements for roots and shoots. Positive root tip counts were prepared for each plant in each treatment and converted into a proportion. The weight of collected roots was divided by the weight of collected shoots to obtain a root:shoot ratio. The data collected were then checked for normality using a Shapiro-Wilk's test followed by a Bartlett's test for equal variance. Positive root tip counts for each treatment group in the greenhouse study were compared using a Chi-Square test, with root tip counts being the Y variable while the treatment was the X. Foliar nitrogen content was analyzed using a one-way ANOVA with treatment being the factor and foliar % N measurements being the response variable; significant results were further analyzed using a Tukey's HSD posthoc test comparing each of the treatments. Basal diameter, height, biomass measurements, and root:shoot ratios were compared individually using a one-way ANOVA with the respective growth metric used as a response variable and treatment as the factor. For all statistical analyses, a significance level of

0.05 (α=0.05) was used. While transformations were tested, no analysis was done using transformed data. All statistical tests and models were created using R version 4.0.3 (The R Foundation for Statistical Computing).

Results:

Field Study Results:

We found no evidence of a correlation between lupine percent cover and basal diameter, nor between lupine percent cover and fir height. There was also no significant observed correlation between distance from edge and basal diameter, however, fir height was significantly correlated with distance from edge when evaluated using the same correlation test (p=0.02, rho= -0.45).

ECM abundance correlated with an increase in cover of lupine by percent area, whereas lupine abundance increased a decrease in ECM abundance was observed (p<0.02, rho= -0.46). There was no correlation between ECM abundance and distance from edge.

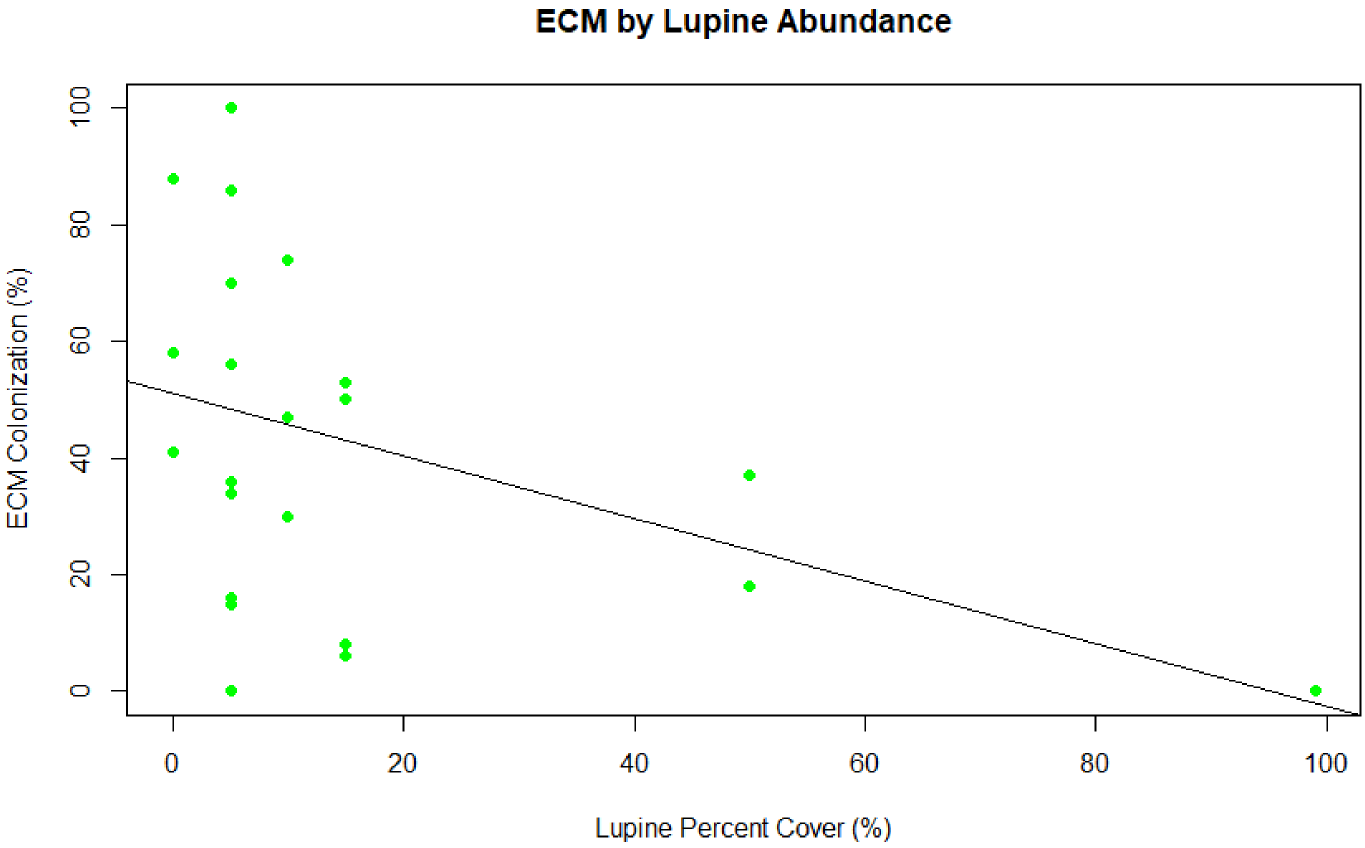

Figure 4: Scatter plot of lupine abundance and ECM colonization demonstrating a significant negative correlation (r = -0.46, df = 25, $p < 0.02$).

