2022-08-09

Development of a Rhizobium Seed Coating to Establish Lupine Species on Reclaimed Minelands

Bridget May Calder
Brigham Young University

Development of a Rhizobium Seed Coating to Establish Lupine Species on Reclaimed Minelands

Bridget May Calder

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

Matthew D. Madsen, Chair
Brad Geary
Joel S. Griffitts

Department of Plant and Wildlife Sciences

Brigham Young University

ABSTRACT

Development of a Rhizobium Seed Coating to Establish Lupine Species on Reclaimed Minelands

Bridget May Calder
Department of Plant and Wildlife Sciences, BYU
Master of Science

Symbiotic interactions among various organisms are often necessary for one or both individual's survival. These symbiotic relationships must be considered in restoration projects to allow for the successful establishment of the species. Rhizobia are nitrogen-fixing bacteria found in symbiotic relations with legumes. By utilizing this relationship, restoration practitioners can establish native legume species more successfully while repopulating soil microorganisms into degraded soils. Despite the potential benefits a rhizobium inoculant can have on restoration efforts, minimal research has been done to understand the impacts this treatment has on specific species and the systems they are employed within. Our research goal was to assess the efficacy of applying a commercial rhizobium product (EXCEED®) and indigenous rhizobium strains on two lupine species (*Lupinus argenteus* Pursh and *Lupinus sericeus* Pursh), commonly used for rangeland seedings in the Great Basin region of the western United States. We conducted laboratory and field trials to meet this research goal, with the results of the laboratory experiments shared in chapter 1 and findings from the field reported in chapter 2. In chapter 1, we evaluated in the laboratory whether indigenous rhizobia strains could be isolated, cultured, and applied as a liquid inoculant or a seed coating to induce root nodulation and increase plant growth. The performance of these inoculums was compared against the commercial rhizobium product. Additionally, we tested in a trial if compost could be applied within the seed coating to improve the efficacy of the rhizobium treatment. Our research demonstrated that the commercial inoculum induced root nodulation, and in one of three trials, this treatment improved plant growth. We also found indigenous strains effectively formed nodules on the plant roots when applied through a liquid culture or a seed coating. However, the number of root nodules and the presence of a pink color (indicating nitrogen fixation) were typically higher in the commercial product than in the indigenous strains when applied through a seed coating. These short-term laboratory studies generally provided minimal evidence that rhizobia impacted plant growth. However, data indicated that having compost in the coating alone improved shoot biomass by 33% ($P = 0.025$).

In chapter 2, research assessed the performance of the same rhizobia inoculums tested in the laboratory trials on a mine in northern Utah at two waste-rock sites, one comprised of crushed waste rock and the other made of waste rock amended with topsoil. One year after seeding, we had high plant recruitment at both study sites, and there were more plants, which were more vigorous, in the amended site ($P<0.001$). These results demonstrate that reclamation efforts on mineland overburden can be improved when topsoil is incorporated into the growing medium. At this stage in the study, there was no difference in plant establishment and vigor between any seed treatments, but future research is planned to assess these metrics in the next growing season.

The lack of improvement in plant growth from a rhizobia treatment in some of our laboratory and field trials may be due to the short period of these studies. Nodules that form on mature root systems provide more nitrogen-fixing benefits than those formed on immature roots. Hence, future research should consider conducting trials for more extended periods to understand how the treatments influence the growth of mature plants. Because we found in the laboratory that the rhizobia inoculums were successful in nodulating the test species, we anticipate that future studies will find that these treatments can improve plant performance and subsequently restoration success.

Keywords: lupine, rhizobia, seed coating, mineland reclamation, rangeland restoration

ACKNOWLEDGEMENTS

I would like to thank my graduate advisor, Matt Madsen (PhD) for mentoring me through this process, as well as my other committee members, Brad Geary (PhD) and Joel Griffitts (PhD), for their help and encouragement throughout this project. I would like to thank Rio Tinto Kennecott for funding this project, and Kate Rubelmann specifically, for helping to facilitate contact and site access. I want to thank Trevor Miller at Three River Seed Co® for providing techniques and advice for applying a rhizobium seed coating. In addition, I would also like to thank all of the students who helped to make this project happen, including Curtis Drake and Alex Benedict (PhD).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

Development of a Rhizobium Seed Coating to Establish Lupine Species on Reclaimed Minelands

Bridget M. Calder, Brad Geary, Joel S. Griffitts, Curtis L. Drake, Kate Ruebelmann, and Matthew D. Madsen
Department of Plant & Wildlife Sciences, Brigham Young University, Provo, UT
Master of Science

ABSTRACT

The establishment of native plant species on degraded and altered landscapes can be a widespread problem. A contributing factor to the success of a plant's survival may be symbiotic partners that inhabit the plant rhizosphere. These symbiotic partners may aid in nutrient acquisition, pathogen protection, stress tolerance, and many other things. Unfortunately, symbiotic microbes often have little to no presence in altered landscapes, so they must be re-integrated into the area to increase establishment and survival. We evaluated within a laboratory setting the ability of commercial and indigenous rhizobia strains to form nodules on lupine species used for rangeland seedings in the Great Basin region of the western United States and ascertained if these strains could be applied through a seed coating. We also evaluated if a compost amendment applied via seed coating could further enhance the performance of the rhizobia strains. Analysis showed successful nodulation could occur using commercial and wildland-collected indigenous strains through either a liquid culture applied to seedlings or as a dry seed coating. However, the number of root nodules and the presence of a pink color (indicating nitrogen fixation) were typically higher in the commercial product than in the indigenous strains. Compost did not improve nodulation or the performance of the nodules; however, this treatment alone improved shoot growth. Longer-term studies are now merited for

assessing how the rhizobia strains evaluated in this study influence plant growth, particularly in a field setting.

INTRODUCTION

Organisms of all kinds interact daily in ways we may not realize (Dimijan 2000). These symbiotic interactions can be necessary for survival and are especially important for sessile organisms such as plants (Baskett et al., 2009). Plant symbiotic partners may reside in the rhizosphere or directly within the tissues as endophytes. These microorganisms, such as mycorrhizal fungi and nitrogen-fixing bacteria, can help with pathogen protection, stress tolerance (Verma et al., 2021; Berendsen et al., 2012), and nutrient acquisition (Chibucos & Tyler, 2009). Because these microorganisms offer so many benefits, their presence in the soil can be a contributing factor to the successful establishment of native plant species (Ryan et al., 2008; Bent & Chanway 1998; Chanway, 1997).

Most often, these microorganisms are not present in areas with degraded soils or altered landscapes because they rely on the presence of their plant host species (Jacoby et al., 2017; Thrall, 2005; Murray et al., 2001). Landscape alterations may come from overgrazing, soil tillage, chemical fertilizers and pesticides, mineral extraction, and urban expansion (Cao, 2007; Killebrew & Wolff, 2010; Haddaway et al., 2019). A necessary step in restoring native landscapes is re-integrating these symbiotic microorganisms into the soil with the plants (Fofana et al., 2020; van der Heijden et al., 2016).

The reintroduction of nitrogen-fixing bacteria (rhizobia), in combination with their associated legume host plant, can affect successional trajectories by facilitating colonization and growth of subsequent species that are limited by soil organic matter and the availability of

critical nutrients such as nitrogen (Chapin et al., 1986; Ritchie & Tilman 1995; Chaer et al., 2011). Nitrogen-fixing bacteria provide the greatest source of fixed nitrogen to terrestrial environments (Maluk et al., 2022). When near their specified partner, rhizobia will induce nodulation of the legume roots. In the nodule, the rhizobia will fix atmospheric nitrogen into ammonium, and the plant will provide the necessary sugars to feed the bacteria (Bennett, 2016). This coalescence of metabolisms allows both partners to thrive in less-than-optimal conditions (Biswas et al., 2000; Udvardi & Poole, 2013; Thrall et al., 2005).

Seed coating technologies that deliver rhizobia at the time of planting may be especially useful in improving restoration efforts in severely degraded landscapes (Cao, 2007; Ma et al., 2019). Seed coating optimizes the delivery of bacteria by placing it in proximity to the host plant (Kavusi et al., 2022; Pawar et al., 2014; Horikawa & Ohtsuka, 1996). Despite the potential benefits rhizobia-coated seeds could provide to restoration efforts, minimal research has been directed towards optimizing this technology for this purpose. Restoration efforts could benefit from research that identifies rhizobia strains that are compatible with the host plant being sown and the site being restored. When there is no compatibility between the host plant and the rhizobia, there may be no nodulation or nodulation but no nitrogen-fixation (Yang et al., 2010; Wang et al., 2018). Additionally, even when the rhizobia are compatible, some strains may perform better with respect to such processes as nodule formation and nitrogen fixation (Irisarri et al., 2019; Karaca & Uyanoz, 2012; Singleton & Tavares, 1986). Commercial strains of rhizobia are available for most legume restoration species. However, there is minimal research showing how these strains will perform outside of an agricultural system. Additionally, in many systems, sufficient research has not been performed to understand if a commercial rhizobia inoculum is adequate or if indigenous rhizobia strains need to be collected and cultured to meet

restoration goals. Because indigenous strains are adapted to their native soils and environment, they may perform better than non-native strains (Singleton & Tavares, 1986). However, commercial rhizobia strains are often selected for their ability to produce nitrogen and be delivered through a seed coating (Bennett, 2016). If the commercial rhizobia strains were compatible with the species and site they were sown, they might outperform indigenous inoculum.

Research is also lacking in wildland systems on methods for delivering rhizobia through a seed coating. Unlike in agriculture, where the seeds are sown during a period that is conducive to germination, seeds sown in wildland systems may sit dormant in harsh conditions for several months before they germinate (Hoose et al. 2022). Seed coating methods and materials that maximize the survival of the rhizobia over the dormant season could aid in the success of this treatment in ecological restoration projects.

Ground and sterilized peat is a favorable medium for the growth of bacteria and is a useful carrier for the organisms within a seed coating (van Schreven, 1970; Thompson, 1980; Casteriano et al., 2013). More recently, biochar has been shown to be a suitable inoculant carrier, which is thought to be due to its ability to protect microorganisms, hold moisture, and provide a potential source of energy and minerals (Glodowska et al. 2016). Compost has also been shown to have a synergistic effect when combined with plant-associated microbes, such as rhizobia (Ben-Laouane et al., 2020). The increased rhizobium activity produced by the compost may be due to the material acting as a carbon and nutrient source (Otieno et al., (2007). Additionally, including compost in a seed coating may further aid in the success of a restoration project by supplying the seed with a diversity of beneficial microbes (Ingham et al. 1985) that can suppress plant diseases (Gamliel et al., 2000) and aid in plant growth (Cogger, 2005).

The objectives of our research were to 1) determine if indigenous rhizobia strains induce nodulation of native lupine species and compare their performance against a commercial rhizobium inoculant; 2) ascertain if these strains could be applied through a seed coating while maintaining bacterial viability; and 3) determine if incorporating a blend of biochar and compost into the coating process will aid in the effectiveness of rhizobia coatings. The research was conducted on silky lupine (*Lupinus sericeus* Pursh) and silvery lupine (*Lupinus argenteus* Pursh). Lupine species, in general, are useful in restoring severely degraded rangelands, as their symbionts help increase soil nitrogen levels, promoting the growth of other native species and advancing successional processes (Chaer et al., 2011; Tilley et al., 2019). Silky and silvery lupine are favored for their bright purple flowers and ability to add biodiversity, benefit pollinators, and provide forage for wildlife (Jones et al., 2016). Seeding trials were conducted in a laboratory using nitrogen-free media. We hypothesized that 1) commercial and indigenous rhizobia strains would successfully nodulate the roots of lupine when applied directly to an immature seedling or when added to a seed coating; 2) the performance of the rhizobia strains would vary in their ability to promote plant growth; and 3) the application of a blend of compost and biochar in conjunction with a rhizobia coating would further enhance seedling establishment and plant growth.

MATERIALS & METHODS

Our research was conducted under three separate trials; the first study (hereafter referred to as EXCEED® Trial) was implemented to test the efficacy of the commercial rhizobium product EXCEED® H Type Inoculant for Lupine (Visjon Biologics, Henrietta, TX, USA) in its ability to improve plant growth when delivered through a seed coating. The next two trials were

focused on understanding the performance of four indigenous rhizobia strains to each other and that of the commercial strain tested in the EXCEED® trial. In the Inoculation Trial we tested the inoculants when they were delivered as a liquid culture onto pre-germinated seeds. This study allowed us to evaluate the rhizobium treatments' performance without the interaction of the seed coating. In our last trial (Coating Trial), we evaluated the inoculants when applied through a seed coating. Additionally, within this seed coating trial, we assessed if adding a compost amendment within the seed coating would enhance the rhizobia's performance and the plant's subsequent growth.

Collection, Isolation, and Preparation of Indigenous Rhizobia Strains

The roots of indigenous lupine plants were excavated from the soil and their nodules were harvested at three different locations: Rio Tinto Kennecott's Bingham Canyon Mine, South Jordan, UT, USA (40.501556, -112.141833); Lupine Hiking Trail, Draper, UT, USA (40.464417, -111.828694); and near Strawberry Reservoir, Heber, UT, USA (40.15440, -111.20236). At each site, we obtained nodules from 5-10 individual plants. The Rio Tinto Kennecott site was a historic mining area now covered in waste rock comprised of weathered monzonite, quartzite, and limestone rock (Borden 2001; Borden 2003). Its elevation is approximately 2000 m. Due to the extended time the waste rock has been deposited on site, it has been colonized by a small group of species, one of which is tailcup lupine (*Lupinus caudatus* Kellogg), from which we collected root nodules. The Lupine Hiking Trail site has an elevation of 1724 m. This Mountain Loam (Mountain Big Sagebrush) ecological site (https://wildlife.utah.gov/range-trend.html) has a soil pH of 7.0, and the soil texture is classified as a loam (Web Soil Survey 2022). Root nodules were collected from silvery lupine (*Lupinus argenteus* Pursh), one

of the site's dominant forbs. Our last collection site, near Strawberry Reservoir, was classified as a Mountain Loam ecological site and is located at an elevation of 2430 m, with a loamy soil texture and pH of 7.2 (Web Soil Survey 2022). This site contained multiple lupine species, and nodules were collected from silky lupine (*Lupinus sericeus* Pursh), silvery lupine, and bigleaf lupine (*Lupinus polyphyllus* Lindl.).