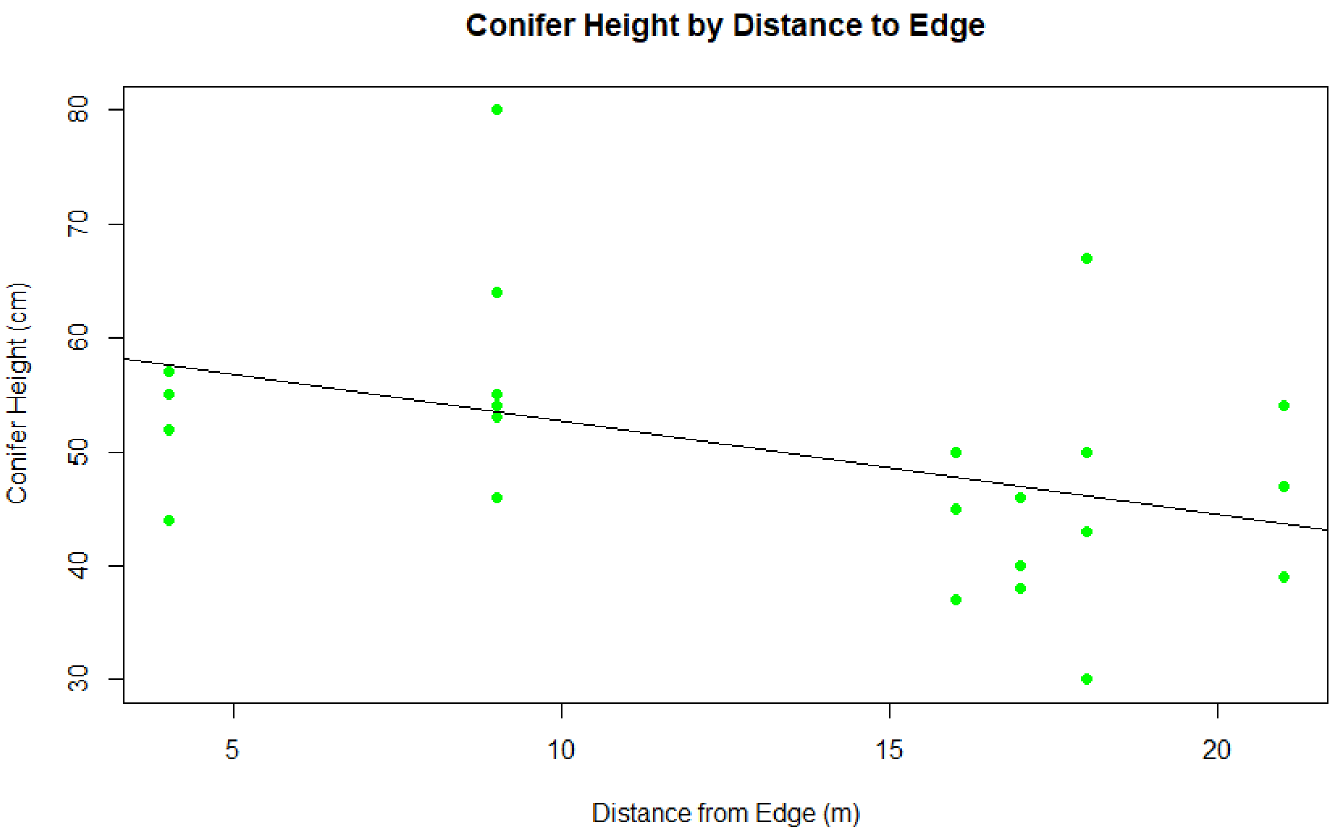

Figure 5: Scatter plot of grand fir height by distance from a forested area demonstrating a significant negative correlation (r = -0.45, df = 25, p = 0.02).

Table 1. The species composition of 24 field samples was analyzed following sequencing the ITS region of the fungal genome and recorded in the following table by the percentage of the sequenced samples that returned a positive match.

ECM SPECIES	% Abundance	Function
Thelephora terrestris	30.43	ECM
Wilcoxina mikolae	26.09	ECM
Sebacina sp.	13.04	ECM
Laccaria sp.	8.70	ECM
Trichoderma sp.	4.35	ECM
Suillus lakei	4.35	ECM
Helotiaceae	4.35	Endophyte
Paraphaeosphaeria sporulosa	4.35	Endophyte
Oidiodendron citrinum	4.35	Ericoid

Greenhouse Study Results:

The most abundant species of ECM fungi found on greenhouse roots were identified as *Thelephora terrestris*, *Wilcoxina mikolae*, and *Sebacina* species (Table 1). ECM species that were less abundant (< 5% root colonization) include *Laccaria* species, *Trichoderma* species, *Suillus lakei*, and *Oidiodendron citrinum*. In addition, two non-ECM species were sequenced, *Paraphaeosphaeria sporulosa*, and Helotiaceae.

The proportion of root tips colonized by ECM fungi was lowest in the nitrogen treatment (χ^2= 116.2, df = 3, P >0.001).

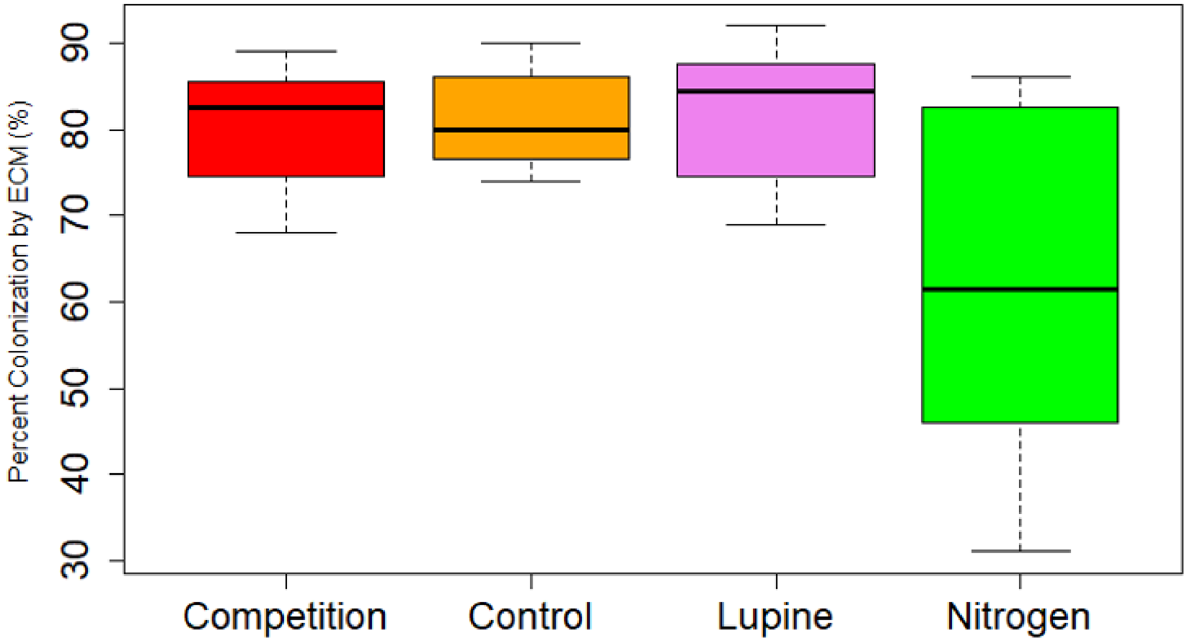

Figure 6: Bar plot depicting the proportion of root tips colonized with ECM (1) compared to uncolonized roots (0) among the four greenhouse treatments: Control, Lupine, Nitrogen, and Competition. There was a marked decrease in colonization observed in nitrogen treatment when compared against other groups (χ^2= 116.2, df = 3, P >0.001).

Species composition was analyzed following the sequencing of the ITS region of the fungal genome. The most abundant species were *Wilcoxina mikolae (50%) and Thelephora terrestris (%)*. (Table 2). Less abundant species include *Sepultariella sp. (12.5%) and Tomentella sublilacina (6.25%).*

Table 2. The species composition of 24 greenhouse isolates was analyzed following sequencing the ITS region of the fungal genome and recorded in the following table by the percentage of the sequenced samples that returned a positive match.

ECM SPECIES	% Abundance	Function
Wilcoxina mikolae	50	ECM
Thelephora terrestris	31.25	ECM
Sepultariella sp.	12.5	ECM
Tomentella sublilacina	6.25	ECM

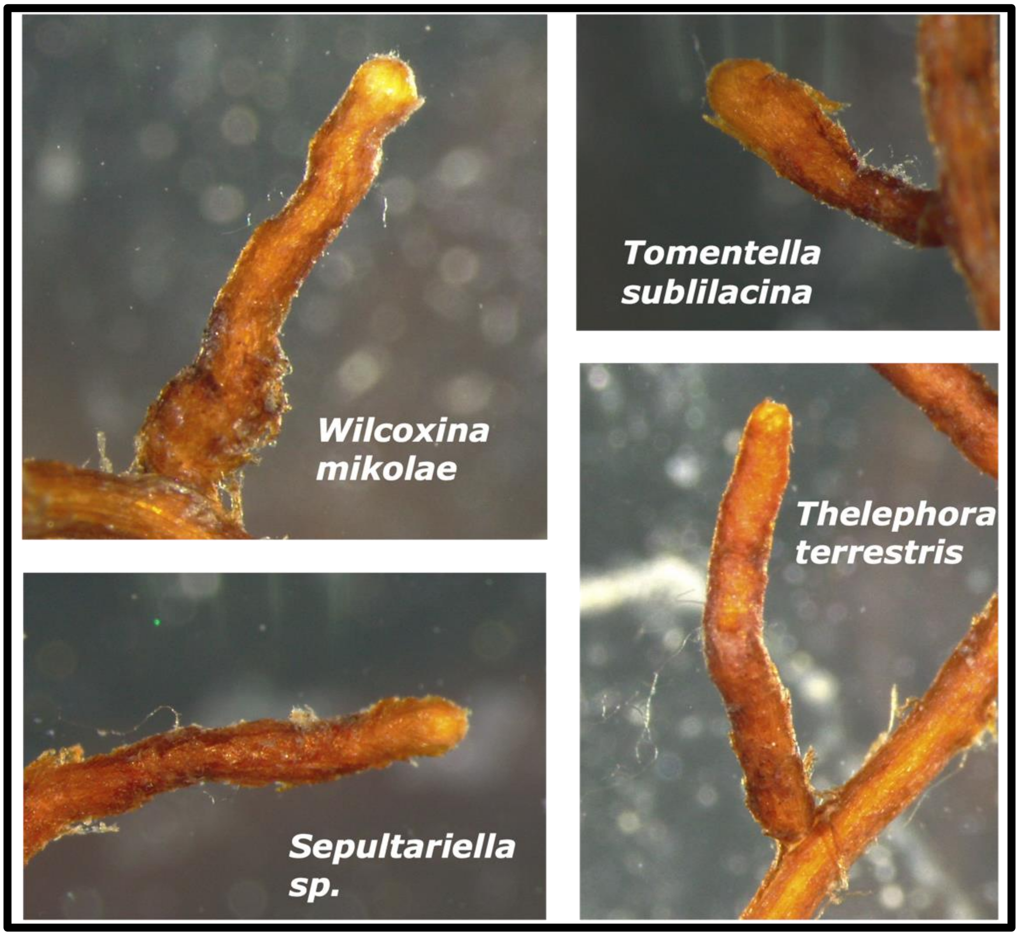

Figure 7: Photograph of the four different greenhouse ECM isolates being morphotyped.