Upon collection, individual nodules were surface sterilized by swirling for 5 s in 75% ethanol, and then 25% bleach, followed by three consecutive dishes filled with autoclaved, distilled water (ddH_2O). Following sterilization, nodules were crushed with a sterile pestle in an Eppendorf tube containing 100 uL tryptone yeast (TY) broth (Table 2). Once the bacteria had been released from the nodules, 5 uL of this liquid was spread onto a TY agar plate (Table 2) and placed in an incubator at 30ºC. Rhizobia strains were then identified to the species level using 16s sequencing (Kim & Chun, 2014). Strains which were identified as rhizobia were transferred from the agar into 100 uL TY liquid broth solution and incubated for 4 d at 30˚C. From here, we selected one rhizobia strain from each site to be used in the study, with the selection of rhizobia based on their ease of growing in liquid culture. For each rhizobia strain, we determined colony counts from optical density (OD) measurements and plated liquid culture CFU counts. From there, we estimated CFU based on the corresponding OD of our final culture, using this knowledge we could add the correct dilution of liquid culture to our peat mixture to get a desired CFU count. This was used to match our commercial product's (Exceed® H Type Inoculant for Lupine (Visjon Biologics, Henrietta, TX, USA)) count of 2×10^9 colony-forming units.

EXCEED® Trial: Evaluation of a Commercial Rhizobium Product

Silky lupine seeds were coated in a rotary drum seed coater (Universal Coating Systems, Independence, OR, USA), with the rotary pan set at 20% of the maximum speed. Coating was performed on a 200 g batch of seed. At the start of the coating procedure, the seeds were initially wet with 20 mL of polyvinyl alcohol (PVA) binder (Ashland Inc., Covington, KY, USA), which was prepared with a 20% solid content. This step was followed by the addition of 25 g of calcium bentonite. Polyvinyl alcohol and calcium bentonite provided a base to help adhere the next coating layer, which was comprised of a blend of wood flour (160 g), powdered cellulose (30 g), and calcium bentonite (10 g). While applying this coating layer, we also simultaneously used ~160 mL of PVA binder. Next, we evenly added 2.5 g of EXCEED® inoculum without the addition of PVA binder. Binder was withheld from this step to reduce wetting of the rhizobium bacteria and their subsequent activation. We then added on top of the inoculum an additional layer of the same blend and amount of wood flour, powdered cellulose, and calcium bentonite as was previously applied, with the same amount of PVA binder. Seeds were dried at room temperature (~21˚C) in a forced air dryer (Braceworks Automation and Electric, Lloydminster, SK, CAN) for ~30 minutes. Coated seeds were stored in the fridge at 4ºC until planting.

The performance of the coated rhizobium seeds was compared against untreated seeds (control). Seeds of each treatment were sown separately in 7.62 cm^2 X 10.16 cm deep polycarbonate plant tissue culture boxes (Plantmedia, Dublin, OR, USA) filled with a sterilized 4:1 mixture of Turface® (Profile Products LLC, Buffalo Grove, IL, USA) and ground vermiculite. Each box had four 3 mm holes, and one 8 mm hole drilled in the bottom to allow for water drainage. To maintain moisture in the box and prevent contamination, we inverted an additional plant tissue culture box over the top of the boxes that contained the plants and growing

medium. This “lid” had an 8 mm hole drilled on the top, which was covered with filter tape to allow for air circulation while maintaining sterility.

Plant tissue culture boxes were planted with eight seeds (5 mm deep) and placed in a walk-in growth chamber (Environmental Growth Chambers, Chagrin Falls, OH, USA), held at a constant temperature of 15°C. This incubation temperature was chosen to mimic spring conditions when seedlings are actively growing during the early seedling stages in the sagebrush steppe of the Great Basin, USA. Lights in the chamber provided a 12 h photoperiod, with a maximum photosynthetically active radiation flux density of approximately 780 μmol $m^{-2}s^{-1}$, at plant height. Boxes were arranged by treatment to decrease the likelihood of contamination and randomized weekly. Plants were watered as needed with a nitrogen-free watering solution (Table 1) for 10 weeks.

After ten weeks, we measured plant height from ground level and then harvested above and below ground biomass for analysis of nitrogen content. During harvest, the presence of nodules was noted for each treatment. Biomass was harvested by washing the growing medium from the roots and then drying in an oven at 105°C for four days. To have enough biomass for measuring nitrogen content, replicate samples were combined into one. Total nitrogen was analyzed by combustion using the Dumas method (LECO TruSpec CN Determinator, LECO Instruments, St. Joseph, MI, USA).

The effect of the commercial rhizobium inoculum on germination and plant height was evaluated using generalized linear mixed-effect models (Sileshi, 2012). In the model, germination and plant height data were fit to a Beta and normal distribution, respectively. Student t-test was used to test the level of difference between treatments. For all comparisons, a significance level of $P < 0.05$ was used.

Inoculation Trial: Evaluation of Indigenous Rhizobia Strains on Root Nodulation and Plant Growth when Applied as a Liquid Culture

We evaluated six different treatments: control (no rhizobia applied), Exceed® H Type Inoculant for Lupine (commercial peat inoculum), a liquid culture of the rhizobia in the Exceed® H Type Inoculant for Lupine (commercial liquid inoculum), and liquid cultures from each of the three native strain collections (Rio Tinto, Strawberry, Draper). The research was performed on silvery lupine. Prior to the application of the inoculum, seeds were pre-germinated in 15-cm diameter Petri dishes with two layers of blue blotter paper (Anchor Paper Co., St. Paul, MN, USA). Germinates were transplanted into the plant tissue culture boxes described above with the same growing medium. After 7 d, the seedlings were treated with one of the six treatments described above. For the commercial peat inoculum, we applied 1 g of the product per box (0.5 g per plant). Liquid inoculums were diluted to an OD of 0.05 nm and resuspended in ddH_2O. These solutions were applied directly to the box using a sterile pipet. Depending on the treatment, each plant was given 500 uL of inoculated water or ddH_2O. The boxes containing the different treatments were arranged in the growth chamber under a completely randomized design with six replicates per treatment. Boxes were re-randomized each week. Plants were watered with ddH_2O for the first two weeks (until seedlings had begun to establish), and for the remainder of the study, a nitrogen-free fertilizer (Table 1) was included with the water. After ten weeks, plants were harvested and we measured shoot length, above and below ground biomass, the number of root nodules, and total nitrogen content in the plant tissue. Biomass was assessed by washing the growing medium from the roots and then drying it in an oven at 105°C for two

days. Total nitrogen is currently being analyzed by combustion using the Dumas method (LECO TruSpec CN Determinator, LECO Instruments, St. Joseph, MI, USA), with results forthcoming.

We evaluated the effect of the rhizobium inoculum treatments on shoot length, above and below ground biomass, number of nodules, and total nitrogen using generalized models with a Poisson distribution (JMP® Pro 16; SAS Institute Inc., Cary, NC, USA). There were four plant tissue culture boxes that did not produce plants and that were not included in the analysis. We performed pairwise comparisons between treatments using the Tukey HSD pairwise comparison test. For all comparisons, a significance level of $P < 0.05$ was used.

Coating Trial: Evaluation of Indigenous Rhizobia Strains when Applied with Different Seed Coatings

As with the previous trial, this research was performed on silvery lupine. Seeds were scarified to weaken the hard seed coat and improve germination uniformity by soaking in 98% sulfuric acid for 4 min, followed by thorough washing with distilled water for 10 min. After scarification, seeds were left to dry under ambient conditions prior to coating for 24 h. Seeds were either coated with the materials used in Trial 1 (hereafter referred to as Standard Coating) or with this same coating except for the wood flour, powdered cellulose, and calcium bentonite was replaced with LifeCube Compost & Soil Builder (TeaLab, Eureka, CA, USA) (hereafter referred to as Compost Coating). Lifecube Compost & Soil Builder is composed of a blend of compost, biochar, worm castings, insect frass, biokashi, kelp meal, rock dust, and alfalfa meal (Table 3). These two different seed coating treatments were either applied to the seed alone or with one of the four different rhizobia inoculum strains described in the Inoculation Trial: Exceed® H Type Inoculant for Lupine, and indigenous strain collections at Rio Tinto,

Strawberry, and Draper (11 treatments total; Table 4). The indigenous inoculum strains were applied within the PVA binder.

The trial was run in the same plant tissue culture boxes, growing medium, growing conditions (i.e., light, temperature, water, and fertilizer), and arrangement in the growth chamber described previously. Plant tissue culture boxes contained 16 seeds each. The study was arranged in the growth chamber in a completely randomized design with 11 replicates per treatment. After two weeks from planting, each box was thinned to three plants. We chose the top three healthiest-looking plants to remain in the boxes. Seedlings that emerged after thinning over the remainder of the study were removed. After 10 weeks, we measured the same metrics as described in the Inoculation Trial, plus we also ranked the nodule color from 1-5. Plants with no nodules were ranked 0, and plants with dark pink nodules were ranked 5. Older nodules that were brown or green were given a value of 1, as well as those which were white. As with the Inoculation Trial, total nitrogen content is currently being analyzed and will be reported in a future publication.

We evaluated the effect of the rhizobium inoculum treatments on seedling emergence, number of nodules, shoot length, and above and below-ground biomass using generalized regression models (JMP® Pro 16; SAS Institute Inc., Cary, NC, USA). In the models, emergence, number of nodules, and shoot length were fit with beta, Poisson, and log-normal distributions, respectively. Above and below-ground biomass were both fitted with normal distributions. For above and below-ground biomass the model included all the treatments, and a second analysis was also performed with just the treatments that did not contain a rhizobia treatment, to assess the effects of the coatings alone. A Tukey HSD, pairwise comparison test, was used to analyze differences among all treatments. In addition, Dunnett's multiple comparison test was used to

assess differences in treatment means to the control. For all comparisons, a significance level of $P < 0.05$ was used.

RESULTS

EXCEED® Trial: Evaluation of a Commercial Rhizobia Product

There was no difference in seedling emergence between the control (uncoated) and coated seed with germination at 44.4 ± 8.1 % and 50.0 ± 5.1 %, respectively ($P = 0.075$; $t = 1.92$). Plants grown from coated seeds had a 53% increase in shoot height relative to the control ($P = 0.008$; $t = 3.09$; Figure 1). While harvesting the study, we observed that the plants grown from coated seed had nodules. In contrast, plants from untreated seed generally lacked nodules and were relatively smaller in size when present compared to the seed coating treatment. An analysis of plant biomass with all replicates combined showed that plants grown from coated seed had a 40% increase in shoot nitrogen content (control = 1.11 ppm, coated = 1.55) and 29% increase in root nitrogen content (control = 1.44 ppm, coated = 47), respectively, relative to the control.

Inoculation Trial: Evaluation of Indigenous Rhizobia Strains on Root Nodulation and Plant Growth when Applied as a Liquid Culture

Rhizobia strains from Draper (Dr), Strawberry Reservoir (St), and the commercial inoculums had more root nodules in comparison to the control ($P < 0.05$; Figure 2). While higher on average, the number of nodules produced from plants grown with Rio Tinto (RT) inoculum was not significant from the control ($P = 0.0503$; Figure 1). There was no difference between the inoculation treatments for shoot height ($P = 0.566$) or root weight ($P = 0.066$), but there was a

difference in shoot weight ($P = 0.017$). Here the St isolate produced shoots that were 57% larger than the control ($P = 0.031$), but no other treatments were significant for this metric (Figure 3).

Coating Trial: Evaluation of Indigenous Rhizobia Strains Applied with Different Seed Coatings

The standard coating (SC) with no added bacteria and the compost coating (CC) + Exceed® were the only two treatments that showed a statistical improvement in emergence, with a 24.5% ($P = 0.039$) and 23.6% ($P = 0.0291$) increase over the control, respectively (Figure 4). All the treatments had an overall effect on shoot height ($P = 0.025$). However, Tukey analysis showed no difference between any two treatments, but the Dunnett's test showed increased shoot height over the control for SC, CC + EXCEED®, SC + St, SC + Dr, and SC + EXCEED®, with lengths ranging between 22-27% higher than the control ($P = 0.014$-0.046; Figure 5).

There was a drastic difference in the number of root nodules produced by the different seed coating treatments ($P < 0.001$; Figure 6). The SC and CC coatings with EXCEED® were the top performing treatments, with EXCEED® having between 4.4-14.1 and 3.6-27.3-fold more nodules than the indigenous coatings in the SC and CC groups, respectively. In addition to EXCEED® coatings, nodule counts were higher than the control for SC+St ($P < 0.0001$), SC+RT ($P = 0.0207$), and CC+St ($P = 0.0002$).

In general, all the treatments with rhizobia strains had pink nodules indicating that active nitrogen fixation was occurring, while the few nodules growing on plants in the control or coatings without rhizobia were lacking in this color (Figure 7; $P < 0.001$). The degree of pink in the nodules was highest on average for EXCEED® although statistically the other rhizobia treatments had a similar color to this product. However, only SC+EXCEED®, SC+St and CC+EXCEED® were higher than the control ($P = 0.002$-0.008; Figure 7).

Lastly, no overall treatment effect was detected for shoot and root biomass (shoots P = 0.067; roots P = 0.183). However, when the rhizobia treatments were excluded from the analysis, there was a significant effect on shoot biomass (P = 0.025) but not root biomass (P = 0.192). The CC treatment had a 33% increase in shoot weight in comparison to the control (P = 0.026), while the SC showed an intermediate response between the control and the CC treatment (Figure 8).

DISCUSSION

The establishment and long-term survival of native plant species on degraded and altered landscapes can be a major challenge to restoration efforts. A contributing factor may be the absence of vital soil symbionts that allow for species success in low-nutrient areas (Chibucos & Tyler, 2009). By incorporating microorganisms directly into a seed coating, restoration practitioners can better ensure site-specific inoculation into the plant rhizosphere (Cao, 2007). We hypothesized that applying a commercially produced inoculum (EXCEED®) through seed coating would induce nodulation. We also hypothesized that wildland-collected indigenous rhizobia strains would nodulate the roots of lupine as successfully as a commercial inoculum. Our research that added inoculum to pre-germinated seeds via liquid culture allowed us to evaluate the performance of commercial and indigenous strains without the interaction of the coating process and seed coating materials. The results of this study showed successful nodulation can occur using commercial inoculum and generally for wildland-collected indigenous strains. In this study, there was an increase in nodule numbers from all the treatments except for the RT strain, which was not quite significant from the control (P = 0.0503; Figure 2). When the different rhizobia treatments were applied through a seed coating the commercial inoculum far surpassed the indigenous strains in the number of root nodules that were produced

(Figure 6). The quality of the nodules, as assessed by ranking them by color showed the indigenous rhizobia were statistically comparable to commercial inoculum (Figure 7). However, it may be important to note, that the color ranking for the commercial inoculum was higher on average than the indigenous rhizobia strains.