Differences in foliar nitrogen percent were analyzed using a one-way ANOVA followed by a Tukey's HSD posthoc test. Significantly higher foliar nitrogen percentages were observed in fir needle tissue growing with both the lupine and competition treatment (1.31%, and 1.77%, respectively) when compared to the nitrogen (0.75%) and control treatments (0.59%) (Figure 2; $F_{(3,28)} = 8.92$, $P<0.001$).

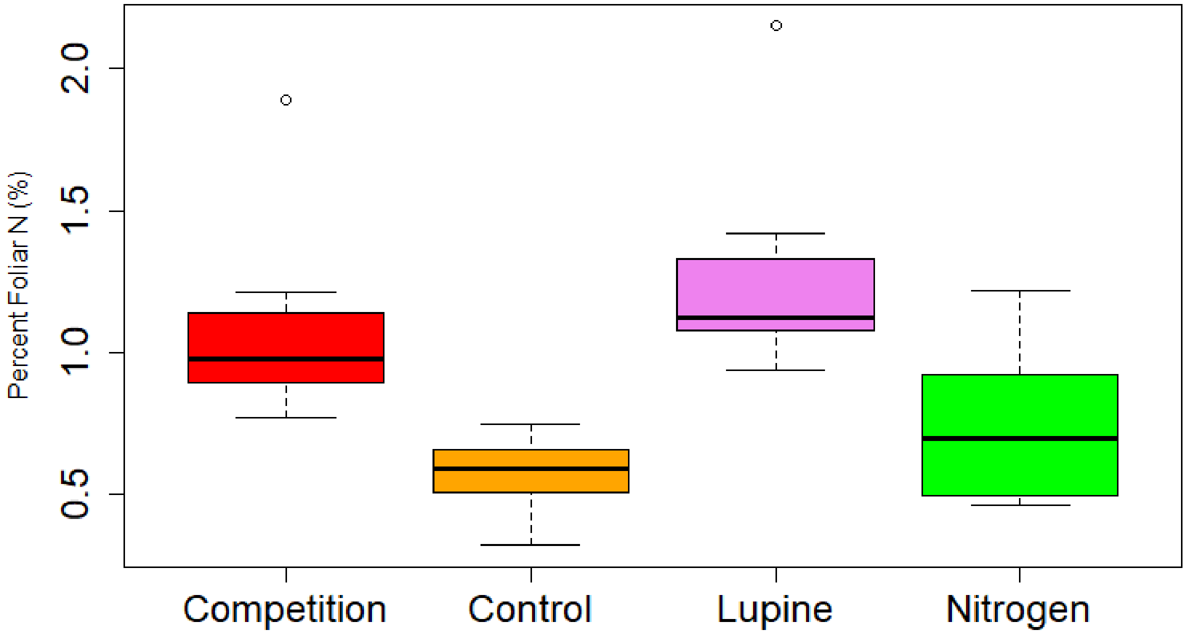

Figure 8. Average foliar N percent by treatment with standard error bars, individual data points, and post-hoc grouping superimposed among the four treatments of Competition, Control, Lupine, and Nitrogen. Average foliar N in new growth foliage was found to be higher in Competition and Lupine treatments than other groups $F_{(3,28)} = 8.92$, $P < 0.001$.

Growth metrics of basal diameter and height were also compared with a one-way ANOVA, but no significant differences between treatments were identified. Additionally, the root:shoot ratio of each plant in the greenhouse study was calculated and compared across treatment groups with a one-way ANOVA. Following analysis, no significant differences between treatments were observed at the 0.05 level.

Discussion:

Observed effects on ECM colonization:

Surveys conducted in the field allowed us to test the hypothesis that lupine growing around grand fir seedlings influences ECM symbioses. We predicted that growing in proximity to riverbank lupine would result in increased root collar diameter and height while reducing ECM colonization rates as the breakdown of lupine biomass into bioavailable forms of nitrogen altered the optimal investment dynamic. Analysis of collected data identified a negative correlation between the abundance of lupine defined as percent cover, and conifer root ECM in the field. These results are consistent with those observed in a previous Elwha study comparing three treatments of relative abundance of lupine and ECM colonization (Kardouni 2020). In contrast to our field study, following a five-month growing period under greenhouse conditions, neighboring lupine did not reduce ECM, however, there was a significant decrease in ECM colonization in response to additions of synthetic N.

Role of Nitrogen in ECM:

As discussed above, field studies have also documented this decrease in ECM. In addition, these findings agree with greenhouse studies that looked at the effect that nitrogen additions had on ECM colonization (Mahood, 2003; Arnebrant, 1994; Kainulainen et al., 1996).

Mahmood (2003) documented the colonization rates of two isolates of *Piloderma* spp. on the roots of spruce seedlings in response to varying levels of N and wood ash in a five-month greenhouse trial. In their study, colonization rates were dependent on wood ash levels, amount of N, and species isolate with a high amount of nitrogen significantly reducing ECM colonization. In another study, Arnebrant (1994) compared the effect that three different nitrogen sources had on the colonization of pine seedlings by five ECM isolates. All nitrogen amendments resulted in a reduction in mycelial growth, and the degree of reduction when compared to the control was dependent on the ECM isolate. These studies provide evidence that the availability of soil nitrogen impacts the colonization rates of ECM fungi in greenhouse settings, supporting the model that nutrient dynamics play an important role in maintaining a functioning symbiosis in controlled environments.