There are many factors that may influence the survival and viability of bacterial isolates. It is possible that modifications to the seed coating procedures could improve the efficacy of the treatment for applying indigenous rhizobia. In our study, the rhizobia were applied during the coating process through a liquid inoculant. While we had high nodule number after coating, the drying process after coating may negatively influence survival rates of the bacteria (Hartley, et al., 2012). The commercial product is applied to the seed with the rhizobia incorporated within a peat-based medium. In the application of the rhizobia to the peat-based medium, methods could be used to optimize the survival of the rhizobia, such as by controlling how fast the peat is dried after receiving the organism. Furthermore, storage of the bacteria in the peat medium has improved their survival. Hartley et al. (2012) showed that while a more mature inoculum may have lower bacteria cell numbers, they tend to have greater organism survival. Immature bacterial colonies are often focused most entirely on growth. They may not be able to handle the stresses of the coating process as well as those with more established growth and colonies (Hartley et al., 2012). Future research could test various methods of applying indigenous isolates and growing them in a medium for a time before incorporating them in the seed coating.

We also saw some nodules form on treatments that did not have an inoculum. Nodules such as those could be due to other bacterial strains that cause nodulation but do not provide nitrogen fixation. Not only do these bacterial strains not fix nitrogen, but they also may inhibit the nodulation of rhizobia (Bent & Chanway 1998). Undesirable bacteria could have come from

the seed as well as the coating process. While seedlings were grown in a sterilized environment, the coating process was not sterile. In addition, these bacteria could inhibit the nodulation of desired rhizobia strains; in addition, some rhizobia strains may nodulate and fix nitrogen but at such a low rate that the benefits are minimal. Not only are these strains poor nitrogen fixers, they also may work to inhibit strains that would fix nitrogen more efficiently (Hartley et al., 2012). Future research may warrant sterilizing the seeds prior to planting. It may also be necessary to compare various strains against each other to see which will provide higher nitrogen fixation benefits while also being competitive against other strains. In addition, the selection of strains that would better survive the coating process would be necessary (Hartley et al., 2012).

Our research provided only moderate evidence that a rhizobium inoculant would improve plant growth during the early stages of plant development. We saw improved growth in the first trial that compared a commercial seed coating inoculum to untreated seed. Additionally, we recorded increased shoot weight being produced from the St isolate. No improvement in plant growth was recorded for any isolates when applied through a seed coating in our third study. Had this study been allowed to continue for longer, we may have seen a more considerable difference in plant growth. Specifically, one reason for this is because nitrogen production can increase as the plant matures and forms nodules. Mature nodules will fix more nitrogen than those found in immature root systems (Hardarson et al., 1989). Additionally, the lack of response in our coated seeds could be due to the time it takes to release the bacteria from the coating and have the organism infect the plant's roots and cause nodulation. This would take longer than the process of our inoculation trial, where we applied liquid inoculum to pre-germinated seeds. Hence, this data collectively may indicate that future studies should be conducted for longer to evaluate the rhizobia inoculum's impact on plant growth.

Our research indicated that the rhizobia inoculum had a minimal impact on seedling emergence. This lack of response in emergence suggests that the effects of rhizobia are not evident until more mature plant stages (Hardarson et al., 1989). However, it is interesting to note that most of the coating treatments had higher emergence on average in comparison to the control and for a couple of treatments, germination was statistically higher (Figure 4). Improvements in germination may be due to the seed coating process conditioning the seed coat to allow for more rapid germination.

Our final hypothesis was that the use of compost in conjunction with rhizobia would further enhance seedling growth and establishment. We found that compost did not improve nodulation or the performance of the nodules; however, this treatment alone improved shoot growth (Figure 8). This may be due to a higher level of nutrients present in compost that is not present in other coatings or our growing medium (Cogger, 2005). However, the minuscule amount of compost applied within the seed coating would provide minimal nutritional benefit to the plant. A more substantial advantage may be that the compost inoculates the growing medium with various microorganisms that can bring many benefits such as increased nutrient mobilization, retention of water and nutrients, disease suppression, and improved soil function (Abbasi et al. 2002; Jacoby et al., 2017).

In conclusion, this study provides evidence that both commercial and indigenous strains of rhizobia can successfully nodulate the model lupine species tested in this study but for some metrics, the commercial product appears to outperform the indigenous strains. Future research is merited for furthering the development of the rhizobia coating for indigenous species. Establishing rhizobia back into the plant rhizosphere at the time of planting will help to provide a long-term solution to establishing species in poor soils. They will also work to establish other

plant species by modifying soils back to pre-altered landscapes (Chapin et al., 1986; Ritchie & Tilman 1995; Chaer et al., 2011).

LITERATAURE CITED

Abbasi, P.A., Al-Dahmani, J., Sahin, F., Hoitink, H.A.J., Miller, S.A. (2002). Effect of compost amendments on disease severity and yield of tomato in organic and conventional production systems. *Plant Dis*. **86**:156–161.

Baskett, M. L., Gaines, S. D., & Nisbet, R. M. (2009). Symbiont diversity may help coral reefs survive moderate climate change. *Ecological Applications*.

Bennett, G. M. (2016). Seed Inoculation, Coating and Precision Pelleting: Science, Technology and Practical Applications. New York, USA: CRC Press Taylor and Francis Group.

Bent, E., & Chanway, C. P. (1998). The growth-promoting effects of a bacterial endophyte on lodgepole pine are partially inhibited by the presence of other rhizobacteria. *Canadian Journal of Microbiology*, **44**:980–988.

Ben-Laouane, R., Ait-El-Mokhtar, M., Anli, M., Boutasknit, A., Rahou, Y. A., Raklami, A., . . . Meddich, A. (2020). Green compost combined with mycorrhizae and rhizobia: A strategy for improving alfalfa growth and yield under field conditions. *Gesunde Pflanz,* **73**, 193-207.

Berendsen, R. L., Pieterse, C. M., & Bakker, P. A. (2012). The rhizosphere microbiome and plant health. *Trends in Plant Science*, **17**:478-486.

Biswas, J. C., Ladha, J. K., & Dazzo, F. B. (2000). Rhizobia Inoculation Improves Nutrient Uptake and Growth of Lowland Rice. *Soil Science Society of America*, **64**:1644-1650.

Borden, R. (2001). Geochemical evolution of sulphide-bearing waste rock soils at the Bingham Canyon Mine, Utah . *Geochemistry: Exploration, Environment, Analysis*, **1**:15-21.

Borden, R. K. (2003). Environmental geochemistry of the Bingham Canyon porphyry copper deposit, Utah. *Environmental Geology*, **43**:752-758.

Casteriano A, Wilkes MA, Deaker R. (2013). Physiological changes in rhizobia after growth in peat extract may be related to improved desiccation tolerance. *Applied Environmental Microbiology,* **79**:3998-4007.

Cao, X. (2007). Regulating mine land reclamation in developing countries: The case of China. *Land Use Policiy*, **24**:472-483.

Chaer, G. M., Resen, A. S., Campello, E. F., de Faria, S. M., & Boddey, R. M. (2011). Nitrogen-fixing legume tree species for the reclamation of severely degraded lands in Brazil . *Tree Physiology*, **31**:139–149.

Chanway, C. P. (1997). Inoculation of Tree Roots with Plant Growth Promoting Soil Bacteria: An Emerging Technology for Reforestation. *Forest Science*, **43**:99–112.

Chapin III, F. S., Vitousek, P. M., & Cleve, K. V. (1986). The Nature of Nutrient Limitation in Plant Communities. *American Naturalist,* **127**, 48-58.

Chibucos , M. C., & Tyler, B. M. (2009). Common themes in nutrient acquisition by plant symbiotic microbes, described by the Gene Ontology. *BMC Microbiology*, **9**:S6.

Cogger, C. G. (2005). Potential Compost Benefits for Restoration of Soils Disturbed by Urban Development. *Compost Science & Utilization,* **13**, 243-251.

Dimijian, G. G. (2000). Evolving together: the biology of symbiosis, part 1 . *Baylor University Medical Center Proceedings* , **13**:217–226.

Fofana, B., Sacande, M., Blagna, F., Dibloni, T. O., Compaore, E., Sanon, K. B., . . . Ouattara, A. S. (2020). Boosting land restoration success in the Great Green Wall through the use of symbiotic microorganisms for propagated tree seedlings . *International Journal of Biological and Chemical Sciences*.

Gamliel, A., Austerweil, M., & Kritzman, G. (2000). Non-chemical approach to soilborn pest management - organic amendments. *Crop Protection,* **19**, 847-853.

Glodowska, M., Husk, B., Schwinghamer, T., & Smith, D. (2016). Biochar is a growth-promoting alternative to peat moss for the inoculation of corn with a pseudomonad. *Agronomy for Sustainable Development,* **36**, 21.

Haddaway, N. R., Cooke, S. J., Lesser, P., Macura, B., Nilsson, A. E., Taylor, J. J., & Kaisa, R. (2019). Evidence of the impacts of metal mining and the effectiveness of mining mitigation measures on social–ecological systems in Arctic and boreal regions: a systematic map protocol. *Environmental Evidence*, **8**:9.

Haigh, M., Desai, M., Cullis, M., D'Aucourt, M., Sansom, B., Wilding, G., . . . Jenkins, R. (2019). Composted Municipal Green Waste Enhances Tree Success in Opencast Coal Land Reclamation in Wales. *Air, Soil, and Water Research*.

Hardarson, G., Golbs, M., & Danson, S. K. (1989). Nitrogen fixation in soybean (Glycine max L. Merrill) as affected by nodulation patterns. *Soil Biology and Biochemistry*, **21**:783-787.

Hartley E. J., Gemell L. G., Deaker R. (2012) Some factors that contribute to poor survival of rhizobia on preinoculated legume seed. *Crop and Pasture Science,* **63**:858-865.

Hoose, B.W., Call, R.S., Bates, T.H., Anderson, R.M., Roundy, B.A., Madsen, M.D. (2022). Seed conglomeration: a disruptive innovation to address restoration challenges associated with small-seeded species. *Restoration Ecology,* **27**:959-965.

Horikawa, Y., & Ohtsuka, H. (1996). Effects of Coating and Adhesive on the Inoculation of Rhizobium metiloti to Alfalfa (Medicago sativa L.) Seeds for Nodulation and Seedling Growth. *Japanese Society of Grassland Science*, **41**:275-279.

Ingham, R. E., Trofymow, J. A., Ingham, E. R., & Coleman, D. C. (1985). Interactions of bacteria, fungi, and their nematode grazers - effects on nutrient cycling and plant-growth. *Ecological Monographs,* **55**, 119-140.

Innovative Uses of Compost Reforestation, Wetlands Restoration, and Habitat Revitalization. (1997, October). Retrieved from EPA: https://www.epa.gov/sites/default/files/2015-08/documents/reforest.pdf

Irisarri, P., Cardozo, G., Tartaglia, C., Reyno, R., Gutiérrez, P., Lattanzi, F. A., . . . Monza, J. (2019). Selection of Competitive and Efficient Rhizobia Strains for White Clover. *Frontiers in Microbiology*.

Jacoby, R., Peukert, M., Succurro, A., Koprivova, A., & Kopriva, S. (2017). The Role of Soil Microorganisms in Plant Mineral Nutrition—Current Knowledge and Future Directions. *Frontiers in Plant Science*, **8**:1617.

Jones, C. D., Stevens, M. R., Jolley, V. D., Hopkins, B. J., Jensen, S. L., Turner, D., & Stettler, J. M. (2016). Evaluation of thermal, chemical, and mechanical seed scarification methods for 4 Great Basin lupine species. *Native* Plants, **17**:5-17.

Karaca, Ü., & Uyanöz, R. (2012). Effectiveness of native Rhizobium on nodulation and growth properties of dry bean (Phaseolus vulgaris L.). *African Journal of Biotechnology*, **11**: 8986-8991.

Kavusi, E., Ansar, B. S., Dehghanian, Z., Lajayer, B. A., Nobaharan, K., Ma, Y., & Glick, B. R. (2022). Delivery of Beneficial Microbes via Seed Coating for Medicinal and Aromatic Plant Production: A Critical Review. *Journal of Plant Growth Regulation*, **10**:1357.

Killebrew, K., & Wolff, H. (2010). *Environmental Impacts of Agricultural Technologies.* Washington: Evans School of Public Affairs.

Kim, M., & Chun, J. (2014). 16S rRNA Gene-Based Identification of Bacteria and Archaea using the EzTaxon Server. In *Methods in Microbiology*, 61-74.

Ma, H., Egamberdieva, D., Wirth, S., & Bellingrath-Kimura , S. D. (2019). Effect of Biochar and Irrigation on Soybean-Rhizobium Symbiotic Performance and Soil Enzymatic Activity in Field Rhizosphere. *Agronomy*, **9**:626.

Maluk, M., Ferrando-Molina, F., Lopez del Egido, L., Langarica-Fuentes, A., Yohannes, G. G., Young, M. W., . . . Squire, G. R. (2022). Fields with no recent legume cultivation have sufficient nitrogen-fixing rhizobia for crops of faba bean (Vicia faba L.). *Plant and Soil*, 345–368.

Murray, B. R., Thrall, P. H., & Woods, M. J. (2001). Acacia species and rhizobial interactions: Implications for restoration of native vegetation. *Ecological Management and Restoration*, **2**:213-219.

Otieno, P. E., Muthomi, J. W., Cheminingwa, G. N., & Nderitu, J. H. (2009). Effect of rhizobia inoculation, farmyard manure and nitrogen fertilizer on growth, nodulation and yield of selected food grain legumes. *Journal of Biological Sciences,* **9**, 326-332.

Pawar, V. A., Pawar, P. R., Bhosale, A. M., & Chavan, S. V. (2014). Effect of Rhizobium on Seed Germination and Growth of Plants. *Journal of Academia and Industrial Research (JAIR)*, **3**:84-88.

Ritchie, M. E., & Tilman, D. (1995). Responses of Legumes to Herbivores and Nutrients During Succession on a Nitrogen-Poor Soil. *Ecology,* **76**, 2648-2655.

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., & Dowling, D. N. (2008). Bacterial endophytes: recent developments and applications . *FEMS Microbiology Letters*, **278**:1-9.

Sileshi, G. W. (2012). A Critique of Current Trends in the Statistical Analysis of Seed Germination and Viability Data. *Seed Science Research,* **22**, 145-159.

Singleton, P. W., & Tavares, J. W. (1986). Inoculation Response of Legumes in Relation to the Number and Effectiveness of Indigenous Rhizobium Populations. *Applied and Environmental Microbiology*, **51**:1013-1018.

Thompson, J. A. (1980). Production and Quality Control of Legume Inoculants. In F. Bergersen, *Methods for Evaluating Biological Nitrogen Fixation*, 489-533.

Thrall, P. H., Millsom, D. A., Jeavons, A. C., Waayers, M., Harvey, G. R., Bagnall, D. J., & Brockwell, J. (2005). Seed inoculation with effective root-nodule bacteria enhances revegetation success. *Journal of Applied Ecology*, **42**:740–751.

Tilley, D., Tilley, N., Fund, A. J., & Wolf, M. (2019). *Seedling growth and competition of a late-seral, native perennial grass and two early-seral, native forbs in the presence of two densities of the invasive annual grass Bromus tectorum L.* Aberdeen: USDA.

Tokgöz, S., Lakshman , D. K., Ghozlan, M. H., Pinar, H., Roberts, D. P., & Mitra, A. (2020). Soybean Nodule-Associated Non-Rhizobial Bacteria Inhibit Plant Pathogens and Induce Growth Promotion in Tomato. *Plants,* **9**, 1494.

Udvardi, M., & Poole, P. S. (2013). Transport and Metabolism in Legume-Rhizobia Symbioses. *Annual Review of Plant Biology*, **64**:781-805.

van der Heijden, M. G., de Bruin, S., Luckerhoff, L., van Logtestijn, R. S., & Schlaeppi, K. (2016). A widespread plant-fungal-bacterial symbiosis promotes plant biodiversity, plant nutrition and seedling recruitment. *The ISME Journal*, **10**:389–399.

Van Schreven, D.A., Lindenbergh, D.J., Koridon, A. (1970). Effect of several herbicides on bacterial populations and activity of persistence of these herbicides in soil. *Plant and Soil,* **33**: 513-532.

Verma, S. K., Sahu, P. K., Kumar, K., Pal, G., Gond, S. K., Kharwar, R. N., & White, J. F. (2021). Endophyte roles in nutrient acquisition, root system architecture development and oxidative stress tolerance. *Journal of Applied Microbiology*, **131**:2161-2177.

Wang, Q., Liu, J., & Zhu, H. (2018). Genetic and Molecular Mechanisms Underlying Symbiotic Specificity in Legume-Rhizobium Interactions. *Frontiers in Plant Science*, **9**:313.

Web Soil Staff. (n.d.). Retrieved from USDA NRCS Web Soil Survey: https://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx

Yang, S., Tang, F., Gao, M., Krishnan, H. B., & Zhu, H. (2010). R gene-controlled host specificity in the legume–rhizobia symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, **107**:18735–18740.

FIGURES

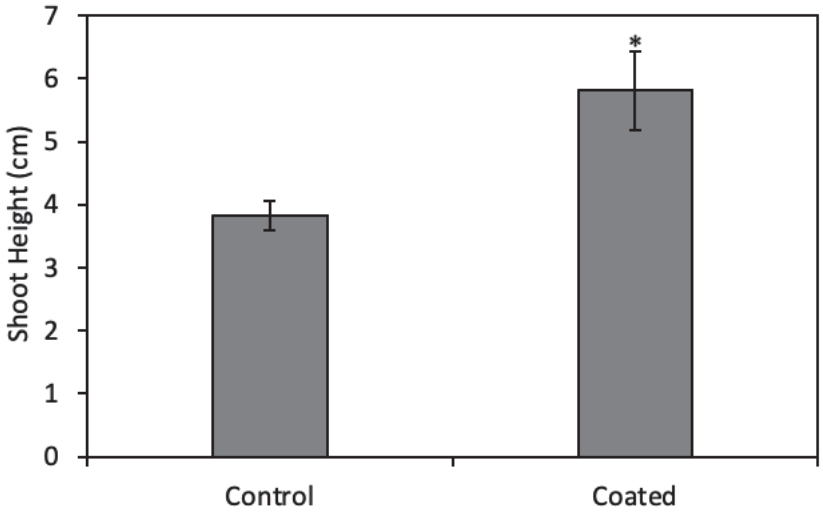

Figure 1 – Silky lupine (*Lupinus sericeus* Pursh) mean (±SE) shoot height. Asterisks represent significance ($P < 0.05$)

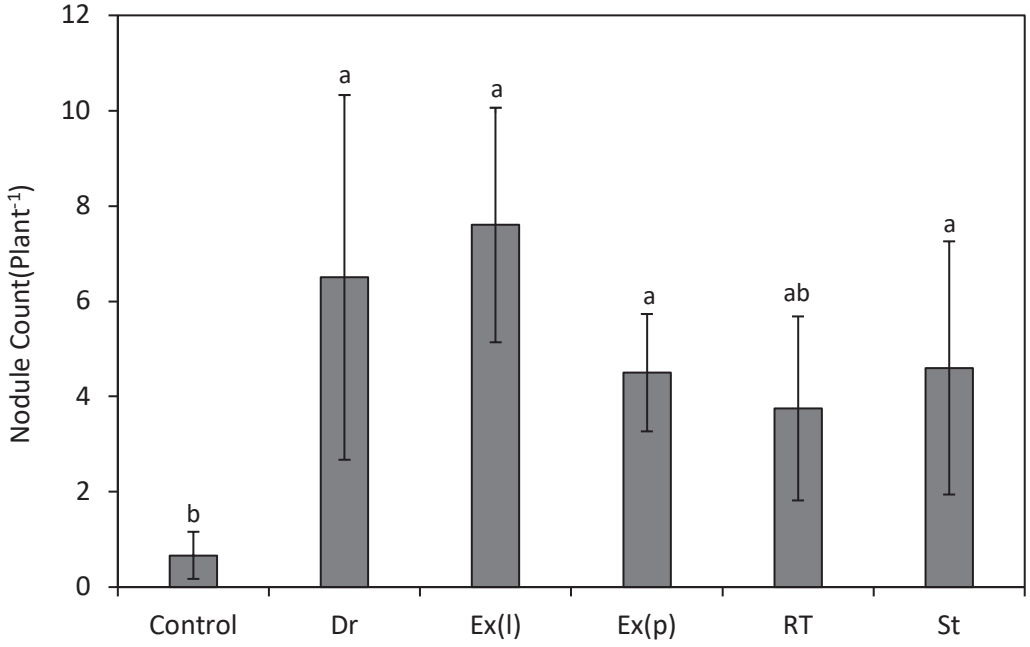

Figure 2 – Mean (±SE) number of root nodules on silvery lupine (*Lupinus argenteus* Pursh) seedlings inoculated with liquid cultures isolated from Draper, UT (Dr), Rio Tinto Kennecott Copper Mine, Herriman, UT (RT), Strawberry Reservoir, Heber, UT (St), and EXCEED® (Nampa, ID, USA) liquid (Ex(l) and peat based (Ex(p) commercial inoculum. Differing lower case letters indicate a significant difference between treatments ($P < 0.05$).

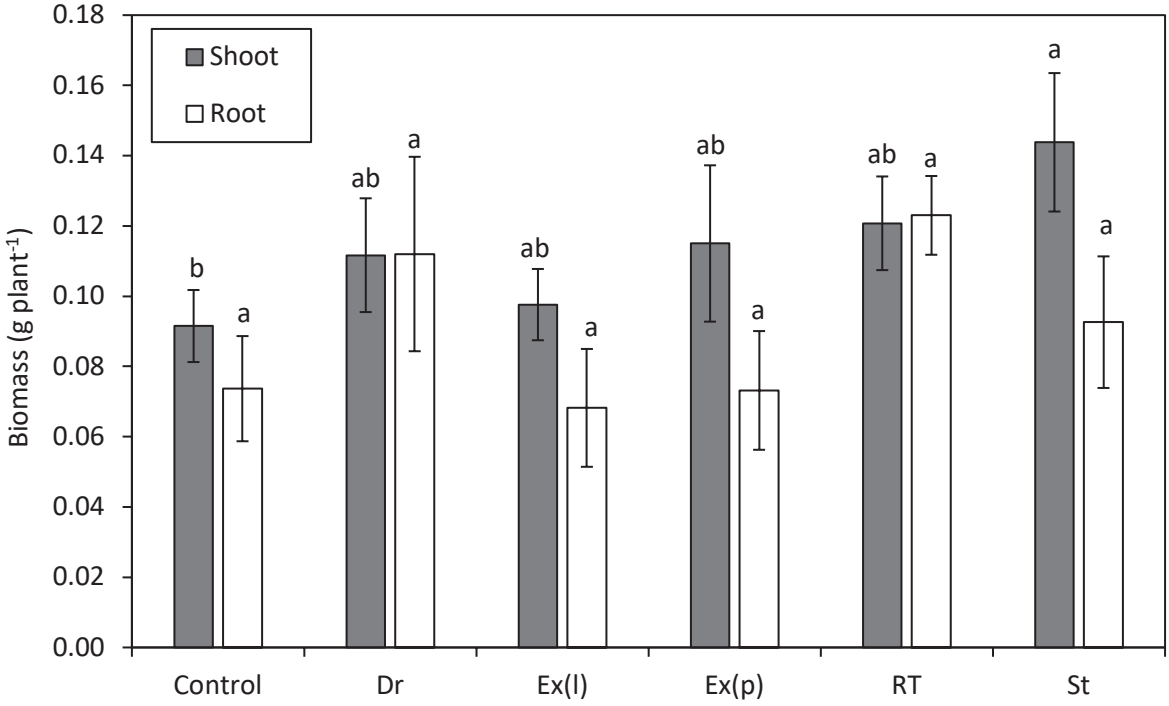

Figure 3 – Mean (±SE) shoot and root dry weight of silvery lupine seedlings inoculated with liquid cultures of rhizobium isolated from plants collected near Draper, UT (Dr), Rio Tinto Kennecott Copper Mine, Herriman, UT (RT), Strawberry Reservoir, Heber, UT (St), and from the commercial inoculum EXCEED® (Nampa, ID, USA) delivered to the seed as either a liquid inoculum (Ex(l) or a peat based inoculum (Ex(p). Differing lower case letters indicate a significant difference between treatments (P <0.05).

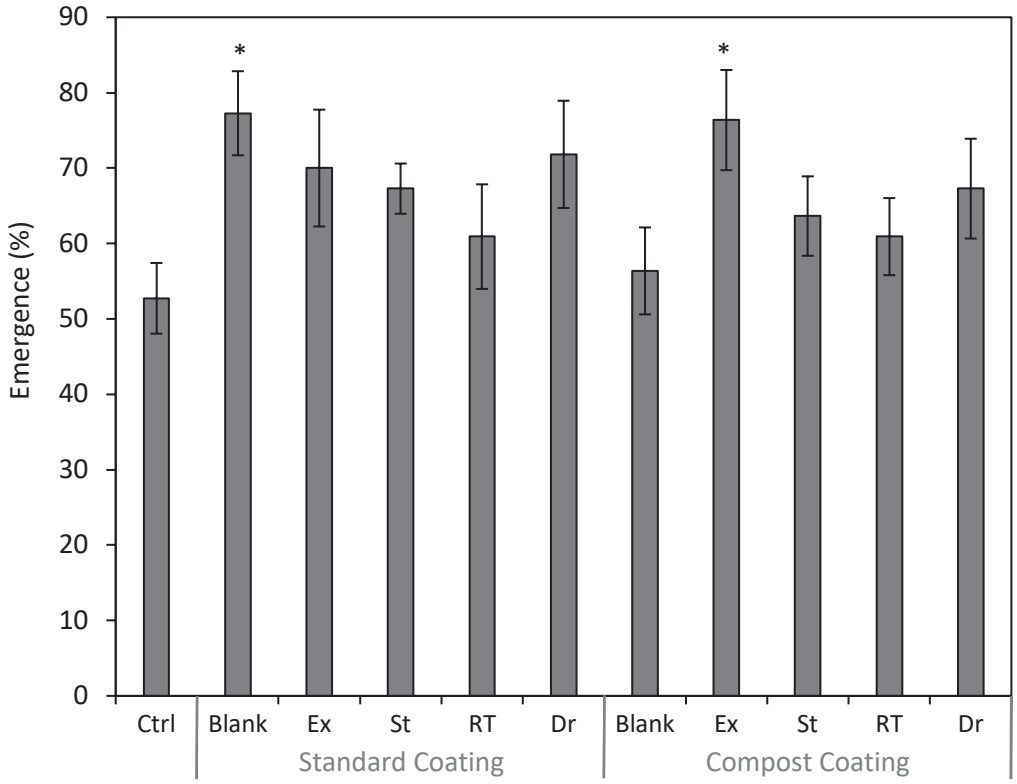

Figure 4 – Mean (±SE) seedling emergence of silvery lupine (*Lupinus argenteus* Pursh) from seeds coated with the commercial rhizobium strain EXCEED® (Ex) and rhizobium strains collected near Strawberry Reservoir, Heber, UT (St), Rio Tinto Kennecott Copper Mine, Herriman, UT (RT), Draper, UT (Dr). Asterisks represent significant differences from the control ($P < 0.05$).