While we did find that synthetic N additions impact ECM, as mentioned, we were unable to show that the presence of lupine also decreased ECM. This observation is in opposition with previous studies that demonstrated lupine and other N-fixers significantly increase the soil nitrogen content in surrounding soils (Halvorson et al., 1991, Myrold and Huss-Danell, 2003, Nuhn 1987), and thus, would influence the nutrient dynamics between plants and mycorrhizae. One plausible explanation for this discrepancy is that since most bioavailable N is transferred to the rhizosphere by the decomposition of biomass (Vitousek et al., 2002) and more than one growing season would be required to see N inputs from biological nitrogen fixation. While root exudates can transfer some N to the rhizosphere (Coskun et al., 2017), the majority of N is released via the process of mineralization, where organic matter is broken down by microbes into the readily available form of ammonium (Robertson and Groffman, 2007). Additionally, while ammonium is the direct product of mineralization, nitrification, or the microbial respiration n of ammonium into nitrate, occurs simultaneously. Therefore, the specific form of nitrogen available for plant usage can change based on soil biochemistry.

Previous research has also demonstrated that the form in which soil nitrogen exists also impacts these ECM symbioses (Holopeinen, 1993, Arnebrant, 1994). While Arnebrant (1994) was able to demonstrate that all forms of nitrogen resulted in decreases in mycelial growth, other studies suggest otherwise. Holopeinen (1993) compared three different forms of added nitrogen, NO_3-, NH_4+, and urea, and found that all nitrogen treatments demonstrated reduced root dry biomass when compared against the control group, however, only NO_3- was shown to reduce ECM colonization rates on *Pinus sylvestris* root tips. Since the processes that produce both of these forms of nitrogen occur simultaneously, we elected to use ammonium nitrate as our synthetic nitrogen source, therefore, if any form of biologically available nitrogen were to have an impact, it would be detectable as both types would be present.

Our greenhouse study occurred over only five months and lupine may not have decomposed and therefore did not release N bound in tissue to the rhizosphere. In contrast, while the greenhouse growing period lasted only five months, the field study took place over three growing seasons. The longer duration of the field study likely allowed for the decomposition of lupine biomass, which in turn could result in higher local soil N levels than those that would be present in our five-month lupine greenhouse treatment. To truly test the influence of N on soil symbiont dynamics would require more time to account for decomposition to illustrate this antagonistic relationship between lupine-derived N and ECM colonization. Therefore, while our greenhouse N treatment supported the functional equilibrium model predicting symbiotic success, our lupine treatment did not.

Observed Effects on Conifer Growth:

We identified no significant relationships between lupine abundance and conifer growth. Neither root collar diameter nor conifer height appeared to be affected by the presence of lupine. These findings contrast with studies done on Norway spruce, European beech, and Scots pine which demonstrated that sowing lupine in study plots increased height and reduced the incidence of chlorosis after a five-year growing period (Mauer et al., 2013). Kardouni (2020) found this relationship to be most beneficial in Elwha sediments at moderate levels of lupine cover. However, my study showed neither a positive nor quadradic response to the lupine cover, which would indicate moderate levels of lupine increasing growth. This may indicate that the additional N produced by neighboring lupine may have also been masked by environmental effects such as water scarcity. Similar effects have been demonstrated in sunflowers (*Helianthus annuus*), where even high loads of N fertilization did not yield a significant effect on growth when soil moisture was below 50% saturation (Abayomi and Adefila, 2008). If so, even direct fertilization may have been of little consequence if there was simply not enough water available during the growing season to allow for nutrient uptake (Lipiec et al., 2013).

Further, we did not observe a positive correlation between ECM colonization rates and conifer growth metrics. These findings contrast with much of the existing literature, as successful ECM symbiosis is typically associated with increased plant growth (Nara and Hogetsu, 2004, Bauman et al., 2012, Smith and Read, 2009). However, other studies have found that limiting factors, such as light and water availability, may not be ameliorated by high rates of ECM colonization (Trocha et al., 2016). In my study, water is the limiting factor on the novel coarse terraces, and the lack of water may simply have been a larger impediment to growth than the lack of nitrogen (Chenoweth 2011). Some studies have shown ECM symbioses help to relieve water stress (Smith and Read 2009, Sebastiana et al., 2018), although host species and level of water stress likely determine if ECM benefit hosts. Boyle and Hellenbrand (2011) demonstrated that ECM colonization increased growth metrics on Black spruce (*Picea mariana*) under simulated drought conditions, but not Jack Pine (*Pinus Banksiana*). Kennedy and Peay (2007) demonstrated that ECM fungi did not improve overall plant performance under 7% soil moisture with ECM performing similarly to non-ECM seedlings, but that benefits were demonstrated when soil moisture was increased to 13 % (Kennedy and Peay 2007). Similarly, Dosskey et al. (1991) found that as water levels in soils decreased, ECM colonization also decreased indicating that water-stressed plants may be unable to support high levels of ECM colonization due to carbon reduction driven by stomatal closure. Therefore, water stress presents an alternative mechanism describing the interaction between ECM, conifers, and neighboring lupine. It is possible that as lupine competes with neighboring conifers for water resources, conifers are not able to maintain their ECM partners due to the reduction of carbon associated with the resulting decreased photosynthetic rate during the growing season when water is scarce.