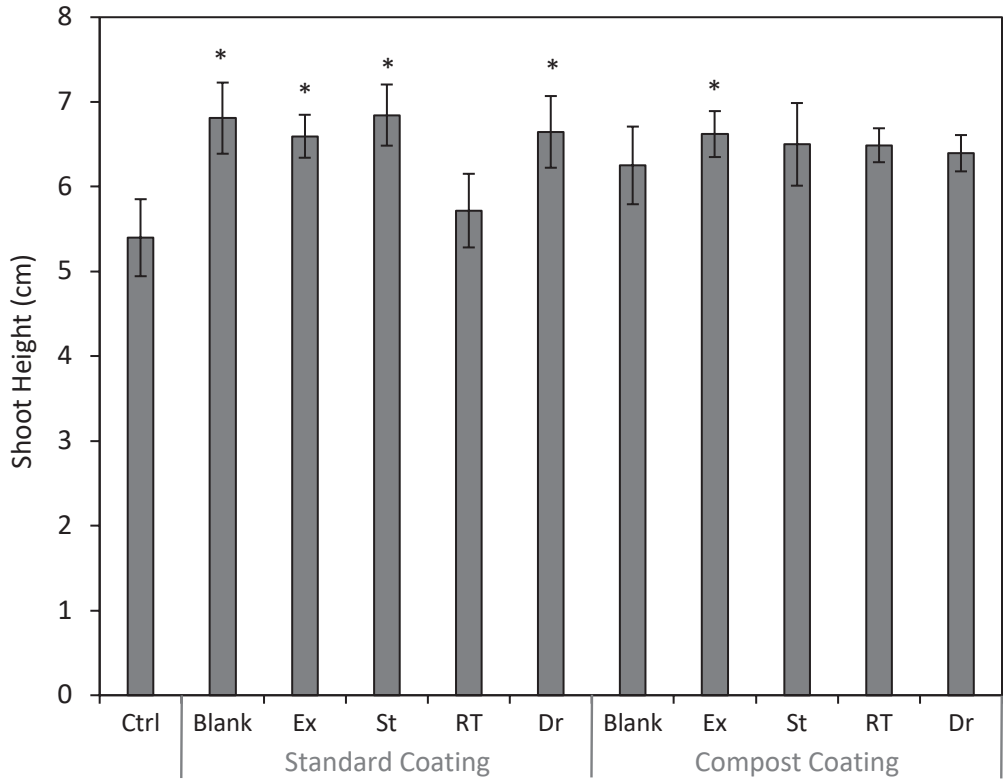

Figure 5 – Mean (±SE) shoot height (cm) of silvery lupine (*Lupinus argenteus* Pursh) from seeds coated with the commercial rhizobium strain EXCEED® (Ex) and rhizobium strains collected near Strawberry Reservoir, Heber, UT (St), Rio Tinto Kennecott Copper Mine, Herriman, UT (RT), Draper, UT (Dr). Asterisks represent significant differences from the control ($P < 0.05$).

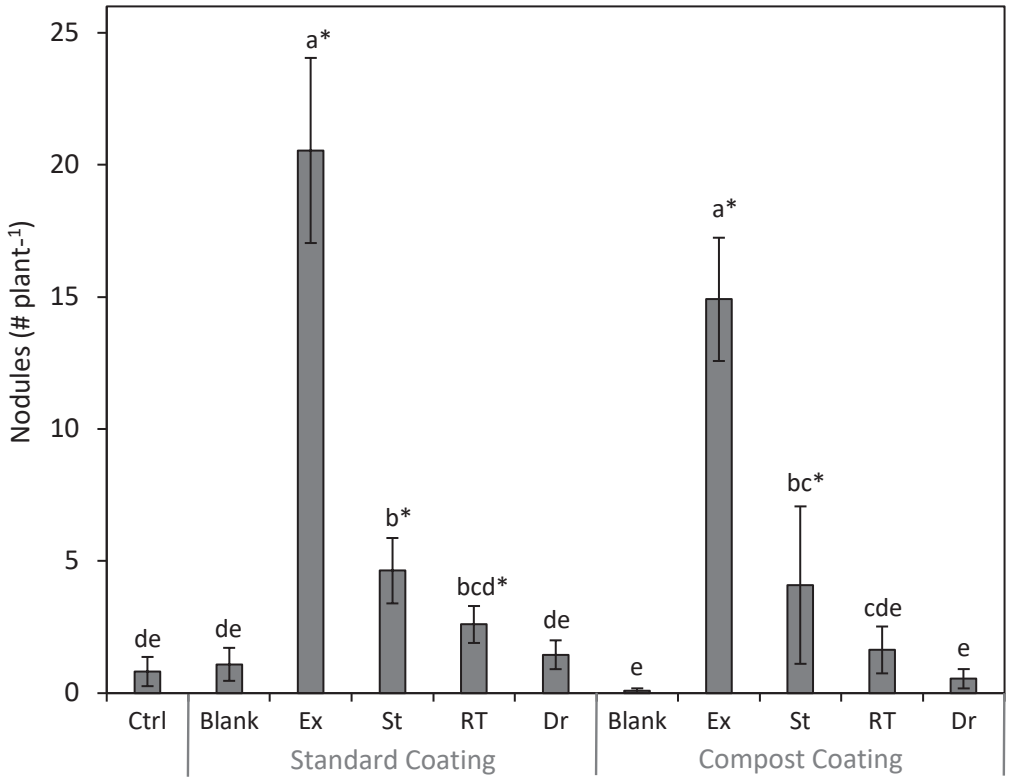

Figure 6 – Mean (±SE) nodule count on silvery lupine (*Lupinus argenteus* Pursh) roots from seeds coated with the commercial rhizobium strain EXCEED® (Ex) and rhizobium strains collected near Strawberry Reservoir, Heber, UT (St), Rio Tinto Kennecott Copper Mine, Herriman, UT (RT), Draper, UT (Dr). Differing lower case letters indicated a significant difference between treatments (P <0.05). Asterisks represent significant differences from the control (P <0.05).

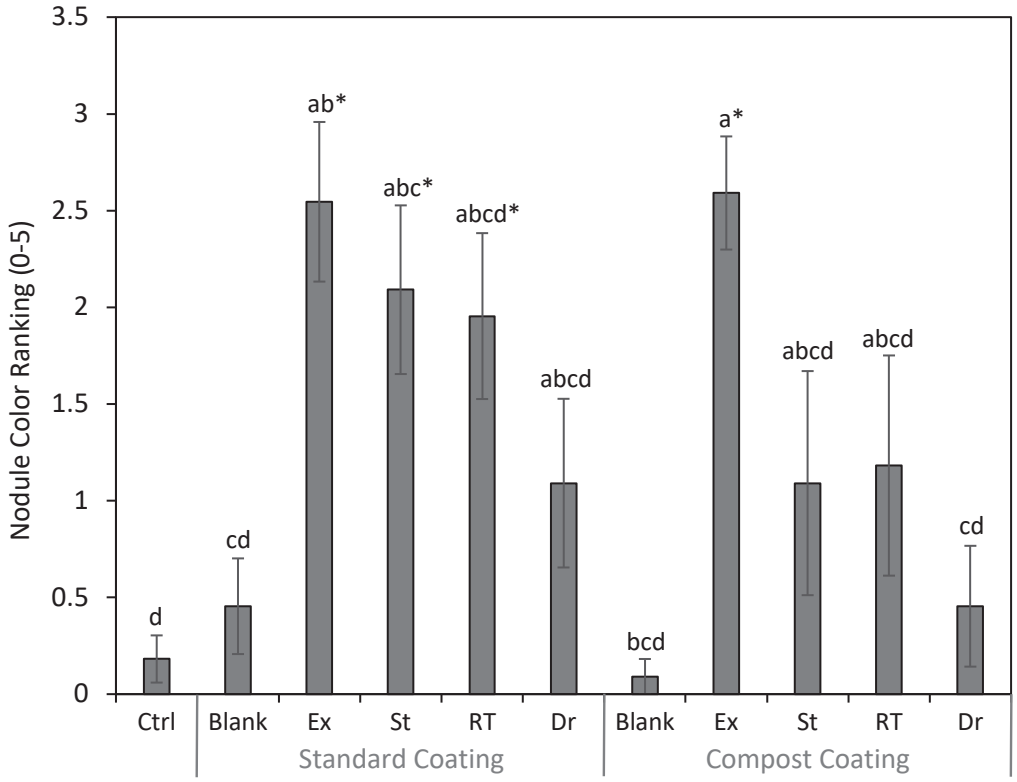

Figure 7 – Mean (±SE) ranking of nodule color of silvery lupine (*Lupinus argenteus* Pursh) from seeds coated with the commercial rhizobium strain EXCEED® (Ex) and rhizobium strains collected near Strawberry Reservoir, Heber, UT (St), Rio Tinto Kennecott Copper Mine, Herriman, UT (RT), Draper, UT (Dr). Plants with no nodules were given a rank of zero, if the nodules were white, brown, or green they were given a value of 1, and plants with a pink/red color were given a score of 2-5 depending on the darkness of the nodule. Differing lower case letters indicate a significant difference between treatments (P <0.05). Asterisks represent significant differences from the control (P <0.05).

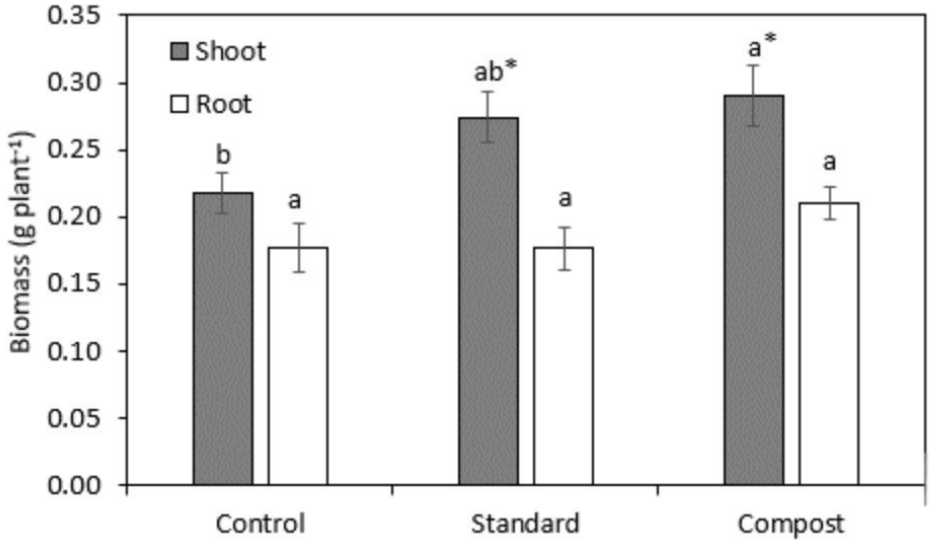

Figure 8 – Mean (±SE) shoot and root weight among different coatings on silvery lupine (*Lupinus argenteus* Pursh). Seeds were either left uncoated or coated with a standard coating or a compost coating (Table 3). Differing lower case letters indicate a significant difference between treatments (P <0.05). Asterisks represent significant differences from the control (P <0.05).

TABLES

Table 1 – Nitrogen-free fertilizer solution; each ingredient was autoclaved prior to use except for the micronutrients, which were filter sterilized.

Ingredient		Amount
Autoclaved, ddH_2O		500 mL
1M KH_2PO_4 [pH ~ 7.0]		1 mL
1M $CaCl_2 \cdot 2H_2O$		0.25 mL
1M $MgSO_4 \cdot 7H_2O$		0.50 mL
3M KCl		0.50 mL
Micro (see below)		0.50 mL
Micro	*EDTA*	230 mg
	$FeCl_3$	100 mg
	$ZnSO_4$	50 mg
	H_3BO_3	5 mg
	$CuSO_4$	5 mg
	Na_2MoO_4	5 mg
	$CoCl_2$	5 mg
	$MnSO_4$	50 mg

Table 2 – Batch recipe used to make tryptone yeast (TY) from which 250 ml of the solution is removed and added with 3 g of bacterial agar and poured into Petri dishes.

Ingredient	Amount
Autoclaved, ddH_2O	1 L
Tryptone	5 g
Yeast extract	2.5 g
$CaCl_2 \cdot 2H_2O$	0.4 g
$MgSO_4 \cdot 7H_2O$	0.4 g
KOH	300 ul

Table 3 – Standard coating (SC and compost coating (CC) recipes applied to 90 g of silvery lupine seed (*Lupinus argenteus* Pursh).

Coating	Ingredient	Amount (g)
SC	*Wood flour (System 3)*	72
	Powdered cellulose (J Rettenmaier)	13.5
	Calcium Bentonite	4.5
	Peat moss (Black Gold), blended	27.5
CC	*Compost (TeaLab), sieved to #12 Tyler mesh sieve*	129.6
	Peat moss (Black Gold)	24.75

Table 4 – Treatments used in the seed coating trial; applied to silvery lupine (*Lupinus argenteus* Pursh) seeds.

Treatment	Description
Control	*Uncoated seed*
SC	*Seed coated with no added inoculum*
CC	*Seed coated with compost and no added inoculum*
SC+Ex	*SC with EXCEED®*
SC+RT	*SC with the native strain collected from Rio Tinto Kennecott Mine*
SC+Dr	*SC with the native strain collected near Draper, UT*
SC+St	*SC with the native strain collected near Strawberry Reservoir, UT*
CC+Ex	*CC with EXCEED®*
CC+RT	*CC with the native strain collected from Rio Tinto Kennecott Mine*
CC+Dr	*CC with the native strain collected from Draper, UT*
CC+St	*CC with the native strain collected near Strawberry Reservoir, UT*

CHAPTER 2

Development of a Rhizobium Seed Coating to Establish Lupine Species on Reclaimed Minelands

Bridget M. Calder, Brad Geary, Joel S. Griffitts, Curtis L. Drake, Kate Ruebelmann, and Matthew D. Madsen
Department of Plant & Wildlife Sciences, Brigham Young University, Provo, UT
Master of Science

ABSTRACT

Restoration and reclamation efforts are widespread, and there is often varying success. One major challenge is the long-term establishment of mature plant species. This challenge is largely influenced by symbiotic partners residing in the soil. These microorganisms are often a contributing factor to the success of their host species. This project targeted one specific symbiotic relationship, that is between rhizobia (nitrogen-fixing bacteria) and leguminous plants. Specifically, we evaluated if a rhizobium inoculant coating will increase the establishment and growth of silky lupine (*Lupinus sericeus* Pursh), and if native strains will perform better than a commercial product. Research was conducted on a mine in northern Utah on two waste-rock sites, one comprised of crushed waste rock and the other made of waste rock that had been amended with topsoil. Seeds were sown in November and by June, we recorded high plant recruitment at both study sites, but there were more plants, which were more vigorous, in the amended site ($P<0.001$). There was no difference between any of the seed treatments ($P=0.754$). These results demonstrate that reclamation efforts on mine land overburden can be improved when topsoil is incorporated into the growing medium. In addition, we showed that silky lupine is a useful species for mineland reclamation in this area. Because nodules formed in mature root systems are a greater contributor to nitrogen than those on immature plants, it may take more time to see an effect from the rhizobium inoculum treatments evaluated in this study. We will

continue to monitor the test plots into the next year and anticipate that treatment differences will become apparent through time, particularly in the non-amended site, which has limited nutrients and microbial activity.

INTRODUCTION

Symbiotic relationships (i.e., prolonged associations between organisms that are often widely separated phylogenetically) are more common than previously thought (Dimijan 2000). They are being discovered in all parts of life and are often vital for the survival of their associates (Baskett, Gaines, & Nisbet, 2009). The plant rhizosphere hosts a diverse array of symbiotic partners that influence its health and establishment, for example, through nutrient acquisition (Chibucos & Tyler, 2009), pathogen protection, and stress tolerance (Verma et al., 2021; Berendsen et al., 2012). Many of these symbionts live within the plant tissues (endophytes) and can increase emergence, growth, and establishment (Ryan et al., 2008; Bent & Chanway 1998; Chanway, 1997).

Symbiotic microorganisms could be used as a tool for the restoration of systems that are in ecological disrepair (Fofana, et al., 2020). Degraded landscapes are becoming an increasing problem, 23% of the Earth's land area is already degraded, with an increase of 5-10 million hectares per year (Stavi & Lal, 2015). Anthropogenic disturbances can come from many sources, such as: overgrazing, soil tillage, the use of chemical fertilizers and pesticides, mineral extraction, and urban expansion (Killebrew & Wolff, 2010). The loss of native plants through these disturbances results in a decline in soil microbial diversity because the survival of symbiotic soil microorganisms is often dependent on the presence of their plant host species (Jacoby et al., 2017; Thrall, 2005; Murray et al., 2001). Inoculating soils with host-specific

microorganisms at the time of revegetation can speed up the natural process of microbial re-colonization and enhance the likelihood of plant establishment and survival (van der Heijden et al., 2016).

Rhizobia are nitrogen-fixing bacteria that are found in relationship with legume plants (Bennett, 2016). When in close connection with their specific host, the host will capture these soil bacteria and house them in root nodules. This allows for the "integration" of their metabolisms, where the plant provides carbohydrates to the bacteria and in turn, the bacteria fix atmospheric nitrogen into ammonium that the plant can use (Udvardi & Poole, 2013). These bacteria can be essential for host survival in degraded landscapes where ammonium is limited (Thrall et al., 2005). As a result, inoculating plants at the time of sowing has been shown to aid in the establishment and growth of legume crops (Biswas et al., 2000). Some impediments to this may be host specificity. Specific bacteria strains can only nodulate specific host species (Yang et al., 2010). When there is no compatibility, there may be no nodulation, or nodulation but no nitrogen-fixation (Wang et al., 2018).

Not only are these symbiotic partners specific to their host, but some strains may perform better than others (Irisarri, et al., 2019). For example, Karaca and Uyanoz (2012) demonstrated that inoculating with higher concentrations of rhizobia would increase nodule formation on the roots, but certain strains will fix nitrogen at a higher rate. Singleton and Tavares (1986) showed that the inoculation of legumes with rhizobia does not produce healthier plants or higher nitrogen content when native strains are already present in the soil. Because specific strains are adapted to their native soils and environment, they should perform better than non-native strains and may also provide other benefits not yet understood (Singleton & Tavares, 1986).

Due to the benefits of rhizobia with their host plants, native legume species are essential for promoting successional processes in a plant community (Chaer et al., 2011). Legumes and their associated bacteria reestablish nitrogen levels to what is needed for later successional species. Additionally, many legumes are early seral species that can help decrease the presence of invasive species after a disturbance (Tilley et al., 2019). For example, in North America's sagebrush steppe, various lupine (*Lupinus*) species are an important component of the system. These species can aid in establishing other plants, add biodiversity to the surrounding landscape, offer benefits to pollinators, and provide forage for wildlife (Jones et al., 2016).

Seed coating technologies that deliver rhizobia at the time of planting could be especially valuable for mineland reclamation (Cao, 2007). This is particularly true in areas where overburden has been deposited on the outside of the mine. This mine waste can be physically, chemically, and biologically different from topsoil, with low organic matter, soil nutrients, and biological activity (Sheoran et al., 2010; Birnbaum et al., 2017; Borůvka et al., 2012). The scarcity of rhizobium and other microbial propagules in the soil can decrease plant establishment and overall productivity of the site, particularly in low-nutrient ecosystems (Herrera, et al., 1993). Ideally, practitioners will cover areas containing overburden with crushed rock and stockpiled topsoil as part of their reclamation efforts (Merino-Martín et al., 2017; Bateman et al, 2019). This approach can provide a growing medium that contains the microbial propagules needed to sustain plant life. However, many mines lack access to stockpiled topsoil (Golos and Dixon, 2014; Merino-Martín et al., 2017; Bateman et al, 2019). Furthermore, even if the topsoil is available, this material is still highly disturbed as it is removed from its original location, stored in stockpiles, and transported on site (Lauber et al., 2008; Birnbaum et al., 2017). In these

instances, seed coatings and other seed enhancement technologies could be particularly advantageous for delivering microbiota with the seed (Erickson et al. 2019; 2021).

The objectives of our research were to 1) determine if a rhizobium inoculant will improve plant growth and vigor of silky lupine (*Lupinus sericeus* Pursh) on mineland overburden sites; and 2) compare the effectiveness of a commercial rhizobium inoculate to indigenous strains of rhizobia. We hypothesized that a rhizobium inoculant delivered through a seed coating would improve plant growth and establishment, with the greatest treatment effect on overburden sites that did not contain a growing medium. In addition, we hypothesized that native rhizobium strains would be better adapted to local conditions and their association with the host plant, allowing this treatment to outperform commercially produced strains.

MATERIALS & METHODS

Research Sites

Field research was conducted at the Rio Tinto Kennecott's Bingham Canyon Copper Mine near Salt Lake City, UT, USA. This mine began operations in 1903 and has produced more than three billion metric tons of overburden, deposited on the slopes of the Oquirrh Mountain Range (Borden & Black, 2005). Extensive efforts are underway to reclaim these overburden sites. We conducted research on two waste-rock sites located on the mine, one that had been amended (40.49443, -112.13392) and one that had not been amended (40.541205, -112.129868) (hereafter referred to as amended and unamended research locations). These sites are associated with the Mountain Loam (Oak) (R047XA432UT) ecological site (Soil Survey Staff 2022). This area is part of the Mountain Brush zone, which forms a transition zone between coniferous forests above and pinyon-juniper woodlands below. The mean annual precipitation in the area

the research was conducted was 612 mm, with an average temperature of 8.5˚C (PRISM Climate Group 2022). Prior to mining operations, the site would have been dominated by tall shrubs such as Gambel oak (*Quercus gambelii* Nutt.), shrub live oak (*Quercus turbinella* Greene), curlleaf mountain mahogany (*Cercocarpus ledifolius* Nutt. (Rosaceae)), alderleaf mountain mahogany (*Cercocarpus montanus* Raf.), bigtooth maple (*Acer grandidentatum Nutt.*), oakleaf sumac (*Rhus trilobata* Nutt.), and Stansbury cliffrose (*Purshia stansburiana* (Torr.) Henrickson). (Banner, 1992). The overburden, waste rock materials that were deposited at the research sites contain different amounts of monzonite, quartzite, and limestone (Borden 2001; Borden 2003). Approximately 10 soil samples were taken at each location and then consolidated for analysis by the BYU Environmental Analytical Lab. At the unamended research location, the growing medium had a high rock content, with a clay-loam soil texture and pH of 7.5.

The amended site was reclaimed by decreasing the slope to a 12% incline and covering the area with a ~1 m cap of topsoil. Once the cap was laid, a bulldozer was used to rip the soil perpendicular to the slope, which created small terraces ~0.5 m wide, spaced ~1 m apart. At this site, there were few rocks with a silt-loam soil texture and pH of 7.3.

Collection, Isolation, and Preparation of Native Rhizobia Strains

Lupine nodules were collected from three different sites in Utah: Rio Tinto Kennecott's Bingham Canyon Mine, South Jordan, UT, USA (40.501556, -112.141833); Lupine Hiking Trail, Draper, UT, USA (40.464417, -111.828694); and near Strawberry Reservoir, Heber, UT, USA (40.15440, -111.20236). At each site, we harvested nodules from 5-10 mature plants. The Bingham Canyon Mine nodule collection site was covered by waste rock from historic mining activities. The elevation of the collection site is 2000 m, soils were formed from weathered

monzonite, quartzite, and limestone rock (Borden 2001; Borden 2003). The site had been colonized by native ruderal forbs, including tailcup lupine (*Lupinus caudatus* Kellogg), from which our nodules were collected. The Lupine Hiking Trail collection site is characterized as a Mountain Loam (Mountain Big Sagebrush) ecological site (UDWR 2022), with an elevation of 1724 m, soil pH of 7.0, and soil texture classified as loam (Web Soil Survey 2022). As implied by the name of the hiking trail, the site has an abundance of silvery lupine, from which we collected nodules. The collection site at Strawberry Reservoir is within the Mountain Loam (Mountain Big Sagebrush) ecological site (UDWR 2022). This site has an elevation of 2430 m, a loamy soil texture and a soil pH of 7.2 (Web Soil Survey 2022). There were many lupine species present in this collection area. We obtained nodules within a 20 m distance of the coordinates shared above from 1-3 silky lupine (*Lupinus sericeus* Pursh), silvery lupine, and bigleaf lupine (*Lupinus polyphyllus* Lindl.) plants.

Individual nodules were surface sterilized in the laboratory by swirling for 5 s in a Petri dish filled with 75% ethanol, followed by a Petri dish with 25% bleach, and then three individual Petri dishes with autoclaved, distilled water (ddH_2O). Surface sterilized nodules were placed in an Eppendorf tube containing 100 uL tryptone yeast (TY) broth (Table 1) and crushed with a pestle. We then spread 5 uL of the crushed nodule liquid on a TY agar plate (Table 1) and incubated at 30°C. Samples of the bacteria that grew on the agar were identified at the species level using 16S sequencing, which targets the rRNA gene (Kim & Chun, 2014). From this work, we positively identified multiple rhizobia strains from our Rio Tinto, Strawberry, and Draper collections, respectively. We then chose one strain from each of these sites to be used in the study. These strains were selected based on their ease of growing in liquid culture.

Rhizobia strains that were chosen for the study were transferred from the agar back into a 100 uL TY liquid broth and incubated for about 4 days at 30°C. We then added a select amount of rhizobium TY broth solution to 5 g of finely milled peat substrate (Black Gold, Sun Gro Horticulture, Agawam, MA, USA). The amount of rhizobium TY broth solution, and peat used in our study was chosen so we could replicate what was being applied by Exceed® H Type Inoculant for Lupine (Visjon Biologics, Henrietta, TX, USA) on 200 g of seed. Within 1 g of this commercial product, there are 2 x 10^9 colony-forming units (CFU) of rhizobium. We employed optical density measurements (and their corresponding CFU counts) on our rhizobium TY broth solution to determine the amount of this liquid culture that should be added to the peat, so it matched the commercial product. The peat used in our native rhizobia coatings was milled using a Nutrimill® flour mill (Nutrimill, St.George, UT, USA), then sieved through a 0.21 mm mesh sieve (#35 U.S. standard sieve). Milled peat was pH adjusted to ~ 7 (6.4-6.6) by the addition of calcium carbonate. The pH levels were obtained by suspending peat and calcium carbonate in a water solution and then measured using a Mettler Toledo® pH probe (Columbus, OH, USA). Dry, pH amended peat was then autoclaved in a closed container.

Field Evaluations

Silky lupine (*Lupinus sericeus*, Pursh) seeds were coated with a recipe provided from a commercial supplier – Three River Seed Company (Nampa, ID, USA) and treatments described in Table 2. The coating is composed of 158.4 g wood flour, 29.7 g powdered cellulose, and 9.9 g calcium bentonite that had been previously blended. The protocol described was used for 198 g of seed and applied with a 45% polyvinyl alcohol (PVA) binder (Ashland Inc., Covington, KY, USA). Seeds were coated in a rotary drum seed coater (Universal Coating Systems,

Independence, OR, USA) set to 20% maximum speed. The process involved wetting the seeds with 19.8 g binder before adding 49.5 g of calcium bentonite. After the calcium bentonite was applied, we slowly added ~ 99 g of our coating material in conjunction with approximately 300 g of binder. Next, we added our peat without adding any additional binder. Once the peat was evenly applied, we added the second half of our dry ingredients (~ 99 g) with an additional 300 g of binder. Seeds were dried under an air dryer (Braceworks Automation and Electric, Lloydminster, SK, CAN) with ambient heating for about 30 minutes and laid out to dry under ambient conditions overnight. Seeds were then stored in a fridge until the time of planting.

In November 2021, the study was installed in a randomized block design with five blocks at each site. Seeds were hand sown in 2 m rows with 282 seeds per row or block. Plant density data was taken in spring and summer of 2022, and plant vigor was assessed in the summer of 2022 on a scale from 0-5, with 0 being dead and 5 being dark green and healthy. Data will be taken again the following spring for plant density, vigor, and nodule count.

Data were analyzed using JMP® version 16 (SAS Institute Inc., Cary, NC). A mixed model analysis was used to analyze plant density and vigor. Here, blocks were considered a random effect, treatment and planting location were considered fixed effects. Mean values were separated when significant effects were found using the post hoc Tukey-Kramer honestly significant difference tests. Differences were considered significant when $P < 0.05$.

RESULTS

In the month prior to planting (October), precipitation was well above average (251% of normal) but decreased to only 21% of normal in the month the seeds were sown. Precipitation was a 130% of normal in December and for the remainder of the study precipitation was

typically well below average (Figure 1). Air temperatures tended to be slightly warmer than normal in the early winter (November – December) but remained relatively close to normal for the remainder of the study with exception of the month of February and March when temperatures were below average. (Figure 1).

At this stage in the study, there was no difference between the rhizobium inoculum treatments for plant density ($P = 0.753$) or vigor ($P = 0.595$) at both research locations. However, there was a difference in plant density ($P < 0.001$) and vigor ($P = 0.010$) between the different field sites (Tables 3 and 4). The amended field site had 185% more seedlings than the unamended field site (Figure 2) and the vigor of the plants growing on the amended site was 26% greater (Figure 2). We are waiting to receive analysis on shoot and root nitrogen content. Data is currently being processed.