The only growth correlation detected in the study was a positive relationship between grand fir height and distance from forest edge; taller seedlings were found to be growing closer to an actively growing forested area. These observed growth differences could be due to abiotic components such as increased organic matter and water-holding capacity in the soil closer to the forested area resulting in substrate conditions more conducive to conifer growth (Murcia 1995, Minasny and McBratney, 2018; Chen et al., 2004). Additionally, proximity-dependent biotic components such as mycorrhizal propagules and soil bacteria likely played a role in this observed growth pattern. Studies have shown that the abundance of mycorrhizal propagules and ECM infection rates decrease as the distance from an established forested community increases

(Dickie and Reich 2005; Davis and Smaill, 2009). Further, it has also been reported that soil bacteria can directly influence plant growth through the production of plant growth hormones such as gibberellin, auxin, and cytokines and by thwarting phytopathogenic organisms (Hayat et al., 2010). Though we did not have the data to further examine this, what may be of interest will be future studies that identify ECM species that eventually replace the greenhouse fungi. Specifically, whether field ECM species offer greater growth and survival benefits than those that are associated with less stressful greenhouse environments. We speculate that the environmental fungal isolates confer a competitive advantage over the greenhouse-derived ECM when placed into these harsher environments, with different resource abundances.

Foliar N:

Growth differences did not vary by treatment, however, foliar N levels did. Grand firs growing within the Lupine and Competition treatments had higher concentrations of foliar N than those growing within the synthetic Nitrogen and Control treatments. We posit that this increase in foliar N is due to root density and architecture reducing the rate at which remnant N leached out of the sediments. This phenomenon can be seen in Bergeron et al., (2011) where rates of nitrate and dissolved organic nitrogen leaching decreased in loam clay soils when actively growing tree roots were present, and undisturbed by trenching. These findings are supported by Ehdaie et al., (2010) who found that when wheat crops were grown in sand tubes for two years at both optimal and low nutrient conditions, root biomass is associated with increased N uptake and reduced leaching. Therefore, increased root density in the Lupine and Competition treatments likely reduced the rate that the remnant slow-release N additions leached, and increased N uptake. In contrast, the nitrogen and control treatments simply did not have the below-ground density to trap this remaining N, and therefore quickly lost both the remnant N, as well as all N added during the study. When these results are put into the context of the observed decrease in ECM within our nitrogen treatment, it becomes difficult to conclude that these reductions in ECM symbioses are due to excess N shifting the optimal investment dynamic away from maintaining symbionts, as quite notably, the nitrogen and control treatments had the lowest measured foliar N.

ECM species

Nine ECM species were identified colonizing grand fir roots in the field, while only four ECM species were identified from the roots of the greenhouse seedlings, marking a reduction in the species richness found in the greenhouse. These greenhouse isolates included *Wilcoxina mikolae*, *Thelephora terrestris*, *Sepultariella sp*., and *Tomentella sublilacina*. Of these, *Thelephora terrestris* and *Wilcoxina mikolae* were the most abundant. These species have been noted as some of the most ubiquitous greenhouse ectomycorrhizas (Hilszczańska and Sierota, 2013; Mikola, 1988). Notable isolates identified from the field were *Sebacina* sp., *Laccaria* sp., *Suillus lakei*, *Trichoderma* sp., *Helotiaceae, Paraphaeosphaeria sporulosa*, and *Oidiodendron citrinum*, an ericoid fungus. The dominance of these species is consistent with other studies, such as Kardouni (2020) who observed many of the same isolates. In Danielson (1991), which tracked growth and mycorrhizal colonization as well as mycorrhizal species abundance on Jack pine (*Pinus banksiana)* growing in both oil and coal spoils, *Thelephora terrestris* was by far the most dominant species of ectomycorrhizal fungi at the four-year mark, while *Suillius* sp. became the most dominant by year ten. We believe that this progression could demonstrate a shift in the relative benefit derived from a symbiotic relationship with specific species appearing at differing biotic and abiotic conditions present in the rhizosphere. In addition to different species likely being more competitive at earlier successional stages, some species of fungi have been shown to confer additional benefits to plants when co-infected with two species (Yin et al., 2014). Yin et al. (2014) demonstrated that *Pinus sylvestris* seedlings inoculated with *Trichoderma virens* 30 days after inoculation with *Suillius luteus* had increased collar diameter when compared against seedlings inoculated with either species alone, or against a sterile medium control. In addition to environmental conditions and species-specific responses, there is also evidence that different ECM species are favored during different life stages, and that the competitive benefit of a particular species may be dependent on the specific life stage of its host plant (Kennedy et al., 2011). Taken together, these studies provide evidence that which ECM species are dominant in a given rhizosphere is heavily context-dependent and subject to shifts in response to changing environmental, temporal, and biotic conditions, and host plant life stages.

Thelephora terrestris and *Wilcoxina mikolae* were the most common species present in both the greenhouse and field, underscoring their ability to survive in both environmental conditions. All grand firs used in the field and greenhouse study were grown from the same field nursery and these data provide evidence that these two abundant ECM species found on the field conifers presumably came from the greenhouse. In contrast, species such as *Sepultariella* sp., and *Tomentella sublilacina* may not be able to survive out planting in the coarse sediments in the field. Identifying greenhouse species also provides evidence that environmental isolates such as *Sebacina* sp., *Laccaria* sp., *Suillus lakei*, and *Trichoderma* sp. may be early successional species. The appearance of these species also provides further evidence that locally sourced ECM propagules are present, as documented in Cortese and Bunn (2017). Further, local ECM can successfully colonize root tips of establishing conifers, even when under the less favorable conditions presented by the novel terraces with little to no biological legacy.