DISCUSSION

While germination is a major milestone for a seed to overcome, survival in mine-altered landscapes can prove just as difficult (Haddaway, et al., 2019). Altered soils may be low on necessary plant nutrients due to a lack of soil microorganisms that provide these nutrients (Chibucos & Tyler, 2009). While it is possible to re-establish microorganisms in the area with large deposits of topsoil, it can be difficult and expensive (Golos and Dixon, 2014; Merino-Martín et al., 2017; Bateman et al, 2019). We hypothesized that a rhizobium inoculant delivered through a seed coating would improve plant growth and establishment, with the greatest treatment effect on overburden sites that did not contain a growing medium. In addition, we hypothesized that native rhizobium strains would be better adapted to local conditions and their association with the host plant, allowing this treatment to outperform commercially produced

strains. To date, we have found no indication that a rhizobium seed coating can improve plant growth and establishment within the early stages of plant development. Additionally, there was no difference in the performance of the different rhizobium inoculants evaluated in the trial. We did find that our test species (silky lupine) had higher plant densities and greater vigor on the amended waste rock dump compared to the waste rock dump that had not been amended (Figures 2 & 3).

A lack of treatment response from the rhizobium seed coatings may be due to the short period this study has been conducted. Because rhizobia aid in the long-term establishment of legume species, it may be difficult to see its effects until the plants are more mature. We plan to continue to monitor this study for an additional year. It may be that a treatment response will be expressed as the plants in our research mature. Previous studies have shown that crown nodules – or those present in the immature root systems do not fix near as much nitrogen as nodules that form in the mature root systems of the plant (Hardarson, et al., 1989). These mature nodules are used in later stages of plant development when nutrients are most needed (Wolyn, et al., 1989). Lopetinksy et al. (2014) showed that the highest grain yield in lupine was one to two years after inoculation with rhizobium. This indicates that we may see significant differences between treatments when tested in the following year when plants and nodules have matured to the point that they can provide a substantial amount of nitrogen to the plant.

This lack of response from the rhizobia seed coating may also be due to the process of applying bacteria to the seed. Many factors are associated with the seed coating procedure that could reduce the viability of rhizobium (Maurice et al. 2001). For example, the type of polymer used and its application in the coating process can significantly impact rhizobia activity (Rocha et al. 2019). We used a PVA binder in the coating process, which we had predetermined using a

zone of inhibition test to not impact rhizobia growth. However, we observed that the coating did not break down well in the field; a less rigid coating may allow a better release of the rhizobium into the soil. A more friable coating could be achieved by lowering the percentage of solids used in the PVA binder or using a different binder. Products such as methyl cellulose, carboxymethyl cellulose, gum Arabic, xantham gum, or polysaccharide Pelgel are commonly used in commercial seed coatings and have been shown to maintain microbial viability (Jambhulkar et al. 2016; Rocha et al. 2019). Future research is merited for evaluating these different binders for use in applying rhizobium to legume seeds that are used in mineland reclamation.

In addition, our method of applying the rhizobium inoculum and other associated seed coating ingredients could be further developed. For example, an increased number of rhizobia could be applied to the seed to improve the chances of plant inoculation in the harsh mineland sites. Similarly, additional carrier material, i.e., ground peat moss, could be added to improve the distribution of the inoculum during coating and a medium for the bacteria to persist on until it has a chance to infect a host plant. Reducing the impact of the coating process on rhizobium from wetting and drying may also improve the efficacy of the seed treatment. As typical for applying rhizobia, the bacteria are cultured in a liquid medium, applied to a peat carrier, and then applied to the seed (Rocha et al. 2019). This process results in the bacteria being dried after they are applied to the inoculum and then again after the seeds are coated. Less impact may occur to the bacteria if they are applied as a liquid culture at the time of coating and thus only need to be dried down once during the coating process. In this approach, a peat carrier would still be used, but it would be applied to the seed at the same time the liquid culture of rhizobium bacteria are being coated onto the seed. Additionally, because a liquid is typically applied more uniformly in the coating process than are dry powders, delivering the bacteria as a liquid culture could

improve the distribution of the inoculum on the seeds. Given that most methods for applying rhizobium to seed are designed for agriculture systems, future work is merited for testing novel techniques for use in mineland applications.

Not only is it necessary to further develop the seed coating process to produce more viable bacterial colonies (Maurice et al., 2001), but it is also necessary to select for optimal strains. Testing indigenous strains previously could help select for strains which would better handle competition pressures, as well as stresses from the coating process. While some strains may nodulate quickly, they may not provide maximum nitrogen fixing benefits. Additionally, due to a non-sterile environment other bacteria may induce nodulation thus inhibiting our indigenous strains from being housed in nodules (Hartley et al, 2012).

While we did not see significant results among coating treatments, we saw differences between our amended and non-amended sites (Figures 2 and 3). The amended site contained more plants that were substantially more vigorous in appearance (i.e., larger in size with greener leaves; Figures 2 and 3). These differences among the sites are most likely due to a host of reasons, such as the amended site having increased soil organic matter, greater water and nutrient availability, enhanced flora of beneficial microorganisms, and a deeper growing medium (Sheoran, et al., 2010; Khan, et al., 2016).

It is evident from the high numbers of plants established in the research sites (Figure 2) that the lupine species used (silky lupine) is an excellent candidate for reclaiming amended and non-amended mine sites in the area the research was conducted. Lupine species are often among the initial colonists in severely disturbed regions of North America (Halvorson, et al., 2005). Their extensive tap root system allows them to access deep water and nutrient sources in severely degraded soils (Chen, et al., 2014). After lupine colonizes a site, it helps to facilitate the

establishment of other plants (Halvorson, et al., 2005), by advancing successional processes such as improving soil fertility, increasing the growth of microorganisms, and improving the physical environment of the site (Morris & Wood 1989; Braatne and Bliss 1999; Fagan et al. 2004; Gosling; 2005). The findings of our research provide evidence that silky lupine is an ideal candidate for use in a seed mix on waste-rock sites at the Kennecott Copper Mine, and back other studies that lupine species are effective at helping reclaim highly disturbed areas (Gosling; 2005; Halvorson, et al., 2005; Pietrzykowski et al., 2017).

In summary, this ongoing study needs careful monitoring to determine if the rhizobium treatments applied will produce a treatment effect as the plants mature. We have made progress in developing methods of culturing and inoculating native rhizobium strains onto seeds; however, we anticipate that further improvements could be made to optimize the performance of a rhizobium seed coating for mineland applications.

LITERATURE CITED

Banner, R. E. (1992). Vegetation Types of Utah. *Rangelands*, **14**: 109-114.

Baskett, M. L., Gaines, S. D., & Nisbet, R. M. (2009). Symbiont diversity may help coral reefs survive moderate climate change. *Ecological Applications* **19**: 3-17.

Bateman, A. M., Erickson, T. E., Merritt, D. J., & Muñoz-Rojas, M. (2019). Inorganic soil amendments alter seedling performance of native plant species in post-mining arid zone rehabilitation. *Journal of Environmental Management* **241**:179-186.

Bennett, G. M. (2016). Seed Inoculation, Coating and Precision Pelleting: Science, Technology and Practical Applications. New York, USA: CRC Press Taylor and Francis Group.

Bent, E., & Chanway, C. P. (1998). The growth-promoting effects of a bacterial endophyte on lodgepole pine are partially inhibited by the presence of other rhizobacteria. *Canadian Journal of Microbiology*, **44**:980–988.

Berendsen, R. L., Pieterse, C. M., & Bakker, P. A. (2012). The rhizosphere microbiome and plant health. *Trends in Plant Science*, **17**:478-486.

Bingham Canyon, UT Weather. (n.d.). Retrieved from USA.com: http://www.usa.com/bingham-canyon-ut-weather.htm

Birnbaum, C., Bradshaw, L. E., Ruthrof, K. X., & Fontaine, J. B. (2017). Topsoil Stockpiling in Restoration: Impact of Storage Time on Plant Growth and Symbiotic Soil Biota. *Ecological Restoration*, **35**:237-245.

Biswas, J. C., Ladha, J. K., & Dazzo, F. B. (2000). Rhizobia Inoculation Improves Nutrient Uptake and Growth of Lowland Rice. *Soil Science Society of America*, **64**:1644-1650.

Borůvka, L., Kozák, J., Mühlhanselová, M., Donátová, H., Nikodem, A., Němeček, K., & Drábek, O. (2012). Effect of covering with natural topsoil as a reclamation measure on mining dumpsites. *Journal of Geochemical Exploration*, **113**:118-123.

Borden, R. (2001). Geochemical evolution of sulphide-bearing waste rock soils at the Bingham Canyon Mine, Utah . *Geochemistry: Exploration, Environment, Analysis*, **1**:15-21.

Borden, R. K. (2003). Environmental geochemistry of the Bingham Canyon porphyry copper deposit, Utah. *Environmental Geology*, **43**:752-758.

Borden, R. K., & Black, R. (2005). Volunteer Revegetation of Waste Rock Surfaces at the Bingham Canyon Mine, Utah . *Journal of Environmental Quality*, **34**:2234-2242.

Braatne, J. H., & Bliss, L. C. (1999). Comparative Physiological Ecology of Lupines Colonizing Early Successional Habitats on Mount St.Helens. *Ecology,* **80**:891-907.

Cao, X. (2007). Regulating mine land reclamation in developing countries: The case of China. *Land Use Policiy*, **24**:472-483.

Chaer, G. M., Resen, A. S., Campello, E. F., de Faria, S. M., & Boddey, R. M. (2011). Nitrogen-fixing legume tree species for the reclamation of severely degraded lands in Brazil . *Tree Physiology*, **31**:139–149.

Chanway, C. P. (1997). Inoculation of Tree Roots with Plant Growth Promoting Soil Bacteria: An Emerging Technology for Reforestation. *Forest Science*, **43**:99–112.

Chen, Y. L., Palta, J., Clements, J., Buirchell, B., Siddique, K. H., & Rengel, Z. (2014). Root architecture alteration of narrow-leafed lupin and wheat in response to soil compaction. *Field Crops Research*, **165**:61-70.

Chibucos , M. C., & Tyler, B. M. (2009). Common themes in nutrient acquisition by plant symbiotic microbes, described by the Gene Ontology. *BMC Microbiology*, **9**:S6.

Dimijian, G. G. (2000). Evolving together: the biology of symbiosis, part 1 . *Baylor University Medical Center Proceedings* , **13**:217–226.

Erickson, T. E., Kildisheva, O. A., Baughman, O. W., Breed, M. F., Ruiz-Talonia, L., Brown, V. S., . . . Ritchie, A. L. (2021). Seed Enhancement Technologies. In *Florabank Guidelines,* **2**:27.

Erickson, T. E., Munoz-Rojas, M., Guzzomi, A. L., Masarei, M., Ling, E., Bateman, A. M., . . . Merritt, D. J. (2019). 'A case study of seed-use technology development for Pilbara mine site rehabilitation', in AB Fourie & M Tibbett (eds), Mine Closure 2019: Proceedings of the 13th International Conference on Mine Closure. *Australian Centre for Geomechanics*, pp. 679-692.

Fagan, W. F., Bishop, J. G., & Schade, J. D. (2004). Spatially structured herbivory and primary succession at Mount St Helens: field surveys and experimental growth studies suggest a role for nutrients. *Ecological Entomology,* **29**:398-409.

Fofana, B., Sacande, M., Blagna, F., Dibloni, T. O., Compaore, E., Sanon, K. B., . . . Ouattara, A. S. (2020). Boosting land restoration success in the Great Green Wall through the use of symbiotic microorganisms for propagated tree seedlings . *International Journal of Biological and Chemical Sciences*, **14**:110-125.

Golos, P. J., & Dixon, K. W. (2014). Waterproofing Topsoil Stockpiles Minimizes Viability Decline in the Soil Seed Bank in an Arid Environment. *Restoration Ecology*, **22**:495-501.

Gosling, P. (2005). Facilitation of Urtica dioica colonisation by Lupinus arboreus on a nutrient-poor mining spoil. *Plant Ecology,***178**:141-148.

Haddaway, N. R., Cooke, S. J., Lesser, P., Macura, B., Nilsson, A. E., Taylor, J. J., & Kaisa, R. (2019). Evidence of the impacts of metal mining and the effectiveness of mining mitigation measures on social–ecological systems in Arctic and boreal regions: a systematic map protocol. *Environmental Evidence*, **8**:9.

Halvorson, J. J., Smith, J. L., & Kennedy, A. C. (2005). Lupine Effects on Soil Development and Function During Early Primary Succession at Mount St. Helens. In: Dale V.H., Swanson F.J. and Crisafulli C.M. (eds), Ecological Responses to the 1980 Eruption of Mount St. Helens.Springer-Verlag, New York, NY, pp. 243-254.

Hardarson, G., Golbs, M., & Danson, S. K. (1989). Nitrogen fixation in soybean (Glycine max L. Merrill) as affected by nodulation patterns. *Soil Biology and Biochemistry*, **21**:783-787.

Herrera, M. A., Salamanca, C. P., & Barea, J. M. (1993). Inoculation of Woody Legumes with Selected Arbuscular Mycorrhizal Fungi and Rhizobia To Recover Desertified Mediterranean Ecosystems. *Applied and Environmental Microbiology,* **59**:129-133.

Horikawa, Y., & Ohtsuka, H. (1996). Effects of Coating and Adhesive on the Inoculation of Rhizobium metiloti to Alfalfa (Medicago sativa L.) Seeds for Nodulation and Seedling Growth. *Japanese Society of Grassland Science*, **41**:275-279.

Irisarri, P., Cardozo, G., Tartaglia, C., Reyno, R., Gutiérrez, P., Lattanzi, F. A., . . . Monza, J. (2019). Selection of Competitive and Efficient Rhizobia Strains for White Clover. *Frontiers in Microbiology*, **10**:768.

Jacoby, R., Peukert, M., Succurro, A., Koprivova, A., & Kopriva, S. (2017). The Role of Soil Microorganisms in Plant Mineral Nutrition—Current Knowledge and Future Directions. *Frontiers in Plant Science*, **8**:1617.

Jones, C. D., Stevens, M. R., Jolley, V. D., Hopkins, B. J., Jensen, S. L., Turner, D., & Stettler, J. M. (2016). Evaluation of thermal, chemical, and mechanical seed scarification methods for 4 Great Basin lupine species. *Native* Plants, **17**:5-17.