Nitrogen fertilization has also been demonstrated to impact ectomycorrhizal community structure. (Lilleskov et al. 2008 and Ostonen et al. 2011; Taylor et al., 2000, Kjøller et al. 2012, Cox et al. 2010, Avis et al., 2003, Lilleskov et al., 2001). Avis (2003) demonstrated that following N addition in the field, total sporocarp richness decreased by 50%, while sporocarps belonging to *Russula* spp. increased five-fold. These results were repeated by Lilleskov et al. (2001) in a study documenting the effects of nitrogen deposition across a gradient in Alaska, which demonstrated that *Laccaria* sp. appeared to be unaffected, and possibly even helped by increasing environmental nitrogen. Further insights into how nutrient gradients impact ECM species abundance can be derived from studies such as Corrales et al. (2017), which determined that long-term N additions reduced ECM colonization of *Oreomunnea mexicana*, and caused a shift in the ECM community composition. N fertilization increased the abundance of *Laccaria* and *Lactarius* species while *Cortinarius* declined (Corrales et al. 2017). These differential effects, in conjunction with previously done field research, suggest that the response of each symbiosis to changes in N abundance may be species-specific and have much to do with their individual biochemistries (Corrales et al., 2017). Unfortunately, not enough species were identified from the greenhouse plants to detect changes in plant communities in my study.

Conclusions and Implications for Restoration:

Forests worldwide are experiencing anthropogenic climate change, as well as artificial and biological additions of N. Understanding if N influences the dynamics that govern ECM symbioses will be of vital importance as we plan effective land management. These insights become even more vital when placed into a restoration context, as ECM symbioses allow for increased seedling recruitment and subsequent forest resiliency in the contexts of drought and nutrient limitations such as those present at the Elwha River Basin.

While our greenhouse and field study disagree in terms of their implications for the impact that lupine has on ECM colonization, they both provide profound insights into the dynamic nature of ECM symbioses. The field study suggests that lupine and lupine abundance have an antagonistic effect on ECM, while the greenhouse study was unable to show this relationship. The greenhouse study was, however, able to show that increased N loads reduced ECM colonization rates, irrespective of the levels of foliar nitrogen. While overall, this provides support for the hypothesis that nutrient conditions play a large role in determining the success of mycorrhizal symbiosis, to what level the observed ECM reduction is due to reduced carbon allocation when nutritional needs are met remains unclear. These results could potentially inform restoration land management practices regarding how nitrogen amendments impact valuable mycorrhizal symbioses on conifers.

Interestingly, companion planting also played a major role in the N dynamics in the greenhouse. The treatments that were comprised of native vegetation increased the foliar N content of the conifers that were growing amongst them. As posited earlier, we believe this is due to below-ground root systems trapping remnant N, allowing for increased N uptake by reducing N lost to leaching effects. It is also worth noting that under greenhouse conditions, the conifers and their companion plants were not under drought conditions like those present in the field. This hints at an interesting paradigm where some companion planting may allow for increased N retention in these coarse sediments, while too much neighboring vegetation may induce fierce competition for already limited water resources. To connect back to Kardouni’s (2020) work, the moderate levels of lupine, not the most abundant lupine cover was most beneficial to the growth of neighboring conifers This poses the question of whether the observed decrease in ECM could be due to water stress under intense competition. Under this paradigm, conifers along the Elwha experiencing a reduction in their photosynthetic rate as a result of water limitation would no longer be able to allocate carbon to symbionts regardless of soil nutrient levels. As such, this presents an alternate hypothesis to purely optimal resource allocation explaining a reduction in ECM, and instead, posits that competition and water stress are the dominant factors determining the relative benefit of maintaining an ECM symbiosis.

When water stress and competition are added to the equation, we get a balancing act between native herbaceous vegetation and conifer establishment, where more research is needed to determine where the tipping points of facilitation or inhibition lie. The subsequent impacts that this neighboring vegetation has on the conifer likely play a role in determining the success of a given ECM symbioses, either by altering nutrient dynamics as we believe is the case for lupine, by increasing nutrient availability, or by inducing competition for water. While more work still needs to be done, ultimately, we find that when considering interventions that would provide the most benefit to establishing conifers, companion planting may offer more benefits than fertilization alone and that the ECM symbioses these conifers are engaged in are dynamic and will respond to changes in the biotic and abiotic conditions.

Future areas of study that would further elucidate the relationship between lupine and ECM symbioses in restoration confers may include conifer growth surveys under varying moisture contents, lupine's influence on varying moisture contents, and isotope labeling to track the flow of nutrients into the rhizosphere. This additional information would allow for increased confidence regarding whether the form of accessible N plays a role in symbiotic stability, the influence of water availability, the influence of below-ground root density, the degree to which soil microbiomes influence establishment and growth in restoration contexts, and finally whether or not certain fungal taxa confer competitive advantages depending on the nutrient conditions present in sediments with little biological legacy.