Karaca, Ü., & Uyanöz, R. (2012). Effectiveness of native Rhizobium on nodulation and growth properties of dry bean (Phaseolus vulgaris L.). *African Journal of Biotechnology*, **11**: 8986-8991.

Kavusi, E., Ansar, B. S., Dehghanian, Z., Lajayer, B. A., Nobaharan, K., Ma, Y., & Glick, B. R. (2022). Delivery of Beneficial Microbes via Seed Coating for Medicinal and Aromatic Plant Production: A Critical Review. *Journal of Plant Growth Regulation*, **10**:1357.

Khan, M. A., Gemenet, D. C., & Villordon, A. (2016). Root System Architecture and Abiotic Stress Tolerance: Current Knowledge in Root and Tuber Crops. *Frontiers in Plant Science*, **7**:1584.

Killebrew, K., & Wolff, H. (2010). *Environmental Impacts of Agricultural Technologies.* Washington: Evans School of Public Affairs.

Kim, M., & Chun, J. (2014). 16S rRNA Gene-Based Identification of Bacteria and Archaea using the EzTaxon Server. In *Methods in Microbiology,* **41**:61-74.

Lauber, C. L., Strickland, M. S., Bradford, M. A., & Fierer, N. (2008). The influence of soil properties on the structure of bacterial and fungal communities across land-use types. *Soil Biology and Biochemistry*, **40**:2407-2415.

Lopetinsky, K. J., Lupwayi, N. Z., Olson, M. A., Akter, Z., & Clayton, G. W. (2014). Contrasting Rhizobium inoculation requirements of zero-tannin faba bean and narrow-leafed lupin in western Canada. *Canadian Journal of Plant Science*, **94**:1117-1123.

Ma, H., Egamberdieva, D., Wirth, S., & Bellingrath-Kimura , S. D. (2019). Effect of Biochar and Irrigation on Soybean-Rhizobium Symbiotic Performance and Soil Enzymatic Activity in Field Rhizosphere. *Agronomy*, **9**:626.

Madsen, M. D., Davies, K. W., Boyd, C. S., Kerby, J. D., & Svejcar, T. J. (2016). Emerging seed enhancement technologies for overcoming barriers to restoration. *Restoration Ecology*, **24**:S77-S84.

Merino-Martín, L., Courtauld, C., Commander, L., Turner, S., Lewandrowski, W., & Stevens, J. (2017). Interactions between seed functional traits and burial depth regulate germination and seedling emergence under water stress in species from semi-arid environments. *Journal of Arid Environments*, **147**:25-33.

Morris, W. F., & Wood, D. M. (1989). The Role of Lupine in Succession on Mount St. Helens: Facilitation or Inhibition? *Ecology,* **70**:697-703.

Murray, B. R., Thrall, P. H., & Woods, M. J. (2001). Acacia species and rhizobial interactions: Implications for restoration of native vegetation. *Ecological Management and Restoration*, **2**:213-219.

Pawar, V. A., Pawar, P. R., Bhosale, A. M., & Chavan, S. V. (2014). Effect of Rhizobium on Seed Germination and Growth of Plants. *Journal of Academia and Industrial Research (JAIR)*, **3**:84-88.

Pietrzykowski, M., Gruba, P., & Sproull, G. (2017). The effectiveness of Yellow lupine (Lupinus luteus L.) green manure cropping in sand mine cast reclamation. *Ecological Engineering,* **102**:72-79.

Rice, W. A., Clayton, G. W., Lupwayi, N. Z., & Olsen, P. E. (2001). Evaluation of coated seeds as a Rhizobium delivery system for field pea. *Canadian Journal of Plant Science*, **81**:247-253.

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., & Dowling, D. N. (2008). Bacterial endophytes: recent developments and applications . *FEMS Microbiology Letters*, **278**:1-9.

Sheoran, V., Sheoran, A. S., & Poonia, P. (2010). Soil Reclamation of Abandoned Mine Land by Revegetation: A Review. *International Journal of Soil, Sediment and Water*, **3**:13.

Singleton, P. W., & Tavares, J. W. (1986). Inoculation Response of Legumes in Relation to the Number and Effectiveness of Indigenous Rhizobium Populations. *Applied and Environmental Microbiology*, **51**:1013-1018.

Standard Test Method for Sieve Analysis of Fine and Coarse Aggregates. (2015). Retrieved from ASTM International, West Conshohocken, PA: www.astm.org

Stavi, I., & Lal, R. (2014). Achieving Zero Net Land Degradation: Challenges and opportunities. *Journal of Arid Environments*, **112**:44-51.

Thrall, P. H., Millsom, D. A., Jeavons, A. C., Waayers, M., Harvey, G. R., Bagnall, D. J., & Brockwell, J. (2005). Seed inoculation with effective root-nodule bacteria enhances revegetation success. *Journal of Applied Ecology*, **42**:740–751.

Tilley, D., Tilley, N., Fund, A. J., & Wolf, M. (2019). *Seedling growth and competition of a late-seral, native perennial grass and two early-seral, native forbs in the presence of two densities of the invasive annual grass Bromus tectorum L.* Aberdeen: USDA.

Udvardi, M., & Poole, P. S. (2013). Transport and Metabolism in Legume-Rhizobia Symbioses. *Annual Review of Plant Biology*, **64**:781-805.

van der Heijden, M. G., de Bruin, S., Luckerhoff, L., van Logtestijn, R. S., & Schlaeppi, K. (2016). A widespread plant-fungal-bacterial symbiosis promotes plant biodiversity, plant nutrition and seedling recruitment. *The ISME Journal*, **10**:389–399.

Verma, S. K., Sahu, P. K., Kumar, K., Pal, G., Gond, S. K., Kharwar, R. N., & White, J. F. (2021). Endophyte roles in nutrient acquisition, root system architecture development and oxidative stress tolerance. *Journal of Applied Microbiology*, **131**:2161-2177.

Wang, Q., Liu, J., & Zhu, H. (2018). Genetic and Molecular Mechanisms Underlying Symbiotic Specificity in Legume-Rhizobium Interactions. *Frontiers in Plant Science*, **9**:313.

Web Soil Staff. (n.d.). Retrieved from USDA NRCS Web Soil Survey: https://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx

Wolyn, D. I., Attewell, J., Ludden, P. W., & Bliss, F. A. (1989). Indirect measures of N2 fixation in common bean (Phaseolus vulgaris L.) under field conditions: The role of lateral root nodules. *Plant and Soil*, **113**:181-187.

Yang, S., Tang, F., Gao, M., Krishnan, H. B., & Zhu, H. (2010). R gene-controlled host specificity in the legume–rhizobia symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, **107**:18735–18740.

FIGURES

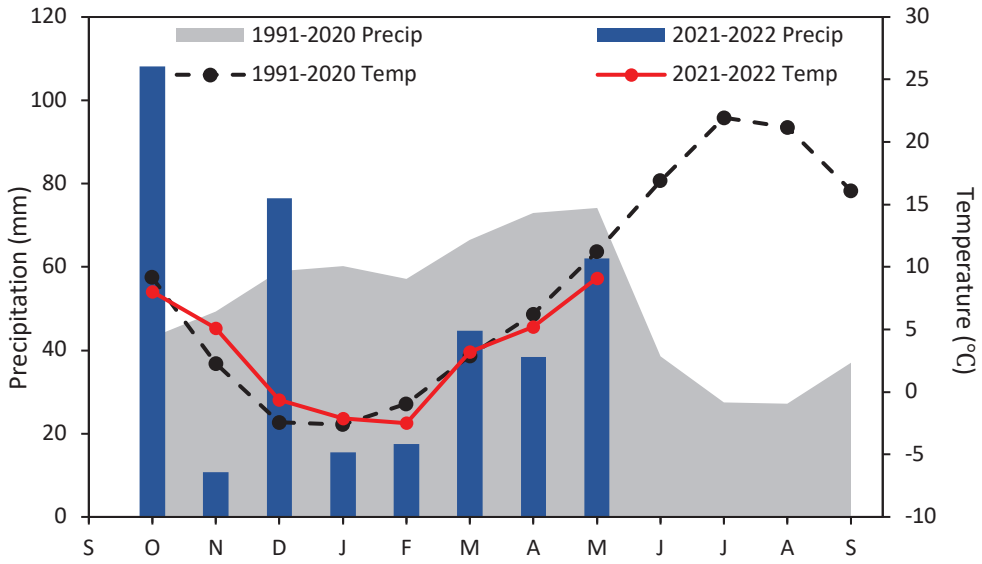

Figure 1 – Monthly precipitation and temperature means experienced throughout the study (2021 -2022), compared to the 30-year long-term averages (1991-2020).

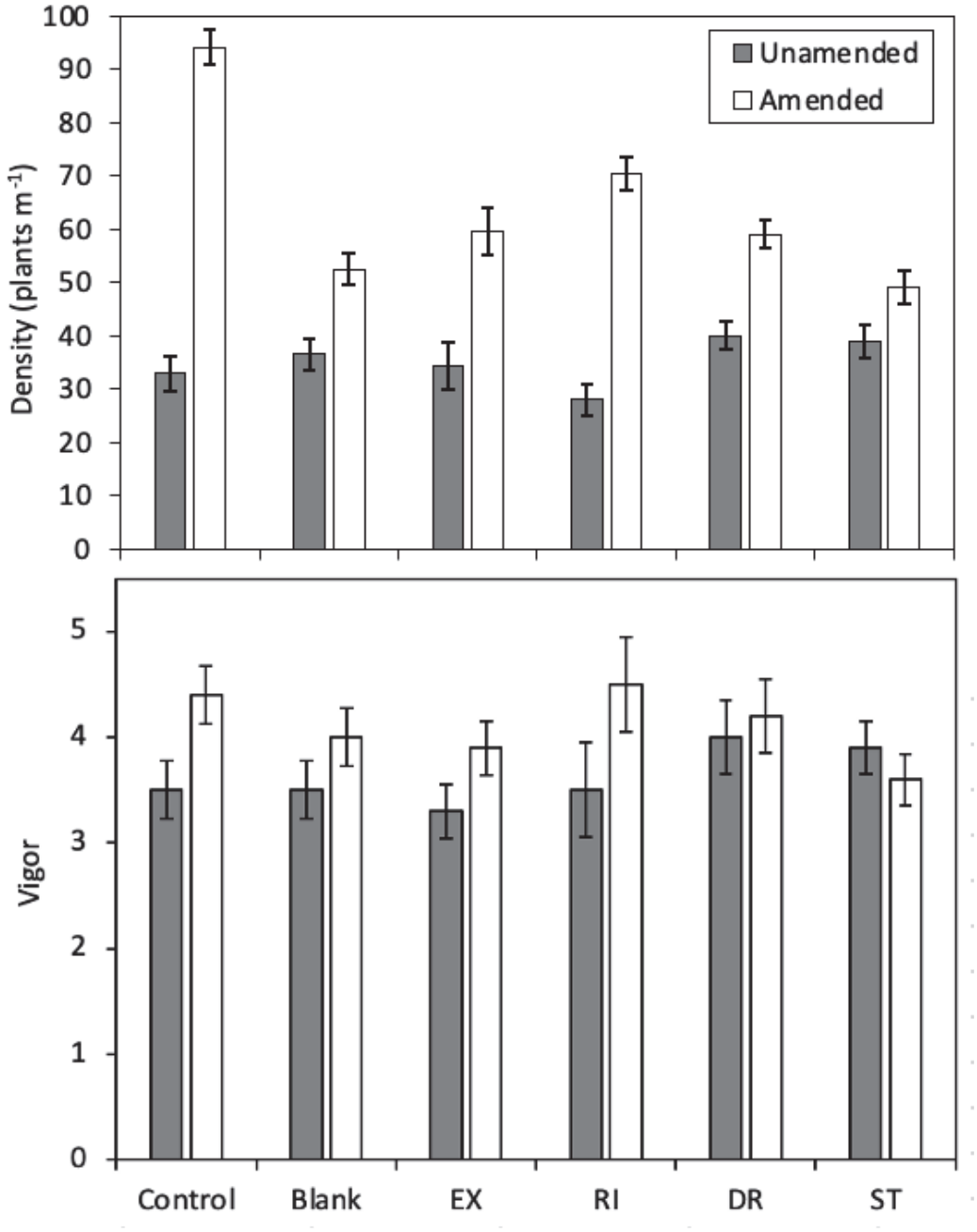

Figure 2 – Mean silky lupine (*Lupinus sericeus* Pursh) plant density and vigor rates (± SE) from October 2021 planting measured in June 2022 at the unamended and amended study mine sites. Treatments include non-treated seed (control), coated seed with no inoculum (blank), and coated with five different rhizobium inoculants: the commercial product Exceed® (EX), native strain from Rio Tinto Kennecott Copper Mine (RT), native strain collected near Draper, UT (DR), native strain collected near Strawberry Reservoir, UT (ST).

TABLES

Table 1 – Batch recipe used to make tryptone yeast (TY) from which 250 ml of the solution is removed and added with 3 g of bacterial agar and poured into Petri dishes.

Ingredient	**Amount**
Autoclaved, ddH_2O	1 L
Tryptone	5 g
Yeast extract	2.5 g
$CaCl_2 \cdot 2H_2O$	0.4 g
$MgSO_4 \cdot 7H_2O$	0.4 g
KOH	300 ul

Table 2 – Seed treatments coated on silky lupine (*Lupinus sericeus* Pursh) for the Fall 2021 planting. Seeds were either left uncoated (control), coated but without an inoculum (blank), or with one of five different rhizobium inoculants.

Treatment	**Description**
Control	*Uncoated seed*
Blank	*Coated seed with no added inoculum*
EX	*Coated seed with commercial EXCEED® inoculum*
RT	*Coated seed with native strain collected from Rio Tinto Kennecott Copper Mine*
DR	*Coated seed with native strain collected from Draper, UT*
ST	*Coated seed with native strain collected near Strawberry Reservoir, Heber, UT*

Table 3 – Degrees of freedom (df), *F*, and *P* (*Pr>F*) values for an analysis of variance for the effect of site, treatment, and their interactions on plant density and vigor. *P* values in bold are statistically significant (*P*<0.05).

	Density			Vigor	
	df	*F Ratio*	*Prob > F*	*F Ratio*	*Prob > F*
Site	1,44	17.9	**<0.001**	7.4	**0.010**
Treatment	5,44	0.5	0.754	0.7	0.595
Site X Treatment	5,44	1.3	0.271	1.2	0.323