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Appendix:

Table of Measured Values:

Measurement	Number of Observations	Median	Mean	Standard Deviation
Difference in Height (cm)	32	18.5	19.159375	6.360556852
Difference in Basal Diameter (mm)	32	3.5	3.65625	0.97911662
Height (mm)/Basal Diameter (mm)	32	52.2333333	55.3752232	23.03724178
Shoot mass (g)	32	24.655	27.040625	10.67442676
Root mass (g)	32	32.285	30.5740625	13.24854316
Root mass (g)/Shoot mass (g)	32	1.10423896	1.15528742	0.395144076
ECM Colonization Percent (%)	32	82	76.3125	14.44106488
Foliar N Percentage (%)	32	0.895	0.9165625	0.39910876

Hoagland’s Solution Composition:

Stock	**FW**	**Stock (M)**	**mL of stock per liter for full strength**	**mL of stock per liter for desired strength**		**Final solution (mM)**	**P(mg/L)**	**P (mg) per pot**	**N (mg/L)**	**N (mg) per pot**	**mL to add for desired volume at desired strength**
$Ca(NO_3)_2$	164.09	1	10	5		5			140.10	3.78	25.000
KNO_3	101.1	1	0	0		0			0.00		0.000
$MgSO_4$	120.37	1	2	1		1					5.000
KH_2PO_4	136.09	1	1	0.5		0.5	15.49	0.418			2.500
FeIII EDTA (dicots)	367.05	1	1	0.5		0.5					2.500
Micronutrient Solution (see below)			1	0.5		see below					2.500

ANOVA Table for Foliar nitrogen comparisons:

	D. F.	Sum Square	Mean Square	F Value	Pr (>F)
Treatment	3	2.412	0.8041	8.915	0.000263
Residuals	28	2.526	0.0902		

Greenhouse Raw Data:

Tree	HT difference (cm)	BD difference (mm)	HT (mm) /BD (mm)	Shoot mass (g)	Root mass (g)	Root mass (g) / Shoot mass (g)	ECM Percent (%)	N (%)	Treatment
L1	14.5	3	48.33333	13.48	15.28	1.133531	92	1.14	Lupine
L2	21.4	4	53.5	24.08	37.91	1.574336	84	1.07	Lupine
L3	28.7	3	95.66667	18.16	18.84	1.037445	85	2.15	Lupine
L5	12.1	4	30.25	26.2	36.27	1.384351	90	1.09	Lupine
L6	15.5	3	51.66667	16.63	41.51	2.496091	75	1.1	Lupine
L7	19.3	4	48.25	18.79	20.56	1.094199	69	1.42	Lupine
L8	25.6	4	64	46.63	48.55	1.041175	74	1.24	Lupine
L9	32.9	3	109.6667	35.81	41.51	1.159173	85	0.94	Lupine
N1	0.3	3	1	13.63	18.66	1.369039	58	0.78	Nitrogen
N2	12.8	4	32	13.61	7.88	0.578986	34	0.52	Nitrogen
N3	12.3	3	41	15.15	14.67	0.968317	61	0.46	Nitrogen
N4	15.4	3	51.33333	35.27	18.13	0.514035	31	0.47	Nitrogen
N5	22.7	5	45.4	36.9	46.51	1.260434	62	0.87	Nitrogen
N6	17.8	5	35.6	18.91	19.2	1.015336	86	1.22	Nitrogen
N7	17.2	3	57.33333	41.74	46.51	1.114279	83	0.62	Nitrogen
N8	28.7	3	95.66667	37.26	44.93	1.205851	82	0.97	Nitrogen
C1	26.4	5	52.8	30.27	38.02	1.256029	76	0.7	Control
C3	18.5	4	46.25	23.71	31.04	1.309152	83	0.53	Control
C4	22.4	3	74.66667	18.7	18.48	0.988235	90	0.62	Control
C5	22.2	5	44.4	23.13	19.06	0.824038	89	0.75	Control
C6	16.7	3	55.66667	34.53	36.33	1.052129	83	0.32	Control
C7	16.8	3	56	22.25	16.93	0.760899	74	0.56	Control
C8	15.4	5	30.8	29.71	48.04	1.616964	77	0.49	Control
C9	19.3	3	64.33333	23.21	27.02	1.164153	77	0.62	Control
O2	18.5	2	92.5	14.39	14.04	0.975678	87	0.77	Competition
O3	24.5	4	61.25	25.23	33.53	1.328973	89	0.97	Competition
O4	20	4	50	36.86	34.95	0.948182	84	0.91	Competition
O5	23	6	38.33333	54.98	54.98	1	68	0.98	Competition
O6	13.6	5	27.2	29.58	22.26	0.752535	79	1.89	Competition
O7	15.2	2	76	16.78	35.09	2.09118	83	1.07	Competition
O8	15.5	3	51.66667	41.5	51.64	1.244337	82	0.88	Competition
O9	27.8	5	55.6	28.22	20.04	0.710135	70	1.21	Competition

Field Raw Data:

Plot	Number	HT (cm)	BD (mm)	HT (mm) /BD (mm)	Distance from Edge (m)	Lupine Cover (%)	ECM Colonization (%)
4	99	43	10	43	18	99	0
4	101	50	10	50	18	99	0
6	136	46	13	35.38462	9	5	0
5	121	53	12	44.16667	9	15	6
5	120	54	10	54	9	15	8
6	138	64	12	53.33333	9	5	15
10	190	44	10	44	4	5	15
6	134	64	12	53.33333	9	5	16
4	100	67	13	51.53846	18	50	18
3	82	40	10	40	17	10	30
2	70	39	10	39	21	5	34
10	191	57	15	38	4	5	36
4	95	30	8	37.5	18	50	37
1	53	37	9	41.11111	16	0	41
3	88	38	10	38	17	10	47
5	118	55	10	55	9	15	50
5	116	80	20	40	9	15	53
2	65	47	11	42.72727	21	5	56
1	52	50	20	25	16	0	58
2	71	54	13	41.53846	21	5	70
3	83	46	12	38.33333	17	10	74
10	199	52	13	40	4	5	86
1	54	45	12	37.5	16	0	88
2	72	39	12	32.5	21	5	100
10	197	55	10	55	4	5	100